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AUTHENTICATION AND TRACEABILITY OF EXTRA-VIRGIN OLIVE OIL BY MEANS OF STABLE ISOTOPES APPLICATIONS

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CONTENTS

ABSTRACT

There is an increasing demand for reliable analytical methods verifying the authenticity of the food we eat. There is, among consumers, a growing enthusiasm for high quality food with a clear regional identity. Numerous food frauds performed by international food producers, have sensitized consumers concerning the authenticity and origin of food products. In the last few years, European Union policy on food has also been orientated towards safeguarding consumers. To achieve this goal the EU has reinforced national control activities, and has driven scientific research to obtain new markers able to control the genuineness and geographical traceability of foods, ensuring their authenticity. Stable isotope ratios of bioelements such as carbon and oxygen have been used for thirty years, in order to check the authenticity of different premium products. With regard to stable isotopes, this ability is based on isotopic fractionation occurring during physical and (bio)chemical processes, as well as along metabolic pathways. For example, in plant material, the different isotope ratios vary according to photosynthetic pathways (plants C3 and C4), climatic, hydrological or geographical conditions. In this study, isotope ratio mass spectrometry (IRMS) analyses have been used to characterise and determine the authenticity of Italian extra virgin olive oils. Furthermore, isotope composition, $\delta^{13}C$ and $\delta^{18}O$, determined in monovarietal extra-virgin olive oils, together with fatty acid compositions, allows distinguishing samples from different cultivars at different maturation degree. Moreover, statistical analyses of obtained results (Multivariate ANalysis Of VAriance (MANOVA), Principal Component Analysis (PCA), and Linear Discriminant Analysis (LDA)), allows grouping oil samples according to their cultivar, demonstrating the usefulness of isotopic composition and fatty acids analysis in differentiating monovarietal olive oils. To conclude the approach used in this study can be proposed for the detection of mislabelling for food products, becoming a tool for checking compliance with the law.

Keywords: stable isotope ratios, IRMS, fatty acid composition, extra-virgin olive oil, cultivar, origin, ripening degree, traceability, authenticity.

1. INTRODUCTION

1.1. Food authenticity

Consumers around the world are increasingly demanding information and reassurance of regarding the origin and content of their food. Furthermore, food manufacturers must provide and confirm the authenticity and point of origin of food products and their components. These increased demands come amid legislative and regulatory drives increasing the complexity and level of regulation imposed on the food supply worldwide. Protecting consumer rights and preventing fraudulent or deceptive practices such as food adulteration are important and challenging issues facing the food industry.

Determining the authenticity of foods means uncovering misdescription of foods not meeting the requirements for legally adopting a certain name, substitution with cheaper but similar ingredients, undeclared processes (e.g. irradiation, freezing) and/or extension of food using adulterants (water, starch), and incorrect origin (e.g. geographic), species or method of production.

Food authenticity issues indeed fall into one of the following categories:

(i) economic adulteration of high value foods;

(ii) misdescription and/or mislabelling of geographical, botanical or species origin;

(iii) non-compliance with the established legislative standards

(iv) implementation of processing practices which are not admitted (e.g. irradiation, freezing). Nowadays, objective assessment of food authenticity has become of paramount importance, as consumers come into daily contact with a wide variety of foods. Globalisation indeed means that more and more foods are traded around the world. Traceability has thus become a cornerstone of the EU's food safety policy, representing a risk-management tool which enables those in the food industry or the authorities to withdraw or recall products which have been identified as unsafe. The increasing complexity and length of the food chain, as well as recent food scares, have also added to public sensitivity regarding the origin and authenticity of food and have underlined the need for tools ensuring that foods are of a high quality and safe to be eaten when they reach the consumer.

In the case of olive oils, the increase in the demand for high-quality olive oils has led to the appearance in the market of olive oils elaborated with specific characteristics. They include oils of certain regions possessing well-known characteristics, that is, olive oils with a denomination of origin, or with specific olive variety composition. There are olive oils obtained from one genetic variety of olive (monovarietal) or from several different varieties (coupage). Monovarietal olive oils have certain specific characteristics related to the olive variety from which they are elaborated. Coupage (or blend) olive oils are obtained from several olive varieties to achieve a special flavour or aroma. The works dealing with olive oil traceability are usually focused on investigating the

botanical or geographical origin. However, the concept of geographical traceability, in which the objective is the geographical location of the olive tree, is slightly different from the concept of botanical traceability, in which the olive used for the olive oil production is the aim. In both cases, the selection of the markers (compounds with discriminating power) to be studied is complicated because the composition of extra virgin olive oils is the result of complex interactions among olive variety, environmental conditions, fruit ripening, and oil extraction technology (Araghipour et al., 2008). Therefore, a careful definition of the geographical or botanical origin of an olive oil based on its chemical composition requires that many factors be taken into account, being very difficult to find an appropriate marker. The aim of this work was to develop new and powerful analytical tools to control the traceability and authentication of olive oils from a certain geographical origin, cultivar and ripening degree of olives used for oil extraction to avoid fraud to the consumers.

1.2. EU legislation

In 1979 European controls on food labelling were introduced with Directive 112/79/EEC of the European Parliament. Additional controls and amendments have since been added, creating an array of labelling requirements. Over two decades, most legislation regarding food safety has been standardised in the European Community. Because of the high administrative costs of standardisation the principle of mutual recognition was used for issues which did not directly endanger consumers' health. European law, and in particular the “Cassis de Dijon” ruling (case120/78, Court of Justice of the European Communities, February 1979), now ensures that, while standards might differ across countries, a country cannot oppose importation from another EU country for technical reasons (except in cases involving sanitary issues or toxic waste issues).

In 2000, the original 1979 directive and its amendments were consolidated into Directive 13/2000/EC of the European Parliament, which focuses on preventing fraudulent or deceptive practices and food adulteration. Directive 13/2000/EC requires detailed food labelling including the exact nature and characteristics of a product, enabling informed consumer choice. It also requires the list of ingredients to include the specific names of all raw materials in descending order by weight. The reliance on mutual recognition in order to avoid trade barriers between countries, together with the need to differentiate quality products from the bulk production generated by the Common Agricultural Policy (CAP) arrangements, has resulted in more importance being given to national labelling. For example, national labels of food quality in France and Italy have appeared more and more attractive to consumers, and therefore to producers, who use them to segment the market. Pressure from countries that had developed a labelling system, largely based on geographical indications, namely France and Italy, led to the definition of a EU-wide system of

labelling, in what is called the '1992 regulation of food quality'. Indeed in 1992 and subsequently in 2006 (2081/92/EEC and 510/2006/EC), the EU Regulations on Protected Designations of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG) provided a set of common rules across countries to register and protect geographical names used to identify food products and traditional production processes. These two sets of regulations are the basis of quality policy system of the EU because they ensure that only those products genuinely originating in a region can be sold as such, eliminating unfair competition and misleading products that may be of inferior quality or made with different components. Finally, the new European Union Traceability Regulation (178/2002/EC) which came into force in January 2005, defines 'traceability' as "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution". While most value chains only allow tracing one step forward and one step back trace, a more detailed traceability system back to the seed is required, for example to ensure that products have not been genetically modified.

1.3. Current Perspective

In the majority of cases paper traceability and livestock tagging systems can guarantee the geographical origin of foods on sale in the retail market. However, increasingly widespread circulation of foodstuffs all over the world can 'lead into temptation' unscrupulous traders who may attribute false descriptions and incorrect origin labelling to foodstuffs. The prospect of high profits combined with a low risk of detection and the lack of stringent penalties associated with food fraud has attracted crime syndicates into illegal food trading. Thus, the food industry urgently needs methods screening non-targeted food samples to provide proof of origin and prevent deliberate or accidental undeclared admixture to food samples.

Isotopic analysis have been applied to a range of foodstuffs for about thirty years, with varying degrees of certainty in order to develop methods that could verify their authenticity. Application of isotopic compositions in order to verify the authenticity of olive oil is based on evidence that these parameters are affected by various complementary factors. In particular stable isotope ratios of organic material mostly depend on botanical, climatic or geographical conditions where the plant grows.

2. STATE OF ART

2.1. Stable isotopes

In bio-organic material, the main elemental constituents (H, C, N, O, S) have different stable isotopes (D, H; ^{13}C, ^{12}C; ^{15}N, ^{14}N; ^{18}O, ^{17}O, ^{16}O; ^{36}S, ^{34}S, ^{33}S, ^{32}S), the main one being the lighter, as evident in Table 1, showing their mean abundance. The natural isotopic composition of organic compounds shows fluctuations around these mean values and these variations, even if on the order of ppm, can be measured precisely and accurately using dedicated analytical techniques such as Isotope Ratio Mass Spectrometry (IRMS). Measurements are reported as relative variation compared to a standard as δ‰ in comparison to international reference standards (Table 1), according to the following equation:

$$\delta‰ = \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1000$$

where R is the ratio between heavier and lighter isotopes. δ is dimensionless and normally indicated in per mill.

Table 1. Mean natural abundance of some stable isotopes and relative international reference standards.

Element	Stable isotope	Mean natural abundance (%)	International reference standard
Hydrogen	^{1}H	99.99	V-SMOW (Vienna –Standard Mean Ocean Water)
	^{2}H (D)	0.01	
Carbon	^{12}C	98.89	V-PDB (Vienna-Pee Dee Belemnite)
	^{13}C	1.11	
Nitrogen	^{14}N	99.63	AIR (Molecular air nitrogen)
	^{15}N	0.37	
Oxygen	^{16}O	99.76	V-SMOW (Vienna –Standard Mean Ocean Water)
	^{17}O	0.04	
	^{18}O	0.20	
Sulphur	^{32}S	95.00	V-CDT (Vienna – Canyon Diablo Troilite)
	^{33}S	0.76	
	^{34}S	4.22	
	^{36}S	0.02	

Natural variation in isotopic composition is due to the different chemical-physical characteristics of the different isotopes of a certain element, owing to different weight and nuclear spin. In a chemical/biochemical reaction or in a phase transition, these differences can both interfere with the speed of reaction (kinetic effect) and affect the energetic state of the system (thermodynamic effect). Due to the smaller weight the lighter isotopes have greater mobility and smaller bound strength and consequently lower activation energy (kinetic effect). The thermodynamic effect is due to the different free energy of isotopically different molecular species: heavier molecules have

lower free energy, so they have greater inertia in reaction and tend to concentrate in the condensed phase. Isotopic fractionation can also be due to situations with an altered reaction equilibrium, such as an instantaneous change in temperature, removal of a reactant or of a reaction product. This kind of fractionation (of non equilibrium, such as enzymatic reactions) determines the enrichment of a particular isotopic species, but without pre-established rules. Subsequently, factors affecting the variability of the isotopic ratios investigated in this thesis (D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) were broadened, in order to clarify their applicative capability.

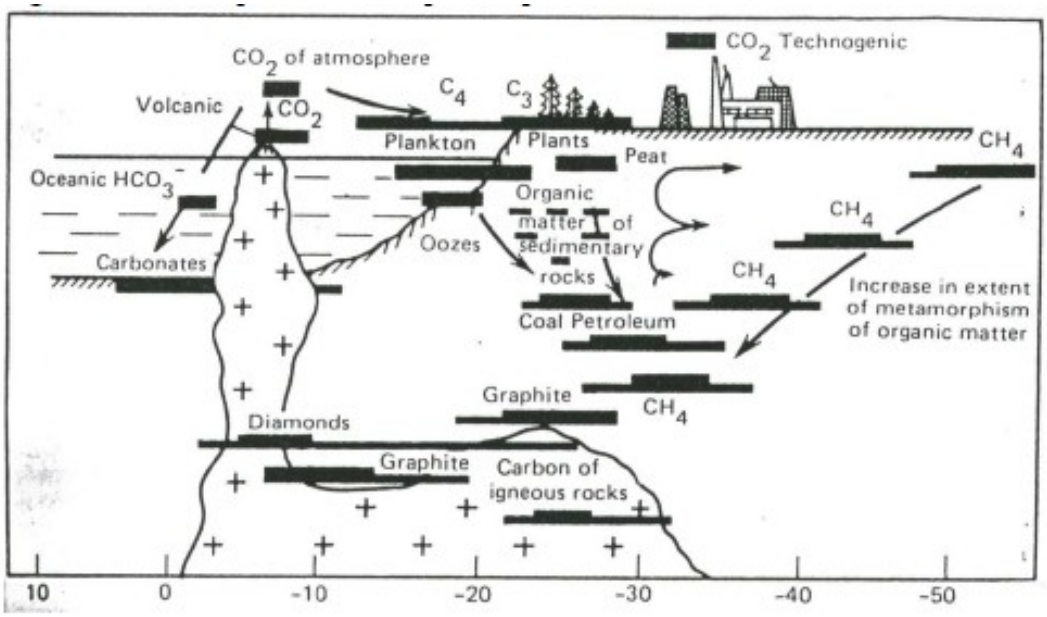
2.2. Natural variation in carbon and oxygen stable isotope abundance

CARBON

Figure 1 shows the carbon isotopic composition of some natural substances. There are essentially three fundamental processes in the carbon cycle involved in isotopic fractionation:

- intake of carbon from the environment by organisms with ^{13}C depletion,
- exchange between atmosphere and hydrosphere leading to an enrichment of ^{13}C (Galimov, 1985),
- group of reactions in animal and plant bio-systems causing further fractionation. Uptake of CO_2 through photosynthesis is the main reaction involved in biological fractionation. In the formation of C-C bound, lighter and more movable carbon atoms are favoured, so photosynthetic products are enriched in ^{12}C and depleted in ^{13}C as compared to atmospheric CO_2 ($\delta^{13}C$ of CO_2 is at present around -8‰). Furthermore, photosynthetic products show different ranges of $\delta^{13}C$ according to the different pathways of photosynthetic cycle (C3, C4 or CAM) because of the different CO_2 concentrating mechanisms and different isotopic effects associated with different primary carboxylase enzymes involved in CO_2 fixation, in different plants types.

Figure 1. Scale of qualitative isotopic composition of carbon in some natural substances. (Galimov, 1985)

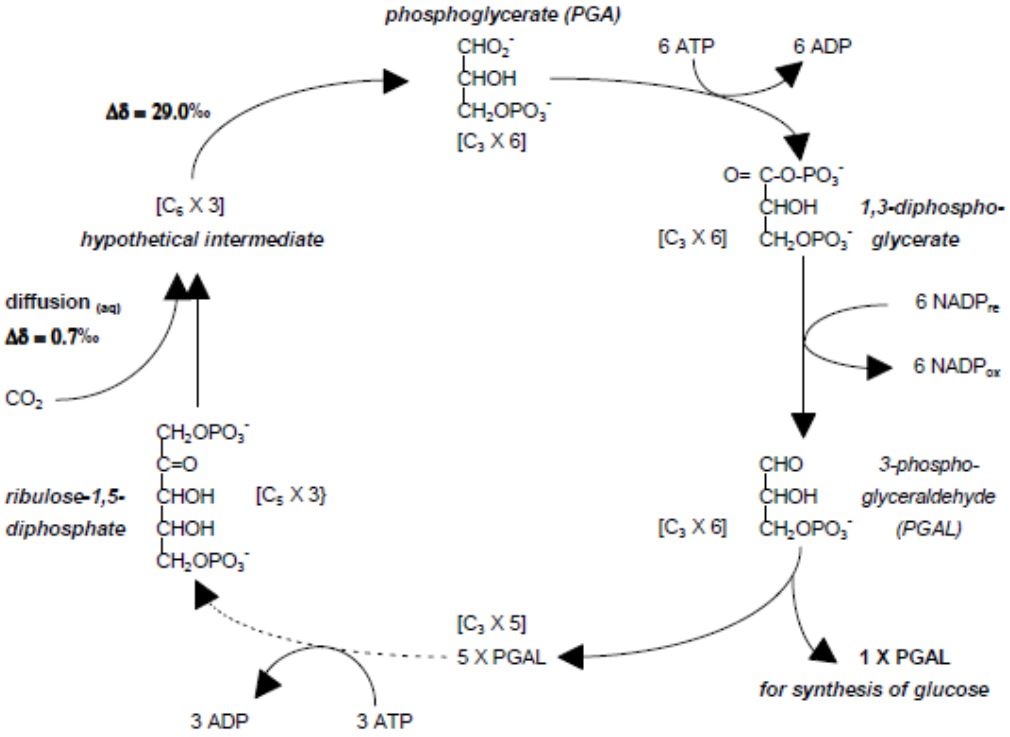


C3 cycle

The main CO_2 assimilation pathway described by Calvin, called C3 because CO_2 is fixed in intermediate products with three atoms of carbon, is characteristic of plants from cold temperate areas (e.g. tomatoes, potatoes, beetroot, wheat, rice, oats, barley, rye, soya bean, grapes, oranges and apples). Atmospheric CO_2 enters into leaf' parenchyma through the stomata, crosses the cell wall, dissolves in the liquid phase, is transported through the plasmalemma into the cytoplasm, and finally enters the chloroplast (Hatch and Slack, 1966; Smith and Epstein, 1971). In the chloroplast stoma, with a series of reactions catalysed by the enzyme riblose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) CO_2 binds to ribulose-1,5-bisphosphate (RuBP), producing two molecules of phosphoglycerate (PGA), which are subsequently reduced to phosphoglyceraldehyde (PGAL), the first sugar of photosynthesis (Figure 2) (Taiz and Zeiger, 1998). Five out of six new PGAL molecules are used to synthesise more RuBP via a series of complex reactions driven by ATP (not shown in Figure 2). The sixth molecule of PGAL may be used to synthesise glucose (usually regarded as the end product of photosynthesis) via combinations

and rearrangements. The glucose may be subsequently used to synthesise complex carbohydrates such as sucrose, structural materials such as cellulose, or energy storage compounds such as starch and lipids (Ting, 1982). Although the initial reaction of CO_2 with RuBP produces the overriding ^{13}C isotope effect associated with these species, there are many other factors which contribute to the final $\delta^{13}C$ value of plant material, such as water availability, evaporative demand of the atmosphere, temperature, fertilisation, salinity, CO_2 concentration, light intensity and photorespiration (O'Leary, 1981). The interplay of all of these factors results in $\delta^{13}C$ ‰ values between -22‰ and -34‰ for 80% to 90% of plants utilising the C3 pathway (Krueger and Reesman, 1982).

Figure 2. Synthesis of carbohydrate by the Calvin (C3) cycle.

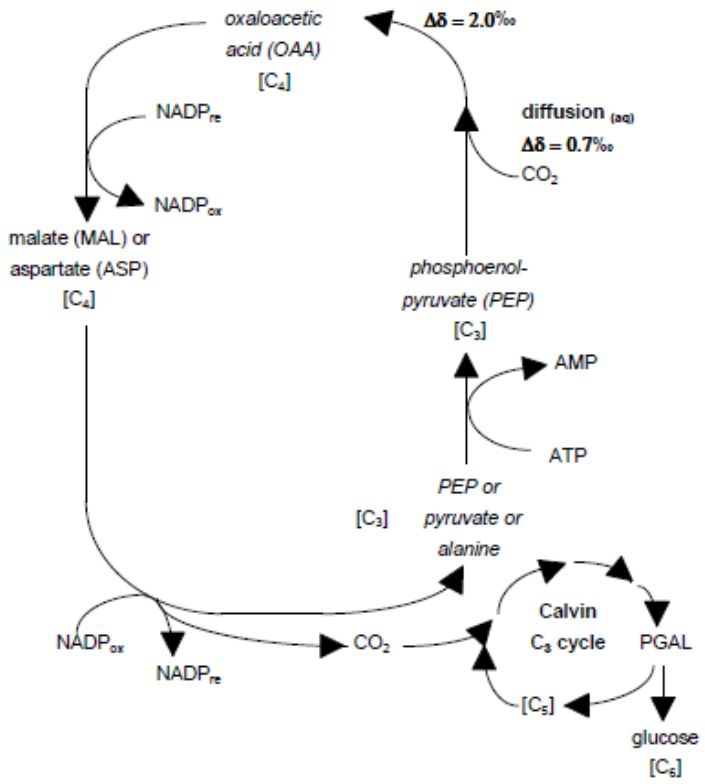


Hatch-Slack or C4 cycle

The carboxylase enzyme Rubisco responsible for carbon fixation in the Calvin cycle is also capable of catalysing the oxidation of RuBP with atmospheric oxygen and this process is known as photorespiration. In other words CO_2 and O_2 are both competing substrates for the carboxylase enzyme (Taiz and Zeiger, 1998). Consequently, the C3 mechanism will not operate with atmospheric CO_2 concentrations of less than approximately 50 ppm. Below this level of concentration the plant photorespires more CO_2 than it assimilates and will eventually die (Krueger and Reesman, 1982). However, there is another group of plants that can carry out photosynthesis with conditions of high temperature, intense light, low moisture, low CO_2 and high O_2 concentrations. The mechanism of carbon fixation used by these plants was elucidated independently by Kortschak (1965) and by Hatch and Slack in the late 1960s, and it is called the C4 pathway (Hatch and Slack, 1970). C4 plants are able to utilise CO_2 concentrations as low as 0.1 ppm. This is achieved by two sequential carboxylation reactions shown in Figure 3, occurring in two separate leaf compartments, namely the mesophill cells (MC), and the bundle sheet cells (BSC). When CO_2 initially enters leaf stomata diffuses in the liquid phasein the MC. CO_2 in the liquid phase is in equilibrium with bicarbonate (HCO_3^-) which combines with a 3-carbon compound, phosphoenolpyruvate (PEP), to form oxaloacetate (OOA), a 4-carbon acid, which is the origin of the term C4 synonymous with the Hatch-Slack cycle. This reaction is catalysed by the enzyme PEP carboxylase whose substrate is HCO_3^- in contrast with Rubisco which uses gaseous

CO_2 as a substrate. Adding up the various isotope effects associated with dissolution and liquid phase diffusion of CO2, the equilibrium fractionation for the hydration of CO2 to bicarbonate and PEP carboxylation results in a much smaller fractionation against ^{13}C as compared to the C3 cycle, δ-variation ~ 2‰ (O'Leary, 1981). OAA is then rapidly reduced by NADPre to form malate (MAL, a C4 acid) or aminated to form aspartate (ASP, a C4 acid). These acids are transported deeper to the BSC into the C4 plant leaves. These acids are then decarboxylated using NADPox to form a C3 compound and CO_2. The CO_2 released in such confined compartment causes a high CO_2 concentration and the entire process can be regarded as a CO_2 concentrating mechanism (CCM). Such high CO_2 concentration can serve as a substrate for Rubisco without any significant photorespiration and then enters the Calvin Cycle (Figure 2). The C3 compound is converted to PEP, pyruvate or alanine and then to PEP by the action of ATP.

Figure 3. The Hatch-Slack pathway of C4 photosynthesis.



It is important to note that although C3 carboxylase enzyme Rubisco shows extensive ^{13}C isotope fractionation (30‰), this is not expressed in the Hatch-Slack photosynthetic pathway. This is because the BSC constitute a relatively gas-tight system and the CO2 decarboxylated there has little opportunity to express it intrinsic fractionation because it has to fix all the CO2 available, independent on its isotopic composition. The only fractionation eventually expressed is due to the fact that BSC and therefore Rubisco has the opportunity to discriminate against 13C, to an extent

depending on the fractionation of BSC CO2 leakage. Consequently, plant biomass formed by C4 plants shows relatively enriched $\delta^{13}C‰$ values ranging between -10‰ and -14‰ (Winkler, 1984). Examples of C4 plants are sugar cane, corn, sorghum, millet and some types of pasture grasses.

Crassulacean Acid Metabolism (CAM) Plants

There is a third group of plants which utilises an adaptive modification of the basic photosynthetic metabolism. This carbon metabolism is characterised by the storage of high concentrations of organic acids during the night and is known as Crassulacean Acid Metabolism (CAM). This term derives from a large succulent plant family, the Crassulaceae, many of which use the CAM metabolism (Ting, 1982). CAM plants tend to be grow in hot and dry climates and as a general rule, they have their stomata closed during the day and open at night. Consequently, the plant strongly reduces its transpiration in the hot daytime hours and only transpires and fixes CO_2 at night with lower air temperature and this causes a reduction in starch and other storage glucans that energise the metabolism. However, if the daytime temperature is relatively low, the stomata may open and the plant will adopt direct C3 metabolism of CO_2. When daytime temperatures are high the stomata remain closed to prevent water loss through transpiration (Krueger and Reesman, 1982). At night, when temperatures drop, the stomata open and CO2 is fixed by PEP carboxylase, in a process similar to that occurring in C4 mesophyll cell. The organic acids are then converted back to CO_2 for C3 photosynthesis during daytime with closed stomata. Therefore, while the two processes are separated in two spatial compartments in C4 species, in CAM plants they are separated in time between night and daytime. The metabolism adopted by CAM plants is therefore linked to local climatic conditions and in extreme cases may be predominantly C3 or C4. Consequently, the $\delta^{13}C‰$ value of CAM plant material varies widely between -30‰ and -12‰ (Winkler, 1984). Pineapple, vanilla and cacti are the most well known plants that use this metabolism.

Secondary carbon metabolism

Further fractionation between plant components from the same primary source (even at specific positions within plant compounds) is a result of kinetic isotope effects (KIEs) in metabolic processes involving side reactions or branching (Schmidt & Kexel, 1999). As a result secondary metabolites such as protein and lipid show systematic deviations from their carbohydrate precursors. As a general rule secondary metabolites tend to be relatively depleted in ^{13}C. This is most notable in lipid fractions that may differ from whole leaf carbon by as much as 10‰. This depletion is known to be caused by an isotope effect associated with the decarboxylation of pyruvic acid and the formation of the C2 substrate for acetyl coenzyme A (De Niro and Epstein, 1977). The repetition of this process during fatty acid chain construction results in alternating ^{13}C depletions (Monson and Hayes, 1982).

OXYGEN

The compositional variability of $\delta^{18}O$ in meteoric water is based on cycles of its evaporation from oceans and subsequent condensation in terms of precipitation. The oxygen isotopic composition of oceanic water (from -1 to 0.7‰) (Clark and Fritz, 1997) is close to that of V-SMOW (0‰), the international reference standard for the measurement of $\delta^{18}O$ (Table 1), although it has changed considerably during different geological eras. The evaporative flux of oceanic water is mainly governed by temperature, and most tropospheric vapour (more than 70%) originates in sub-tropical oceans. During evaporation there is an enrichment of the lighter isotope in vapour, calculated in a hypothetical situation of equilibrium as:

$\delta^{18}O$ = -10.0‰ for oceans at 20°C

$\delta^{18}O$ = -11.6‰ for oceans at 10°C

which leads, according to these theoretical estimations, to the following values for precipitation, considering cooling of 15°C and 5°C:

$\delta^{18}O$ = 1.5‰ for precipitations at 15°C

$\delta^{18}O$ = 2.2‰ for precipitations at 5°C

These calculations do not agree with the mean isotopic composition of world precipitation ($\delta^{18}O$ = -4‰), proving that from an isotopic point of view evaporation and condensation are non-equilibrium processes (mainly determined by low humidity level, temperature, wind and degree of salinity). Precipitation occurs when humidity is 100%, so isotopic fractionation is mainly due to temperature. Oceanic vapour from subtropical areas moves towards the poles, becoming cooler and condensing in the form of precipitation, becoming depleted in heavier isotopes that concentrate in initial rainfall (Figure 4). Consequently, latitude is a factor discriminating oxygen isotopic fractionation. This situation is clear in Figure 5, where it can be seen that ^{18}O depletion in precipitation increases with latitude. Thus, equatorial water is richer in heavier isotopes than water at the poles with an intermediate situation of continuum according to latitude. However, in the same figure considerable deviations can be noted (e.g. on the east coast of South America or in the Atlantic Ocean between Mexico and Scandinavia), due to warm oceanic currents. In addition to the 'latitude' effect there is a 'continental' effect due to the distance from the sea, related to the vapour masses moving over continents (with the increasing distance from the primary source of vapour the $\delta^{18}O$ of precipitation decreases due to previous precipitation) causing precipitation along the coasts

which is isotopically richer than that in continental areas (mean depletion of -2.8‰/1000 km from the coast). Moreover, different altitudes inland lead to ^{18}O depletion of around - 0.15‰ to -0.5‰ per 100 meters of height, because at higher altitude there is lighter vapour. Finally, deviation is also due to seasonal trends; during the summer there is ^{18}O enrichment, especially inland.

Figure 4. Isotopic fractionation of vapor mass in relation to temperature (Clark and Fritz, 1997).

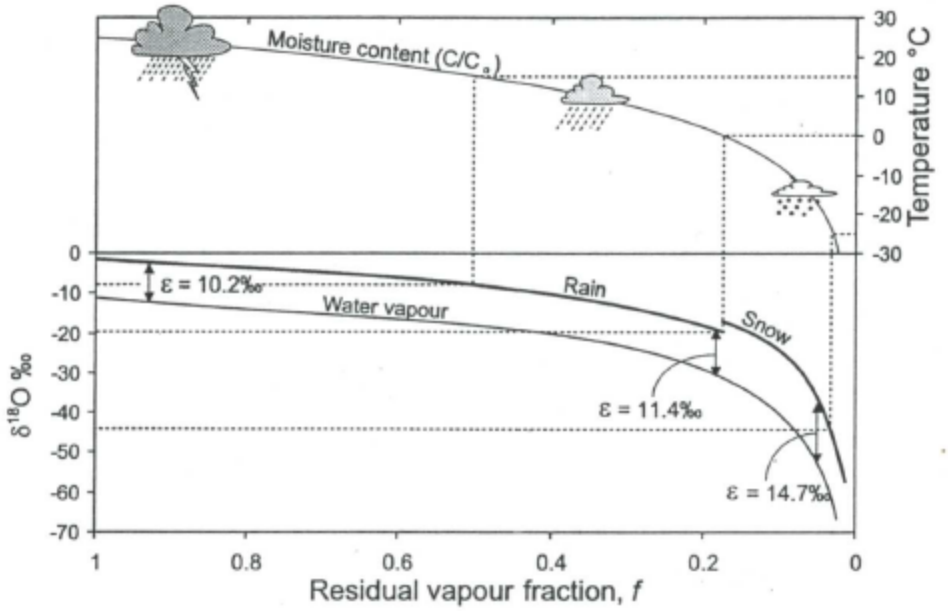
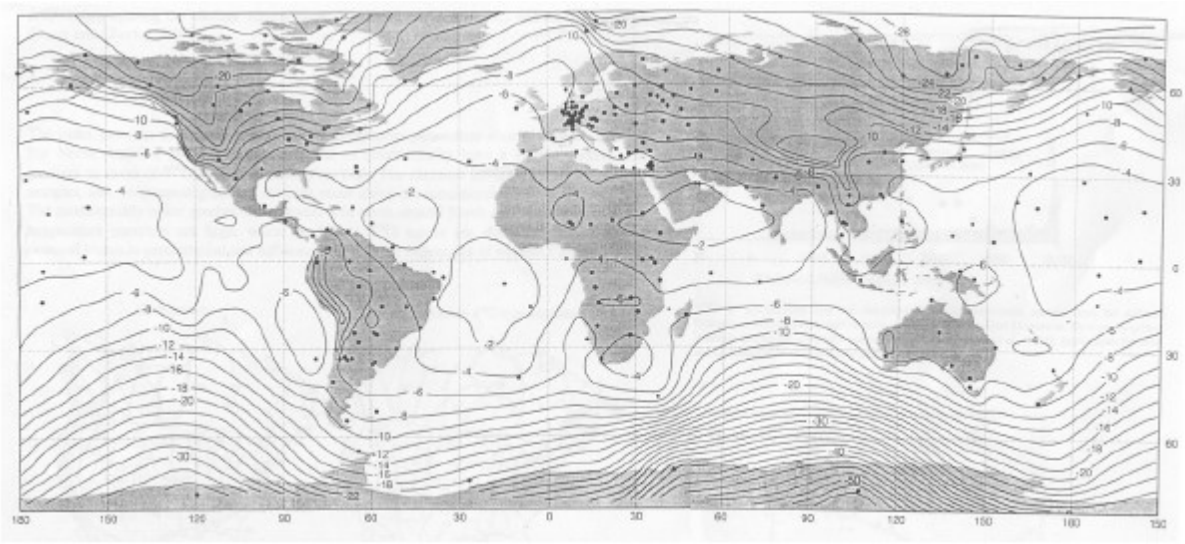


Figure 5. Distribution of mean $\delta^{18}O$ values for precipitation in 1992 and 1993 (Rozanski, 1993)



To summarize, the factors affecting $\delta^{18}O$ content in meteoric water are latitude, distance from the sea, altitude and seasonal trends. Ground water has an isotopic composition related to the mean annual isotopic composition of precipitation water and its $\delta^{18}O$ depends only on geographical factors (altitude, latitude, distance from the sea) but not on the season. In plants, the isotopic composition of plant water is related to the water absorbed from the soil, so it is affected by the factors mentioned above. Furthermore, leaf water is subjected to an isotopic fractionation during evapotranspiration processes which are affected by temperature and relative humidity and leading to an enrichment in heavier isotopes. Plant compounds derive their oxygen atoms from vegetal water but also from air CO_2 and O_2 with $\delta^{18}O$ values that are essentially constant and stand at around

+40.3/+42.5‰ and +23.5/+23.8‰ respectively. Oxygen integration in organic compounds through metabolic processes causes considerable isotopic fractionation. For example, the $\delta^{18}O$ of cellulose is correlated with the $\delta^{18}O$ of leaf water, with an enrichment of around 27‰ caused by the isotopic fractionations occurring during exchanges between the carbonylic group and water (Schmidt et al., 2001; Barbour, 2007).

2.3. Stable isotopes composition in food authentication

Stable isotope analysis, performed using Isotope Ratio Mass Spectrometry (IRMS) has taken on increasing importance in determining the authenticity of fruits, vegetable and food of animal origin for producers and food controls.

Olive oil (together with wine) is one of the most studied food items with regards to commercial frauds and adulteration. Angerosa et al. (1999) made measurements of $\delta^{13}C$ and $\delta^{18}O$ of the whole oil and some of its fractions in order to gain information about the geographical origin of olive oil produced in Greece, Morocco, Spain, Italy, Tunisia, and Turkey. By applying statistical procedures they demonstrated that oil samples show the trend to cluster according to the different climatic areas of growing environment of fruits. Some confusion were observed for samples coming from neighboring countries having similar climates. Kelly and Rhodes (2002) highlighted emergent techniques such as compound and position specific-isotope mass spectrometry. These latter developments offer the potential to provide more rapid and improved detection of the economic adulteration of vegetable oils.

In wine IRMS can be used both for origin assignment of samples and to detect adulteration. Gremaud et al. (2004) characterized Swiss vineyards and were able to distinguish five main production zones. They obtained this result by combining via a multivariate approach δ 18O variations with elemental (Mn, Al, B, Ba, Ca, Fe, Mg, Na, Rb, Sr, Zn) and FT-IR (ethanol, pH, total acidity, volatile acidity, malic acid, fructose, tartaric acid, lactic acid, succinate, citric acid, glycerol, 2,3-butandiol, dry matter and relative density) analyses. Not only carbon and oxygen ratios can be useful for wine authenticity certification, as a matter of fact Almeida and Vasconcelos (2001) set up an ICP-MS for 87Sr/86Sr determination and showed that the isotopic profile of strontium is a promising fingerprint of wine origin. Adulteration of wine with glycerol is considered to be a problem in European wine-producing countries. As there is little chance of being able to identify glycerol from different sources on the basis of a method which uses only one isotope, Rossmann et al. (1998) developed a multielement approach using NMR. Glycerol from wine showed the lowest relative enrichment with deuterium, was mainly in position C-2, and had a relatively high 18O content, together with very negative $\delta^{13}C$ values, which significantly correlated with those of

ethanol from the same wines. Isotopic data of glycerol samples from different sources were in agreement with those given by indices of origin. These data allowed identification of the origin of these glycerol samples, i.e. whether they were produced industrially or synthesized by animals or plants. Glycerol of plant origin was most similar to glycerol found in wine. The combination of several isotopic data by discriminant analysis yielded clusters of data obtained from glycerol samples of similar origin. Taking into account the characteristics of possible mixtures, proof that wine has been adulterated depended on the origin and isotope levels of the added compound. It was shown that it is possible to prove that wine has been adulterated with glycerol from other sources when the latter is present at a concentration of 15% of total glycerol content.

As proved by Brescia et al. (2001) IRMS can be a tool suitable to provide information about the geographical origin of durum wheat. As a matter of fact though the application of chemometrics to isotopic determinations they performed the discrimination of semolina by cultivar and geographical origin.

Due to the huge consumption and to the consequently large commercial movements the authenticity of orange juice is an issue too. Pupin et al. (1998) used isotopic analyses to determine the authenticity of Brazilian orange juice. The mean ratios found for these parameters in authentic hand-squeezed orange juice were as follows: $\delta^{13}C$ = -26.6‰; and $\delta^{18}O$ = +2.27‰. Simpkins et al. (2000) found similar values for Australian orange juices. The mean of their (273) samples was $\delta^{13}C$ -24.77 (min. -27.3‰, max. -22.5‰).

3. OLIVE OIL

3.1. Introduction

European and Italian law require that the origin of some premium products, such as virgin and extra virgin olive oils must be declared on the label. This is particularly important with the growth and promotion of "added value" regional foods such as those produced under "Designated Origin" labels. But at the moment no analytical methods exist that are capable of verifying the truthfulness of the assertions on labels so, to achieve this aim the EU is urging to investigate new markers able to support food characterization and geographical traceability assuring its genuine origin. World olive oil production currently stands at around 2.9 million tons (2009 data provided by FAO, http://faostat.fao.org/) and the olive oil industry employs about 2.7 million people, of whom approximately two million are 'family producers' living in the less economically favored regions of southern Europe (http://www.oliveoiltimes.com). Indeed European producers on the Mediterranean basin – Greece, Spain, Portugal, Italy and France – supply the bulk of overall production. Thus olive oil is a fundamental part of the farmers' income and represents as much as 15% of the gross agricultural domestic product in countries such as Greece (Luchetti, 2002). Extra virgin olive oil commands a high retail value (2 to 4 times that of other oils) because of its organoleptic qualities and the purported health benefits of consuming oils high in mono-saturated fatty acids (Gurr, 1999). These factors and the increasing demand for olive oil have encouraged many Mediterranean countries to invest in olive oil production. Consequently throughout Europe there is an enormous range of quality olive oils. However, when a product acquires a reputation extending beyond national borders it can find itself in competition with products which pass themselves off as the genuine article and take the same name. This unfair competition not only discourages producers but also misleads consumers. This is why the European Union created the PDO and PGI systems in 1992 to promote and protect foodstuffs of particular quality (Regulation 2081/92/EEC subsequently replaced with Regulation 510/2006/EEC). Furthermore in February 2009 labelling of origin for virgin and extra virgin olive oils became compulsory by European law (Regulation 182/2009/EC). Nevertheless, within the framework of limits prescribed by European regulations there is potential for mislabeling olive oil without risk of detection. Indeed, official olive oil quality control methods are based on maximum or minimum limits of certain chemical components, e.g. fatty acids, sterols, alcohols or stigmastadiene (Regulation 2568/91/EEC and amendments), and it is impossible to verify the real geographical origin of olive oil using these. This situation can influence consumers' perception of the benefits of consuming olive oil and the current image of uncontrolled provenance in the olive oil market poses a considerable risk to the opportunity for economic growth in many Mediterranean countries. In particular, Italy has the highest number of PDO and PGI registered

foodstuffs in Europe (N=206) and extra virgin olive oil is the second most important commodity, with forty recognized brands (Wesseler, 2010). So this commodity is very important for the Italian economy, being Italy the second largest producer in the world (588,000 tons in 2009, data provided by FAO, http://faostat.fao.org/) after Spain and the largest consumer (International Olive Oil Council, http://www.internationaloliveoil.org). This situation highlights the increasing demand for analytical methods and statistical tools capable of effectively verifying claims of origin.

3.2. Stable isotopes composition in olive oil authentication

Initial studies of the stable isotope ratios of olive oils focused on their use for detecting the adulteration of olive oil with cheaper oils. In particular, $^{13}C/^{12}C$ measured using an Elemental Analyzer – Isotopic Ratio Mass Spectrometer (EA-IRMS) or Gas Chromatography/Combustion/Isotopic Ratio Mass Spectrometer (GC/C/IRMS) in bulk olive oil or in some sub-components (individual fatty acids or aliphatic alcohols and sterols) has been shown to be useful for detecting the adulteration of olive oil with cheaper *Pomaceae* olive oil or with other vegetable oils (Angerosa et al., 1997; Spangenberg et al., 1998). Subsequently, stable isotope ratio analysis, in particular $^{13}C/^{12}C$ in combination with the $^{18}O/^{16}O$ of bulk oil, proved to be a good tool for characterizing geographical origin. Royer and co-workers (1999) studied the $^{13}C/^{12}C$ of palmitic, oleic and linoleic fatty acids of olive oils, observing differences between French and Italian olive oils as compared to Greek ones and obtaining a regional classification of Greek olive oils. Some authors (Bréas et al., 1998; Angerosa et al., 1999) found that both the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ of olive oils from Italy, Greece, Spain, Tunisia, Morocco and Turkey change according to latitude, suggesting distance from the sea and environmental conditions during the growing of plants (water stress, atmospheric moisture and temperature) as co-factors of variability. Finally, Aramendia et al. (2007) observed that the $^{18}O/^{16}O$ values of bulk olive oils were influenced by the variety of the olives and by their geographical origin, but not by the altitude, ripening degree and harvesting date of olives.

3.3. Fatty acids in olive oil

Fatty acids are simple structures made up of long chains of various numbers of carbon atoms, with a carboxylic acid group at one end. The main fatty acids present in olive oils are presented in Table 2.

Table 2. Percentage of the different Fatty Acids present in olive oil (Rev Esp Cardiol. 2009; 62:294).

Common Name (Numerical Symbol)	Percentage
Myristic (14:0)	0.0-0.05
Palmitic (16:0)	7.5-20
Palmitoleic (16:1n7)	0.3-3.5
Margaric (17:0)	0-0.3
Heptadecenoic (17:1)	0-0.3
Stearic (18:0)	0.5-5
Oleic (18:1n9)	55-83
Linoleic (18:2n6)	3.5-21
α-linolenic (18:3n3)	0-0.9
Arachidic (20:0)	0-0.6
Eicosenoic (20:1n9)	0-0.4
Behenic (22:0)	0-0.2
Lignoceric (24:0)	0-0.2

High amounts of monosaturated fatty acids are present in olive oils, which confer to them a high nutritional value. Moreover, oil characteristics are influenced by the proportions of fatty acids present. As it can be seen in Table 2, the studies on the composition of fatty acids in olive oils were usually performed by gas chromatography (GC) coupled with flame ionization detection (FID) (Di Bella et al., 2007; D'Imperio et al. 2007; Mannina et al., 2003). In most papers collected in Table 2, the use of chemometric tools was required to perform an olive oil differentiation according to the fatty acid composition. The fatty acids content was used by some researchers to differentiate olive varieties (Tsimidou et al., 1993; Alonso et al., 1993; D'Imperio et al.,2007). For example, D'Imperio et al. established a qualitative similarity among olive oils of different cultivars and showed that these varieties were grouped together by discriminant analysis. This fact revealed that the fatty acid composition of olive oils was strongly influenced by several factors such as cultivar, maturation stage of fruit, and zone of origin (Alonso et al. 1993). To relate the fatty acid composition of olive oils with the cultivar influence, Mannina et al. (2003) studied olive oil in a well-limited geographical region, neglecting the pedoclimatic factor (soil characteristics such as temperature and humidity) and finding a relationship between the fatty acid composition and some specific cultivars. However, Di Bella et al. (2007) maintained that although the effect of the cultivar was significant in an olive oil classification based on the fatty acid composition, a predominant and well-defined geographical effect was also present. It is important to consider that chemometric tools can be used not only to describe the characteristics of oils and classify them but also to select the best variables to obtain satisfactory results (Gurdeniz et al., 2009). For example, variability in fatty acid and triglyceride concentrations among olive oil samples was used by some authors (Olliver et

al., 2003; Aranda et al., 2004) applying chemometric methods. Moreover, in comparison with other markers, the fatty acid and triglyceride composition allowed a better differentiation of olive oils than the sterol composition (Brescia et al., 2003).

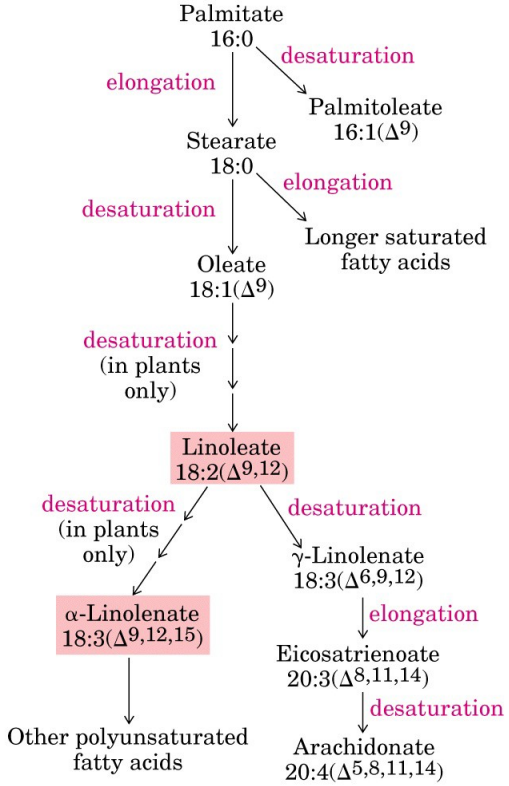
3.4. Metabolic pathway of main fatty acids in olive oil

Fatty acid biosynthesis involves the cyclic condensation of two-carbon units in which acetyl-CoA is the precursor. The enzymes of the pathway are thought to be held together in a complex that is collectively referred to as fatty acid synthase.

Some 16:0-ACP is released from the fatty acid synthase machinery, but most molecules that are elongated to 18:0-ACP are efficiently converted to 18:1- ACP by a desaturase enzyme. The repetition of this sequence of events makes 16:0-ACP and 18:1-ACP the major products of fatty acid synthesis in plastids. Fatty acids may undergo further modification after they are linked with glycerol to form glycerolipids. Additional double bonds are placed in the 16:0 and 18:1 fatty acids by a series of desaturase isozymes.

The methabolic pathway in the synthesis of fatty acids is shown in figure 4.

Figure 6. Metabolic pathways in the synthesis of fatty acids.



3.5. Fatty acid and isotopic composition in olive oil

Although the vast majority of plants (>300,000) belongs to the C3 group with $\delta^{13}C$ values of <−24‰, differences in enzyme kinetics of biochemical pathways (mainly caused by environmental

factors, such as climate and geographical location) result in subtle variations in the ^{13}C signature at natural abundance level of bioorganic compounds, such as fatty acids. The information locked into organic molecules due to these kinetic isotope effects provides valuable information on origin, authenticity and food webs, to name but a few, and can only be accessed by GC/C–IRMS techniques.

It has been established that the fatty acids, particularly oleic acid, are formed in the chloroplasts of leaves or proplastids of seeds (Stumpf, 1980). Acetyl co-enzyme A is the basic unit for fatty acid biosynthesis. While the olive oil accumulation occurs in the fruit, the precursors for its synthesis have to be imported from the leaves. The carbohydrates in the olive fruit pericarp evidently reflect the influx of metabolites from the leaves to the fruits and serve as a source for the acetyl needed for fatty acid biosynthesis, as demonstrated in castor bean seeds (Simcox et al., 1979).

The isotopic fractionations involved in the biosynthesis of lipids has been the subject of many investigations based on the IRMS determination of overall isotope ratios (Meinschein, et al.1974; De Niro, M.J. and S. Epstein, 1977; Monson and Hayes, 1980). It has been observed that ^{13}C contents of saponifiable lipids are lower than those of corresponding carbohydrates. Using appropriate degradations reactions it has been also shown that kinetic isotope effects occurring in the oxidation of pyruvate to acetyl-CoA by Pyruvate dehydrogenase are the source of enhanced ^{13}C depletion at the carbonyl site and that fatty acid synthesis may than introduce some alteration in ^{13}C distribution along the chain. It has been claimed that, in addition, kinetic isotope effects in the reaction leading to 18:2, causing ^{13}C to pass preferentially from 18:1 to 18:2, yielding a net depletion of carbon 13 at the carboxyl position in 18:1 lipid (Monson and Hayes 1981). Another isotopic effect is associated with the elongation pathway leading from 16:0 to 18:0 and may be responsible for the enrichment of ^{13}C in 18:0 (Monson and Hayes 1981).

Woodbury et al found the saturated 16:0 fatty acid in maize oil to be more depleted in ^{13}C than the unsaturated fatty acids (18:1 and 18:2) from the same oil. In addition, consistent differences were observed for $\delta^{13}C$ values of vegetable oils from the same plant species but from different geographical regions. For example, the major fatty acids in maize oil (16:0, 18:1 and 18:2) from Argentinean maize showed on average +2‰ higher $\delta^{13}C$ values than those from maize grown in Italy. In subsequent work, Woodbury et al. determined fatty acid composition and $\delta^{13}C$ values of the major fatty acids of more than 150 vegetable oils, thus establishing a database that provides isotopic information for authenticity control of vegetable oils. Variability in $\delta^{13}C$ values could be related to geographical origin, year of harvest and the particular variety of oil. Their findings suggest that ultimately $\delta^{13}C$ values of fatty acids are determined by a combination of environmental and genetic factors.

4. AIMS AND OUTLINE OF THE THESIS

4.1. Aims of the thesis

The general aim of this thesis was to verify the applicability of the analysis of multielement stable isotope ratios using IRMS (Isotopic Ratio Mass Spectrometry) in food authenticity. Especially, analytical approaches based on these techniques were developed for the traceability of different extra-virgin olive oils. The origin of this product must be declared by law, but to date no analytical methods exist that are capable of verifying the truthfulness of the assertions and description given on labels.

In detail the research focused on:

- characterization of Italian extra virgin olive oils: creation and validation of a dataset with isotopic values;
- Analysis of isotopic and fatty acid composition of Mediterranean monovarietal olive oils collected in the same experimental field: multivariate statistical analysis usable to verify the authenticity of compound specific cultivar;
- Study of the effect of ripening stage and climatic conditions on the isotopic and fatty acid composition of mono-varietal olive oils in two experimental fields.

4.2. Outline of the thesis

The first introductory chapter gives an overview of the question of food authenticity and the provisions of EU law. Furthermore, natural variation in the stable isotopes of bio-elements (C, O) is illustrated, with the potential implications for food authenticity control.

In Chapter 2 analysis of the stable isotope ratios of bioelements is described in general.

Chapter 3 is dedicated on olive oil describing stable isotopes and fatty acids compositions as powerfull markers in traceability studies.

In this chapter 4 the scope and the outline of the thesis are illustrated.

In Chapter 5 materials and methods used in this work are presented.

Subsequent chapter is devoted to three individual experimental works, presenting the results obtained during the PhD thesis.

Specifically, Chapter 6.1 focuses on the possibility of improving traceability of the geographical origin of extra virgin olive oils using carbon and oxygen isotope ratios. This analytical technique was applied to authentic Italian oils collected at nine regions for three different years of harvest. The isotope ratios of monovarietal olive oils collected in the same experimental field, combined with fatty acids composition, were investigated in Chapter 6.2 providing direct evidences of the relationship between the Fatty Acid (FA) and isotopic composition of olive oils and specific

cultivars grown within a well-limited geographical region. In particular the appropriate multivariate statistical analysis combining the used variables can be used as a tool for verifying the cultivar of origin. In Chapter 6.3 the isotopic and fatty acid composition of different olive oil cultivars grown in the same olive orchard at different maturation degree was studied to investigate the effect of the degree of ripeness of the olive on carbon and oxygen isotopic composition in relation with fatty acid composition.

5. MATHERIALS AND METHODS

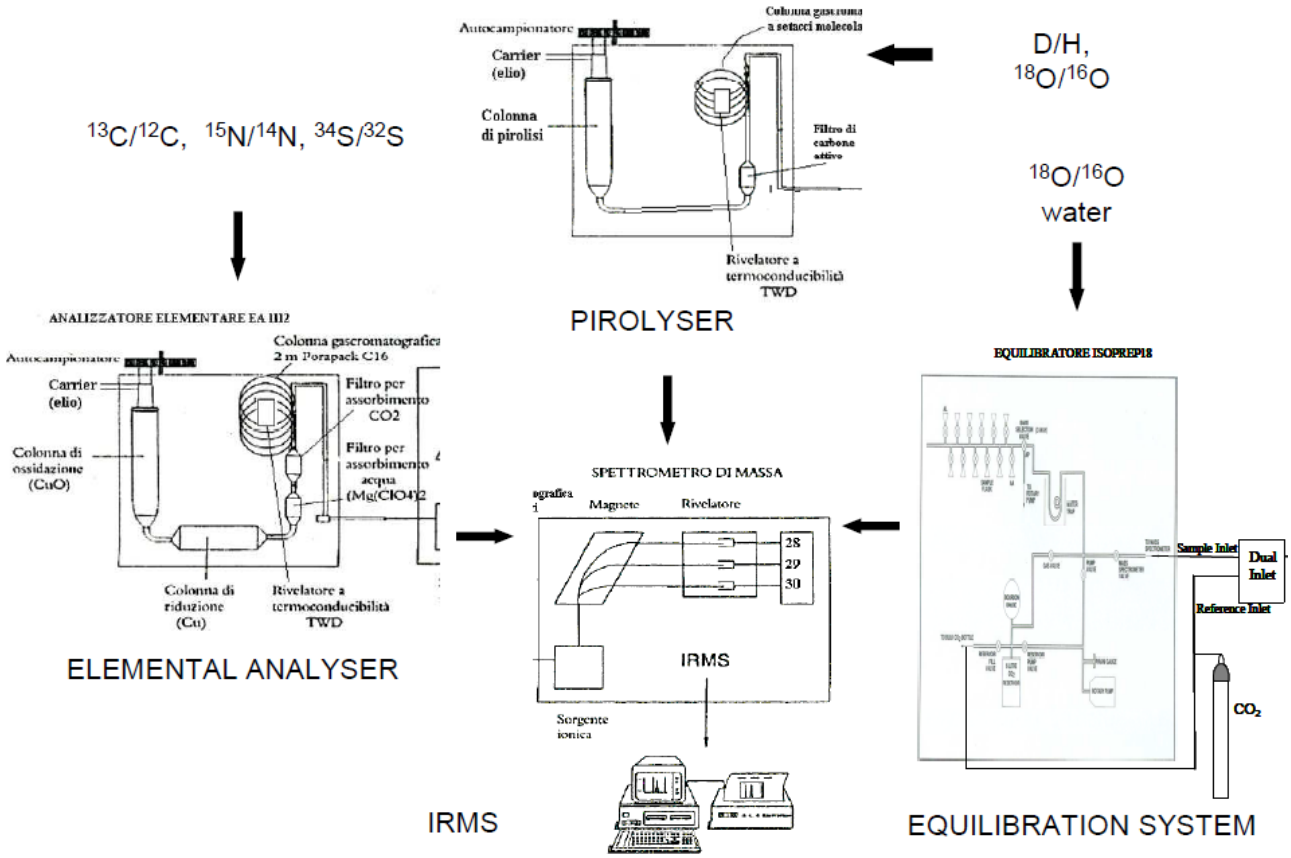
5.1. Preparation of the samples

Isotopic ratios were performed on bulk sample for determination of $\delta^{13}C$ and $\delta^{18}O$ in olive oil.
The detailed preparation procedures are described in the relative chapters.

5.2. Isotope ratio mass spectrometry method

For analysis of stable isotope ratios, the sample was weighed in tin or silver capsules. The quantities of sample weights ranged from around 0.3 mg to 1 mg depending on the commodity and the isotope ratio analyzed. More details on the methods are provided in the specific chapters.
Analysis was carried out using an IRMS (Isotope Ratio Mass Spectrometer, Isoprime, Cheadle, UK) equipped with an elemental analyzer (NA1500, Carlo Erba, Milan, Italy) for $\delta^{13}C$ and a pyrolyser (Euro Pyr-OH, Euro Vector Instruments & Software, Milan, Italy) for $\delta^{18}O$ measurement (Figure 7). In detail, for determination of $\delta^{13}C$ the sample was dropped into an oxidation reactor inside the elemental analyzer, where it combusted (1050°) with development of CO_2.
For measurement of $\delta^{18}O$ the sample was dropped into a high-temperature conversion/elemental analyzer where it was subjected to high temperature pyrolysis (1450°) with development of carbon monoxide gas. Then the weighed samples were put in the carousel of the auto-sampler equipped with a suitable cover.

Figure 7. IRMS diagram



The developed gases (CO_2, CO) were then separated in a GC column at 80°C and transferred from the elemental analyzer or pyroliser after the separation into the ion source of an IRMS by the gas carrier helium (100 mL/min). In each analytical batch, every ten samples, a working in-house standard was analyzed and used to calculate the isotope ratios values of the samples (see below). In IRMS the gases are ionized in the ionic source and ions pointed through a semicircular flight tube. In the flight tube they are subjected to a constant magnetic field and, depending on the acceleration potential applied, only ions with specific masses can hit the electron multiplier detector (Table 2).

Table 2. Masses measured using IRMS to determine stable isotope ratios

Isotope	Molecular specie determined	Mass
H	H_2	2
D	HD	3
^{12}C	$^{12}CO_2$	44
^{13}C	$^{13}CO_2$	45
^{14}N	$^{14}N_2$	28
^{15}N	$^{14}N^{15}N$	29
^{16}O	$C^{16}O$	28
^{18}O	$C^{18}O$	30

The isotope ratios were expressed as ‰ referred to the international standard V-PDB (Vienna—Pee Dee Belemnite) for $\delta^{13}C$ and V-SMOW (Vienna - Standard Mean Ocean Water) 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, and δ indicates the isotopic composition for C ($\delta^{13}C$) or O ($\delta^{18}O$). The standard deviation of replicate measurements of standards was 0.1‰ for $\delta^{13}C$ and 0.3‰ for $\delta^{18}O$ measurements.

6. RESULTS AND DISCUSSION

6.1. Carbon and Oxygen stable isotopes composition in olive oils: pedoclimatic traceability

The importance of assessing food authenticity is increasing due to expanding global markets. Additionally, fast and cheap transportation, as well as various subvention schemes within a growing EU, are stimulating the need for reliable monitoring systems on the food sector (Rossmann, 2001; Luikx, 2008). Besides conventional analysis, isotope ratio measurements have proved especially useful in this field (Rodrigues, Maia, Lauteri, Brugnoli & Máguas, 2013). Certain biological and environmental processes characteristically discriminate against light or heavy isotopes (Aramendia et al., 2007). Consequently, the stable isotope composition of different elements in biological products provides information about geographical and ecological origin (Bréas, Guillou, Reniero, Sada & Angerosa, 1998; Angerosa et al., 1999; Rossmann et al., 2000; Manca et al., 2001; Camin et al., 2004). In particular, isotope signatures can serve as indicators of geographical origin (Schwertl, Auerswald, Schäufele, & Schnyder, 2005; Rodrigues et al., 2009, Camin et al., 2010a,b). The most relevant advantage of stable isotope techniques, as applied to foodstuffs, is time-consuming pre-treatments are not usually required. Carbon isotope composition in plants varies systematically, largely determined by the photosynthetic pathways, (C3, C4 or CAM [crassulacean acid metabolism]), used by plants to fix atmospheric CO_2 in organic matter (Farquhar et al., 1989; O'Leary, 1995; Brugnoli & Farquhar, 2000). Olive trees belong to the C3 group, where carbon isotope composition ($\delta^{13}C$) ranges from about -22 to -33‰. The variation in $\delta^{13}C$ is also related to genetic determinants at the inter- and intra-specific level (Lauteri et al. 2004), although a number of environmental factors are known to affect isotopic fractionation, causing enrichment or depletion of certain isotopes during growth. Hence, within the same species, and within the same genotype, a wide variation in $\delta^{13}C$ is expected, especially with changing environmental conditions during the vegetative and reproductive (fruit setting and ripening) stages (Scartazza et al., 2004).

In details, the overall fractionation along the C3 assimilation path with respect to the air CO_2 isotopic composition (carbon isotope discrimination, $\delta^{13}C$) is linearly related to the ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a). Such a robust model (Farquhar, O'leary & Berry, 1982) explains how environmental or genetic factors driving the C_i/C_a ratio in a C3 plant is reflected in the levels of discrimination against the heavy isotope ^{13}C. Thus, any factors affecting the level of stomatal closure or the activity of carboxylating enzymes is expected to influence the isotopic composition of photo-assimilates and the composition of (plant) organic matter.

Many studies performed under controlled and field conditions have shown that plants grown in drought conditions are relatively enriched with $\delta^{13}C$ (O'Leary, 1993; Barbour et al. 2005). The

$^{18}O/^{16}O$ ratio in plant material reflects the (a) isotopic ratio of the water sources (linked to latitude, elevation, distance from the evaporation source, temperature, amount of precipitation) (Clark et al. 1997; Van der Veer et al. 2009), (b) isotopic effects during transpiration (affected by relative humidity, temperature, isotope composition of water vapor) (Hermann et al. 2008, Gavrichkova et al. 2011) and (c) biosynthetic pathways including isotopic exchange between organic molecules and plant water (Barbour 2007, Schmidt et al 2001). A variation in stomatal conductance affects the isotopic signal of leaf water and, hence, of photosynthetic products and related oil (Scheidegger, Saurer, Bahn, & Siegwolf, 2000). In fact, photosynthetic products (carbohydrates) are exported from the leaves to the fruits to fulfil the metabolic requirements for oil synthesis (Sànchez & Harwood, 2002). Moreover, a number of studies have shown that variation in $\delta^{18}O$ of leaf water is reflected in the organic matter of the fruit (Barbour, 2007).

The aim of this work was to elucidate the influence of environmental and geographical factors on the stable isotopes (C and O) to ascertain the geographical origin of virgin olive oil. With this objective in mind, isotope measurements were extended to a large number of Italian olive oil samples, originating from regions with different climatic conditions along a north-south transect. $\delta^{13}C$ and $\delta^{18}O$ were compared with climatic and environmental factors including mean annual temperature, amount of rain, humidity, elevation, and xerothermic index (Xi).

6.1.1. Samples

A total of 387 virgin olive oil samples produced in different Italian regions and from three different years of production (2009, 2010, 2011) were analyzed. The olive growing areas were differentiated by latitude, longitude, altitude and climatic conditions. Mean values for temperature, total rainfall and relative humidity for the different sites in 2009, 2010 and 2011 were provided by colleagues from the Institute of Atmospheric Sciences and Climate of CNR (CNR-ISAC). A xerothermic index (Xi) for each site was calculated using the formula (taking into account each growing season month): $Xi = \sum (2TM - P)$ if $2TM > P$ or $Xi = 0$ if $2TM \leq P$, where, TM is the monthly mean temperature and P is the monthly precipitation. Latitude and Longitude were taken from Google Earth; the farmers provided altitude, based on their local knowledge. The geographical origin of the samples, climatic data and the geographical parameters are shown in Table 3.

Table 3. Geographical and climatic characteristics of sites of origin of olive oil samples analyzed. Number of samples, geographical (latitude, longitude) and climatic data (mean annual temperature, amount of precipitations) of the provenience area. (Institute of Atmospheric Sciences and Climate of CNR-ISAC).

Region	N	Lat	Long	Altitudo	Mean annual temperature				Annual precipitation				Relative humidity				Xerothermic index			
		deg	deg	m a.s.l.	°C				mm				%							
		Mean	Mean	Mean	Mean	DS	Min	Max	Mean	DS	Min	Max	Mean	DS	Min	Max	Mean	DS	Min	Max
2009																				
Lombardia	2	45.6	10.4	310	9.9	0.3	9.5	10.1	1126.6	208.3	970.9	1394.3	69.3	1.9	67.9	70.6	8	11	0	16
Toscana	10	42.8	11.0	150	15.7	0.5	15.6	17.1	817.2	45.0	697.2	832.2	72.1	0.3	71.2	72.2	77	16	72	125
Lazio	33	41.8	12.9	376	14.7	0.5	13.4	14.9	930.8	58.6	883.3	1095.0	74.2	0.7	73.0	75.5	55	30	2	122
Molise	18	41.7	14.8	310	16.1	0.5	14.9	17.0	784.0	62.0	755.6	930.8	73.3	0.6	73.1	74.9	83	14	46	88
Puglia	32	41.2	16.5	130	16.7	0.7	15.0	17.0	736.8	65.5	693.5	887.0	70.2	2.5	68.5	74.2	117	32	48	132
Calabria	25	40.0	14.7	272	16.4	1.0	14.2	17.8	787.1	112.9	631.5	1043.9	73.5	1.0	71.3	74.9	121	34	46	178
Sicilia	24	37.4	13.5	214	17.1	0.3	16.5	17.3	636.0	8.7	624.2	646.1	71.1	0.3	70.7	71.7	166	16	150	189
Sardegna	7	40.6	8.5	250	16.8	0.2	16.2	16.9	544.4	11.1	507.4	547.5	73.5	0.7	72.4	73.9	159	2	158	162
2010																				
Lombardia	6	45.8	10.0	225	8.8	0.2	8.6	9.1	1328.6	184.0	1200.9	1565.9	71.4	1.6	70.1	73.4	0	0	0	0
Liguria	10	44.0	9.1	189	13.1	1.5	10.0	15.1	1143.5	177.8	865.1	1430.8	73.8	2.1	72.3	78.3	20	14	0	32
Toscana	9	42.8	11.1	100	15.1	0.5	14.9	16.4	960.4	53.5	817.6	978.2	73.6	0.5	72.2	73.8	45	19	39	96
Lazio	10	42.1	12.9	395	14.3	0.5	13.0	14.4	1064.7	24.5	1054.9	1131.5	76.1	1.4	73.4	79.1	37	13	10	59
Molise	10	40.6	15.1	397	15.7	0.5	14.4	16.8	707.7	124.2	649.7	985.5	73.6	1.2	73.0	76.1	50	13	44	89
Puglia	27	41.2	16.3	160	16.3	0.7	14.7	16.7	668.5	62.3	598.6	784.8	69.1	2.5	67.4	75.5	85	19	44	102
Calabria	25	40.0	14.7	281	16.2	1.1	13.8	17.6	877.6	132.9	649.7	1054.9	73.7	1.4	71.2	77.7	102	45	16	178
Sicilia	8	37.5	13.6	224	17.5	0.7	16.5	18.4	596.8	15.5	580.4	624.2	73.1	0.4	72.5	73.7	160	10	153	173
Sardegna	13	40.1	8.8	198	15.9	0.2	15.7	16.5	699.7	32.2	613.2	715.4	75.7	0.5	74.0	75.9	116	5	110	129
2011																				
Lombardia	15	45.8	10.0	250	10.1	0.3	9.9	10.5	884.5	151.6	693.5	1003.8	68.7	2.1	65.7	70.3	0	0	0	0
Liguria	11	44.7	9.6	216	13.6	1.0	10.8	14.0	829.5	89.5	693.5	1058.5	70.9	3.0	64.6	75.6	84	30	24	121
Toscana	15	42.9	11.1	174	15.6	0.4	15.5	17.0	566.0	28.4	474.5	573.1	71.1	0.1	70.6	71.1	68	0	68	68
Lazio	24	42.0	12.7	369	15.0	0.9	13.6	16.4	661.6	77.3	584.0	839.5	73.1	1.5	69.7	76.4	80	32	38	136
Molise	10	41.8	14.7	397	15.7	1.0	14.3	16.5	627.8	109.9	547.5	803.0	73.6	1.6	72.6	76.4	76	34	32	102
Puglia	13	41.8	15.7	82	17.1	0.4	16.5	17.4	491.3	32.0	474.5	547.5	69.0	2.1	67.9	72.6	119	9	102	123
Sicilia	12	37.6	13.3	248	17.6	0.6	17.1	18.3	517.1	37.6	474.5	547.5	70.2	0.5	69.8	70.7	164	30	140	199
Sardegna	14	40.1	8.8	217	16.9	0.3	16.8	18.0	525.0	32.7	463.6	562.1	72.7	0.5	72.3	73.3	159	18	138	184

6.1.2. Statistical treatment of data

For isotopic data, mean values, standard deviation, minimum, maximum and the confidence interval (95%) were calculated. Pearson coefficients were calculated for isotope compositions against other climatic and geographical parameters. These values were compared with the critical value (F) for a confidence level of 95%. In the case of a significant correlation, the equation for the isotopic values and climatic factors was determined. Two-way ANOVA, applying Fisher's test, was performed to determine if the distribution of the isotope parameters for the 2009, 2010 and 2011 was specific to the year of production. The data were statistically evaluated using Statistica 8 (StatSoft Italia s.r.l, Padova, Italy).

6.1.3. Results and Discussions

The mean $\delta^{13}C$ and $\delta^{18}O$ values for olive oil samples, along with the standard error, minimum and maximum, and confidence interval (CI) are shown in Table 4.

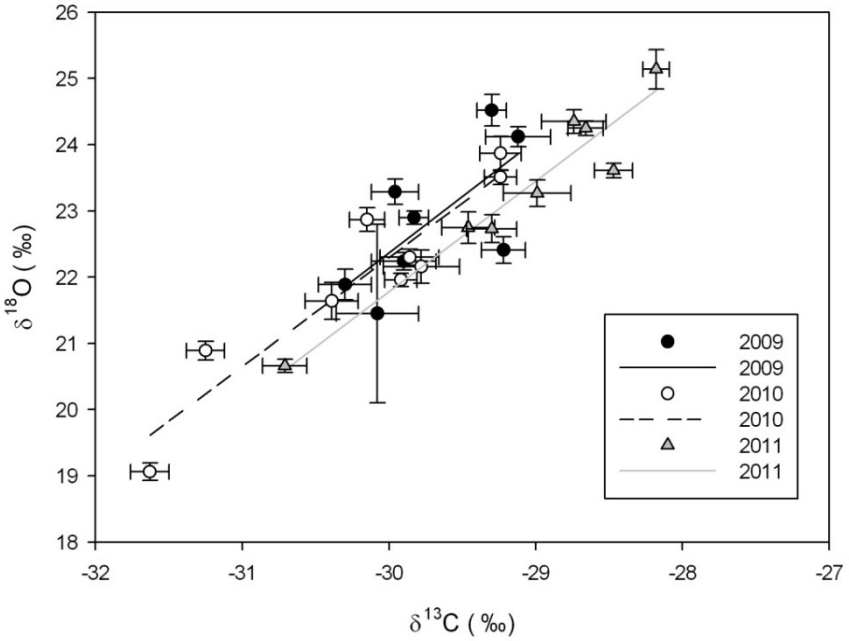
Table 4. Mean, Standard Error (SE), minimum and maximum values and 95% mean confidence interval (CI) of the $\delta^{13}C$ and $\delta^{18}O$ values for each region of oil origin, in different years production.

			$\delta^{13}C$					$\delta^{18}O$				
Regione	**N**	**year**	**Mean**	**SE**	**min**	**max**	**CI**	**Mean**	**SE**	**min**	**max**	**CI**
Lombardia	2	2009	-30.1	0.3	-30.4	-29.8	-	21.5	1.4	20.1	22.8	-
Toscana	10	2009	-29.2	0.2	-30.0	-28.4	0.3	22.4	0.2	21.4	23.4	0.5
Lazio	31	2009	-29.8	0.1	-31.0	-28.5	0.2	22.9	0.1	21.7	24.2	0.2
Molise	18	2009	-29.9	0.2	-31.1	-28.0	0.5	22.2	0.1	21.0	22.9	0.3
Puglia	13	2009	-30.3	0.2	-31.3	-29.0	0.4	21.9	0.2	20.7	23.9	0.5
Calabria	24	2009	-30.0	0.2	-31.3	-28.9	0.3	23.3	0.2	20.9	24.9	0.4
Sardegna	6	2009	-29.1	0.2	-29.9	-28.4	0.6	24.1	0.2	23.5	24.5	0.4
Sicilia	23	2009	-29.3	0.1	-30.2	-28.5	0.2	24.5	0.2	21.1	26.3	0.5
Lombardia	6	2010	-31.6	0.1	-32.2	-31.3	0.3	19.1	0.1	18.7	19.5	0.3
Liguria	10	2010	-31.3	0.1	-31.6	-30.2	0.3	20.9	0.1	20.3	21.4	0.3
Toscana	9	2010	-29.9	0.2	-30.9	-29.1	0.5	22.3	0.1	21.7	22.8	0.3
Lazio	10	2010	-29.8	0.3	-31.4	-28.4	0.6	22.2	0.3	20.5	23.2	0.6
Molise	10	2010	-30.4	0.2	-30.9	-29.0	0.4	21.6	0.3	20.9	23.8	0.6
Puglia	30	2010	-29.9	0.1	-31.1	-28.8	0.2	22.0	0.1	20.8	23.2	0.2
Calabria	25	2010	-30.2	0.2	-31.2	-28.9	0.3	22.9	0.2	20.9	25.5	0.4
Sardegna	13	2010	-29.2	0.1	-29.9	-28.3	0.2	23.5	0.1	22.7	24.2	0.3
Sicilia	8	2010	-29.2	0.1	-30.0	-28.8	0.3	23.9	0.3	22.9	24.9	0.6
Lombardia	15	2011	-30.7	0.2	-31.6	-29.8	-0.3	20.7	0.1	20.0	21.1	-0.2
Liguria	11	2011	-29.5	0.2	-30.3	-28.5	-0.4	22.8	0.2	22.1	24.4	-0.5
Toscana	15	2011	-28.5	0.1	-29.4	-27.7	0.3	23.6	0.1	22.9	24.7	0.2
Lazio	24	2011	-28.7	0.1	-29.9	-27.8	-0.2	24.3	0.1	23.3	25.3	-0.2
Molise	10	2011	-29.3	0.1	-29.9	-28.2	-0.4	22.7	0.2	21.3	23.4	-0.5
Puglia	13	2011	-29.0	0.2	-30.5	-27.8	0.5	23.3	0.2	21.7	24.5	0.4
Sardegna	14	2011	-28.7	0.2	-30.2	-27.2	-0.5	24.4	0.2	23.3	25.2	-0.4
Sicilia	12	2011	-28.2	0.1	-28.8	-27.5	-0.2	25.1	0.3	24.2	27.9	-0.7

The range of variation in $\delta^{13}C$ mean was about 3.5‰, (-31.6‰ to -28.2‰). A wider range was found for ^{18}O mean = 6‰, ranging from 19.1‰ to 25.1‰).

Correlation of $\delta^{18}O$ vs $\delta^{13}C$ for means from olive oil samples, collected in different regions during 2009, 2010 and 2011, are plotted in Figure 8.

Fig. 8. Relationships between the $\delta^{13}C$ and $\delta^{18}O$ mean values of olive oil samples collected in different regions during the years 2009, 2010 and 2011. Error bars indicate S.E.



Correlation coefficients (r) between $\delta^{18}O$ and $\delta^{13}C$ were 0.70 for 2009, 0.90 for 2010 and 0.96 for 2011. The relatively low r obtained in 2009 is probably due to the limited number of samples available from Northern Italy (Table 4). Nevertheless, the correlation was always significant for each year. Therefore, one or more environmental and/or geographical factors influenced both the isotopic variables.

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 olives growing in drought prone sites (southern regions) can be due to both environmental factors (such as temperature and precipitation) and plant-related parameters (such as stomatal conductance) and highlights a strong genotype x environment interaction. In fact Italian oils from northern regions had significantly lower isotope ratios than those from southern regions, due to higher precipitation and lower temperatures (Table 3).

The markedly dry Mediterranean climate in Sicily caused $\delta^{18}O$ values up to 27.9‰ in 2011 as well as relatively high $\delta^{13}C$ values (up to -27.5‰), indicating the effect of drought and related

physiological responses by olive plants. Data for oils from Lombardia, a region with significantly lower average temperatures and higher average precipitation, particularly in 2010, produced (as expected) the lowest isotope compositions for both elements (minimum 18.7‰ for $\delta^{18}O$ and -32.2‰ for $\delta^{13}C$).

The climatic influence of harvest year on stable isotope ratios in olive oil is shown in Table 5.

Table 5. ANOVA and Post Hoc Fisher test results, comparing the isotopic parameters $\delta^{13}C$ and $\delta^{18}O$ in olive oil for the three different years. Number of samples for different years and p values. Values marked in bold indicate significant differences.

	Number of samples		$\delta^{13}C$	$\delta^{18}O$
Region	**2009**	**2010**	**p**	**p**
Lombardia	2	6	**0.003**	**0**
Toscana	10	10	**0.025**	0.753
Lazio	33	9	0.808	**0.007**
Molise	18	10	**0.045**	**0.046**
Puglia	32	30	0.201	0.818
Calabria	25	25	0.288	0.055
Sardegna	7	13	0.729	0.093
Sicilia	24	8	0.807	0.023
	2010	**2011**		
Lombardia	6	15	**0.002**	**0**
Liguria	10	11	**0**	**0**
Toscana	10	15	**0**	**0**
Lazio	9	24	**0**	**0**
Molise	10	10	**0**	**0.001**
Puglia	30	13	**0**	**0**
Sardegna	13	14	**0.033**	**0.002**
Sicilia	8	12	**0**	**0**
	2009	**2011**		
Lombardia	2	15	0.177	0.134
Toscana	10	15	**0.003**	**0**
Lazio	33	24	**0**	**0**
Molise	18	10	**0.013**	0.075
Puglia	32	13	**0**	**0**
Sardegna	7	14	0.154	0.452
Sicilia	24	12	**0**	**0.024**

These data show of the variation in stable isotope compositions for both carbon and oxygen in extra-virgin olive oil. The inter-annual variability of stable isotope fractionation reflects the variation in annual meteorological conditions, such as precipitation and temperature during the ripening. This was confirmed by meteorological data (Table 1); the maximum value for precipitation and the minimum of temperature was observed in 2010, calculated across the whole sampling network. The lowest $\delta^{18}O$ and $\delta^{13}C$ mean values (22.0‰ and -30.2‰, respectively) were

observed in 2010. In contrast, the $\delta^{18}O$ and $\delta^{13}C$ mean values for oils in 2011, a year characterized by low precipitations and high temperatures, were as high as 23.4‰ and -29.1‰ respectively. The relatively low precipitation and high temperature in 2011 were associated with greater enrichment of $\delta^{18}O$ and $\delta^{13}C$ values, except for two samples of Lombardia 2009, likely owing to a peculiarity in the local climatic conditions around Lake Garda. Therefore, the annual variation of isotopic values was more or less pronounced, depending on the meteorological conditions, affecting the isotopic fractionations during the photosynthesis and transpiration.

Values for $\delta^{18}O$ and $\delta^{13}C$ in the oils were compared with environmental and geographical parameters, and are reported in Table 6.

Table 6. Correlation between $\delta^{13}C$, $\delta^{18}O$ and the geographical and climatic parameters of latitude, longitude, altitude, mean annual temperature, amount of precipitation, relative humidity and xerotermic index of all three years (Pearson coefficient, r, and significance, p).

	$\delta^{13}C$		$\delta^{18}o$	
	r	p	r	P
$\delta^{13}C$	**-**	**-**		
$\delta^{18}O$	**0.88**	**0**	**-**	**-**
Latitude	**-0.50**	**0.010**	**-0.74**	**0**
Longitude	**-0.028**	0.895	0.03	0.872
Altitude	-0.04	0.876	0	0.995
Temperature, °C	**0.67**	**0.003**	**0.79**	**0**
Rainfall, mm	**-0.80**	**0**	**-0.80**	**0**
RH %	0.00	0.982	0.14	0.5
Xi	**0.67**	0.000	**0.84**	0.000

$\delta^{18}O$ are highly correlated with latitude (negative r), mean annual temperature (positive r) and annual precipitation (negative r). No correlation was found between $\delta^{18}O$ and elevation, longitude and relative humidity. The $\delta^{13}C$ was significantly correlated with latitude (negative r), longitude (positive r), mean annual temperature (positive r) and the annual amount of precipitation (negative r). No correlations were found between $\delta^{13}C$ and elevation or relative humidity.

The oxygen isotopic composition of plant material is dependent on both the ^{18}O composition of the source water taken up by the plants and on enrichment of ^{18}O of leaf water arising from evaporation. Photosynthetic products are in isotopic equilibrium with the water in which they are formed, and should, therefore, reflect leaf water enrichment (Barbour, 2007). The isotopic signature of the source water is strictly dependent on ^{18}O composition of local precipitation, which is affected by the temperature of droplet formation (the 'temperature effect') and by the amount of precipitation (the 'amount effect'). More specifically, the ^{18}O content of precipitation decreases with the condensation temperature and the amount of precipitation. Hence areas with high air temperatures and low rainfall are, generally, characterized by heavier precipitation. This could, in

part, explained the strong relationships observed between olive oil $\delta^{18}O$ from different Italian regions and environmental parameters, such as the mean annual temperature and the amount of precipitation (Table 6).

However, low rainfall and high air temperatures are typical of dry sites and are expected to cause partial stomatal closure. This induces an increase in leaf temperature, related to an increase of water-vapor pressure in the intercellular spaces, which is reflected in further ^{18}O enrichment of leaf water during transpiration (Barbour et al. 2005). Hence, both latitudinal variation in precipitation $\delta^{18}O$ and the higher ^{18}O enrichment at dryer sites, could explain the strong positive relationship between olive oils $\delta^{18}O$ and xerothermic indices. Moreover, a reduced stomatal conductance in dry environments causes a decrease in the ratio of intercellular and atmospheric concentration of CO_2, inducing decreased leaf carbon isotope discrimination. This should be reflected in higher values of $\delta^{13}C$ in olive oils genotypes growing in dry environments compared with those in wetter areas.

Linear regression was used to analyze isotope variation against latitude, mean annual temperature, annual precipitation and xerothermic index, in order to obtain predictive equations for both $\delta^{13}C$ and $\delta^{18}O$ (Table 7).

Table 7. Regression coefficients, slopes and intercepts of the isotope variables and the climatic and geographical parameters best correlated with $\delta^{13}C$ and $\delta^{18}O$ values.

	$\delta^{13}C$			$\delta^{18}O$		
	r^2	Slope	Intercept	r^2	Slope	Intercept
Latitude	0.25	-0.17	-22.40	0.54	-0.43	40.52
Temperature	0.45	0.22	-33.01	0.63	0.44	16.13
Rainfall, mm	0.64	-0.003	-27.32	0.64	-0.005	26.63
Xerothermic index	0.39	0.010	30.53	0.71	0.022	20.81

The equation “Temperature- $\delta^{18}O$” shows that changes of 1°C in mean annual temperature corresponded to 0,4‰ $\delta^{18}O$. This shift is similar to that calculated by Iacumin et al (2009). The slope obtained is twice that for $\delta^{13}C$, indicating the degree of oxygen isotopic enrichment caused by temperature variation is much stronger. The equation “Rainfall- isotopes” shows changes of 100 mm of rain per year was associated with -0.5‰ $\delta^{18}O$ and -0,3‰ $\delta^{13}C$ in the olive oils selected. Increasing isotope compositions of olive oil with decreasing precipitations clearly reflects the significant effect of drought and warm seasons. The oils originating from drought prone areas were significantly more positive for both $\delta^{13}C$ and $\delta^{18}O$, compared with those from more mesic regions. This observation is supported by the significant, positive relationship between $\delta^{18}O$ and xerothermic index obtained from climatic data of native areas of the plants.

6.1.4. Conclusions

Stable isotope ratios of carbon and oxygen can be used for authentication and traceability of olive oil. Indeed, stable isotope fractionations of carbon and oxygen integrate both biotic and abiotic factors, producing isotope patterns capable of characterizing geographic origin, year of harvest, and meteorological conditions. The plot of $\delta^{13}C$ versus $\delta^{18}O$ mean values for the oils indicated a strong correlation, driven by variations in latitude, temperature, rain and xerothermic index. No correlation was found between the isotopic compositions of olive oils and elevation, longitude and atmospheric relative humidity. Significant differences were found harvest year. In particular, the $\delta^{18}O$ and $\delta^{13}C$ values for 2011 were generally higher than in other years. Moreover the environmental conditions could not be considered the only drivers affecting oil isotopic variability; further investigations are necessary especially with respect to possible effects of ripening and genotype on isotopic fractionation in olive oil.

6.2. Isotopic and fatty acid composition of Mediterranean monovarietal olive oils

Olive oil, a major constituent of the Mediterranean diet, has received prominent attention during the last decades due to the protective effect that its constituents exhibit against different types of cancer and cardiovascular diseases [Owen et al. 2004]. Given its production cost, olive oil is more expensive than other types of vegetable oils and is thus a target for adulteration and market fraud. The need to determine the origin of an olive oil has become necessary after the introduction of the "Protected Designation of Origin" (PDO) concept to olive oils [European Commission No. 1187/2000, Off. J. Eur. Union L133 (2000) 21]. Classification of an olive oil as a PDO product involves a thorough knowledge of the physical and chemical characteristics as well as properly defined cultivar names [E. Stefanoudaki, F. Kotsifaki, A. Koutsaftakis, (1997)].

The verification of the cultivar employed to produce an olive oil sample may contribute to certify the oil origin. Actually, the olive cultivar or variety clearly affects the oil composition in fatty acids, phenolics, and volatile fraction, as well as oil color (Angerosa et al. 2004). Olive cultivars differ in seasonal crop load, fruit size, flesh/pit ratio, and ripening pattern, as well as in their adaptive response to water stress or high temperatures during fruit growth and ripening. The selection of olive varieties has been largely driven by its adaptation to climatic conditions and soils. Such differences could provide chemical and physiological markers for cultivar identification. Indeed, the present situation of cultivate olive cultivars is very complex. More than 1,200 cultivars are cultivated worldwide, but 3 cultivars cover 63% of Spanish production, 24 cultivars account for 58% of the olive-cultivated area in Italy, 3 cultivars cover more than 90% of the olive area in Greece and Portugal, and a single cultivar represents 97% of the cultivated area in Morocco. Many cultivars have only a very local diffusion (Inglese and Famiani 2008). In addition, whereas some cultivars are characteristic of a given zone, others can be found in several countries (Japòn-Luján et al. 2006). As a consequence, one olive variety can be cultivated in distinct geographical locations with different names (several synonyms), making the differentiation of olive varieties in olive oils quite complex. Traditionally, the identification of olive cultivars has been supported by numerous morphological and pomological (related to the fruit) traits. In the last decade the progress in DNA fingerprint techniques has provided reliable genetic markers for the identification of olive cultivars and for solving cases of cultivar misnaming (omonimy and synonimy) [Muzzalupo et al. 2009]. On-line databases have been set up, which provide agronomic description and DNA profile of the main olive cultivars, such as OleaDB [www.oleadb.it, Bartolini 2014, DOI: 10.7349/OLEA_databases] and Certolio [www.certolio.org];

Nowadays, different efforts have been focused on the investigation of one or several compounds present in olive oils with capability to differentiate olive varieties. In the past 20 years, several

studies have addressed the systematic analysis of components of extra virgin olive oils and on their relationship with the geographical origin and the olive cultivar (Mannina et al. 2001). It was shown that the choice of suitable components of olive oils allows the observation of two well-separable major effects, namely the pedoclimatic effect and the cultivar effect (Mannina et al. 1999). Even limited to fatty acids, the compositional analysis can provide a very useful means for olive oil characterization (Alonso et al. 1993). Unfortunately there are potential adulterants, such as genetically modified sunflower oil, with a fatty acid composition similar to olive oils, and in these cases other techniques are needed for authentication.

Stable isotope analysis of fatty acids (FA) was developed in the 1970s to study the pathways of lipid biosynthesis (Meinschein, et al.1974; De Niro, M.J., and S. Epstein, 1977; Monson and . Hayes, 1980) but the isotope ratio mass spectrometry (IRMS) technique has been applied only recently to the authentication of olive oils. Few studies demonstrated the existence of a varietal effect on isotopic composition of bulk olive oils. Some authors (Aramendia et al. 2007, Jacumin et. al 2009; Royer, Gerard, Nautlet, Less, & Martin, 1999) studied the $^{13}C/^{12}C$ ratio of the palmitic, oleic, and linoleic fatty acids of olive oils, observing differences between French and Italian olive oils as compared to Greek ones and achieved regional classification of the Greek olive oils.

Although FA composition of olive oil has been studied extensively (Maria and Out, 2003, Salvador et al., 2001 and Shela et al., 2003), until now, no direct evidence had been found relating the fatty acid and isotopic composition of olive oils to the cultivar within a well limited geographical region. In the present study we investigate the cultivar effect on the fatty acid and isotopic composition, neglecting the pedoclimatic factor. The study was conducted on monovarietal olive oils obtained from 15 cultivars grown in the same experimental field, in Umbria region (Perugia, Italy). IRMS and GC techniques, in conjunction with a multivariate statistical have been applied. The relationship between the isotopic compositions and FA content of the selected olive oils are determined and discussed.

6.2.1. Material and methods

Monovarietal olive oils of cultivars Leccino, Frantoio, Moraiolo, San Felice, Carolea, Ottobratica, Coratina, Ogliarola, Casaliva, Nocellara, Arbequina, Manzanilla, Picholine, P. Marochaine and Kalamon, were harvested and analyzed in 2012. All the cultivars were grown in the germplasm collection of the Department of Agricultural and Environmental Sciences of the University of Perugia in the Umbria region (Central Italy; http:// www.oleadb.it/collections/cultivar_coll_list.php?mastertable= collections&masterkey1=027) (43°04′54.58″N, 12°22′53.41″E). The collection is located on a hill at 320 m a.s.l.. The trees were 20 years old, trained according to the vase training system and spaced m 5 × 5. The soil was

managed with a spontaneous green cover mowed 2−3 times/year. The olives were hand-piked at random from the crown of 2-4 trees per cultivar and oil samples were extracted by micromill from batches of 1-2 kg of olives, corresponding to a single tree.

Fruit Characteristics. The following fruit characteristics were determined: pigmentation (50 drupes/tree) using the Ripening Index (Jaèn pigmentation index), ranging from 0 to 7, with 0 for green olives and 7 for olives with superficial pigmentation on 100% of the epicarp and 100% pigmentation on the pulp; oil content (1 sample/tree) using the Foss-let 1531 apparatus (Foss Electric, Hilleröd, Denmark) on fresh and dry weight (by drying the samples at 105°C).

NIR Spectra Collection. A Luminar 5030 miniature Hand-held NIR Analyzer (Brimrose Corporation, Baltimore, 92 MD), based on the AOTF-NIR principle, was used for spectral detection This is a portable device that can be used directly in the field on-tree, even though in our study the spectral detection was conducted under laboratory conditions. Two different measurements were performed on each intact olive through contact between the external gun of the NIR device and the epicarp of the fruit, using the diffuse reflectance detection method. The raw spectra were detected and recorded in transmittance, as reported by other authors. Detection was conducted in the 1100−2300 nm range, with 2 nm wavelength increments and 10 spectra per average, which represented a single measurement. The average of the two measurements was recorded as the spectral response of the fruit.

Gas Chromatographic Analysis. The fatty acid composition was released as methyl ester by the official GU of the CEE methylation procedure (European Community Regulation, 1991) and analyzed by gas chromatography (GLC). A Shimadzu GC 17A (Milano, Italy) instrument, equipped with a split/splitless injector (split ratio 70:1) and flame ionization detector, was used. A Mega 10 fused silica capillary column, 50 m 0.32 mm i.d., 0.25 ím film thickness (Legnano, Milano, Italy), was employed. The following chromatographic conditions were used: column temperature, 170 °C; injector and detector temperatures, 250 and 280 °C, respectively; carrier gas, hydrogen, linear velocity) 50 cm/sec. Peak areas of 13 fatty acids (C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C24:0) were calculated using a HP 3394A integrator.

6.2.2. Statistical analysis

Statistical analysis were carried out using Statistica.8 software. One-way analysis of-variance (ANOVA) and post-hoc Fisher comparison test were performed on both isotopic and the fatty acid composition data. The ANOVA and Fisher tests were performed in test whether the isotopic and fatty acid composition were correlated with the plant cultivar and ripening degree. For multivariate

statistical analysis, three different procedures were used: Hierarchical cluster analysis, principal component analysis and linear discriminant analysis. Hierarchical cluster analysis (HCA), a method of unsupervised learning of a dataset partitioning into classes or categories, consisting of elements of comparable similarity. Tree Clustering Analysis (TCA). TCA allows to classify samples without any a priori hypothesis. Different amalgamation rules can be used to determine when two clusters have to be joined together. In particular, in this work the complete-linkage (GC data) and the unweighted pair-group average (NMR data) methods have been used. Principal component analysis (PCA), making an a priori unspecified number of clusters, allows us to obtain linear combinations of the selected variables which capture their "essence" and maximize the variability among groups.
Linear Discriminant Analysis (LDA). LDA allows us to classify samples with the a priori hypothesis, that is, the number of groups suggested by TCA, and to find the variables with the highest discriminant power. This analysis is used to determine whether the model (with all variables) leads to significant differences between the a priori defined groups and which variables have significantly different means across the groups. The selected variables are submitted to linear combinations to give rise to discriminant canonical functions, whose number is equal to the number of groups minus one: the first function provides the most overall discrimination between groups, the second provides the second-most, and so on. The discriminant power of the variables is evaluated using Wilks' ì, F, and p-level parameters. The Wilks' λ is computed as the ratio of the determinant of within-group variance/covariance matrix to the determinant of the total variance/ covariance matrix: its value ranges from 1.0 (no discriminatory power) to 0.0 (perfect discriminatory power).
We aimed at creating a classification of samples with the same characteristics of cultivar and ripening stages. Furthermore, multivariate regression analysis was performed on oil sample data, in order to obtain indication about correlations between isotopic and fatty acid composition.

6.2.3. Results and discussion

Table 8. Fruit Characteristics of the Olive Cultivars Considered: Colonna n = number of samples, Jean Index (JI), oil content on fresh (fw) and dry (dw) weight and production efficiency

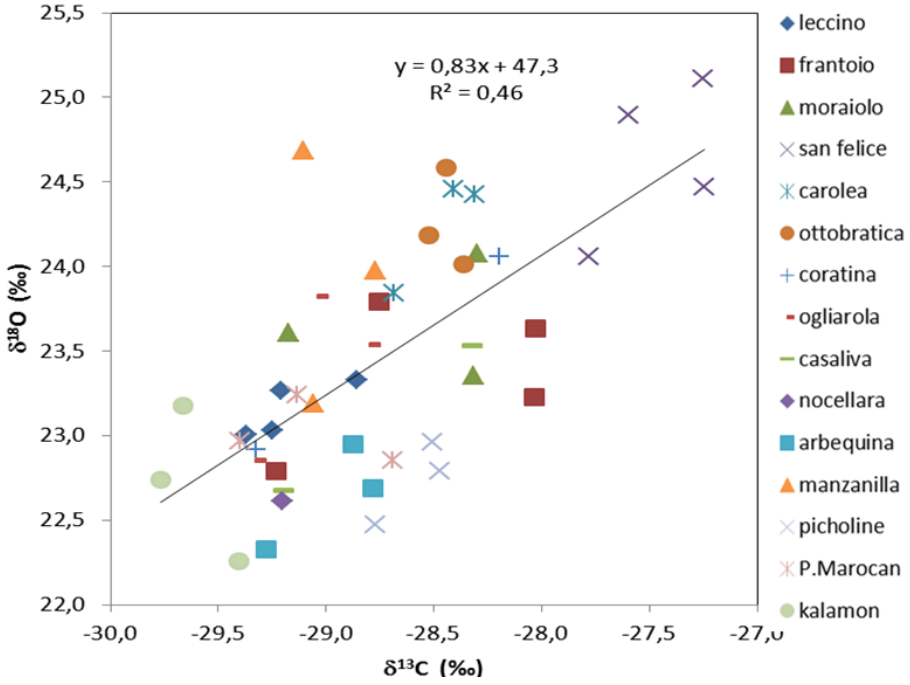
Cultivar	Origin	N	JI	Oil content (%/fw)	Oil content (%/dw)	Production efficiency (kg/m^3)
Leccino	Umbria	4	3.4	16.2	37.1	0.5
Frantoio	Umbria	4	1.1	15.1	31.2	0.6
Moraiolo	Umbria	3	3.1	18.6	38.8	1.7
San Felice	Umbria	4	1.1	17.5	39.2	2.2
Carolea	Calabria	3	0.9	21.9	44.6	1.0
Ottobratica	Calabria	3	2.2	23.2	51.3	0.4
Coratina	Puglia	2	0.7	16.2	32.9	0.3
Ogliarola	Puglia	3	2.2	17.6	37.6	0.5
Casaliva	Lombardia	2	1.7	25.2	49.0	0.2
Nocellara	Sicilia	1	2.9	12.0	32.6	0.8

Arbequina	Spagna	3	0.7	18.4	41.3	1.2
Manzanilla	Spagna	3	1.3	15.1	37.7	1.3
Picholine	Francia	3	0.7	14.8	34.6	0.8
P.Marochaine	Marocco	3	0.7	15.7	34.3	1.2
Kalamon	Grecia	3	3.1	17.1	40.2	1.1

The olive fruit sampling was carried out on the same date to obtaining a more homogeneous pool of samples between the varieties studied. Nevertheless, it should be noted that, the fruit characteristics of the different cultivars showed significant differences among them.

The $\delta^{13}C$ and $\delta^{18}O$ values found for olive oils of all the varieties are shown in Figure 9.

Figure 9. Linear regression between $\delta^{13}C$ and $\delta^{18}O$ of all the olive oil samples from the experimental field of Perugia.



The range of variation for the $\delta^{13}C$ mean values was about 2.6‰, going from -29.8‰ to -27.3‰. Higher range was found for ^{18}O, it was about 2.8‰ ranging from 22.3‰ to 25.1‰. A significant positive correlation was found between $\delta^{13}C$ and $\delta^{18}O$ (R^2=0.46). This correlation can be considered representative of many cultivars originated from the Mediterranean area and grown in common garden. Therefore, as environmental conditions (e.g. water stress, atmospheric moisture, temperature) are common, the differences between cultivars have arisen from varietal genetic effect on both $\delta^{13}C$ and $\delta^{18}O$.

This positive correlation is likely explained physiological drivers, mainly stomatal control of transpiration, photosynthesis and by complex genotype x environment interactions. Such

interactions have been deeply investigated in many tree species (e.g., Lauteri et al., 2004; Nguyen Queirens, 2005) and are known to involve differences in phenology, plant structure and allocation pattern, plant carbon - water relationships, primary productivity and relationships along the hydraulic soil – plant – atmosphere continuum (SPAC). Any genotypic difference in phenological, physiological and structural traits has the potential to affect both the carbon and the oxygen isotopic fingerprint in plant material and in the fatty acids of the oil. For example, under constant atmospheric vapor pressure, carbon and oxygen isotopic enrichment of the primary photosynthetic products is dependent on stomatal conductance, that affects both the internal to atmospheric CO_2 concentration ratio (C_i/C_a) and the atmospheric to internal vapor pressure ratio (e_a/e_i). Both ratios, finally, are known to exert a mechanistic determinism on both $\delta^{13}C$ and $\delta^{18}O$ of plant material (Barbour, 2007; Brugnoli & Farquhar 2000). Thus, the strong correlation between the two isotopic ratios (Fig. 9) can be interpreted as a combined genotypic answer, involving on one side the available water source (shallow or deep soil water), affecting and $\delta^{18}O$ and, on the other side, the plant water status and its photosynthetic performance, linked to $\delta^{13}C$. Such an interpretation contains the potential of selecting drought tolerant versus mesic genotypes or varieties. Particularly, in the comparative field, the variety Kalamon displayed a deep rooting character (as inferred by the low $\delta^{18}O$ values) associated with sustained transpiration, active photosynthesis and productivity (as inferred by the $\delta^{13}C$ values). On the opposite extreme of the distribution (Fig. 1), the variety San Felice showed an opposite pattern, possibly related to a less deep rooting system, which can interpreted as a relatively higher susceptibility to summer drought.

The mean $\delta^{13}C$ and $\delta^{18}O$ values of olive oil samples along with Jean index (JI), the standard deviation, minimum and maximum values shown in table 9.

Table 9. Mean, Standard Deviation(SD), minimum and maximum values of the $\delta^{13}C$ and $\delta^{18}O$ values for each variety from Perugia experimental field.

Table 2. Isotopic ratio $\delta^{13}C$ and $\delta^{18}O$ observed in monovarietal olive oils from cultivars grown in Perugia experimental field. n=number of samples, JI, Jean ripening index; SD, standard deviation.

				$\delta^{13}C$				$\delta^{18}O$			
Cultivar	n	Origin	JI	Mean	Min	Max	SD	Mean	Min	Max	SD
Leccino	4	Umbria	3.4	-29.2	-29.4	-289	0.2	23.2	23.0	23.3	0.2
Frantoio	4	Umbria	1.1	-28.5	-29.2	-28.0	0.6	23.4	22.8	23.8	0.4
Moraiolo	3	Umbria	3.1	-28.6	-29.2	-28.3	0.5	23.7	23.4	24.1	0.4
San Felice	4	Umbria	1.1	-27.5	-27.8	-27.2	0.3	24.6	24.1	25.1	0.5
Carolea	3	Calabria	0.9	-28.5	-28.7	-28.3	0.2	24.2	23.8	24.5	0.3
Ottobratica	3	Calabria	2.2	-28.4	-28.5	-28.4	0.1	24.0	24.0	24.6	0.3
Coratina	2	Puglia	0.7	-28.8	-29.3	-28.2	0.8	22.9	22.9	24.1	0.8
Ogliarola	3	Puglia	2.2	-29.1	-29.3	-28.8	0.3	22.9	22.9	23.8	0.5
Casaliva	2	Lombardia	1.7	-28.8	-29.2	-28.3	0.6	22.7	22.7	23.5	0.6

Nocellara	1	Sicilia	2.9	-29.2	_	_	_	22.6	_	_	_
Arbequina	3	Spagna	0.7	-29.0	-29.3	-28.8	0.3	22.3	22.3	23.0	0.3
Manzanilla	3	Spagna	1.3	-29.0	-29.1	-28.8	0.2	23.2	23.2	24.7	0.7
Picholine	3	Francia	0.7	-28.6	-28.8	-28.5	0.2	22.5	22.5	23.0	0.2
P.Marocaine	3	Marocco	0.7	-29.1	-29.4	-28.7	0.4	22.9	22.9	23.2	0.2
Kalamon	3	Grecia	3.1	-29.6	-29.8	-29.4	0.2	22.3	22.3	23.2	0.5

In this study the influence of environmental factors is common, thus, the intrinsic fractionation processes of each cultivars remain the only factors affecting the variation of olive oil isotopic ratio among cultivars.

To analyze the isotopic discrimination which occur during the metabolic pathway involved in the transformation of palmitic to oleic acid (elongation step) and then linoleic (unsaturation step) (Harwood, 1988; Williams, et al. 1993), differences in the contents of fatty acids are compared to that of the isotopic ones. In table 10 the main fatty acids composition mean values along with the standard deviations are shown for the same oils.

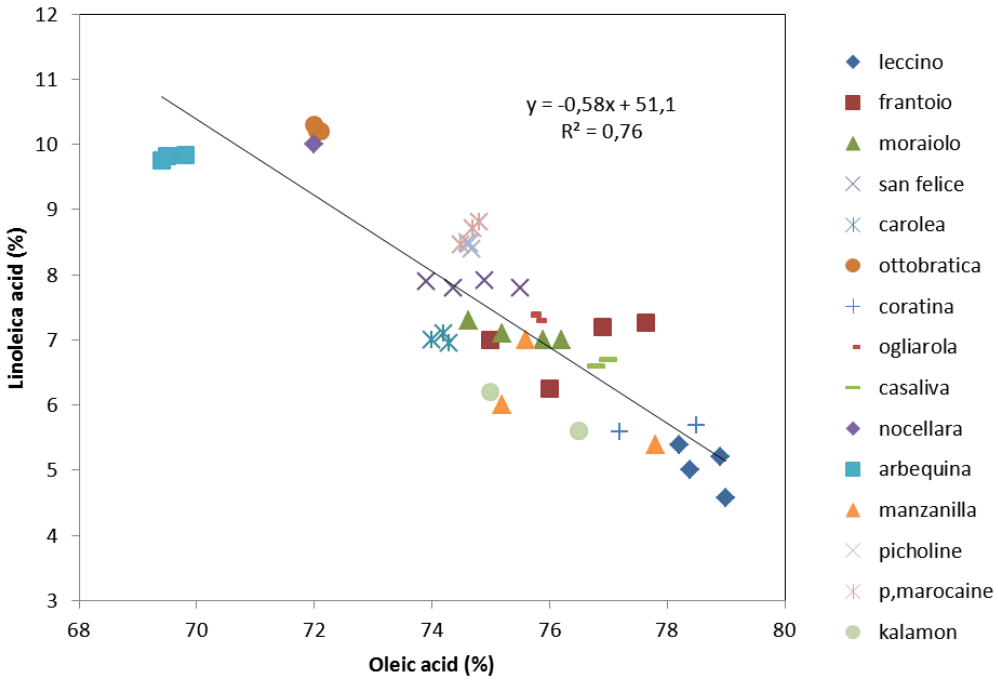
Table 10. Fatty Acid Concentrations in monovarietal olive oils from cultivars grown in Perugia experimental field (Mean and Standar Deviation)

Cultivar	16:0 %	16:1 %	17:0 %	17:1 %	18:0 %	18:1 %	18:2 %	18:3 %	20:0 %	20:1 %	22:0 %
Leccino	12.7 (0.2)	1.1 (0.1)	<0.01	0.1 (0.01)	1.6	78.6 (0.4)	5.0 (0.3)	0.5 (0.01)	05 (0.01)	0.2(0.01)	0.1 (0.1)
Frantoio	13.7 (0.4)	0.9 (0.1)	<0.01	0.1 (0.01)	2.1	76.4 (1.1)	6.9 (0.5)	0.6 (0.1)	0.4(0.01)	0.2(0.01)	0.1(0.1)
Moraiolo	13.4 (0.1)	0.9 (0.1)	<0.01	0.1 (0.01)	1.9	75.5 (0.7)	7.1 (0.1)	0.7 (0.01)	0.3(0.01)	0.3(0.01)	0.1(0.01)
San felice	13.0 (0.1)	0.8 (0.1)	<0.01	0.1 (0.01)	2.2	74.7 (0.7)	7.9 (0.1)	0.7 (0.01)	0.4(0.01)	0.3(0.01)	0.1(0.01)
Carolea	13.7 (0.1)	1.2 (0.1)	0.20 (0.01)	0.3 (0.01)	2.1	74.2 (0.2)	7.0 (0.1)	0.6 (0.01)	0.4(0.01)	0.3(0.01)	0.1(0.01)
Ottobratica	13.3 (0.1)	0.9 (0.1)	0.20 (0.01)	0.2 (0.01)	1.7	72.1 (0.1)	10.2 (0.1)	0.7 (0.01)	0.5(0.01)	0.3(0.01)	0.0(0.1)
Coratina	12.7 (0.1)	0.8 (0.1)	<0.01	0.1 (0.01)	1.5	77.9 (0.9)	5.6 (0.1)	0.1 (0.01)	0.5(0.01)	0.3(0.01)	0.2(0.01)
Ogliarola	12.5 (0.1)	0.8 (0.1)	<0.01	0.1 (0.01)	2.1	75.7 (0.1)	7.4 (0.1)	0.7 (0.01)	0.5(0.01)	0.3(0.01)	0.1(0.01)
Casaliva	12.6 (0.1)	0.8 (0.1)	<0.01	0.1 (0.01)	1.7	76.9 (0.1)	6.7 (0.1)	0.7 (0.01)	0.3(0.01)	0.2(0.01)	0.2(0.1)
Nocellara	13.5 (-)	1.0 (-)	<0.01	0.3 (-)	2.1 (-)	72.0 (-)	10.0 (-)	0.8 (-)	0.4 (-)	0.3 (-)	0.1 (-)
Arbequina	15.5 (0.4)	1.9 (0.1)	0.6 (0.4)	0.2 (0.01)	1.7	69.6 (0.2)	9.8 (0.1)	0.7 (0.01)	0.4(0.01)	0,3(0.01)	0.0(0.01)
Manzanilla	13.4 (0.7)	0.9 (0.1)	<0.01	0.1 (0.01)	1.7	76.2 (1.4)	6.1 (0.1)	0.5 (0.3)	0.5(0.01)	0.3(0.01)	0.2(0.1)
Picholine	12.7 (0.1)	0.8 (0.1)	<0.01	0.1 (0.01)	1.8	74.6 (0.1)	8.5 (0.1)	0.9 (0.1)	0.4(0.01)	0.3(0.01)	0.1(0.1)
P.Marocaine	12.8 (0.1)	0.8 (0.1)	0.10 (0.01)	0.1 (0.01)	1.8	74.7 (0.2)	8.7 (0.2)	0.8 (0.1)	0.4(0.01)	0.3(0.01)	0.0
Kalamon	14.9 (0.9)	0.6 (0.1)	0.1 (0.1)	0.2 (0.01)	1.7	76.0 (0.9)	5.8 (0.3)	0.6 (0.01)	0.5(0.01)	0.3(0.01)	0.0(0.01)

The preliminary descriptive analysis gave evidence that oils from Leccino, Coratina, Frantoio and Kalamon had higher concentrations of C18:1and low concentrations of C18:2 fatty acids than the other samples. On the other hand, samples from Arbequina, Ottobratica and the only one sample from Nocellara showed lower amounts of C18:1.

A high correlation was found between oleic and linoleic acid from the oils collected (figure 2).

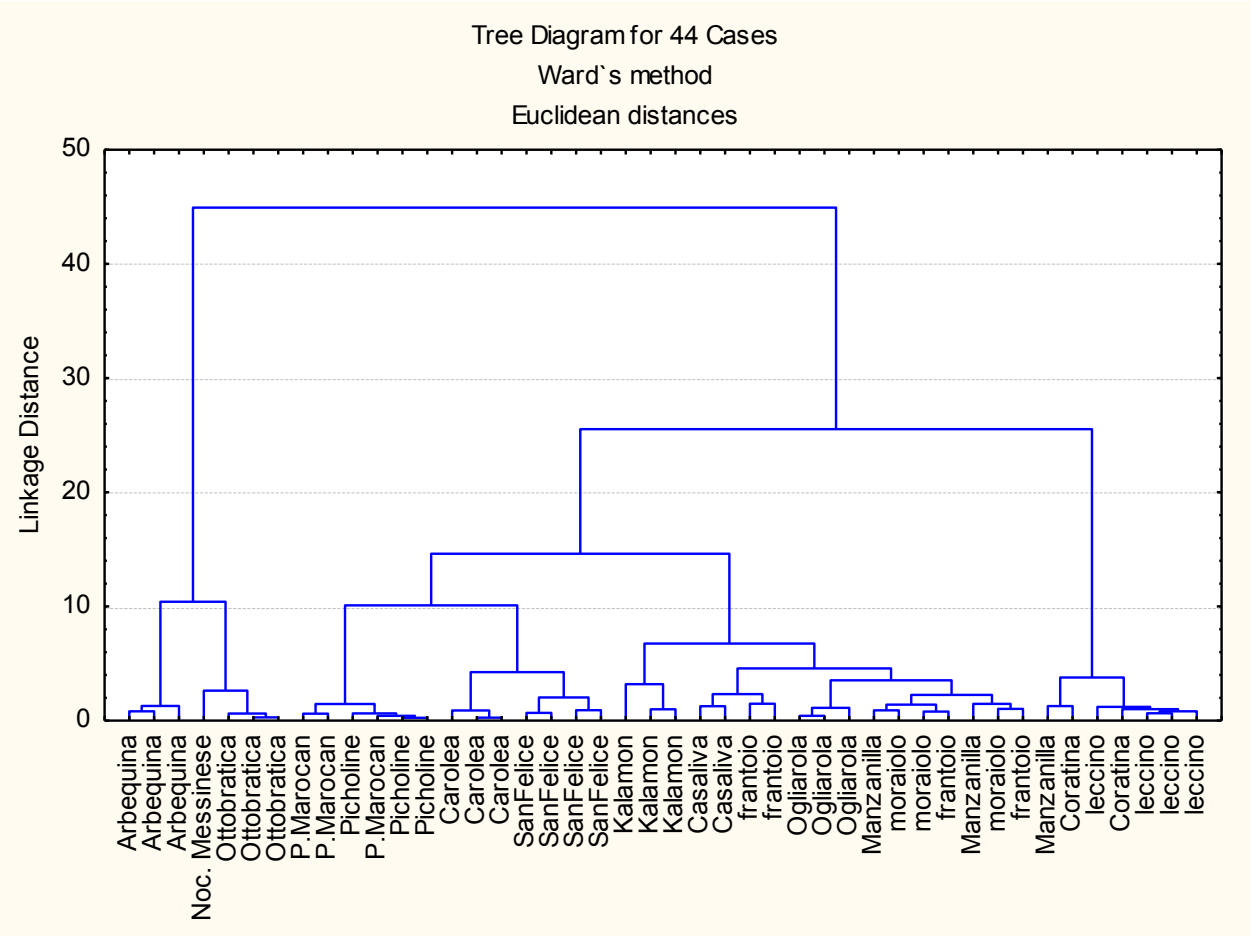
Figure 10. Linear regression between oleic and linoleic acid composition of the oil collected from the different cultivars.



Three cultivar (Arbequina, Ottobratica and Nocellara) are well separated from the others which shown a trend similar to that of isotopic one (Figure 9).

To have an immediate evaluation of the clustering ability of the single variables, the squared Euclidean distance calculation was applied on our analytical data, δ13C, δ18O and fatty acids (figure 10). The results of TCA are reported as a dendrogram, which can be cut at different levels (see Figure 2A). The linkage distance is reported on the y-axis; this distance is proportional to the dissimilarity among samples. The dataset exhibited a good clustering of the extra-virgin olive oils grouped on the basis of their cultivar.

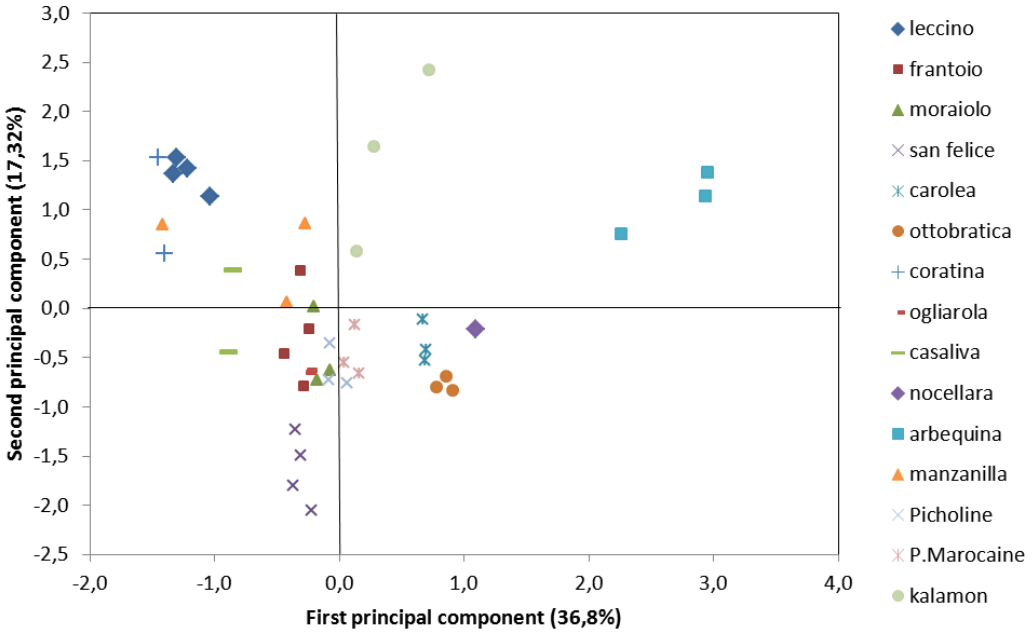
Figure 11. Cluster dendogram for $\delta^{13}C$, $\delta^{18}O$ fatty acid compositions of olive oil samples collected in 2013 (inputs variables = $\delta^{13}C$, $\delta^{18}O$, fatty acids; method = ward; distance = squared – Euclidean).



Cutting the dendrogram at a first level, two big groups are obtained: the first group consisting of the 3 olive oils cultivar (Arbequina, Nocellara messinese and Ottobratica) and the second one consisting of all the other cultivars. The second cluster can be further partitioned in different subgroups containing well clustered varieties.

To explore the meaning of the obtained clustering and, to reduce further the number of chemical indices, PCA was applied on our data set, combining the fatty acid and isotopic composition of the bulk oil, which had been previously autoscaled (zero mean and unit variance) to exclude the variance ascribed to the different measurement units. The PCA map is shown in figure 12.

Figure 12. Scatterplot of the scores from the first two principal components for the olive oil samples.



Loadings of the principal components are given in table 3.

Table 11. Principal Component Analysis Performed on the isotopic and Fatty Acid composition of bulk extra-virgin olive oil samples.

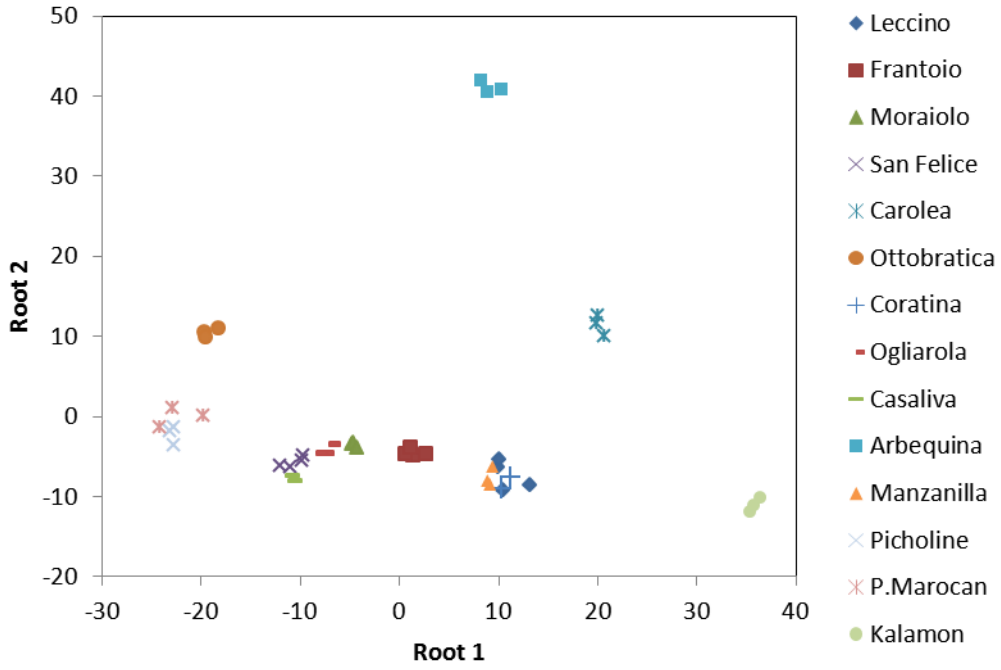
Variance proportion loadings	Principal component			
	1	2	3	4
$\delta^{13}C$	-0.01	**-0.32**	-0.24	-0.16
$\delta^{18}O$	-0.04	-0.25	**-0.45**	0.04
16:0	0.14	0.17	-0.10	-0.15
16:1	0.13	0.12	-0.11	-0.30
17:0	0.15	0.08	-0.08	-0.12
17:1	0.14	0.04	-0.16	0.16
18:0	0.01	-0.27	-0.04	-0.26
18:1	**-0.18**	0.10	0.04	-0.07
18:2	0.14	-0.18	0.11	0.17
18:3	0.08	-0.19	0.42	0.00
20:0	-0.05	0.15	-0.30	0.46
20:1	0.11	-0.12	-0.02	0.43
22:0	-0.12	0.00	-0.12	-0.24

The PC1 and PC2 scores, explaining 53,9% of the total variance, allow a good separation of the different cultivars studied. In PC1 (explaining 36,8% of the total variance) oleic acid is the most discriminant parameters (Table 11): in fact, samples from Arbequina, Nocellara and Ottobratica, plotted along positive PC1 values, whereas samples from Leccino, Casaliva, Ogliarola and one sample from Manzanilla show a high contento of oleic acid. The most discriminant parameters for

PC2 was the carbon isotope $\delta^{13}C$, showing a high value in the samples from San Felice and a low value in that of Kalamon (Figure 9).

Using the same 13 variables, LDA was performed, giving the map shown in Figure 13 (the only one sample from Nocellara cultivar was not included in LDA) .

Figure 13. Linear discriminant analysis resulting from data expressing isotopic and fatty acids composition (independent variables) of olive oils from different cultivars (grouping variables).



The group separation obtained with LDA was improved with respect to the PCA map. The LDA map, in fact, being per se a classification model, confirms the compositional differences among the varieties. The Wilks' λ factor for a forward stepwise LDA analysis, applied to the 13 variables, shows that palmitoleic, 17:1 and palmitic acids have the highest discriminating power (see Table 12).

Table 12. Linear Discriminant Analysis of the selected variables: $\delta^{13}C$, $\delta^{18}O$, and fatty acids.

Trait	Wilks' λ	F-remove (13.17)	p-level	Tolerance
$\delta^{13}C$	**0.000**	**2.557**	**0.036**	**0.480**
$\delta^{18}O$	**0.000**	**4.559**	**0.002**	**0.217**
16:0	**0.000**	**55.454**	**0.000**	**0.077**
16:1	**0.000**	**60.315**	**0.000**	**0.605**
17:0	0.000	0.913	0.559	0.637
17:1	**0.000**	**58.894**	**0.000**	**0.325**
18:0	**0.000**	**11.292**	**0.000**	**0.143**
18:1	**0.000**	**2.433**	**0.044**	**0.352**
18:2	**0.000**	**37.757**	**0.000**	**0.122**
18:3	**0.000**	**14.305**	**0.000**	**0.161**

20:0	**0.000**	**19.596**	**0.000**	**0.474**
20:1	**0.000**	**6.735**	**0.000**	**0.470**
22:0	**0.000**	**2.642**	**0.031**	**0.503**

The fairly good discrimination shows a light overlap between Manzanilla, Coratina and Leccino and between Ogliarola, Moraiolo and san felice. Such discrimination is highly statistically significant (Wilks' λ value < 0.00001), according to a high discriminant power of the model, and the F ratio is high (60.315), indicating significant differences between means across the groups (p-level <0.00001). Globally TCA, PCA and LDA consistently (see Figure 6A,B) discriminated Arbequina, Ottobratica and Nocellara (the last noe not included in LDA) from the other cultivars.

To analyze the relationship between isotopic compositions and fatty acids and to explore the effects of the main fatty acid composition on the carbon and oxygen isotope fingerprinting in bulk olive oil, an analysis a linear regression was performed (Pearson coefficient, R, and significance, p) between the variables considered. At first step, all sample were considered and no correlation was found concerning $\delta^{13}C$ and $\delta^{18}O$ with fatty acid composition of olive oil (data not shown). In a second step, the analysis of correlation was repeated with the same variables, but after the exclusion of cultivars Arbequina, Ottobratica and Nocellara, characterized by a extreme value of 18:1 and 18:2 fatty acids. The results are shown in table 13.

Table 13. Correlation between $\delta^{13}C$, $\delta^{18}O$ and the main fatty acid composition of olive oil (Pearson coefficient, r, * p<0.01; ** p<0.05).

Fatty acid (%)	$\delta^{13}C$	$\delta^{18}O$
16:0	-0.19	-0.14
16:1	0.02	0.18
17:0	0.03	0.13
17:1	-0.11	0.12
18:0	**0.44***	**0.46***
18:1	**-0.39****	-0.26
18:2	**0.39****	0.03
18:3	0.10	-0.23
20:0	-0.27	0.05
20:1	0.04	0.03
22:0	0.08	0.18

The variation in carbon-isotope composition among olive oil varieties was correlated, although minimally, with variation in oleic and linoleic acid composition (the stearic acid is too few abundant in olive oil). In particular, ^{13}C was more abundant in linolenic than in oleic acid, because of the presence of double bonds. In fact, there is a kinetic isotope effect in the reaction leading to 18:2, causing ^{13}C to pass preferentially from 18:1 to 18:2, yielding a net depletion of carbon 13 at the carboxyl position in 18:1 lipid (Monson and Hayes 1981). Another isotopic effect is associated with the elongation pathway leading from 16:0 to 18:0 and may be responsible for the enrichment of

13C in 18:0 (Monson 1981). However any genetic difference between cultivars in phenological, physiological and structural traits has the potential to affect both the carbon and the oxygen isotopic fingerprint in the fatty acids of the oil. E.g., under constant atmospheric vapor pressure, carbon and oxygen isotopic enrichment of the primary photosynthetic products is dependent on stomatal conductance, that affects both the internal to atmospheric CO_2 concentration ratio (Ci/Ca) and the atmospheric to internal vapor pressure ratio (e_a/e_i). Both ratios, finally, are known to exert a mechanistic determinism on both $\delta^{13}C$ and $\delta^{18}O$ of plant material (Barbour, 2007; Brugnoli & Farquhar 2000). The low regression coefficient founded between carbon isotope and oleic and linoleic acid (r = -0.39 and r = 0.39 respectively) lead to conclude that the scatter of the $\delta^{13}C$ values of the C3 vegetable oils may be attributed to variation of the isotope effect during fixation of carbon dioxide.

6.2.4. Conclusions

Carbon and oxygen isotopic and fatty acid composition of 45 monovarietal extra virgin olive oils from plants grown in common garden revealed interesting features of these chemical traits. The isotopic variation observed among the cultivars was significant, but no not so large as that caused by environmental factors (Portarena et al. 2014). Statistical analysis (TCA, PCA and LDA), applied to these data, was successfully used for cultivar discrimination of these extra virgin olive oils.

The carbon and oxygen isotopic composition of olive oil were dependent on olive cultivar, but was minimally related to the main fatty acid components. In particular, both carbon and oxygen isotopic composition were positively correlated with stearic acid, whereas only carbon isotope was negatively related with oleic and positively with linoleic. These results suggest the $\delta^{13}C$ values of the olive oil may be attributed to isotopic fractionation effect during carbon dioxide fixation.

This isotopic shift is partially and minimally explained by factors affecting the chemical distribution of the fatty acids, and particularly by the physiological processes and enzymatic reactions occurring in the plants cells (Vogel, 1993; O'Leary, 1993; Jackson et al., 1993). Some of the variations of the isotopic composition of the olive oil samples may be due to different olive cultivars and plant growth conditions. The present results, can provide scientific basis for an authentication procedure. In addition, further studies on the isotopic fractionation among the olive oil compounds may improve the efficiency of the cultivar characterization of monovarietal olive oils.

6.3. Effect of ripening stage and climatic conditions on the isotope composition of monovarietal olive oils

Maturation of olive oil fruit is rather low process: after fruit set, olives grow rapidly for 30 or 40 days, after that the seed is formed and the pit hardens. From then on, seed weight hardly changes. Pulp weight increases slowly during summer, if enough water is available, and then more rapidly from mid-September, when structural changes in the fruit indicate the approach of the color change stage. Various authors (Va´zquez et al., 1971; Agramont et al., 1986; de la Torre et al., 1985; Solinas et al., 1987) have studied structural changes occurring during olive ripening which has strong effects on the different components of the fruit and extracted oils. However, those studies deal with individual components, and no studies on oil isotope composition have been published so far. Owing to the seasonal transition imposing a shift of environmental conditions in this period, the plant primary activity usually changes from a limiting condition to a condition favoring CO_2 diffusion trough the stomata, towards the carboxylation sites into the chloroplasts (Diaz-Espejo et al, 2007). In other terms, along the ripening stage, the olive trees generally experience a progressive mitigation of the hot and arid summer conditions, towards the fresh and humid autumn conditions. Under such circumstances, the photosynthetic carbon uptake is expected to shift from enriched to depleted carbon isotope compositions ($\delta^{13}C$), due to the well-known relationship linking the carbon isotope fractionations along the photosynthesis assimilation to the intercellular to atmospheric CO_2 concentration ratio, Ci/Ca (Farquhar et al. 1982; Brugnoli et al., 1988). Under mild condition the lighter isotopes are favored leading to higher discrimination.

Several authors (Kelly, S. et al., 1997; Spangenberg et al., 1998) claimed about putative differences in carbon isotope composition among different fatty acids of olive oil, attributing such evidence to differential fractionation effects along the fatty acids biosynthetic pathway. However, at least to our knowledge, little information exists on the possible effects of seasonal shift on the fatty acids and isotopic composition.

The isotopic fractionations involved in the biosynthesis of lipids has been the subject of many investigations based on IRMS determination of overall isotope ratios (De Niro & Episten 1977, Monson & Hayes 1982, Stenberg et al., 1984).

In this work the isotopic and fatty acid composition of different olive oil cultivar grown in the same olive orchard at different maturation degree were studied.

The aim of the present work was to investigate the effect of the degree of ripeness of the olive on carbon and oxygen isotopic composition in relation with fatty acid composition of the selected olive cultivar.

6.3.1. Plant material and oil samples

Extra virgin olive oil was extracted from individual olive cultivars grown in two experimental olive orchards, located in Central Italy, Umbria region, but characterized by different climatic condition. The first named Perugia, the second one Orvieto.

Four cultivars (Leccino, Frantoio, Moraiolo and San Felice) are grown in the germplasm collection of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia (43°04′54.58″N, 12°22′53.41″E; http://www.oleadb.it/collections/cultivar_coll_list.php?mastertable=collections&masterkey1=027), located nearby Perugia town. This collection is placed on a hill at 320 m a.s.l. . The trees are 18 year old, 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.

Four cultivars (Leccino, Frantoio, San Felice, Nostrale di Rigali) and one selected clone (IX 90) were grown in the experimental field of ONAT (Orvietano, Narnese, Amerino, Tuderte) Mountain Community in Orvieto (42°55′00″N, 12°03′00″E). The trees are 8 year old. The olive growing area is situated at 500 m above sea level. The soil was managed with a spontaneous green cover moved 2 times/year. Leccino and Frantoio are two of the main Italian olive cultivars, cultivated all over the world; while Moraiolo, San Felice and Nostrale di Rigali are local olive cultivars.

The olives were randomly hand-picked from the crown of 4 trees per cultivar and oil samples were extracted using a hammer mill, 30 minutes of kneading, centrifugation without extra water from batches of 1-3 kg of olives, corresponding to a single tree. The experiment was conducted for 3 months (October, November and December) during the ripening stage of olives in 2012 year. The degree of ripening was measured by Jaèn pigmentation index (JI) on 100 drupes/tree, ranging from 0 to 7, with 0 for green olives and 7 for olives with superficial pigmentation on 100% of the epicarp and 100% pigmentation on the pulp (Uceda & Hermoso, 1998). Fruit and tree characteristics, fatty acid composition were determined only on tree, fruit and oil samples collected from adult olive tree in Perugia olive orchard. 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 drying (at 105 °C) the samples used for fresh weight determination in an oven until constant weight; oil content (2 sample/tree) using an InfraAlyzer apparatus (SpectraAlyzer Zeutec BRAN+LUEBBE, Rendsburg, Germany) both on fresh and dry weight basis. Before harvesting, olive detachment force (DF; N) was determined on each tree using a dynamometer (Somfy Tec, Ademva, Cluses Cedex, France). fruit drop was determined by counting every 15 days the fruits present on labelled twigs, one per

tree per each cultivar, compared to those present at the beginning of the ripeness. Yield (kg olive / tree), canopy diameter (D; m) and height (H; m) were measured for each of the harvested trees. The meteorological data of the different fields were obtained from the Italian Central Office for Agriecology (UCEA) and are shown in Table 14.

Table 14. Climatic characteristics of Perugia and Orvieto experimental field at different sampling dates: cumulated amount of precipitations from 28th of August, (UCEA), mean of maximum daily temperature over five days before the harvest.

Field Site	Harvest 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

Different botanical (variety and ripeness of the fruit) and environmental (amount of rain and temperature) factors were considered in order to investigate their influence on the physico-chemical parameters of the oils: carbon isotopes, oxygen isotopes and fatty acids composition.

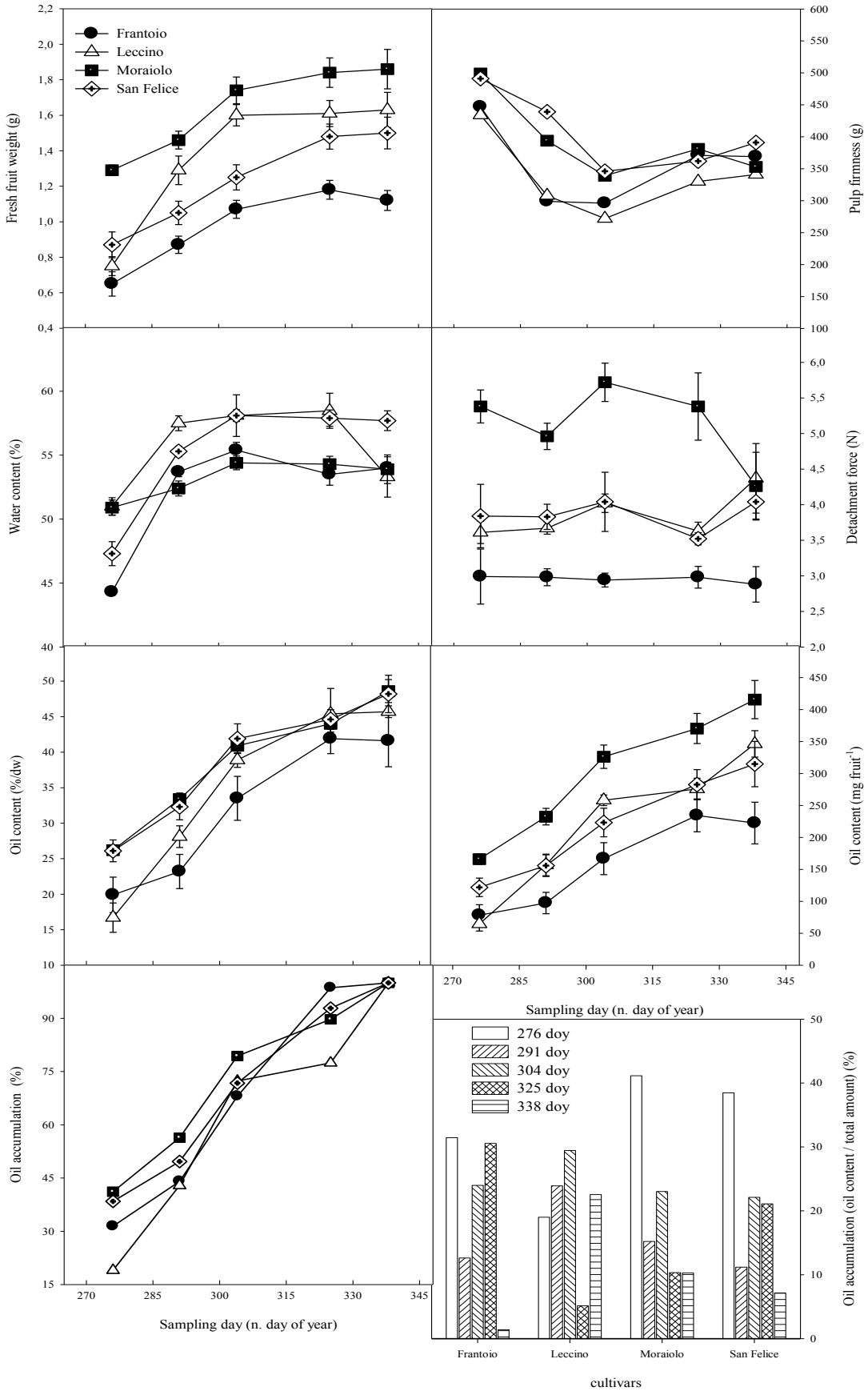
6.3.2. Statistical analysis

For isotopic and fatty acid data, mean values, standard deviation, minimum and maximum have been calculated. Factorial analysis of-variance (ANOVA) and Post Hoc Fisher multiple comparison tests were performed on isotopic and the fatty acid using Statistica 8 software (StatSoft Italia s.r.l,, Padova, Italy). Oil samples were classified according to cultivar and ripening stages. Furthermore, multivariate regression analysis was performed on oil sample data, in order to identify significant correlations between isotopic and fatty acid composition.

6.3.3. Results and Discussion

Fruit characteristics, ripening index, yield and canopy volume were shown in Figure 14.

Figure 14. Fruit characteristics, ripening index, yield and canopy volume (means ± standard errors)



During ripening the firmness of the pulp and the detachment force of the fruits decreased in all varieties, while the oil content increases. The period of oil accumulation in olives resulted to be different among the 4 cultivars. In specific Moraiolo and San Felice cultivars accumulate, respectively, 41% and 39% of total oil in September, Frantoio 32%, and Leccino only 19%. Leccino cultivar accumulates oil in the fruits especially in October, 53.3% of the total, while Moraiolo, Frantoio and San Felice cvs at the same time accumulate 38.2%, 36.6% and 33.3%, respectively. The accumulation of oil in the fruits of cv. Frantoio is completed in the second week of November, when it had been accumulated 98.6% of the oil present at the olive harvest. On the contrary at the same period, fruits of cv. San Felice had been accumulated 92.9% of total oil; those of cv. Moraiolo the 89.7% and those of cv. Leccino only 77.5%. From 325 to 338 day of year Leccino cv. accumulates 22.54 % of total olive oil; Moraiolo cv. the 10.27% and San Felice only the 7.14%. During ripening process the pigmentation index of the olives of the different varieties increased correlating with the Day of Year (DOY), the amount of precipitation and the decrease of temperatures (Table 15 a,b).

Table 15 (a,b). Correlation between Jean Index and the climatic parameters of mm of rain (amount of rain from the last week of august) and mean of maximum daily temperature (taken five days before the harvest) and Day Of Year (Pearson coefficient, r, the * values show significance at 1% level).

15a)

Perugia Orchard			
Cultivar	DOY	rain (mm)	t (°C)
Frantoio JI	0.71*	0.69*	-0.73*
Leccino JI	0.92*	0.88*	-0.93*
Moraiolo JI	0.83*	0.81*	-0.83*
San Felice JI	0.89*	0.93*	-0.80*

15b)

Orvieto Orchard			
	DOY	rain (mm)	t (°C)
Frantoio JI	0.95*	0.96*	-0.97*
Leccino JI	0.98*	0.98*	-0.97*
San Felice JI	0.98*	0.97*	-0.95*
Clone JI	0.92*	0.95*	-0.95*
Nostrale JI	0.97*	0.98*	-0.99*

The values of $\delta^{13}C$ and $\delta^{18}O$ isotope ratio for olive oils of all the varieties in the two experimental fields are shown in Table 16 and 17.

Table 16. Isotopic ratio of $\delta^{13}C$ and $\delta^{18}O$ in olive oil (n is the number of samples) of different varieties from Perugia experimental field at different harvest dates (the Day Of Year, DOY, is considered) and Jean Index (JI).

				$\delta^{13}C$				$\delta^{18}O$			
Cultivar	n	DOY	JI	Mean	Min	Max	St.Dev.	Mean	Min	Max	St.Dev.
Frantoio	4	276	0.2	-27.6	-27.8	-27.4	0.2	24.8	24.5	25.1	0.3
Frantoio	4	291	0.8	-27.9	-28.0	-27.8	0.1	23.7	23.5	24.2	0.3
Frantoio	4	304	1.1	-28.5	-29.2	-28.0	0.6	23.4	22.8	23.8	0.4
Frantoio	4	325	1.5	-29.5	-29.8	-29.2	0.2	22.3	22.2	22.4	0.1
Frantoio	4	338	1.6	-30.0	-30.6	-29.5	0.5	21.5	21.4	21.9	0.3
Leccino	4	276	0.7	-27.7	-27.8	-27.6	0.1	24.5	24.1	24.9	0.3
Leccino	4	291	2.3	-28.6	-28.8	-28.4	0.2	24.2	24.0	24.3	0.1
Leccino	4	304	3.4	-29.2	-29.4	-28.9	0.2	23.2	23.0	23.3	0.2
Leccino	4	325	3.9	-30.2	-30.5	-30.0	0.2	21.5	21.3	22.0	0.3
Leccino	4	338	5.9	-30.1	-30.4	-29.3	0.6	21.3	20.3	22.0	0.8
Moraiolo	4	276	1.1	-28.0	-28.6	-27.4	0.6	25.2	24.9	25.5	0.3
Moraiolo	4	291	2.3	-28.4	-28.7	-28.2	0.2	24.3	24.0	24.6	0.3
Moraiolo	4	304	2.8	-28.7	-29.2	-28.3	0.5	23.7	23.4	24.1	0.4
Moraiolo	4	325	3.8	-29.4	-29.7	-29.2	0.2	24.0	23.7	24.1	0.2
Moraiolo	4	338	5.3	-29.6	-29.9	-29.3	0.3	22.4	22.1	22.8	0.3
San Felice	4	276	0.4	-27.1	-27.7	-26.8	0.4	26.8	26.3	27.4	0.5
San Felice	4	291	1.4	-27.5	-28.0	-27.3	0.3	25.2	24.8	25.5	0.3
San Felice	4	304	1.1	-27.5	-27.8	-27.2	0.3	24.6	24.1	25.1	0.5
San Felice	3	325	3.5	-28.7	-28.9	-28.4	0.3	23.4	22.3	24.2	1.0
San Felice	4	338	4.8	-28.9	-29.2	-28,7	0.2	23.3	22.3	23.7	0.6

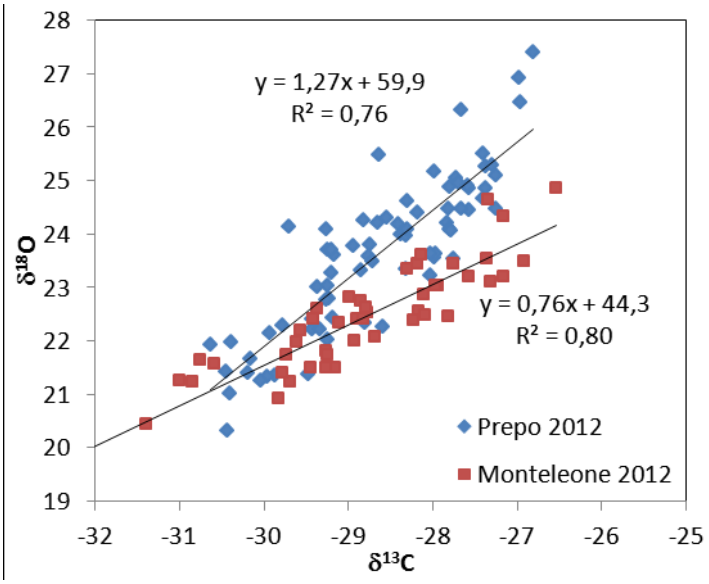
Table 17. Isotopic ratio of $\delta^{13}C$ and $\delta^{18}O$ in olive oil (N is the number of samples) of different varieties from Orvieto experimental field at different harvest dates (the Day Of Year, DOY, is considered) and Jean Index (JI).

				$\delta^{13}C$				$\delta^{18}O$			
Cultivar	N	DOY	JI	Mean	Min	Max	St.Dev.	Mean	Min	Max	St.Dev.
Frantoio	2	278	1.0	-28.5	-28.9	-28.2	0.5	22.5	22.4	22.5	0.1
Frantoio	2	292	1.3	-29.1	-29.4	-28.8	0.4	22.6	22.5	22.6	0.1
Frantoio	2	310	1.6	-29.4	-29.7	-29.2	0.4	21.4	21.2	21.5	0.2
Frantoio	2	327	2.5	-30.2	-30.9	-29.5	1.0	21.4	21.2	21.5	0.2
Leccino	2	278	2.0	-29.0	-29.3	-28.7	0.4	21.9	21.8	22.1	0.2
Leccino	2	292	2.9	-29.0	-29.1	-28.9	0.1	22.2	22.0	22.3	0.2
Leccino	2	310	4.0	-29.4	-29.6	-29.3	0.2	21.9	21.5	22.2	0.5
Leccino	2	327	5.0	-30.4	-31.0	-29.8	0.8	21.1	20.9	21.3	0.2
Nostrale	3	278	1.0	-27.1	-27.3	-26.9	0.2	23.3	23.1	23.5	0.2
Nostrale	3	292	1.6	-27.8	-27.9	-27.8	0.1	23.0	22.5	23.4	0.5
Nostrale	3	310	2.1	-28.1	-28.2	-28.1	0.0	22.9	22.5	23.4	0.5

Nostrale	3	327	3.5	-28.5	-29.4	-28.0	0.8	22.6	22.4	23.0	0.4
Clone	3	278	0.5	-28.9	-29.0	-28.8	0.1	22.7	22.4	22.8	0.2
Clone	3	292	0.8	-29.5	-29.8	-29.3	0.3	21.7	21.4	22.0	0.3
Clone	3	310	1.1	-30.4	-30.8	-29.7	0.5	21.6	21.6	21.7	0.1
Clone	2	327	2.3	-31.8	-32.2	-31.4	0.6	20.0	19.6	20.4	0.6
San Felice	3	278	1.0	-27.0	-27.3	-26.5	0.4	24.6	24.3	24.9	0.3
San Felice	3	310	4.5	-27.9	-28.3	-27.4	0.5	23.5	23.3	23.6	0.1
San Felice	2	327	5.3	-28.2	-28.8	-27.6	0.9	22.9	22.6	23.2	0.4

Figure 14 shows the total range of variation founded for carbon and oxygen isotopic values in all olive oil samples studied in Perugia and Orvieto field.

Figure 14. Relationships between the $\delta^{13}C$ and $\delta^{18}O$ values of all olive oil samples collected in Perugia and Orvieto field.



The range of variation for 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 field. Bigger range was found for ^{18}O; it was about 7.1‰ (from 20.3‰ to 27.4‰) and 5,3‰ (from 19.6‰ to 24.9‰) for Prepo and Monteleone respectively.

The correlation between $\delta^{13}C$ and $\delta^{18}O$ across all the cultivars and the two experimental fields was tested by linear regression (Figure 14). A positive and significant ($p < 0.05$) correlation was found between the two variables and both experimental sites. The correlation was slightly stronger in Orvieto than in Perugia. This strong correlation between the two isotopic ratios (Figure 14) 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 and $\delta^{18}O$ and, on the other side, the plant water status and its photosynthetic performance, linked to $\delta^{13}C$. Factorial analyses of

variance (ANOVA) with interaction was carried out to test the influence of ripening stage (Jean Index), cultivar, and field on carbon and oxygen isotopic composition on olive oil. Results are shown in Table 18.

Table 18. Factorial analyses of variance (ANOVA) calculated for carbon and oxygen isotopic composition on olive oil sample from different fields, cultivar and Jean index.

		$\delta^{18}O$				$\delta^{13}C$			
	Freedom Degree	Sum Sq	Mean Sq	F value	Pr(>F)	Sum Sq	Mean Sq	F value	Pr(>F)
index	1	36.42	36.42	76.57	**3.8e-14 ***	21.76	21.76	59.68	**5.5e-12 ***
cultivar	5	141.25	28.25	59.38	**2.2e-16 ***	70.74	14.14	38.79	**2.2e-16 ***
field	1	9.09	9.09	19.13	**2.8e-05 ***	0.01	0.01	0.03	0.863
Index:cultivar	5	6.98	1.39	2.94	0.016	9.87	1.97	5.41	**0.0001 ***
index:field	1	1.32	1.32	2.76	0.099	0.05	0.05	0.14	0.713
cultivar:field	2	2.41	1.2	2.53	0.084	2.35	1.17	3.23	0.043

This analysis shows significant effect of index and cultivar for both $\delta^{13}C$ and $\delta^{18}O$. The effect of field was significant only for oxygen isotopic ratio, and the interaction index X cultivar resulted significant for carbon but not for the oxygen isotopes.

The variation of oxygen isotope fractionation reflects the variability of meteorological conditions between the two field, and any difference in source water. Indeed, the amount of precipitation during the ripening period was almost similar in the two fields; instead Perugia field was characterize by higher temperatures in 2012, together with higher $\delta^{18}O$ values of olive oils (Table 6). The analysis of variance and post hoc Fisher's test were performed to elucidate whether the oil $\delta^{13}C$ and $\delta^{18}O$ values were statistically different $P < 0.5$) between varieties. The results are shown in Table 19.

Table 19 (a,b). Results of Post Hoc Fisher test ($P < 0.05$) for the two isotopic ratio among the olive oils produced from different cultivar in Perugia and Orvieto.

19a)

Perugia	$\delta^{18}O$	$\delta^{13}C$
Variety	Groups	Groups
San Felice	A	a
Moraiolo	Ab	b
Frantoio	Bc	b
Leccino	C	b

19b)

Orvieto	$\delta^{18}O$	$\delta^{13}C$
Cultivar	Groups	Groups
San Felice	A	a
Nostrale	B	a
Frantoio	C	b

Leccino	C	b
Clone	C	b

The results indicate a significant difference between San Felice cultivar and Leccino and Frantoio in both fields. The $\delta^{13}C$ in Moraiolo, in Perugia was statistically similar to those of Leccino and Frantoio but not to that of San Felice; instead for $\delta^{18}O$ Moraiolo was statistically different from Leccino. In Orvieto field, Nostrale di Rigali cultivar was statistically different from all the other cultivars for $\delta^{18}O$ but it was similar to San Felice for $\delta^{13}C$ values. The other cultivars did not show significant variation. In particular the different isotopic mean values of the four cultivar collected in Prepo reflect the different oil accumulation which occurs in September (Figure 14). In fact the carbon and oxygen isotopic ratios of olive oil are affected by the climatic conditions during oil accumulation. The lowest $\delta^{18}O$ and $\delta^{13}C$ mean values were observed in Leccino which accumulate only 19% in September characterize by lower precipitations and higher temperatures than October when it accumulates 53.3% of the total, In contrast, Moraiolo and San Felice cultivars accumulate, respectively, 41% and 39% of total oil in September and are characterize by higher $\delta^{18}O$ and $\delta^{13}C$ values.

Values of $\delta^{18}O$ and $\delta^{13}C$ in all oils analyzed, were compared with environmental and physiological parameters of temperature, mm of rain and Jean Index (JI), in the two experimental fields. Results are reported in Table 20 a and b.

Table 20. Correlation (Pearson coefficient, r,) between $\delta^{13}C$, $\delta^{18}O$, Jean Index (JI), precipitation (cumulated rain from the last week of august), and mean of maximum daily temperature (five days before the harvest) in Perugia (a) and Orvieto (b), esperimental field. Statistical significance, * significant at $p < 0.01$; ** significant at $p<0.05$).

20a)

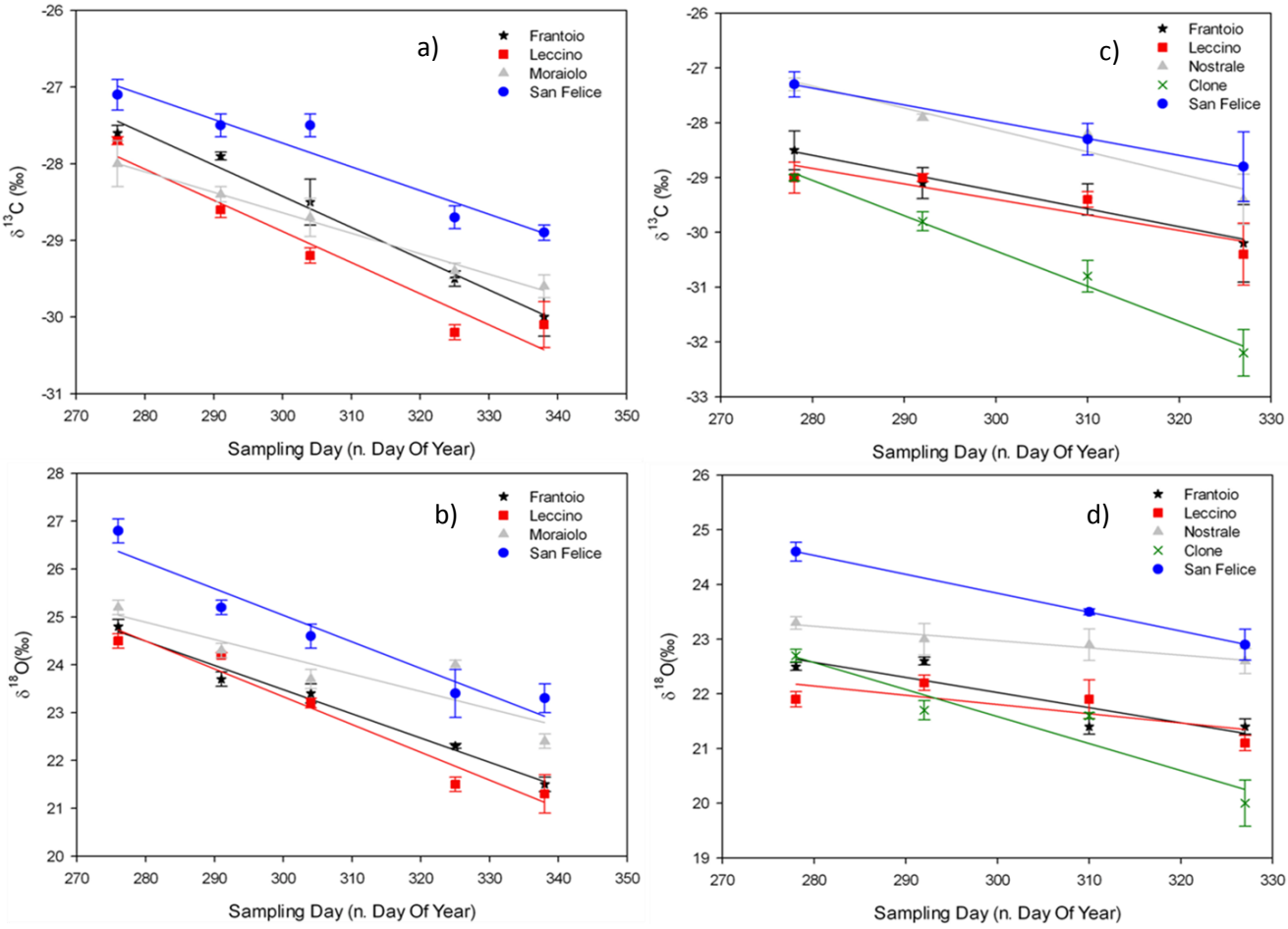
	Frantoio		Leccino		Moraiolo		San Felice	
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$
$\delta^{13}C$	-		-		-		-	
$\delta^{18}O$	0.91*	-	0.92*	-	0.69*	-	0.83*	-
JI	0.56**	0.71*	0.77*	0.83*	0.62**	0.80*	0.82*	0.80*
T, °C	0.88*	0.93*	0.91*	0.88*	0.81*	0.91*	0.80*	0.90*
Rain, mm	-0.93*	-0.96*	-0.92*	-0.95*	-0.87*	0.80*	-0.93*	-0.88*

20b)

	Frantoio		Leccino		San Felice		Clone		Nostrale	
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$
$\delta^{13}C$	-		-		-		-			
$\delta^{18}O$	0.72**	-	0.63*	-	0.95*	-	0.93*	-	0.62**	
JI	0.77**	0.77**	0.77**	0.73**	0.76**	0.95*	0.94*	0.80	0.79*	0.57*
T, °C	0.80**	0.80**	0.80**	0.73**	0.95	0.95*	0.95*	0.90	0.82	0.57**
Rain, mm	-0.79**	-0.88*	-0.82**	-0.76**	-0.97	-0.96*	-0.95*	-0.88	-0.80	-0.55**

Values of $\delta^{18}O$ isotopic ratios were highly and significantly correlated with all the factors studied. The best correlation was found between $\delta^{18}O$ and climatic parameters, especially temperature (positive correlation) and amount of rain (negative correlation). These results indicate that the carbon and oxygen isotopic ratios of olive oil are affected by physiological parameters strongly dependent on environmental conditions during plant growth than the secondary metabolic factors.

Figure 15. $\delta^{13}C$ and $\delta^{18}O$ mean values of olive oil samples collected at different harvest dates (Julian day) at Perugia (a, b) and Monteleone (c, d) experimental field. Error bars indicate Standard Error.



Figures 15 shows the evolution of carbon and oxygen isotopic composition of the different varieties analyzed in both fields (Perugia and Orvieto respectively) along with harvest date (increasing ripening stage). The observed isotopic trends along the ripening stage could be related to climatic factors. Biochemical modification associated to ripening could also affect the isotopic composition of fatty acids, if for example these are derived from different source of carbon (e.g.: soluble sugars, starch). The mean daily temperature decrease from October to December 2012 was associated with an increase in precipitation. This meteorological trend causes variations in plant water source, atmospheric humidity, evaporative demand, photosynthesis and transpiration processes, with effects

on the isotopic composition of the synthesized fatty acids. The range of the isotopic ratio was rather narrow for each cultivar, and slightly decreased during ripening evolution. Thus, despite the variation during ripening evolution the isotopic ratios of the different genotypes were well separated. These data clearly reveal that the isotopic signals of these monovarietal oils are strongly affected by genotype and slightly by ripening degree.

Effects of ripening on FA content and their relation with isotopes

The variation in the olive oil carbon isotope composition could be also linked to intrinsic isotopic effects along the pathway of fatty acid 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 (Royer et al. 1999; Kelly et al.,1997). We analyzed the content of oil fatty acids: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), acids (Table 21). In all the samples, the oleic acid was always the most abundant compound, always higher than 74% of the total fatty acid. Table 21 shows the effect of variety and ripening degree on the major FA content (expressed as per cent of total FA), during ripening of four olive cultivars. Such differences in FA composition probably reflect the metabolic behavior of each cultivar controlled by genetic determinants.

Table 21. Relative fatty acid composition (mean value) for each cultivar at different harvest dates in Perugia experimental field.

Cultivar	Harvest data	Palmitic (16:0 %)	Palmitoleic (16:1 %)	Stearic (18:0 %)	Oleic (18:1 %)	Linoleic (18:2 %)	Linolenic (18:3 %)
Frantoio	02/10/2012	13.3	0.8	1.8	76.7	6.6	0.8
	17/10/2012	12.6	0.8	1.5	77.1	6.9	0.8
	30/10/2012	13.7	0.9	2.1	76.4	6.9	0.6
	20/11/2012	13.7	0.9	2.0	77.9	6.9	0.8
	03/10/2012	14.0	0.8	2.2	77.1	6.6	0.8
Leccino	02/10/2012	12.6	1.1	1.6	78.4	5.1	0.5
	17/10/2012	13.0	1.1	1.5	78.5	5.1	0.5
	30/10/2012	12.7	1.1	1.6	78.6	5.0	0.5
	20/11/2012	12.7	1.1	1.4	79.1	5.0	0.5
	03/10/2012	12.7	1.1	1.4	79.5	5.0	0.5
Moraiolo	02/10/2012	13.2	0.8	1.9	75.2	7.2	0.7
	17/10/2012	13.1	0.8	2.0	75.2	7.2	0.7
	30/10/2012	13.4	0.9	1.9	75.5	7.1	0.7
	20/11/2012	13.6	0.9	1.9	75.6	7.2	0.7
	03/10/2012	13.6	0.9	1.9	76.1	7.2	0.7
San Felice	02/10/2012	12.9	0.8	2.2	74.8	7.9	0.7

17/10/2012	12.9	0.8	2.2	74.9	7.9	0.7
30/10/2012	13.0	0.8	2.2	74.7	7.9	0.7
20/11/2012	12.9	0.8	2.2	74.8	7.7	0.7
03/10/2012	12.9	0.7	2.2	74.7	7.7	0.7

In Table 22 the correlations between fatty acids and isotopic composition in all olive cultivars grown in Perugia experimental field are shown.

Table 22. Correlations between fatty acids and isotopic composition in all olive cultivars grown in Perugia experimental field.

	$\delta^{13}C$	$\delta^{18}O$
C16:0 palmitic	**-0.23****	-0.15
C16:1 palmitoleic	**-0.44***	**-0.42****
C17 margaric	0.10	0.13
C17:1margaoleic	0.08	0.17
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
C20 arachic eicosanoic	-0.04	-0.09
C20:1 eicosenoic	**0.63***	**0.61****
C22:0 behenic	0.11	0.14
C24:0 lignoceric	0.08	0.16

In Table 23 the correlations between fatty acids composition, pigmentation index (Jean Index, JI) and the harvest data (Day Of Year) in olive cultivars grown in Perugia experimental field are shown.

Table 23. Correlations between fatty acids composition, pigmentation index (Jean Index, JI) and the harvest data (Day Of Year) in olive cultivars grown in Perugia experimental field.

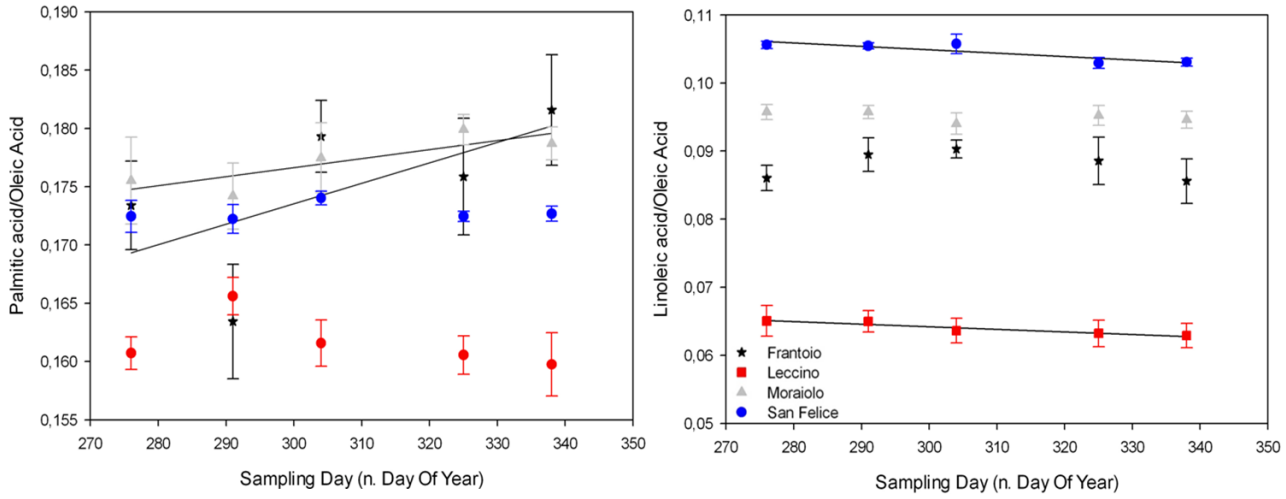
	Frantoio		Leccino		Moraiolo		San felice	
	JI	DOY	JI	DOY	JI	DOY	JI	DOY
C16:0	0.40	**0.64***	-0.01	-0.06	**0.61****	0.45	0.17	0.16
C16:1	0.16	0.21	0.38	**0.48****	0.34	0.47	**-0.81***	**-0.72***
C17:0	0.04	0.32	0.32	0.44	-0.15	-0.10	0.05	0.10
C17:1	-0.40	**-0.50****	-0.39	-0.36	-0.01	0.41	**0.51****	**0.49****
C18:0	0.36	0.43	-0.21	-0.26	-0.25	-0.30	-0.14	0.09
C18:1	0.19	0.23	**0.45****	**0.53****	**0.50****	**0.62****	-0.10	-0.17
C18:2	0.14	-0.02	-0.11	-0.17	0.45	0.19	-0.39	**-0.47**
C18:3	-0.01	0.04	-0.43	-0.44	-0.16	0.21	**0.52****	**0.58***

C20:0	**-0.56***	-0.41	**-0.47****	**-0.51****	-0.25	-0.03	0.32	0.24
C20:1	**-0.54****	**-0.82***	-0.36	**-0.56****	-0.35	-0.28	0.09	0.04
C22:0	**-0.48****	-0.16	-0.15	-0.17	**-0.57****	**-0.60****	-0.36	-0.24
C24:0	0.29	**0.51****	-0.32	-0.41	0.37	0.16	0.22	0.38

These data are shown in figure 16 (a ,b) for the main fatty acid in olive oil.

Figure 16. Palmitic/Oleic and Linoleic/Oleic acid composition of different cultivars during sampling date.

16a) 16b)



The highest oleic acid content (C18:1) was observed in Leccino olive oil with increasing trend during ripening (from 78.4 at green stage to 79.5 at full ripening), whereas San Felice variety showed the lowest one (oleic acid mean value = 74.8 %) with rather constant oleic acid content during ripening. Instead, palmitic acid did not show definite trends during ripening. The content of linolenic acid (C18:2) was stable during ripening for all cultivars. During ripening, the percentages of oleic acid accumulated in the oil were 0.3%; 1.2%; 0.9% for frantoio, leccino and moraiolo respectively, this correspond to a decrease for the carbon of 2.4‰, 2.9‰, 1.6‰ and for the oxygen 3.3‰, 3.2‰ and 2.8‰ for the same cultivar respectively. Therefore, the isotopic composition of olive oil is at least partially explained by the chemical composition of fatty acids, and physiologically related to processes of fruit ripening (Vogel, 1993; O'Leary, 1993). Some of the variation of the isotopic composition of the olive oil samples may be due to different olive variety and climatic and plant growth conditions.

Oleic and linoleic acid showed an opposite pattern between the different varieties,. In detail, oleic acid content was higher in Leccino and Frantoio, instead linoleic acid content was higher in San

Felice and Moraiolo. This result may be linked to a different enrichment in ^{13}C and ^{18}O and can explain the depletion in ^{13}C of some cultivar as Leccino. Moreover the effects of environment factors on the isotopic composition of plant organic material have revealed more significant trends.

Table 24. Correlation between isotopic and fatty acid compositions of oil from olive variety grown in Perugia experimental field. Pearson correlation coefficient, r. Significance level at p<0.01*, and p<0.05 **

	Frantoio		Leccino		Moraiolo		San Felice	
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$
C16:0	**-0.70***	**-0.58***	0.05	0.22	-0.34	-0.22	0.06	-0.06
C16:1	-0.17	-0.18	**-0.54****	**-0.47****	-0.24	-0.35	**0.64***	**0.48****
C17	-0.32	-0.32	**-0.53****	**-0.54****	0.11	0.09	-0.09	0.01
C17:1	**0.59***	0.37	0.29	0.31	-0.43	-0.35	**-0.50****	-0.43
C18:0	-0.38	-0.41	0.24	0.28	0.15	0.24	-0.17	-0.10
C18:1	-0.19	-0.28	**-0.57***	**-0.53****	-0.47	**-0.59****	0.16	0.03
C18:2	0.01	0.03	0.25	0.16	-0.13	-0.04	**0.47****	0.20
C18:3	-0.10	-0.05	0.38	0.44	-0.26	-0.20	**-0.52****	**-0.74***
C20	0.26	0.33	0.44	0.41	-0.08	-0.07	-0.14	-0.30
C20:1	**0.82***	**0.77***	**0.66***	**0.50****	0.22	0.44	0.13	0.22
C22:0	0.03	0.11	0.16	0.23	0.44	**0.53****	0.21	0.26
C24:0	**-0.55****	**-0.46****	0.39	**0.52****	-0.15	0.05	-0.23	-0.20

The correlation values presented in table 24 show that carbon isotope ratio was always more correlated with the same variable, than oxygen isotope. The results demonstrate the importance of elucidating the metabolism and biosynthesis of oil fatty acids, as determinants of $^{13}C/^{12}C$ discriminations observed, to better use the $\delta^{13}C$ values as tracers of different olive oil varieties characterized by specific metabolic pathway. In perspective, the study of isotopic composition of individual fatty acids in extra-virgin olive may be a more sensitive molecular trace of its climatic, genotypic and physiological characteristics.

6.3.4. Conclusions

The differences in carbon and oxygen isotopic composition were observed in olive oil due to olive variety and olive harvesting date.

Carbon and oxygen isotopic and fatty acid composition of monovarietal extra virgin olive oils microextracted from olives selectively collected from different varieties cultivated in the experimental field of Perugia and Orvieto were analyzed at different ripening stages. The isotopic variability obtained for all the varieties was considerable even if it is not so large as that obtained

from environmental factors (Portarena et al. 2014). Regression analysis was applied the obtained data in order to correlate isotopic values with the quantity of fatty acids components present in olive oils. According to the obtained results it was concluded that the carbon and oxygen isotopic composition of olive oil depends on olive variety and they are minimally related with the main fatty acids components. In particular both carbon and oxygen are positive related with stearic, linoleic and eicosenoic acid and negatively related with oleic and palmitoleic.

7. CONCLUSION AND PERSPECTIVES

In this PhD thesis the usefulness of carbon and oxygen stable isotope ratios as markers for extra-virgin olive oil authenticity was extensively studied and verified.

Firstly a dataset with the isotopic values of Italian extra virgin olive oils was created and analyzed, allowing the distinction of samples from different macro areas (mainly North versus South Italy). The isotope ratios of olive oils collected in nine Italian regions with different climatic characteristics (Lombardia, Liguria, Toscana, Lazio, Molise, Puglia, Calabria, Sicilia, Sardegna) were shown to be related respectively to geographical and climatic factors of the sites where the plants were grown.

Verification of a cultivar effect in the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ values and fatty acid composition of olive oils from the same experimental field made it possible to discriminate monovarietal samples. The multivariate statistical analysis subsequently used offered good varietal olive oil traceability. The model developed could be used to verify the authenticity of commercial monovarietal olive oil samples, becoming a tool for ensuring compliance with European law.

Furthermore, $\delta^{13}C$ was shown to be a reliable tool which can be used to characterized olive oil samples with different linoleic and oleic fatty acid composition. Further studies on the isotopic fractionation among the olive oil compounds may improve the efficiency of the geographical and cultivar characterization of olive oils.

The same isotopic and fatty acids variables were analyzed on Italian cultivars from two different experimental field in central Italy at different harvest dates, verifying the only isotopic negative regression during ripening degree of fruits. The statistical model developed allowed good differentiation of olive oil samples harvested at different maturation degree. In conclusion, olive oil traceability is gaining importance due to the development of olive oils with specific characteristics. Consumers demand high-quality olive oils, including olive oils with PDO labels or coupage/monovarietal olive oils with certain properties characteristic of the olive variety from which they are elaborated.

With a view to the future, it may be expected that these results will lead to the development of an analytical control procedure checking on the geographical provenance of olive oil products used in the food industry. The analytical approach could be applied to different premium products and it could be the premise for each traceability study.

Currently isotope ratios analysis definitely offers one of the most promising hypothesis-driven approaches for establishing the authenticity of premium products. This is further highlighted by the fact that in the last thirty years some official methods have been based on these parameters. These analytical approaches are based on large databases, in which the number of analyzed samples has to

be sufficient to truly reflect commercial samples. Furthermore, it is extremely important to evaluate the accuracy and precision of the measurements in order to verify whether the differences found can be deemed to be statistically significant or not. Finally, in the longer term, it is to be hoped that a greater understanding of how meteorological and geochemical signatures are transferred to premium products may reduce the need for expensive comparative databases of authentic commodities. Ultimately, this would allow the generation of isotopic maps for foods, in particular for olive oil, from different geographical locations, which could be incorporated into traceability systems. Overcoming the need for comparative databases in food provenance determination will be the next extremely challenging task.

The future perspectives of my work is determining natural stable carbon (C) and oxygen (O) isotope composition of individual fatty acids in olive oil. For this purpose compound specific isotope analysis (CSIA) by using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) can be applied. This technique is a capable analytical tool to study lipid metabolism of different cultivar in different areas of origin.

The existence of naturally different $\delta^{13}C$ values of individual fatty acids was a prerequisite for tracing metabolic pathways of fatty acids in plants due to isotope fractionation, occurring during the biosynthesis of fatty acids. These differences allowed to trace transformations of fatty acids metabolism in olive oil.

The use of Compound-specific Stable Isotope Analysis (CSIA) is increasing in many areas of science and technology for source allocation, authentication, and characterization of transformation reactions.

It is therefore expected that CSIA may contribute significantly in forthcoming studies to the characterization of geographical origin of food and in prevention of frauds in food industry.

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APPENDIX

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2 Portarena S. (2011). Authentication and traciability of Italian extra-virgin olive oil applying stable isotopes. *16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*. 353-354.

3 Gavrichkova, O., Proietti, S., Moscatello, S., Portarena, S., Battistelli, A., Matteucci, G., Brugnoli, E. (2011) Short-term natural δ13C and δ18O variations in pools and fluxes in a beech forest: the transfer of isotopic signal from recent photosynthates to soil respired CO_2. *Biogeosciences,* 8, 2833-2846.

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6 Portarena S. (2013). Enviromental, phenological and genotypic effects on carbon and oxygen isotopic fingerprinting of extra-virgin olive oil. *18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, 25-27 September, pp. 197-201.

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