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SHORT COMMUNICATION

The use of nitrogen stable isotope ratios to discriminate between organic and conventional olive cultivation

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ABSTRACT

Several studies have been conducted for the authentication of food products and the discrimination between conventional and organic vegetables from different geographical locations, but, the lack of information on the authenticity of olives according to the cultivation and fertilizer type makes its market sensitive to attempted frauds. In the present work, the difference and variation of nitrogen stable isotope ratios have been measured to assess olives authenticity with respect to cultivation practices.The results reported from this case study can be potentially applicable to discriminate between the use of natural organic or synthetic fertilizers in olive orchards.

Keywords: Isotopic ratio mass spectrometry; Nitrogen fertilizers; Olive drupes; Olive leaves; Organic production

INTRODUCTION

Oganic foods, not long ago, could only be found in dedicated stores; today, they are available in most supermarkets. Organic farming is a very fast growing sector of food industry: every year, the area of organic farmland in the European Union has increased by half a million hectares in the last decade. Today, over 186,000 farms are dedicated to organic farming. There are many reasons for choosing to consume organic foods: some people prefer their taste, others want to encourage organic farming practices and contribute to soil conservation, water saving and pollution reduction. In fact, chemicals, which are involved in the production and processing of conventional foods, are basis elements that differentiate organic foods (Schmidt et al., 2005; Laursen et al., 2013; Ehleringer et al., 2015). A food, to be recognized as "organic", must be produced respecting precise rules established in local regulations (Council Regulation (EC) No 834/2007) that describe the farming practices allowed in organic production and the control systems to be carried out in order to guarantee them. Synthetic nitrogen fertilizers are not allowed, however, to guarantee soil fertility, the crop rotation method is used which includes any application of fertilizer selected after inspection of authority (Organic Food and Farming Report, 2003). Fertilizers allowed include animal fertilizers and composting products of both plant and animal origin. The production of conventional foods takes place through agricultural practices that allow the use of many chemicals. These substances have an important impact on the environment, but not only, their residues in food products have dubiously effects on human health. Several studies have been carried out to encourage the use of organic foods, as they are safer and/or richer in nutrients; to date, the most exhaustive study conducted on organic and conventional foods has demonstrated that their nutrient contents are not significant (European Parliamentary Research Service, 2016). Although this study showed that organic food has a 30% lower risk of pesticide residues, it has also shown that residual levels in conventional foods were well below the safety limits established by law. However, many people who pay the huge premium, often more than 100% for organic foods, do so because they are afraid of pesticides (Rembiałkowska and Badowski, 2011; Yu et al., 2018). In the last few years, many cases of serious fraud were reported worldwide in the organic foods sector

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(Johnson, 2014), a scams system, certainly, encouraged by the growing demand for organic products but also lured by loans that the EU regularly bestows to encourage the development of organic farming. A certification center evaluates the complete traceability of organic products at all stages of their production, processing and marketing. However, there are no methods available for assessing the source of the nutrients in organic farming systems to manage the fertility of the soil. Precisely for this reason, when it is required, the soil can be enriched by applying synthetic nitrogen by irrigation; this type of fraud is very difficult to identify. Recent researches have demonstrated that the evaluation of the isotopic composition of nitrogen can be traced back to the presence of synthetic fertilizers (Choi et al., 2003; Ro and Lee, 2003; Russo et al., 2012). The possibility of using isotopes of nitrogen for the discrimination between conventional and organic crops is based on the hypothesis that the application of fertilizers synthesis would result in a lower value of the $\delta\ ^{15}N/^{14}N$ ratio, compared to that found in biological systems (Kohl et al., 1973; Freyer and Aly, 1974; Shearer et al., 1974; Kreitler, 1979; Yoneyama et al., 1990; Rapisarda et al., 2010; Camin et al., 2011; Russo et al., 2012). In the last ten years, considering the importance of the topic, several studies have been undertaken to authenticate food products and to differentiate foods obtained both traditionally and organically in different geographical areas (Benincasa et al., 2007; Rummel et al., 2010; Benincasa et al., 2014) by measuring the nitrogen isotopic ratio, but, to our knowledge, there is a lack of information on its application on olives grown using organic fertilizer from those grown using conventional N fertilizers. Therefore, in this study, nitrogen stable isotope ratios were measured to assess olives with respect to the cultivation and type of fertilizer used. Beyond collecting different types of olives from olive groves where different agronomic practices are carried out, samples of leaves have also been taken to study the effect of the fertilizers. The data obtained in this work can be used to create a preliminary useful database for the identification of organic olives.

MATERIALS AND METHODS

Olives and leaves sampling

For this research, four olive groves were selected containing "Carolea" cultivar olive trees. The experimental plots are all located on the Ionian coast of Crotone, one of the provinces of Calabria, a southern Italian region. The first site (Site 1), facing south at 60 meters a.s.l., is situated on the countryside of Strongoli town, placed between hills (39° 16' 0" N, 17° 4' 0" E). The pedogenetic substrate consists of coarse reddish brown sediments of continental origin, which rest, on silty clay formations from Pliocene era. Due to the intense processes of leaching of the clay itself, the sandy loam of the epipedon becomes clayey. This process is associated to a strong biochemical alteration with a relatively high content of iron and aluminum sesquioxides (fersiallitization process). Clay migration is favored by the absence of carbonates and by the unsaturation of the exchange complex, which allows deflocculation and transmission of the same in the aqueous medium. Soils are deep with a common skeleton well drained, but locally, problems of hydromorphy, due to the suspended stratum that forms on the argillic horizon, can occur. From the chemical point of view, soils are characterized by subacid reaction that, locally, can become acidic due to stronger leaching. In these soils, organic residues undergo rapid oxidation and. In particular, the physico-chemical characteristics average value of the top-soil are the following: Clay 19.32 %; Total Sand 60.55 %; pH (H_2O) 6.86; Effervescence 0.86; Organic Substance 1.18%; Conductivity 0.17 mS/cm; Cation Exchange Capacity (CEC) 13.88 meq/100g and Density 1.29 g/cm^3. The second site (Site 2), placed between hills at 500 meters a.s.l., is situated on the countryside of Cotronei town (39° 10' 0" N, 16° 47' 0" E). The vegetation, consisting of Mediterranean maquis with a prevalence of oaks, guarantees, in most cases, the slopes stability. However, serious erosion phenomena are evident in the areas affected by fires and where extensive olive groves. The substrate consists of sandstone, generally calcareous, from Miocene era. The weaving is frank sandy with common skeleton. The soils have low cation exchange capacity and a limited water reserve. From the chemical point of view, they are characterized by the presence of carbonates and the reaction from sub-alkaline to alkaline. In particular, the physico-chemical characteristics average value of the top-soil are the following: Clay 16.68 %; Total Sand 57.92 %; pH (H_2O) 7.41; Effervescence 1.03; Organic Substance 2.81%; Conductivity 0.19 mS/cm; CEC 18.69 (meq/100g) and Density 1.09 g/cm^3. The first site hosts an olive grove that has never received any kind of fertilizer and it is far from any pollution source (level code 1). The second site, instead, hosts three olive groves. The first olive grove received, for the last two years, a conventional fertilizer (level code 2). The second olive grove received, for the last three years, an organic fertilizer and a copper treatment (level code 3) and, to increase the availability of nitrogen to the plants, leguminous plants were used. The third olive grove receive, each year, a conventional fertilizer (level code 4). From each experimental site, 100 olive drupes and 100 leaves were randomly handpicked from all possible position of twenty trees and immediately brought to the laboratory for sample preparation. Eighty olive drupe samples and eighty olive leaf samples were in total collected, processed and analyzed, each one, three times.

Sample preparation for $\delta^{15}N$ determination

Olive drupes and leaves, from the year crop 2016, immediately after being harvested were freeze dried and grounded, left over night in an oven at 50 °C to facilitate the removal of humidity and stored in light protected plastic flasks until analysis. Considering that an organic solid sample should usually contain 20-150μg of N to obtain reliable results, for the analysis of $\delta^{15}N_{AIR}$ about 2 mg of homogenized pulverized olive drupe samples were weighed directly into tin capsules (3.2 x 4 mm; Thermo Fisher Scientific, Bremen, Germany) and placed in the autosampler for EA-IRMS analysis.

Determination of $\delta^{15}N$ by EA-IRMS

Nitrogen stable isotope compositions ($\delta^{15}N$) were determined by isotope ratio mass spectrometry (IRMS) by mean of a Delta V plus instrument (ThermoFinnigan; Bremen, Germany). Samples were analyzed in triplicate by combustion in a Flash EA 2000 Series elemental analyzer. For nitrogen, the three Faraday cups are arranged to capture isotopologues of m/z 28, 29 and 30 ($^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$). Once the isotope ratios ($^{15}N/^{14}N$) of the samples have been calculated, further data processing is required to report these ratios relative to ratios derived from standard reference substances. This relative deviation is expressed using the isotope delta (δ) notation, described for the first time, for C, by Urey (Urey, 1948) and adapted for other elements by Brand and Coplen:

$$\delta^i [‰] = [(R_i - R_{std})/(R_{std}) - 1]$$

where ^{i} is the mass number of the heavier isotope (^{15}N); R_i and R_{std} are the respective isotope number ratio of the sample and of the international recognized reference materials (Air for N_2), respectively.

To obtain accurate isotope ratios is fundamental the isotopic calibration through the measurement of the ion current ratios relative to that of an international reference standard. The working standards used for the quality control of the EA-IRMS measurements was IAEA-N1 (International Atomic Energy Agency; Vienna, Austria), compositionally similar to our samples. The analytical precision achieved was ± 0.02‰. In addition, the long-term reproducibility (± standard deviation) was ± 0.06‰. Thus, the ^{15}N enrichment in the samples were expressed against working in-house standard (urea) calibrated against the international reference material IAEA-N1. In particular, on the value corresponding to the reference standard, a dilution factor of 2000 have been applied in order to be close enough to the peak observed for the sample.

Statistical analyses

One-way analysis of-variance (ANOVA) and post-hoc Fisher comparison test were performed on nitrogen stable isotope composition data. The ANOVA and Fisher tests were carried out to determine whether the isotope ratios ($^{15}N/^{14}N$) of the samples were specific to cultivation and fertilizer practices. The statistical treatment was performed by using the statistics program STATGRAPHICS Plus Version 5.1 (Statistical Graphics Corporation, Professional Edition - Copyrigth 1994-2001).

RESULTS AND DISCUSSION

During the chemical and physical transformations, in plant material, a process called isotopic fractioning takes place which leads to a variation of the isotopic composition of light elements contained therein. The latitude, as well as the geographical origin, is among the most dominant factors of this process. Nitrogen is one of the main elemental constituents in plant material having two different stable isotopes. The mean natural abundance of these isotopes, ^{15}N and ^{14}N, in respect to the international reference standard Air (molecular air nitrogen) is 99.63% and 0.37%, respectively. Several processes, both biological and geophysical, can alter the ratio of two isotopes ($\delta^{15}N$); the study of these alterations can lead to different conclusions (Schmidt et al., 2005; Sturm et al., 2011; Laursen et al., 2013). In nature, molecular nitrogen of the air is transformed through physical processes and through the activity of microorganisms. The forms present and available in the soil are nitrate and ammonia (inorganic nitrogen) and amino acids and proteins (organic nitrogen). However, soil depth, vegetation and climate affect the values of $\delta^{15}N$ in the ground. In fact, a land with high slope depresses the denitrification process leading to a depletion of the isotope ^{15}N. On the contrary, water stress and proximity to the sea (Heaton, 1987) involve an enrichment of the isotope ^{15}N in the soil. The stable isotopic ratio value of nitrogen in plants is between -0.28‰ and 7.1‰ (Camin et al., 2007; Moreno-Rojas et al., 2008). No significant difference were noticed between C3 and C4 plants (Hübner, 1986; Heaton, 1987). In cropland, the content of ^{15}N is influenced by the fertilization practice. An intensive use of organic fertilizers brings an ^{15}N enrichment in the nitrogen compounds of the soil which values are in a range between +2 and +30 ‰ (Kreitler and Jones, 1975). This very large range of values of $\delta^{15}N$ is due to the different origins of organic fertilizers (Bateman et al., 2007; Flores et al., 2007; Camin et al., 2007; Rogers, 2008). Artificial fertilizers, instead, produced by the fixation of atmospheric nitrogen (urea,

ammonium and potassium nitrates), have values of $\delta^{15}N$ comprised in a narrow range between -4 and +4 ‰. Leguminous and nitrogen tackers plants, such as alfalfa, more prevalent in less fertilized soils, use, as a source of nitrogen, the nitrogen from the air isotopically poorer and thus with lower values of ^{15}N, around 0 ‰ (Yoneyama, 1995). The $\delta^{15}N$ values measured in the monovarietal olive drupe and leaf samples analyzed in this study are listed in Table 1.

Each value represents the average of twenty samples composed of 100 olive drupes and 100 olive leaves handpicked randomly from all possible position of twenty trees, extracted and analyzed in triplicate with the standard deviation (*SD*) and the standard error of the mean (*SEM*). *Sample 1* and *Sample 3*, both olive drupes and leaves, showed a natural enrichment (positive values of $\delta^{15}N$) of heavy nitrogen (^{15}N), whereas *Samples 2* and *Sample 4* showed a pauperization of ^{15}N (negative values of $\delta^{15}N$). The mean $\delta^{15}N$ values of the olive drupes of *Sample 1* and *Sample 3* were 2.103 ± 0.238 ‰ and 1.857 ± 0.272 ‰, respectively. The mean $\delta^{15}N_{AIR}$ values of the olive leaves of *Sample 1* and *Sample 3* were 2.016 ± 0.406 ‰ and 1.900 ± 0.220 ‰, respectively. The mean $\delta^{15}N$ values of the olive drupes of *Sample 2* and *Sample 4* were -2.814 ± 0.592 ‰ and -4.994 ± 0.802 ‰, respectively. The mean $\delta^{15}N$ values of the olive leaves of *Sample 2* and *Sample 4* were -2.150 ± 0.421 ‰ and -2.506 ± 0.661 ‰, respectively. *Sample 1* and *Sample 3* did not showed a statistically significant difference, whereas a statistical discrimination was possible for all the other cases [(One-way ANOVA and post-hoc Fisher comparison test ($p < 0.01$)]. The Box-and-Whisker plot of the observed $\delta^{15}N$ values, in the analyzed samples, in respect to the different cultivation practices (*level code 1 - 4*) are showed in Figure 1.

For olive drupes, the $\delta^{15}N$ values of *Sample 1* (range between 1.582 and 2.780 ‰) and *Sample 3* (range between 1.251 and 2.488 ‰) resulted less dispersive than the $\delta^{15}N$ values of *Sample 2* (range between -4.348 and -1.699 ‰) and *Sample 4* (range between -7.103 and -3.567 ‰). The same trend was observed in olive leaves, where the $\delta^{15}N$ values of *Sample 1* (range between 1.150 and 2.674 ‰) and *Sample 3* (range between 1.074 and 2.349 ‰) resulted less dispersive than the $\delta^{15}N$ values of *Sample 2* (range between -2.921 and -1.472 ‰) and *Sample 4* (range between -3.669 and -1.304 ‰). The $\delta^{15}N$ measurements obtained in this study were in agreement with what has recently been observed in other crops. In particular, the $\delta^{15}N$ values of the organic grown crop samples were higher compared to those of conventional grown crop ones. In fact, olive trees of *Sample 1,* that never received any kind of fertilizer and it is far from any pollution source, can be compared with the olive trees of *Sample 3* that, on the other hand, received for the last three years an organic and cupper treatment. $\delta^{15}N$ values of *Sample 2,* whose olive trees received for the last two years a conventional fertilizer treatment, instead, showed a similar trend than the $\delta^{15}N$ values of *Sample 4*, whose olive trees are treated each year conventionally. Although this study did not take into account $\delta^{15}N$ information of the soil and applied fertilizers, the isotopic composition of nitrogen obtained from the analysis of four thesis have shown that it is possible to distinguish organic from conventional crops.

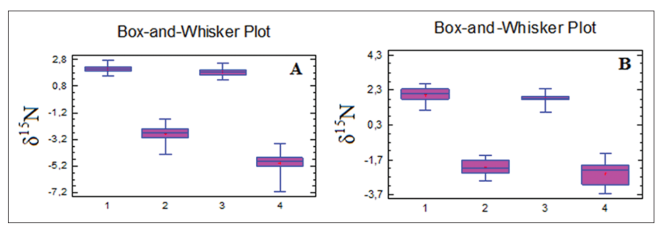

Fig 1: Box-and-Whisker plot for the values of $\delta^{15}N$ observed in the olive drupes (A) and olive leaves (B) analyzed corresponding to the different cultivation practices (level code 1 - 4). For each level code, a rectangle, the "box", is divided into two parts, from which two segments exit. The "box", divided inside by the median, is delimited by the first and third quartiles. The "whiskers" are the segments delimited by the minimum and maximum values.

Table 1: δ15N values observed in monovarietal olives drupes and leaves from Carolea cultivar grown in Calabria in the Ionian coast of Crotone

Samples	Town	Cultivation practice	Olive grove site	Mean value	$\delta^{15}N$		SDZ	SEM
					Min	Max		
Sample 1 drupes	Strongoli (KR)	Organic	Site 1	$2,103^{A}$	1,582	2,780	0,238	0,031
Sample 1 leaves				$2,016^{A}$	1,150	2,674	0,406	0,054
Sample 2 drupes	Cotronei (KR)	Conventional	Site 2	$-2,814^{B}$	−4,348	−1,699	0,592	0,076
Sample 2 leaves				$-2,150^{B}$	−2,921	−1,472	0,421	0,054
Sample 3 drupes	Cotronei (KR)	Organic	Site 2	$1,857^{A}$	1,251	2,488	0,272	0,035
Sample 3 leaves				$1,900^{A}$	1,074	2,349	0,220	0,028
Sample 4 drupes	Cotronei (KR)	Conventional	Site 2	$-4,994^{C}$	−7,103	−3,567	0,802	0,104
Sample 4 leaves				$-2,506^{C}$	−3,669	−1,304	0,661	0,085

CONCLUSIONS

Organic grown products have higher prices on the market because they are safer for consumers and the farming practices are more environmentally friendly. On the other hand, organic fruits and vegetables are, very often, subject to frauds. In fact, false labels classify foods as "organic" when they were cultivated with synthetic fertilizers. Fraud has a very important financial cost, undermines customer and consumer confidence and in the most serious cases can negatively impact consumers' health and well-being. The identification of mislabeled fruit and vegetables represents a challenge as laboratories need techniques that identify fruits and vegetables grown using organic fertilizers and synthetic fertilizers with full confidence in results. The data obtained in this study represent an application to the case of olive tree and allowed us to conclude which olives were grown using organic fertilizers and which ones were grown using synthetic ones by measuring the isotope composition of nitrogen. The measurement of N isotope composition may be suggested as a potential marker to evaluate olive drupes authenticity (e.g., agricultural practice) in order to confirm the possibility of characterizing organic olives.

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