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Stable isotope and fatty acid compositions of monovarietal olive oils: Implications of ripening stage and climate effects as determinants in traceability studies

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ABSTRACT

The quantitative variability of isotopic and fatty acid compositions in extra virgin olive oil was investigated in relation to variety, olive ripeness index and geographical origin. The study was carried out in two environments of Umbria region (central Italy), differing in annual/seasonal temperatures and rainfalls. Significant correlation was found between oil isotope compositions and climatic seasonal changes during fruit ripening. Moreover, significant isotopic differences among cultivars were related to the period of maximum oil accumulation in the fruits and to the fatty acid profile. A carbon kinetic isotopic effect could be associated with the fatty acid elongation from palmitic to stearic acid and to the unsaturation step leading from oleic to linoleic. No correlation was found between stable isotope and fatty acid compositions during fruit ripening. The analyzed cultivar and ripening effects on oil traceability issues are discussed in order to focus the resolution power of stable isotope methodologies and the possibility to distinguish monovarietal oils from the same area.

1. Introduction

Olive fruit is a drupe, which is characterized by a lignified endocarp (stone), which contains a seed, a fleshy mesocarp, where the oil is accumulated, and a thin epicarp. The development of olive fruit, like that of most other stone fruit, is characterized by a double sigmoid growth curve (Tombesi, 1994). After fruit set, olives grow rapidly for 30–40 days, mainly due to cell multiplication, then the growth slows down considerably. During this period, seed and endocarp reach their final size and the endocarp hardens (lignifies). Successively, the fruit has a second period of intense growth, mainly due to cell expansion. During this phase, ripening occurs and the beginning of this process can be defined as the stage when chlorophyll content declines. Afterwards, during ripening, different processes occur: accumulation of anthocyanins firstly in the epicarp and then in the mesocarp, softening of the pulp (mesocarp), accumulation of oil in the flesh and reduction of the fruit detachment force with some fruit drop. Various authors (Agramont, López, Boatella, & de la Torre, 1986; De la Torre, López, Colell, & 1985; Famiani, Proietti, Farinelli, & Tombesi, 2002; Farinelli, Boco, & Tombesi, 2002; Inglese et al., 2011; Solinas, Marsilio, & Angerosa, 1987) studied the structural changes occurring during olive ripening and their effects on the different components of the fruit and extracted oils. However, those studies dealt with

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individual components, and no integrative studies on oil stable isotope compositions have been published so far. Owing to the seasonal changes of environmental conditions during the ripening period, the plant primary activity usually changes from a condition which limits to a condition which favors CO_2 diffusion, through the stomata, to the carboxylation sites into the chloroplasts (Diaz-Espejo et al., 2006). Indeed, the olive trees generally experience a progressive mitigation from hot and arid summer conditions to cool and humid autumn conditions. Under such circumstances, the carbon isotope discrimination occurring during the photosynthetic carbon uptake is expected to increase, with a relatively depleted carbon isotope composition ($\delta^{13}C$) of photoassimilates (Brugnoli, Hubick, von Caemmerer, Wong, & Farquhar, 1988; Farquhar, Ehleringer, & Hubick, 1982). In fact, under mild conditions, the lighter isotope (^{12}C) is favored along the photosynthetic pathway, owing to diffusive and biochemical effects and leading to higher discrimination (Portarena, Gavrichkova, Lauteri, & Brugnoli, 2014; Lauteri et al., 2004). Meanwhile carbon isotope discrimination represents a powerful proxy of plant primary productivity, the oxygen isotope composition ($\delta^{18}O$) of plant organic matter relates to the (a) isotopic composition of water source (linked to latitude, elevation, distance from sea, temperature, amount of precipitation) (Barbour, 2007; Clark & Fritz, 1997) (b) isotopic effects during transpiration (affected by relative humidity, temperature, isotope composition of water vapor) (Hermann & Voerkelius, 2008) and (c) biosynthetic pathways including isotopic exchange between photosynthetic intermediates and metabolic water (Barbour, 2007; Schmidt, Werner, & Rossmann, 2001). A variation in stomatal conductance invariably will affect the isotopic signal in leaf water and, hence, of photosynthetic products related to the fatty acid synthesis (Scheidegger, Saurer, Bahn, & Siegwolf, 2000). In fact, photosynthetic products (carbohydrates) are exported from the leaves to the fruits to fulfill the metabolic requirements for oil synthesis (Sànchez & Harwood, 2002). Hence, applications of stable isotopes technology might highlight some still poorly explored fundaments on the ecophysiology of olive oil traceability.

Several authors (Kelly, Parker, Sharman, Dennis, & Goodall, 1997; Spangenberg, Macko, & Hunziker, 1998) claimed about putative differences in carbon isotope composition among different fatty acids of olive oil. Such evidences have been addressed to differential fractionation effects along the fatty acids biosynthetic pathway. The isotopic fractionations involved in the biosynthesis of lipids have been the subject of many investigations based on applications of isotope ratio mass spectrometry (IRMS) (De Niro & Episten, 1977; Monson & Hayes, 1982; Stenberg, De Niro, & Keeley, 1984). Overall, these studies concerned the analysis of the bulk oil material. However, to our knowledge, little information exists on possible effects of the seasonal shift on the fatty acids and isotopic composition of olive oil.

In this work, isotopic compositions ($\delta^{13}C$, $\delta^{18}O$) and fatty acid profiles were studied on different monovarietal extra-virgin olive oils, derived from cultivars grown in the same orchard and sampled at different ripening degrees. The aim of the present work was to investigate: a) the effect of the stage of olive ripeness on carbon and oxygen isotopic fingerprints of monovarietal extra-virgin olive oils; b) possible relationship among $\delta^{13}C$, $\delta^{18}O$, fatty acid profiles and oil accumulation period of the same oils.

2. Materials and methods

2.1. Olive orchards, fruit sampling and characteristics, oil extraction and composition

Extra virgin olive oil was extracted from individual olive cultivars grown in two experimental olive orchards, located in Perugia and Orvieto, Umbria region, Central Italy, and characterized by different climatic conditions. Referring to long-term averages, Perugia has lower minimum temperatures, higher maximum temperatures and rainfalls than Orvieto (Research unit for Climatology and Meteorology applied to Agriculture – CMA, 2012). In particular, in the year of the experiment (2012), the average annual minimum temperatures were 8.7 °C and 9.8 °C, respectively, whereas the annual maximum temperatures were 23.2 °C and 19.3 °C, respectively. From July to October, main period of oil formation and accumulation, the minimum temperatures were 15.1 and 16.1 °C respectively, whereas, for the same period, the maximum temperatures were 31.5 °C in Perugia and 27.2 °C in Orvieto (CMA, 2012). The annual rainfalls of Perugia and Orvieto were 850 mm and 774 mm, respectively. From July to October, the rainfalls of Perugia and Orvieto were 308 mm and 267 mm, respectively (CMA, 2012). Four cultivars, namely Frantoio, Leccino, Moraiolo and San Felice, were used within the germplasm collection 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) (from here on referred as Perugia). This 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 was rainfed. Fertilization was based on the supply of nitrogen, potassium and phosphorous as chemical fertilizers. The soil was managed with a spontaneous green cover mowed 2–3 times/year. Furthermore, four cultivars (Frantoio, Leccino, Nostrale di Rigali, San Felice) and one clone (IX 90) were used in the experimental field of ONAT (Orvietano, Narnese, Amerino, Tuderte) Mountain Community at Monteleone d'Orvieto (42°55′00″N, 12°03′00″E) (hereinafter referred as Orvieto). The trees were 8 years old (at the beginning of the trial), pruned according to the vase training system and spaced at 5 × 5 m. The orchard was rainfed. Fertilization was based on the supply of nitrogen, potassium and phosphorous as chemical fertilizers. The soil was managed with a spontaneous green cover mowed 2 times/year. The olive growing area is situated at 500 m a.s.l. Frantoio, Leccino and Moraiolo are the main cultivars of the Umbria Region and among the most cultivated in Central Italy. Moreover, Frantoio and Leccino are also grown in the other Italian regions where olive is spread and in countries where olive has been introduced and its cultivation is becoming more and more important (e.g., Australia and Argentina). Nostrale di Rigali and San Felice are local variety.

The olives were randomly hand-picked in each comparative field from four trees per cultivar/clone.

After the collection, the samples (four per cultivar and sampling data; one sample per tree) were divided into 2 subsamples: one, smaller (about 0.5 kg), used to evaluate fruit characteristics and ripening indexes; the other one, 3 kg, utilized to extract the oil.

Oil samples were extracted using a hammer mill, 30 min of kneading and centrifugation without extra water. The experiment was conducted along 3 months (October, November and December) during the ripening stage in 2012. Fruit characteristics and oil fatty acid composition were only determined on samples from Perugia orchard, where adult and relatively large trees ensured enough fruits for all the determinations. The degree of ripening was synthetically evaluated by Jaèn pigmentation index (JI) on 100 drupes/tree. JI ranges from 0 to 7, with 0 for fully green olives and 7 for olives with superficial pigmentation on 100% of the epicarp and pigmentation on 100% of the pulp (Uceda & Hermoso, 1998). As far as the determination of fatty acid composition is concerned, the fatty acids were converted to methyl esters before analysis by shaking a solution of 0.2 g oil and 3 mL of hexane with 0.4 mL of 2 N methanolic potassium hydroxide. Contents of the different methyl esters were analyzed determined by means of GC- FID detector, and

after separation through a 100% dimethyl-polysiloxane VF-wax Varian (30 m length, 0.25 mm i.d. and 0.25 μm film thickness) with a split/splitless injector (1/10 ratio). The temperatures of the injector and detector were held at 220 and 250 °C, respectively. The injection volume was 1 μL. Preliminary peak identification was carried out by comparison of retention times with known standards. Fatty acids were designated according to the standard notation (total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule) as described by Peacock et al. (2001). The following fruit characteristics were determined: fresh weight (two samples of 100 drupes/tree); pulp (epicarp + mesocarp) firmness using a hand-held dynamometer with a 1.0 mm plunger (Effe.gi, Ravenna, Italy) (100 drupes/tree); water content by ovendrying at 105 °C the samples used for fresh weight determination; the oil content was determined (2 samples/tree) by using an InfraAlyzer apparatus (SpectraAlyzerZeutec BRAN + LUEBBE, Rendsburg, Germany) expressed on both fresh and dry weight basis. At each sampling date, olive detachment force was determined on each tree using a dynamometer (Somfy Tec, Ademva, ClusesCedex, France). The mean of maximum daily temperature, over five days before the sampling date, of the different fields were obtained from the Research unit for Climatology and Meteorology applied to Agriculture (CMA), while the cumulated amount of precipitations from 28th of August were determined using a pluviometer (Stocker, Bolzano, Italy). Both temperatures and cumulated rains are shown in Table 1.

Different cultivars and ripening stages along with amounts of rains and temperature during the ripening period were considered in order to investigate their influence on carbon isotope, oxygen isotope and fatty acid compositions.

2.2. Stable isotopes determinations

Analyses of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios in olive oils were performed using an isotope ratio mass spectrometer (Isoprime, GV, Cheadle, UK) connected respectively to an elemental analyzer (NA1500, Carlo Erba, Milan, Italy) and to a pyrolysis system (Euro Pyr-OH, Euro Vector Instruments & Software, Milan, Italy). Isotope compositions were anchored to IAEA international standards on the VPDB and VSMOW scales, respectively for $\delta^{13}C$ and $\delta^{18}O$ determinations. Particularly, gaseous (RM 8562, RM 8563, RM 8564) and solid IAEA standards (NBS-22 fuel oil and IAEA-CH6 Sucrose) were used to refer determinations on the VPDB scale. On the ^{18}O side, isotopic compositions were referred to the VSMOW scale by means of IAEA-CH6 Sucrose ($\delta^{18}O = +36.4‰$). This latter reference value was assigned by Boschetti and Iacumin (2005) and, since then, commonly used. The isotopic determination were expressed as δ notation vs VPDB for $\delta^{13}C$ and vs VSMOW for $\delta^{18}O$, according to the expression: $\delta = (R_s - R_{std})/R_{std} \times 1000$, where R_s is the isotope ratio of the sample and R_{std} is the isotope ratio of the international standard. The precision of measurement, expressed as standard deviation when measuring an oil sample 10 times, was 0.1‰ for $\delta^{13}C$, and 0.3‰ for $\delta^{18}O$.

Table 1
Climatic characteristics of Perugia and Orvieto experimental fields at different sampling dates: cumulated amount of precipitations from 28th of August (UCEA), mean of maximum daily temperature over five days before the sampling date.

Field site	Sampling day	Cumulated rain (mm)	T (°C)
Perugia	October 2	67.0	28.4
	October 17	185.5	22.7
	October 30	221.5	16.9
	November 20	481.0	15.9
	December 3	557.0	11.6
Orvieto	October 4	70.8	24.8
	October 18	131.4	21.0
	November 5	253.8	16.8
	November 22	378.3	13.8

2.3. Statistical treatment of data

Basic statistic has been applied to the studied parameters. Pearson coefficients were calculated for isotope and fatty acid compositions against other climatic and geographical parameters. These values were compared with the critical value (F) for a confidence level of 99% and 95%. Factorial analysis of-variance (ANOVA) and Post Hoc Fisher multiple comparison test were performed to test the influence of ripening index, cultivar and field site on carbon and oxygen isotopic composition of olive oils. Oil samples were classified according to cultivar/clone and ripening stages. All statistical analyses were carried out using Statistica 8 (StatSoft Italia s.r.l, Padova, Italy).

3. Results and discussions

3.1. Effects of cultivar, environment and ripening on the stable isotope signatures of the oil

During ripening, the firmness of the pulp and the detachment force of the fruits decreased in all varieties, while the oil content increased (data not shown). The Jaen Index (pigmentation index) of the olives of the different varieties increased, correlating with the amount of precipitation and the decrease of temperatures (data not shown).

Fig. 1 shows the ranges of variation found for carbon and oxygen isotopic values in all olive oil samples extracted from olives collected in Perugia and Orvieto fields.

The range of variation of the $\delta^{13}C$ values was about 3.8‰ (from −30.6‰ to −26.8‰) and 5.6‰ (from −32.2‰ to −26.5‰) respectively for Perugia and Orvieto fields. A larger range of variation was found for $\delta^{18}O$ values: 7.1‰ (from 20.3‰ to 27.4‰) and 5.3‰ (from 19.6‰ to 24.9‰) for Perugia and Orvieto, respectively.

The correlation between $\delta^{13}C$ and $\delta^{18}O$ was tested by linear regression, by splitting the dataset from all the cultivars for each of the two experimental fields (Fig. 1). Positive and significant ($p < 0.05$) correlations were found between the two variables. The

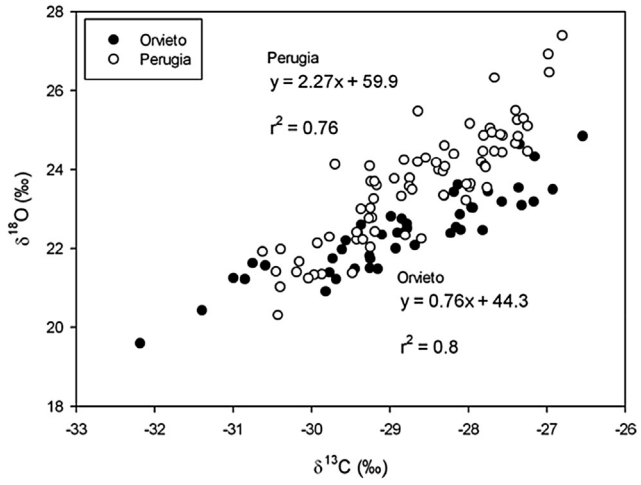

Fig. 1. Relationships between $\delta^{13}C$ and $\delta^{18}O$ values of all olive oil samples extracted from olives collected in Perugia (open circles) and Orvieto (closed circles) experimental fields.

correlation was slightly stronger in Orvieto than in Perugia. This overall strong correlation between the two isotopic variables (Fig. 1) can be interpreted as a common response of olive cultivars to environmental factors, involving on one side the available water source (shallow or deep soil water) affecting the final $\delta^{18}O$ in the oil and, on the other side, the plant water status and its photosynthetic performance, linked to $\delta^{13}C$ (Brugnoli & Farquhar, 2000; Farquhar, Ehleringer, & Hubick, 1989).

A factorial analyses of variance (ANOVA) was carried out to test the influence of ripening stage (Jaen Index), cultivar, and field site on carbon and oxygen isotopic composition of olive oils (Table 2). This analysis shows significant independent effect of Jaen Index and cultivar on both $\delta^{13}C$ and $\delta^{18}O$. The climatic characteristics of the two fields significantly affected only responses in oxygen isotopic composition. The statistical interaction between Jaen Index and cultivar resulted significant for carbon but not for oxygen isotopic composition.

The variation in oxygen isotope composition reflects the variability of meteorological conditions between the two fields (Table 1). Indeed, the amount of rainfalls during the ripening period showed minor variability between the two fields; contrarily, Perugia field showed higher temperatures in 2012, together with higher $\delta^{18}O$ values of olive oils (Fig. 1). Both $\delta^{13}C$ and $\delta^{18}O$ resulted significantly correlated with all the factors studied. Indeed, in both experimental fields $\delta^{18}O$ and $\delta^{13}C$ of all oils were correlated to temperature, mm of rains and Jaen Index (JI) during the ripening period (data not shown). The best correlations were found between $\delta^{18}O$ and temperature (positive correlation) and amount of rain (negative correlation). Fig. 2 shows the evolution of carbon and oxygen isotopic compositions of the different cultivars/clone analyzed in both fields (Perugia and Orvieto) during ripening. From data in Fig. 2, we also calculated the phenotypic plasticity of isotopic responses of the different olive tree genotypes (data not shown). We found several evidences indicating a phenotypic differentiation of the three genotypes, Frantoio, Leccino and San Felice, which are common to both experimental fields. The observed diversity among these cultivars, reveals a clear distinction in the pattern of variation of both carbon and oxygen compositions. Such a phenotypic diversity is especially driven by the Jaen Index, irrespective of diverse location and age of the two comparative fields. The analysis also suggests that San Felice shows an earlier oil accumulation, characterized by relatively enriched isotopic compositions, than the varieties Frantoio and Leccino. This latter shows the latest pattern of oil accumulation (Fig. 3), accordingly with its proverbial frost resistance.

The mean daily temperature decreased from October to December 2012 and was associated to an increase of the precipitations. Such a usual meteorological trend caused variations in plant water source, atmospheric humidity, evaporative demand, photosynthesis and transpiration processes, with effects on both the isotopic compositions (carbon and oxygen) of the synthesized fatty acids. Biochemical modifications associated to the ripening stage could also affect the isotopic compositions of the different oils. Indeed, differences in the ripening earliness among the olive varieties have the potential to influence the isotopic signatures of the oils, given their derivation from sources of carbon with different isotopic compositions along the season (e.g. soluble sugars, starch) (Scartazza et al., 2004). Thus, despite the changes observed during ripening, the isotopic compositions of the different genotypes were well separated. These results clearly show that the isotopic signals of the monovarietal oils are strongly affected by both genotype and ripening degree.

Table 2
Factorial analyses of variance (ANOVA) of carbon and oxygen isotopic compositions of olive oils extracted from olives at different ripening stages (JI) collected from the different considered genotypes in the two experimental fields (Perugia and Orvieto) (** = significant at $p < 0.01$).

	$\delta^{18}O$		$\delta^{13}C$	
	F value	p Value	F value	p Value
JI	76.57	3.8e−14**	59.68	5.5e−12**
Cultivar	59.38	2.2e−16**	38.79	2.2e−16**
Field	19.13	2.8e−05**	0.03	0.863
JI*cultivar	2.94	0.016	5.41	0.0001**
JI*field	2.76	0.099	0.14	0.713
Cultivar*field	2.53	0.084	3.23	0.043

3.2. Effects of ripening on fatty acid profiles and relationships with C and O isotope compositions

The observed variability in carbon isotope composition of the oils could be partially linked to intrinsic isotopic effects along the pathway of fatty acid (FA) synthesis (Nergiz & Engez, 2000; Poiana & Mincione, 2004). Any metabolic isotopic effect could be reflected in the overall isotopic composition of the oil, enhancing or dampening the seasonal isotopic signal over-impressed along the ripening process (Kelly et al., 1997; Royer, Gerard, Naulet, Lees, & Martin, 1999). The content of the main oil fatty acids was analyzed: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) (Table 3). In all the samples, the oleic acid was the most abundant compound, always higher than 74% of the total FA content. Indeed, the relative FA composition was different in the studied cultivars. Leccino had the highest concentration of oleic acid (with increasing trend during ripening, from 78.4% at green stage to 79.5% at full ripening) and the lowest amount of linoleic acid. San Felice and Moraiolo had a high percentage of linoleic acid and a low and constant concentration of oleic acid. Palmitic acid did not show significant differences among the considered cultivars. The content of linolenic acid (C18:3) was rather stable during ripening in the oils of all the cultivars. Differences in FA composition probably reflect the specific metabolic behavior of each cultivar, controlled by genetic determinants.

The main FA percentages of oils did not show important changes among cultivars during the considered olive ripening period. Therefore, the isotopic differences observed in olive oils during ripening cannot be related to the evolution of FA composition.

The correlations between fatty acids and isotopic composition in all olive cultivars grown in the Perugia experimental field are shown in Table 4.

The differences in carbon-isotope composition among olive oil cultivars were significantly related with changes in oleic ($r = -0.50$) and linoleic ($r = 0.43$) acid abundance. Aside the significant relationship ($r = 0.28$), the variation in stearic acid content is too little in olive oil to represent an effective source of variation in carbon isotope composition. It is noteworthy that $\delta^{13}C$ was positively related to the linoleic acid content but negatively to the oleic acid one. Because of the presence of double bonds, in fact, a kinetic isotope effect influences the reaction leading to 18:2 through unsaturated oxidative steps, catalyzed by fatty acyl coenzyme a desaturase. This causes ^{13}C enrichment from 18:1 to 18:2, yielding a net depletion of carbon 13 at the carboxyl position in 18:1 lipid (Monson & Hayes, 1981). Another isotopic effect is associated with the elongation pathway leading from 16:0 to 18:0 (by addition of one acetyl group to palmitic acid) and may be responsible for the enrichment of ^{13}C in 18:0 (Monson & Hayes, 1981).

A similar pattern of relationships was also observed among oxygen isotope compositions and FA profiles. Hypothesizing a unique source of oxygen for the fatty acid biosynthesis, the oxygen

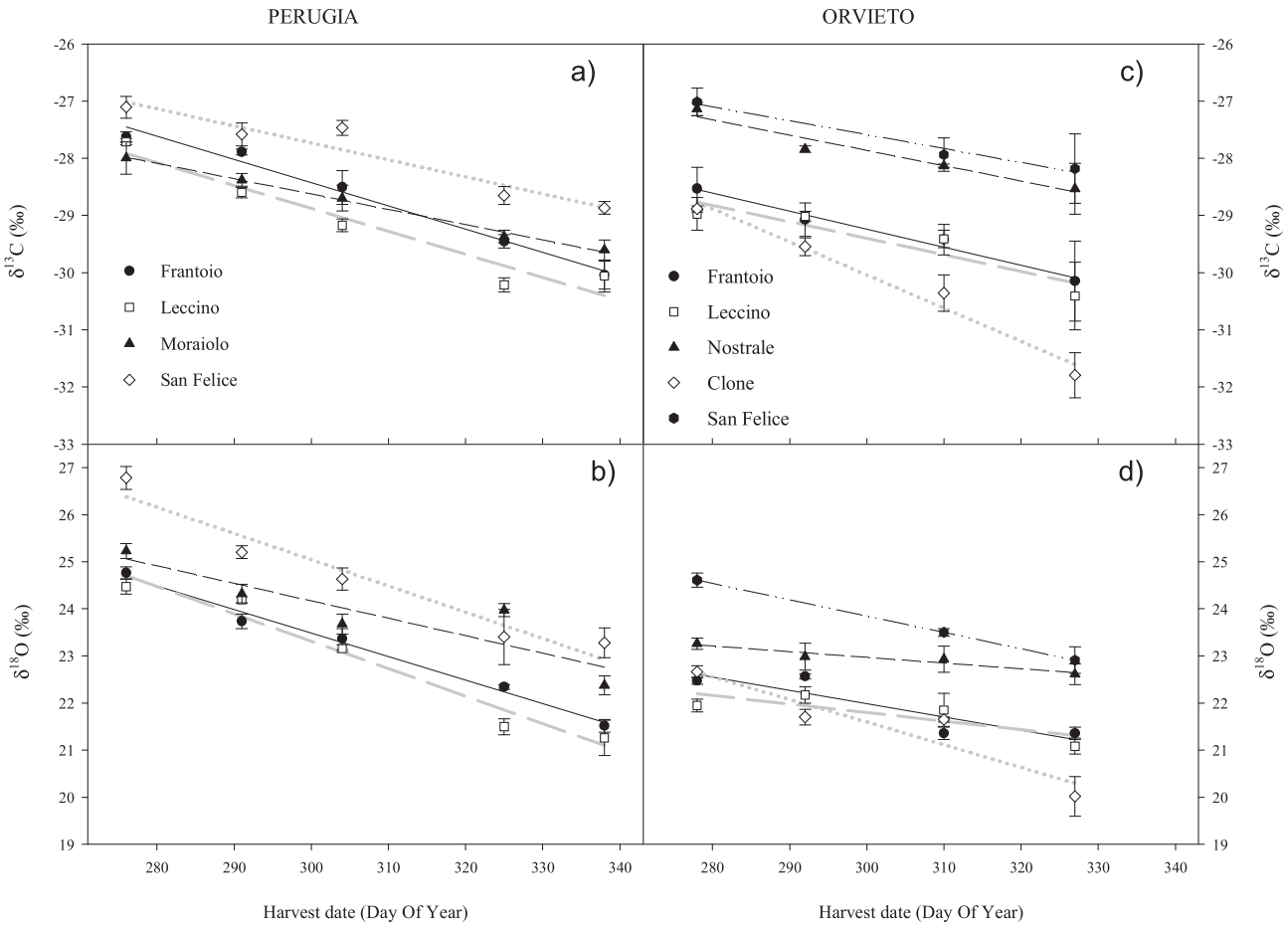

Fig. 2. Mean values of $\delta^{13}C$ and $\delta^{18}O$ in olive oils from different cultivars obtained from progressive harvestings in the experimental fields of Perugia (a, b) and Orvieto (c, d). Bars represent standard errors.

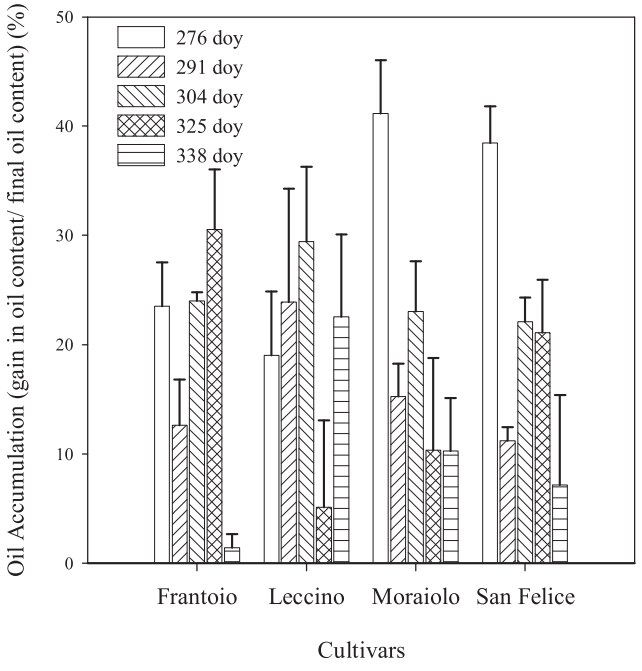

Fig. 3. Oil accumulation (gain in oil content/final oil content, %) in olives harvested at five different DOY (Day Of Year) from the different cultivars grown in Perugia.

isotope compositions could not vary among different fatty acids, its value being associated to the same carboxylic group in the different chains. The effectiveness of any isotopic effects cannot explain the relationship between $\delta^{18}O$ values and fatty acid composition. Such a finding strongly supports the hypothesis that both carbon and oxygen isotope compositions in olive oil are mainly determined by the variation of environmental conditions during the ripening period and oil accumulation. Fig. 3 shows the period of oil accumulation (gain in oil content/final oil content)*100, where gain in oil content is = (oil content at the considered sampling date − oil content at the previous sampling date) in olives from the different cultivars grown in Perugia. The oil accumulation pattern presented large differences among the four cultivar. In September (275 Day of Year, DOY), Moraiolo and San Felice accumulated, respectively, 40% and 39% of total oil, Frantoio 23% and Leccino 19%, only. Leccino accumulated oil in the fruits especially in October (from 276 to 304 DOY) (53.3% of the total), when Moraiolo, Frantoio and San Felice accumulated 38.2%, 36.6% and 33.3% of the total, respectively. The accumulation of oil in the fruits of Frantoio was completed in the second week of November (up to 325 DOY), when 98.6% of the total oil was accumulated. On the contrary, at the same date, fruits of San Felice accumulated 92.9% of total oil, those of Moraiolo 89.7% and those of Leccino only 77.5%. From DOY (Day of Year) 326 to 338, Leccino accumulated 22.54% of total oil in the fruits, Moraiolo 10.27% and San Felice 7.14%.

In particular, $\delta^{13}C$, $\delta^{18}O$ and 18:2/18:1 ratio in olive oil resulted significatively and positively correlated with the ratio between

Table 3
Variations of mean percentages of fatty acid composition for each cultivar along the ripening period (Day Of Year, DOY and Jean Index, JI) in Perugia experimental field. Standard errors in parentheses.

Cultivar	DOY	JI	Palmitic (16:0; %)	Palmitoleic (16:1; %)	Stearic (18:0; %)	Oleic (18:1; %)	Linoleic (18:2; %)	Linolenic (18:3; %)
Frantoio	276	0.02	13.3 (0.1)	0.8 (0.1)	1.8 (0.1)	76.7 (0.2)	6.6 (0.1)	0.8 (0.1)
	291	0.08	12.6 (0.1)	0.8 (0.1)	1.5 (0.1)	77.1 (0.2)	6.9 (0.1)	0.8 (0.1)
	304	1.01	13.7 (0.1)	0.9 (0.1)	2.1 (0.1)	76.4 (0.3)	6.9 (0.1)	0.6 (0.1)
	325	1.05	13.7 (0.1)	0.9 (0.1)	2.0 (0.1)	77.9 (0.2)	6.9 (0.1)	0.8 (0.1)
	338	1.06	14.0 (0.1)	0.8 (0.1)	2.2 (0.1)	77.1 (0.3)	6.6 (0.1)	0.8 (0.1)
Leccino	276	0.07	12.6 (0.1)	1.1 (0.1)	1.6 (0.1)	78.4 (0.1)	5.1 (0.1)	0.5 (0.1)
	291	2.03	13.0 (0.1)	1.1 (0.1)	1.5 (0.1)	78.5 (0.2)	5.1 (0.1)	0.5 (0.1)
	304	3.04	12.7 (0.1)	1.1 (0.1)	1.6 (0.1)	78.6 (0.1)	5.0 (0.1)	0.5 (0.1)
	325	3.09	12.7 (0.1)	1.1 (0.1)	1.4 (0.1)	79.1 (0.1)	5.0 (0.1)	0.5 (0.1)
	338	5.09	12.7 (0.1)	1.1 (0.1)	1.4 (0.1)	79.5 (0.3)	5.0 (0.1)	0.5 (0.1)
Moraiolo	276	1.01	13.2 (0.1)	0.8 (0.1)	1.9 (0.1)	75.2 (0.1)	7.2 (0.1)	0.7 (0.1)
	291	2.03	13.1 (0.1)	0.8 (0.1)	2.0 (0.1)	75.2 (0.1)	7.2 (0.1)	0.7 (0.1)
	304	2.08	13.4 (0.1)	0.9 (0.1)	1.9 (0.1)	75.5 (0.2)	7.1 (0.1)	0.7 (0.1)
	325	3.08	13.6 (0.1)	0.9 (0.1)	1.9 (0.1)	75.6 (0.1)	7.2 (0.1)	0.7 (0.1)
	338	5.03	13.6 (0.1)	0.9 (0.1)	1.9 (0.1)	76.1 (0.1)	7.2 (0.1)	0.7 (0.1)
San Felice	276	0.04	12.9 (0.1)	0.8 (0.1)	2.2 (0.1)	74.8 (0.1)	7.9 (0.1)	0.7 (0.1)
	291	1.04	12.9 (0.1)	0.8 (0.1)	2.2 (0.1)	74.9 (0.1)	7.9 (0.1)	0.7 (0.1)
	304	1.01	13.0 (0.1)	0.8 (0.1)	2.2 (0.1)	74.7 (0.2)	7.9 (0.1)	0.7 (0.1)
	325	3.05	12.9 (0.1)	0.8 (0.1)	2.2 (0.1)	74.8 (0.1)	7.7 (0.1)	0.7 (0.1)
	338	4.08	12.9 (0.1)	0.7 (0.1)	2.2 (0.1)	74.7 (0.1)	7.7 (0.1)	0.7 (0.1)

Table 4
Correlation (r) between fatty acids and isotopic composition in all olive cultivars grown in Perugia experimental field (* = significant at $p < 0.05$, ** = significant at $p < 0.01$).

	$\delta^{13}C$	$\delta^{18}O$
C16:0 palmitic	−0.23*	−0.15
C16:1 palmitoleic	−0.44**	−0.42**
C18:0 stearic	0.28*	0.28*
C18:1 oleic	−0.50**	−0.54**
C18:2 linoleic	0.43**	0.43**
C18:3 linolenic	0.20	0.16

September and October oil accumulation in the drupe ($p < 0.01$, Table 5). The inter-cultivar variability of stable isotope compositions reflects the variation in the period of oil accumulation during September and October months. Such a pattern is well explained by the meteorological data (Table 1). The maximum value of daily temperature (28.4 °C) and the minimum of monthly precipitation (67.0 mm) was 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) and the amount of precipitations much higher (221.5 mm). According to the dual-isotopes conceptual model of Scheidegger et al. (2000), a stomatal response to environmental drivers should be reflected in a positive relationship between $\delta^{13}C$ and $\delta^{18}O$ in plant material. Hence, the enrichment in ^{13}C and ^{18}O in early accumulating oil olives can be due to both environmental factors (such as temperature and precipitation) and plant physiological responses (such as regulation of stomatal conductance to CO_2 and water vapor). These considerations suggest strong genotype × environment interactions in the determinism of the stable isotope profile of olive oils. Indeed, San Felice and Moraiolo oils showed significantly higher isotope ratios than those from Leccino, due to lower precipitation and higher temperatures in September causing higher $\delta^{18}O$ and $\delta^{13}C$ values, indicating the effect of drought and related physiological responses of olives during fatty acid synthesis. Oils from Leccino, being accumulated in the fruits especially in October (53.3% of the total) with lower temperatures and higher precipitations, showed the lowest isotope compositions for both elements.

Table 5
Correlation (r) between $\delta^{13}C$, $\delta^{18}O$, linoleic/oleic fatty acid ratio (lin/ol) in olive oil and the ratio of September and October oil accumulation (Sep.A/Oct.A) in the drupe (** = significant at $p < 0.01$).

	Sep.A/Oct.A
$\delta^{18}O$	0.79**
$\delta^{13}C$	0.84**
lin/ol	0.79**

Bulk isotope composition in olive oil represents a weighted average of multiple, isotopically heterogenous biochemicals, whose relative proportions may vary among different olive varieties and in different climates (Mihailova, Abbado, Kelly, & Pedentchouk, 2015). Establishing the links between biochemical and physiological responses of different cultivars of olive trees (*Olea europaea* L.) under various climatic conditions is a relevant issue for identifying the authenticity and geo-location of olive oils. For example, comparing the isotopic compositions of olive oil from one variety with that obtained from another one with a different metabolism (e.g. oil accumulation pattern) may mask the isotopic differences expected owing to climate gradients, among oil producing regions. In perspective, studying isotopic compositions of individual components in extra-virgin olive oil may be a more informative strategy in traceability studies, especially by investigating those organic compounds that provides a more direct link to environmental factors or that are sensitive to physico-chemical processes during biosynthesis. Interesting perspectives in this respect are open by the wider availability of compound specific IRMS technologies, also referred to as CSIA (Rodrigues, Lauteri, Brugnoli, & Máguas, 2013).

4. Conclusions

Carbon and oxygen isotopic composition of olive oil was dependent on cultivar and fruit ripening stage. The plot of $\delta^{13}C$ versus $\delta^{18}O$ values for the oils indicated a strong correlation, driven by a stomatal response to environmental conditions during the ripening period. The oil accumulation pattern presented large differences among the cultivar and a significant correlation was found between stable isotopes fractionation, fatty acid composition and period of maximum oil accumulation (September or October). In particular, oils obtained from olives which showed an early oil accumulation, such as San Felice and Moraiolo (Fig. 3), showed a relative enrichment in ^{13}C and ^{18}O and higher contents of linoleic

acid. On the contrary, oils obtained from olives which showed a late oil accumulation, such as Leccino (Fig. 3), showed a relative depletion in ^{13}C and ^{18}O, joined with an increased percentage of oleic acid. The relationships among isotopic and fatty acid compositions of the different cultivars were partially explained by the chemical profiles in fatty acids. In particular, both carbon and oxygen isotope compositions were correlated positively to stearic and linoleic acid contents but negatively to oleic acid content.

In its whole, this work highlights that the variability in $\delta^{13}C$ and $\delta^{18}O$ values of olive oil is determined by a complex combination of environmental, physiological and genetic factors. Particularly, the results highlight the importance of the cultivar and ripening stage as determinants of isotope compositions in olive oil. This strongly suggests to take in duly account these determinants in traceability studies. Moreover, the results point out the influence of oil accumulation period in determining the isotope fingerprint of a specific cultivar characterized by a specific metabolic pathway. In perspective, studying isotopic compositions of individual components in extra-virgin olive oil may be a more sensitive tool in traceability studies.

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