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Brief Introduction About Interfering Masses Correction on IRMS Measurements and Considerations on the Workshop “Expression of Isotopic Results, Calibration, Internal and International Standards, Accreditation and Methodologies”, Parma, 2017

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At the GRITIS meeting held in Parma in May 2017, the communications presented concerned both analytical methods - particularly with regard to calibration lines - and examples of applications of stable isotopes to the environment, food and archeology. In particular, the discussion turned to problems concerning the use of standards, calibration and evaluation of analytical uncertainty. In the light of what emerged from the discussion held at that meeting, it is considered useful that GRITIS defines minimum criteria regarding the use of standards, calibration and assessment of analytical uncertainty and invites its members to adapt to these minimum criteria. Although the definition of such minimum criteria may seem useless for those who have sufficient analytical practice, it is nevertheless believed that it may be useful for technical laboratories which for structural or economic reasons can not afford sufficient meditation on such problems.

In this brief report, the minimum criteria used at the Isotopic Geochemistry Laboratory of the University of Parma are presented. They could be a starting point for proposals that could be formulated by a small GRITIS commission and transmitted to all members for additions and comments.
Hydrogen and Oxygen Stable Isotopes to Trace Ecosystem Water Fluxes: Interdisciplinary Challenges and New Opportunities

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Achieving a detailed understanding of the factors controlling the transport and the availability of water in ecosystems in the current context of global climate change and population growth is becoming increasingly important to address key environmental and social problems linked to ecosystem services. This is a critical point to obtain robust predictions of changes in green and blue water quantity, and to promote long-term sustainability of water resources management. The stable isotopes of hydrogen and oxygen are an effective investigation tool to trace water within the hydrological cycle, and have been intensively used in hydrological, ecological and physiological applications in the last decades. Indeed, as an example, a search on the Scopus database querying the terms “hydrogen oxygen isotope plant” in the title, abstract and keywords returned 356 papers published between 1950 and 2017, with a sharp increase in the last decade (Fig. 1). New advancements in isotope-based techniques have recently fostered interdisciplinary research on water movement in ecosystems. However, despite the amount of work that is being currently conducted on this topic, there are still unsolved issues that can affect our interpretation of isotopic data and hamper a thorough conceptualization of the complex functional interrelations between water in soil, vegetation and the atmosphere. These issues were intensively discussed at the workshop on “Isotope-based studies of water partitioning and plant-soil interactions in forested and agricultural environments” held at Villa Montepaldi, San Casciano in Val di Pesa, Florence, Italy on 27-29 September 2017. The workshop attracted scientists with different background (hydrology, soil science, plant physiology) from 12 countries (Fig. 2) who use stable isotopes of hydrogen and oxygen to study water movement through ecosystems. The workshop aimed at sharing experiences and advance knowledge about the hydrological and ecophysiological mechanisms that regulate the water flux exchange in different environments, by exploiting interdisciplinary perspectives on major obstacles (and their potential solutions) in applying isotope analyses in ecohydrological studies. Here we summarize the main themes that were addressed during the workshop discussions, identifying knowledge gaps and trying to convert them into new interdisciplinary research opportunities that can pave the way towards a better understanding of the physical processes governing water flux in natural and anthropic environments.
Fig. 1. Number of papers published between 1950 and 2017 on hydrogen and oxygen isotopes in plants from the Scopus database.

Fig. 2. Provenance of workshop participants
PL3

Application of Stable Isotope Ratios in Wine Official Control: ICQRF-Laboratory Catania’s Activity

Grazia Laura Gambino, Rosario Caruso, Mirella Fiorillo, Maria Gargano, Leonardo Sabatino, Monica Scordino, Pasqualino Traulo, Giacomo Gagliano

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Measurement of stable isotope ratios of H, C and O for grape products has been adopted as the official method by European Union (EU) regulations and by the OIV (The International Organization of Vine and Wine) in order to detect the addition of water and sugar to oenological products as well as mislabelling. It is based on comparison of sample data with that obtained for authentic products with the same origin - European Wine Databank established by UE Reg. n. 1306/2013, 273/2018, 274/2018. While IRMS provides $^{18}\text{O}/^{16}\text{O}$ and $^{13}\text{C}/^{12}\text{C}$ isotopic ratios determination, SNIF-NMR for (D/H)$_I$ and (D/H)$_II$ deuterium/hydrogen evaluation. In this work, a general overview of the most frequent fraud detected in the last 5-years with stable isotopes during Italian official control is reported.

Samples collection. Oenological samples (i.e. wine, grape juice, must concentrate, vinegar) have been collected in Italy regions according an official sampling program promoted by the Italian Ministry of Agriculture from 2013 to 2017.

Samples analyses. Wines were direct distilled to obtain the ethanolic fraction. Sugars contained in musts were fermented and converted in ethanol, which is subsequently extracted after a distillation process. The resulting ethanol was then analyzed via IRMS for carbon and SNIF-NMR for hydrogen isotope ratios respectively [1, 2]. Isotopic $^{18}\text{O}/^{16}\text{O}$ ratio was determined in must and wine after 24 hours equilibration time with reference CO$_2$ from a mixture He/CO$_2$ (99.6/0.4%) at 28°C as described in the OIV-MA-AS2-12, by using a Gasbench II (Manchester, UK) connected to the IRMS Delta V IRMS Thermo Scientific (Bremen, D). Isotopic $^{13}\text{C}/^{12}\text{C}$ ratio was obtained by a Delta V IRMS spectrometer (Thermo Fischer, Bremen, Germany) coupled with a Conflo IV gas controller system (Thermo) and an elemental analyzer EA Flash 2000 (Thermo Scientific).

Deuterium/Hydrogen ratios were determined through an FT-NMR Avance II 400 UltraShield NMR (Bruker Corporation), probe SEX 400 MHz S2 10 mm spinners for $^2$H-NMR lock $^{19}$F spectra registration. The $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ variables are expressed in delta notation, in which stable isotope abundance is expressed relative to a standard, Vienna – PeeDee Belemnite for $\delta^{13}$C and Vienna – Standard Mean Ocean Water for $\delta^{18}$O. (D/H)$_I$ represents the ratio between deuterium and hydrogen of the methyl group in alcohol and(D/H)$_II$ is the corresponding ratio in the alcohol methylene group. (D/H)$_I$ and (D/H)$_II$ values are expressed in ppm.

In Figure 1 are reported the data of isotopic control performed on a total of 785 samples of wine products among the last 5 years. The increase of 150% of the samples analysed from 2013 to 2017 shows an increasing involvement of the ICQRF regarding isotopic analysis in the wine sector controls. Samples not compliant represent about 15%. The distribution of detected frauds during the investigated period show a prevalence of watering with an average of 34%, followed by 16% of sugaring with a decrement in the last two years. Incorrect geographical and vintage origin represent the lower incidence (about 2%).
Fig. 1. Isotopic control in oenological field among the years 2013-2017 (a). Distribution of not compliance among isotopic parameters among the years 2013-2017 (b).

References:
Geographical Characterization of Italian Garlic Using Stable Isotope Ratio Analysis

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Garlic (Allium sativum L.) is a component of lots of cuisines all around the world, including Italy. It contains water, carbohydrates, proteins, free amino acids, fibres and, last but not least, organosulfur compounds, such as allicin, which gives garlic its characteristic pungent flavour. These compounds are related to garlic beneficial effects on health, such as cardiovascular and respiratory benefits; this vegetable is as well considered to have antioxidant, antimicrobial, anticarcinogenic and antimutagenic properties [1,2].

Garlic is produced in several areas of Italy, with an average production of approximately 30.000 tons per year. As demand does not match supply, Italy also imports a large amount of garlic (30.000 tons more), mainly from Spain (50%), Holland (13%) and China (11%). As the price and the quality of the Italian products are higher [3] than the other suppliers, it is important to develop analytical tools able to characterise the Italian garlic and discriminate the imported products.

The use of stable isotope ratio analysis is showing an increasing significance in characterising the geographical origin of different vegetables, including tomatoes [4], lettuce [5], peppers [5], potatoes [5,6], as well as cereals [7], lentils [8] and so on. As for garlic, a previous work carried out in Slovenia showed the possibility to discriminate different varieties of garlic with diverse provenance on the base of stable isotope ratios [9].

In this preliminary work we analysed 65 samples of garlic, coming from different regions of Italy (Sicily, Abruzzo and Lazio). The aim of the study was to define the isotopic composition of Italian garlic, verifying the possibility to discriminate the considered regions. We focused on δ13C, δ15N, δ34S, δ2H and δ18O values and we found that all of them, and particularly δ2H and δ34S, are able to differentiate the production areas.

References:

Isotope Ratio Mass Spectrometry (IRMS) is commonly recognized to be able to provide information about the geographical, chemical, and biological origins of substances on the bases of isotopic abundances of the elements that comprise the material. Commonly a separation is performed prior to isotope ratio analysis using LC or GC techniques. However, for highly complex samples a single chromatographic step could not be effective for the complete purification of target components. Multidimensional chromatographic approaches could enhance the purification power of specific fractions before the detection. LC coupled to GC is a powerful technique when a pre-separation step is required aiming to remove non-volatile components or/and to reduce the complexity of a sample. The triglycerides analysis is often used for the detection of the adulteration of olive oil. The profile is characteristic for each kind of oil, as it depends on the fatty acid composition and on the biosynthesis rules, so that it can be used to check for the presence of extraneous oils. However, the quality of extra-virgin olive oil greatly depends also by the plant cultivar and geographical origin. Applied to olive oil triglycerides, the RP-LC step provides the pre-separation of the fractions based on the partition number (PN) depending on the total carbon number subtracting two for each double bond. The resulting fractions show a greatly reduced complexity avoiding coelution problems respect to direct GC analysis. The present work deals with the development of an LC-GC-MS/IRMS prototype characterized by the improved resolution of the heart-cut mode. Two different chromatographic mechanisms were employed with simultaneous qMS and IRMS detection. After the LC pre-separation, fast GC was applied for the separation of each fraction transferred before the IRMS/qMS step providing qualitative and isotopic ratio information ($\delta^{13}$C). Different origin and cultivar olive oils were analyzed and the results are here presented.
Consumers are increasingly interested in health food, dietary supplements and natural compounds, which command a higher price and are therefore subjected to adulteration. Methods for testing authenticity of these products are therefore required.

Focus of the study is on the development of H, C and O stable isotope ratios analysis methods for this purpose. Analysis of the isotopic ratio of C, O and H in the bulk samples was performed using an isotope ratio mass spectrometer interfaced with an elemental analyser and a pyroliser. Moreover, compound specific analysis of the $^2\text{H}/^1\text{H}$, and $^{13}\text{C}/^{12}\text{C}$ was done using gas chromatography-combustion-pyrolysis-isotope ratio mass spectrometry (GC-C\Py-IRMS). Stable isotope ratio of C can distinguish olive oil squalene from shark squalene, Rosa damascena essential oil from Palmarosa oil, Monacolin K naturally present in Red Yeast Rice from the prescription biosynthetic Lovastatin. Moreover it allows to differentiate natural Vitamin C, Vanillin and Caffeine from the synthetic ones. Stable isotope ratio of H discriminate natural from synthetic Curcumin, and in combination with the stable isotope ratio of O, authentic Serenoa oil from the adulterated one. The stable isotope ratio analysis can be used for verifying the authenticity of health foods, dietary supplement and natural compounds commanding higher price.
The Use of Stable Isotope Ratio Analysis to Characterize
Saw Palmetto (Serenoa repens) Extract

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Saw palmetto extract (SPE) is a nutritional supplement obtained from the fruit of the saw palmetto, a shrubby palm that grown in the southeastern United States. It was proved that SPE has multiple pharmacological effects, such as: digestive, diuretic, reproductive and anti-inflammatory. (Bennett & Hicklin, 1998) (Sultan et al., 1984). As consequence, the request of SPE has steadily increased, but this increasing demand has not been matched by the availability of berries on the market (GIR GlobalInfoResearch, 2017). Indeed, saw palmetto grows in a specific area (Florida peninsula and Georgia), often subject to environmental factors which can reduce crop yield or destroy the harvest (Carrington et al., 2001). That explain the substantial increase in raw material cost and consequently the diffusion of counterfeit SPEs. To meet the high request, adulterated SPE products are put on the market. The adulterated products are obtained diluting the authentic ones with a specially formulated blend of lower-cost vegetable oils or lipids, probably of animal origin, in an attempt to emulate the authentic fatty acid profile (Gafner & Baggett, 2017). Therefore, the current analytical methods verifying the fatty acid profile or the content of specific components such as β-amyril, β-sitosterol are not enough to ensure detection of this adulteration.

To our knowledge, stable isotope ratio analysis has not yet been used to characterise and protect authentic SPEs. Analysis of the H, C and O stable isotope ratios of bulk and of the H and C stable isotope ratios of individual fatty acids, sometimes combined with the fatty acid profile, has proven to be a powerful tool for protecting high-quality oil from adulteration, because it allows identification of the origin of the specific component, whether natural or coming from other sources (Osorio et al., 2014; Paolini et al., 2017; Spangenberg & Ogrinc, 2001; Spangenberg, 2016).

In this work, bulk and fatty acid isotope ratio analysis was performed in 20 authentic and 9 commercial SPEs, 12 meat fats and 4 pure fatty acids in order to investigate if the isotopic analysis can be applied in SPE authentication. The study defined the variability range of δ¹³C, δ²H and δ¹⁸O.
Stable isotope analyses held enormous potential as traceability markers of a vast variety of food products (Camin et al., 2017). This also occurred for extra virgin olive oil, since stable isotope analyses determined on oil bulk have allowed sample differentiation on the basis of geographical origin (Portarena et al., 2014; Camin et al., 2016; Portarena et al., 2017). However, the bulk isotope composition of olive oil represents an average of a relatively large number of isotopically heterogeneous compounds that are differently affected by genotype × environment interaction (Portarena et al., 2015). In this work, δ13C values on the main olive oil fatty acids (FAs) (palmitic, stearic, oleic and linoleic) have been measured from 80 monovarietal EVOOs, similarly grown and harvested at five different olive maturation stages. By collecting oils at a common site, we attempted to minimize first the effects of climate, allowing potential genotypic patterns in the δ13C of FAs to be expressed more strongly. At the same time, the five different olive ripening stages, collected per each cultivar, allowed to link FA δ13C with the seasonal climatic gradient (increasing precipitation and decreasing temperature). The results showed that climatic parameters resulted the main drivers affecting FA δ13C variability during oil accumulation. However, there was a non-homogeneity in carbon isotopic fractionation processes in the different compounds in relation to both genotype and climatic changes during oil accumulation. Oleic acid is the main oil compound produced by olive plants during all harvest season and its isotopic composition was affected, with more extent than other FAs, by seasonal climatic gradient. In particular, its δ13C values resulted mainly dependent on the amount of rain, which is the main water source available for the plants. At the start of oil accumulation, oleic acid showed the highest 13C enrichment in all cultivars. This could be attributable to the use of stored 13C enriched carbohydrates that plant used for oleic biosynthesis. In fact, during the late summer–autumn period, under low rainfall and increased air temperatures, the plant photosynthetic activity was limited and the concentration of leaf soluble sugars at canopy level was low. Indeed, plants could potentially biosynthesize oleic acid mobilizing storage carbohydrates to satisfy the continued metabolic demand (Ryan 2011, Silpi et al. 2007). Proceeding the oil accumulation, the observed oleic 13C depletion reflected the physiological responses to environmental variations. On the contrary, the isotope fractionation relative to palmitic, stearic and linoleic biosynthesis during the ripening season, implied lower isotope discrimination. In addition, the δ13C values of linoleic acid varied among the studied cultivars appearing as a useful tool for studying inter-varietal differences in olive oils. Selecting the specific oil compounds that could provide a more direct link to environmental or genotypic factor will enhance the sensitivity of fingerprinting approach in olive oil traceability studies.

References:


In recent years there has been a growing demand for organic products as consumers consider them as safer and healthier than conventional products. Various researches have been carried out over the years to investigate the authenticity of products obtained using organic cultivation methods. Some studies have focused on the identification of new quality "markers" that allow to differentiate, from field to fork, the organic product respect to the conventional one. In particular, the monitoring of some chemical components, deriving from primary and/or secondary metabolism of organic and conventional products, has highlighted the diversity induced by the two production techniques. It is now well known that the difference in fertilization practices of the two cultivation methods influences the isotopic distribution of some elements present in fruits and vegetables, with particular reference to nitrogen. The possibility of using the $^{15}\text{N}/^{14}\text{N}$ ($\delta^{15}\text{N}$) isotopic ratio as a screening tool for the differentiation of biological products from conventional ones is based on the fact that $\delta^{15}\text{N}$ values of synthetic fertilizers are close to 0 ‰, thus determining higher $\delta^{15}\text{N}$ values of nitrogen metabolites of the organic crops respect to those of crops obtained by conventional agronomic practices.

The INNOVABIO ('Application of innovative methods for the traceability of organic farming products') research project (Italian Ministry of Agricultural, Food and Forestry Policies n. 93173/12/22/2017) aims to improve the understanding of the factors influencing the food quality of organic horticultural products, with particular reference to their differentiation respect to conventional ones and to the traceability from field to fork.

The aim of the project is the implementation, for biological horticultural crops, of a chemical and chemometric investigation system that allows, through the acquisition of isotopic data and other chemical and biochemical parameters, to discriminate between productions obtained with synthetic fertilizers, usually employed in conventional agriculture and not allowed in organic farming, and productions obtained using the organic cultivation method, which involves only the use of organic fertilizers and the application of agronomic methods for soil fertility such as rotations and the introduction of agroecological service crops and legume species. Horticulture has the advantage, compared to fruit growing, of not having pluriannual influences; for this reason, this research will give more immediate results than pluriannual cultivation systems. Multivariate analysis of isotopic data combined with classical quality parameters (macro and micro elements, total nitrogen, inorganic nitrogen ($\text{NO}_3^-$, $\text{NH}_4^+$), ascorbic acid, total polyphenols, antioxidant activity), performed using the "Linear Discriminant Analysis" and the "Partial Least Squares Discriminant Analysis", will give the possibility to discriminate the employed nitrogenous source and the consequent effect on the qualitative parameters which contribute to differentiate organic products from conventional ones. The horticultural crops which will be investigated are the most representative of Italian horticulture (tomato, fennel and cauliflower) and the experiments will be carried out in three different production areas, Vittoria (RG), Metaponto (MT) and Monsampolo del Tronto (AP). Using the existing experimental long-term field devices MOVE LTE and MITIORG, we will quantify the variations of $\delta^{15}\text{N}$ induced by the combination of the different fertilizations together with the use of legumes in the organic production system; evaluating how the agroecological management of the soil influences, in comparison with the traditional organic based system, the soil $\delta^{15}\text{N}$ in the various compartments of the plant.
Measurement of Challenging Matrixes Using the IsoFLOW Headspace Analyser

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The measurement of stable isotopes of challenging samples is made easy using the clean-up and sample processing capacity of the new isoFLOW sample preparation unit. The capability of the system to deal with any type of interference such as ethanol vapours of wines and spirits, as well as VOC’s from fruit juices will be demonstrated. The analysis of headspace samples of nonpure carbonates poses a serious challenge and high precision analysis are really only achieved using dual inlet preparation of the samples. The isoFlow demonstrates a unique capacity to deliver near dual inlet performance using the automatic acidify and purification capabilities of the isoFLOW.
PL5

Chromatography-Based EA-IRMS: Redesigning the Combustion Elemental Analyzer Around Modern Chromatographic Principles

Guido Giazzi, Andreas Hilkert
Thermo Fisher Scientific

The elemental analyzer (EA) was invented by Justus Liebig in 1830 and is deeply rooted in analytical chemistry, but the steps to make it an analytical tool for biology and geochemistry came in 1968, when Carlo Erba company replaced trapping with isothermal gas chromatography using a packed GC column, and 1980, when Tom Preston put a Carlo Erba EA onto an IRMS, inventing "continuous flow-IRMS". The technique was rapidly adopted and the work flow was extended from N to C and then S as well as to H and O. In 2016, Thermo Scientific introduced the EA-IsoLink, a revolutionary change to the elemental analyzer, where every component has been examined and either optimized or redesigned, from the auto-sampler though to the TCD. The Dumas combustion products are resolved on a GC column using variable helium flow rates and temperature ramping, and for the first time, chromatographic terms (e.g. baseline and resolution) are rigorously defined. The result is improved chromatographic performance which leads to improved isotope ratio precision for every mode of measurement and for every sample size, while at the same time improving throughput and greatly reducing helium consumption. Concrete applications of EA-IRMS will be presented.
PL6

Timber Isoscapes. A Stable Isotopes Approach to Fight Illegal Logging

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²Stable Isotopes Center, Institute of Biosciences, São Paulo State University (UNESP), Botucatu Campus, Brazil

Spatial interpolation provides a powerful tool for estimating the value of a variable where data are not available by generating a regular grid surface that takes into account the spatial variability of the data. The word isoscapes come from “isotope” and “landscapes”, thus defining the geospatial predictive power of stable isotopes. Among the various interpolation methods, kriging is a generic term for several techniques that provide best linear unbiased predictions (BLUP), best in the sense of minimum variance [1].

Illegal logging is a major cause of deforestation in many countries and has significant impacts on local communities and biodiversity. Techniques for tracing timber would provide a useful tool to protect local timber industries and contribute to the fight against illegal logging [2, 3].

This study reports accurate spatial distribution of δ¹⁸O and δ²H in timber from north-eastern Italy in order to trace geographical origin and prevent illegal logging [4].

We believe that in the future will be possible to apply these isoscapes effectively on a continental or global scale to identify the likely area of origin of wood, thereby contributing to combatting illegal logging.

References:
Climate change may turn cold biomes from sinks to sources for CO\(_2\) depending on the balance between ecosystem photosynthesis and respiration. One of the most important effect of global warming, in Arctic regions is, indeed, the permafrost thawing with consequent increase of soil active layer. A possible consequence of such degradation may be an increase in soil respiration with release of organic carbon. On the other hand, climate change and, in particular, increasing temperature and atmospheric CO\(_2\) concentration, may have positive feedbacks on photosynthetic activity. Photosynthetic capacity is species-specific and it is important to quantify the contribution of different target species in the Arctic regions such as Svalbard Islands. Moreover, photosynthetic performances may be associated with the carbon isotope composition (δ\(^{13}\)C) of plant material. This, because δ\(^{13}\)C in plants is a function of the δ\(^{13}\)C of the CO\(_2\) entering the plant and of the fractionation processes occurring during CO\(_2\) diffusion in the leaf, photosynthetic metabolism and post-photosynthetic processes. We focused our attention on the most representative species present at the Ny Ålesund site (78°57’ N; 11°34’ E), Svalbard Islands, in Norway. Photosynthetic capacity at different atmospheric CO\(_2\) concentration, light intensity and air temperature were measured on the following four species: Salix Polaris, Dryas octopetala, Saxifraga oppositifolia, Carex rupestris. On the same species, \(^{13}\)C isotope composition was measured, to obtain an overview of the photosynthetic performances of those species during the season.

The photosynthetic parameters of the different species shown that Salix and Dryas exhibited the highest assimilation rates, while Saxifraga showed the lowest. The high value of assimilation in Dryas could be explained with the high stomatal conductance recorded for this species (table 1). The same, but in opposite sense, is valid for Saxifraga, which presents the lowest value of assimilation and stomatal conductance (table 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Assimilation rate, (\mu\text{mol m}^{-2}s^{-1})</th>
<th>Stomatal conductance, (\mu\text{mol m}^{-2}s^{-1})</th>
<th>Maximum rate of RuBisCO, (\mu\text{mol m}^{-2}s^{-1})</th>
<th>Light saturated photosynthetic rate, (\mu\text{mol m}^{-2}s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carex rupestris</td>
<td>3.16(^{bc}) ± 0.30</td>
<td>0.201(^{ab}) ± 0.027</td>
<td>67.8 ± 12.7</td>
<td>4.46(^{a}) ± 0.69</td>
</tr>
<tr>
<td>Dryas octopetala</td>
<td>4.65(^{ab}) ± 0.48</td>
<td>0.361(^{a}) ± 0.063</td>
<td>89.4 ± 16.9</td>
<td>4.67(^{a}) ± 0.29</td>
</tr>
<tr>
<td>Salix polaris</td>
<td>5.11(^{ab}) ± 0.75</td>
<td>0.125(^{bc}) ± 0.018</td>
<td>66.6 ± 8.9</td>
<td>4.16(^{ab}) ± 0.67</td>
</tr>
<tr>
<td>Saxifraga oppositifolia</td>
<td>2.33(^{a}) ± 0.16</td>
<td>0.058(^{c}) ± 0.018</td>
<td>55.8 ± 2.7</td>
<td>2.80(^{b}) ± 0.49</td>
</tr>
</tbody>
</table>

Table 1. Photosynthetic parameters measured at ambient conditions, namely atmospheric CO\(_2\) concentration, 400 \(\mu\text{mol m}^{-2}s^{-1}\); light intensity, 1000 \(\mu\text{mol m}^{-2}s^{-1}\); leaf temperature, 10°C.

The assimilation rates measured at increasing atmospheric CO\(_2\) concentration, at constant light and temperature shown for the four selected species a similar behavior. Despite all species reached remarkable values of assimilation rates (ranging between 15 and 20 \(\mu\text{mol CO}_2\text{ m}^{-2}\text{s}^{-1}\)), none of them showed a clear saturation of assimilation to increasing CO\(_2\) concentration. The assimilation rate
measured at different light intensities showed significant differences among species. Independently of light intensity, *Saxifraga* exhibited the lowest assimilation values, while *Dryas* and *Salix* always exhibited the highest values although, at saturating light levels. When looking at the dependence of the assimilation rate from leaf temperature, all species show a clear decrease of \( A \) at increasing leaf temperatures, although with species-specific patterns. All species considered are perennial vascular plants and dicotyledons (except *C. rupestris*, which is a monocot). Their values of carbon isotope composition (\( \delta^{13}C \)) measured on above- and below-ground parts of the plants show that they are all C\(_3\) species. The \( \delta^{13}C \) value, indeed, varied between -28 and -31‰ in the above-ground dry matter and it was around -29‰ in below-ground matter (figure 1 panel above). These values were reflected in the values of carbon isotope discrimination (\( \Delta \)) calculated considering the \( \delta^{13}C \) of air CO\(_2\) (figure 1, panel below). Moreover, the selected species showed variations in carbon isotope discrimination (\( \Delta \)), reflecting partially differences in the ratio C\(_i\)/C\(_a\) integrated in the period of development of the plant material analysed and then photosynthetic performances.

To conclude, the photosynthetic performances indicated different species responses to light intensity and potential positive species responses to future increases in atmospheric CO\(_2\) concentrations. Species-specific metabolism adaptation to low temperatures could trigger significant feedbacks in a climate change context. This highlight the need to quantify the role of dominant species to the C cycle (sinks or sources) as changes of vegetation or species phenology in response to climate change.
When the Mediterranean Becomes Harsh for *Eucalyptus Camaldulensis*: Changes in C Allocation as Revealed by $^{13}$CO$_2$ Pulse Labelling

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Heat waves in autumn and spring in Mediterranean are mainly caused by the wind Sirocco which lasts from 0.5 to 7 days and carries warm and dry air from Sahara with peaks in air temperature reaching 43-45°C. Mediterranean vegetation in this period is at the peak of metabolic activity: actively growing in spring and recovering after the summer drought in autumn. These phenophases are the most vulnerable and the consequences of the exposure to extreme temperatures could be long-lasting [1]. Climate change forecasts increase in the frequency, duration and severity of such extreme events globally and particularly in the Mediterranean region [2].

Physiology of pure heat stress, its effects on C allocation and functioning in plant-soil-atmosphere continuum is poorly studied. Few available data suggest that during heat waves the NEE, GPP and $g_s$ in some Mediterranean species are negatively affected but plants are often highly resilient and recover fast without any long-term consequences [3]. Mechanistic understanding of C allocation changes in the conditions of the heat stress is lacking. In this study, we aimed to evaluate the effects of strong heat pulses on C allocation in *Eucalyptus camaldulensis* by performing pulse labelling of heated and non-heated branches in $^{13}$CO$_2$.

Branches of 4 yr-old saplings of *Eucalyptus* were subjected to an increase of the air temperature up to 45°C during the day time. Allocation of C was inferred after pulse labelling of heated (H) and ambient-temperature (A) branches in $^{13}$CO$_2$ enriched atmosphere and subsequent tracing of $^{13}$C among the main pools and fluxes. Label dynamic was assessed in leaves, branches, roots and soil and in shoot respiration, trunk respiration and microbial respiration. Compound specific analyses was done by extracting soluble sugars and starch from leaves and branches. The tracing covered the first 48h after the labelling. Post-labelling conditions were similar between the treatments: branches of A plants labelled at ambient temperature were warmed to 45°C after one cycle of measurements between all the components had been completed. Heat was kept on during the day hours and was turned in both treatments to ambient temperatures during the night.

*Eucalyptus* assimilation rates declined during plants exposure to 45°C. Shoot respiration instead boosted by 200% with high temperature, highlighting different temperature optimum for photosynthesis and respiration. Excess of $^{13}$C in shoot respiration increased contemporary to respiration flux (Fig. 1a), indicating that the contribution of recent assimilates is maintained stable with temperature changes. These data suggest that old non-labelled C reserves, which are considered to be mobilized in the conditions of stress, are not involved in covering respiration expenses at elevated temperatures. The shape of shoot isoflux curves evidences instead an involvement of different fast cycling labelled substrates in diurnal respiration course and their variable contribution between $H$ and $A$ treatment. Shoot isoflux in $A$ treatment was characterized by a bell-shape diurnal pattern (Fig. 1a). The highest peak was reached by night which is in line with the release of the labelled transitory starch formed during the day in the chloroplasts and broken down during night [4]. The relative stability of the $\delta^{13}$C in respiration flux at elevated temperatures in $H$ plants suggests that additional C is provided from a well-mixed soluble carbohydrates pool dissolved in the cell’s cytosol which we suggest to act as a primary substrate for respiration in the conditions of a heat pulse.
Downwards transfer of C almost ceased in $H$ treatment (Fig. 1b) as measured by stem $^{13}$C excess, root tissues and microbial respiration. Allocation of $^{13}$C upward in the direction of newly growing leaves was instead independent from treatment and was likely sink-driven (data not shown).

![Graph showing excess of $^{13}$C in shoot respiration and trunk respiration in A and H plants. In grey-night hour. In yellow-labelling event.](image)

**Fig. 2.** Excess of $^{13}$C in shoot respiration (a) and trunk respiration (b) in A and H plants. In grey-night hour. In yellow-labelling event.

Heat pulses occurring in the Mediterranean area strongly affect C gain and allocation in plant-soil-atmosphere continuum. C limitation of heterotrophic organs induced by missing C displacement from leaves should be covered by local C reserves. We expect that prolonged heat pulses during the end of the growing season, characterized for many species by reserves accumulation, may shift the process to reserves depletion, with possible consequences for the next-year spring resprouting.

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Carbon Stable Isotopes and Chemical Composition of Biogas from Stabilized Organic Waste: Insights into Biochemical Reactions

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Biological processes under anaerobic conditions promote the decomposition of municipal solid waste in landfills and drive the production of biogas, namely, a gaseous mixture composed almost entirely of methane (CH₄), carbon dioxide (CO₂) and other trace gases (i.e. H₂S, H₂). Landfill gas is ranked as the third highest source of global anthropogenic methane emissions, responsible for approximately 9–12% of those emissions in 2005.

Although varieties of models were applied to estimate the potential methane production from an active landfill, little is known about processes and the emission from “old” waste and from the stabilized organic waste (SOW), especially for large-scale landfills. The organic fraction of municipal solid waste undergoes mechanical and biological treatments in order to sort and stabilize the biodegradable matter. However, the final stabilization level was seen to be influenced by scaling factors and the 21 days of treatment turned to be not so adequate [1].

In this work, we performed an experiment with SOW from different plants and two excavated wastes dated 2001 and 2007 from a landfill in Tuscany. The material was located in a closed reactor and biogas was collected at 3-7-14-21-91 days of incubation. For each sample, N₂, O₂, Ar, CH₄ and CO₂ was quantified. On these two last gases, the ¹³C/¹²C ratio was measured. The Fig. 1 illustrates the temporal variation of the median values of δ¹³C_CO₂ and δ¹³C_CH₄ for the three groups of samples. Main outcomes from the experimental activity have been discussed, along with main biochemical reactions scheme reconstructing using chemical composition and stable isotopes as tracer of organic and inorganic processes.

Fig. 1. δ¹³C_CH₄ vs δ¹³C_CO₂ median values of the three gas types generated by the SOW (FOS), amendments (INO) and excavated waste (WASTE) (modified from [2]).

References:
OR10

An EA-IRMS Approach for Carbon Speciation in Soils and Sediments: Inferences on Soil Evolution and Anthropogenic Contributions

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The presented study is mainly in the framework of the project SaveSOC2, funded by the Emilia-Romagna region in order to understand and mitigate the Soil Organic Matter (SOM) deterioration processes. Soils from different pedo-climatic contexts have been analyzed for the carbon elemental and isotopic composition of their distinct OC and IC carbon. EA-IRMS analyses, have been carried out by an Elementar Vario Micro Cube-Isoprime 100 system at the University of Ferrara, following the method recently defined by Natali \textit{et al.} (2018) properly designed to characterize, from the elemental and isotopic point of view, the distinct inorganic (IC) and organic (OC) soil/sediment carbon pools (Fig. 1). Results pools in order to characterize the soil/sediment evolution in relation to the distinct environments. These data, integrated with other geochemical analyses (of lithophile, siderophile and calcophile elements) provide important information on metal speciation, and allow to identify natural and anthropogenic contributions to the metal budget. EA-IRMS analyses, have been carried out by an Elementar Vario Micro Cube-Isoprime 100 system at the University of Ferrara, following the method recently defined by Natali \textit{et al.} (2018) properly designed to characterize, from the elemental and isotopic point of view, the distinct inorganic (IC) and organic (OC) soil/sediment carbon pools (Fig. 1). Results have been cross-checked with those obtained by independent (calcimetric and thermogravimetric) analyses performed at the University of Bologna, showing a very good agreement both for IC and OC. Further comparison is obtained with X-Ray Fluorescence (XRF) data that have also been carried out at the University of Ferrara.

\textbf{Figure 2. Schematic representation of the TBS experimental setup (Natali et al., 2018)}
Soils developed on sediment from different depositional environment are characterized by distinct elemental and isotopic features of their IC and OC fractions (Fig. 2).

![Figure 3](image1.png)

**Figure 3.** Carbon speciation by TBS in soils developed from distinct depositional environment of the Padanian Plain (submitted to Chemie der Erde – Geochemistry)

Some metals appear positively correlated with the OC content and negatively correlated with the carbon bulk isotopic composition, suggesting their association within organo-metallic complexes. Of particular interest are cases in which the $\delta^{13}C_{OC}$ is correlated with ratios between distinct metals, suggesting that different organic compounds selectively accumulate specific metals (Fig. 3).

![Figure 4](image2.png)

**Figure 4.** Relationship between the isotopic composition of organic matter and Pb/Cu in sediments from distinct Northern Adriatic coastal lagoon (Natali et al., 2018).

The above mentioned cases demonstrate that a correct carbon speciation in soils and sediments is extremely important because to understand soil dynamics and especially the potential Soil Organic Matter deterioration. This process minimizes soil fertility, increase GHG in the atmosphere and the mobility of some metals potentially harmful.

**References:**


OR11

Isotopic Ratio Mass Spectrometry (IRMS) Analysis for the Isotopic Discrimination of Surfactants.

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In the last years, cosmetics have gained an important role in everyday life. In the cleaning products marketplace, retailers evaluate manufacturers based on product performance, how they compare against other products and sustainability factors. Formulation and environmental regulations, such as REACH and CLP, become increasingly stringent and there is an increase of more environmentally and bio-based detergents, in particular raw materials from biomass. The costs of raw materials from vegetable origin are higher than the same from fossil origin and these formulations are, as a consequence, less competitive. Therefore, it becomes interesting to assess and identify the authenticity of bio-based products, such as using isotopic analysis. The $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio is a chemical parameter with many important applications in several scientific areas and the technique of choice currently used is the isotope ratio mass spectrometry (IRMS).

This study focused on the determination of the ratio of stable carbon isotopes ($^{13}\text{C}/^{12}\text{C}$) to discriminate, innovatively, the origin of surfactants in raw materials and in commercial detergent formulations. This study was conducted on 12 selected commercial surfactants samples and on complete commercial formulation, such as dishwashing, degreasing, hand washing and laundry detergents. These samples were collected within the collaboration of Cle. Pr. In. srl (Italian chemical factory of cleaning products). Samples were used as received without any preliminary treatment. The surfactants samples were analysed by Delta Plus V Isotope Ratio Mass Spectrometer (Thermo Finnigan, Bremen, Germany) equipped with a Flash EA 1112 Elemental Analyzer to measure $\delta^{13}\text{C}$. The $\delta^{13}\text{C}$ isotopic values were calculated using a homogenized in-house protein standard which was itself calibrated against international reference materials: L-glutamic acid USGS 40 (IAEA International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 for $^{13}\text{C}/^{12}\text{C}$. The measurement uncertainty, expressed as 2 SR (standard deviation of reproducibility), was 0.3‰. The isotopic ratio was determined by isotopic mass spectrometry (IRMS) analysis. The vegetal and synthetic surfactants were analyzed without any preliminary treatment. Commercial products, dishwashing detergent, hand washing, degreasing agent, were analyzed in bulk and after quantitative extraction of surfactants from complex matrices to compare the isotopic results. Solutions of pure synthetic surfactants mixed with natural surfactants were also prepared, at different concentrations, in order to evaluate the influence of composition on the $\delta^{13}\text{C}$. The $\delta^{13}\text{C}$ values found for surfactants samples are in agreement with those reported in the literature [1,2], showing isotopic domain characteristic of the particular kind of surfactants. The $\delta^{13}\text{C}$ values of bio-based surfactants, for example, were in the range expected for oil samples from -24.0 to -22.0 ‰. The carbon isotope composition indicated values typical for the C3 photosynthetic pathway and their carbon isotope compositions fall into the range -22 to -34‰. On the other hand, the fossil surfactants showed an isotopic composition ranging from -28.00 to -35.00 ‰ typical isotopic composition of hydrocarbons and fossil samples.

The results obtained showed the possibility to discriminate vegetal surfactants from fossil samples. The intention of this work was to provide information about the origin of surfactants and examination of different products in terms of commodity, variety, composition and chemical nature, demonstrating that isotopic analysis could be an useful techniques for these innovative application.
References:


The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of agricultural produce has been proposed as soil-derived marker to determine its geographical origin. Sr is transferred from the soil to the plant through root uptake without isotope fractionation due to the low mass difference of the Sr isotopes. Therefore, the isotope composition of the plant reflects closely that of the soil and varies according to the geological features of the soil. There are, however, aspects that should be deeply investigated to better explain the potential of this technique and improve its use as traceability marker. For instance, the agricultural management practices, such as the use of irrigation water or the intensive application of fertilizers, may represent additional Sr-inputs that can affect the final $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the soil, and hence loosen the chemical link of the plant with the area of origin. Additionally, the variability of $^{87}\text{Sr}/^{86}\text{Sr}$ ratio among different production areas is low and, at the same time, the variability within samples from the same area is high, the sample classification according to their provenance could not be fully accomplished. To our knowledge, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio variability both intra-tree and intra-orchard has not been yet investigated. With this contribution, we present the results of two studies aimed at clarifying these aspects and at providing more details to support the use of the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio as geographical tracer.

A first experiment with transplanted one-year-old apple trees (cv. Golden Delicious Klon B Lb®, rootstock M9) in pots was set up and carried on for two growing seasons. Trees were divided in groups and underwent to different treatments, each one characterized by a specific isotope ratio, different from that of both trees and soil. The results highlighted that prolonged Sr-inputs can play a role in determining the final Sr isotope composition of tree organs, even though not completely appreciable compared to the large contribution coming from the soil. The second study was performed in two orchards located in different valleys of South-Tyrol to evaluate the variability of the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio. Different organs of the same apple trees and then different trees of the same orchard (cv. Gala Schniga®, rootstock M9) were compared. Moreover, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio change along the soil profile (10-80 cm depth) was also evaluated. The results indicated that the $^{87}\text{Sr}/^{86}\text{Sr}$ range of values highly depends on the soil heterogeneity and is then reflected both intra- and inter-trees.

Our studies proved that there are aspects that should not be underestimated when applying the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio to traceability studies. Additional investigations are required to further explore the potential of this technique that has nonetheless proved to be very powerful as geographical tracer.

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In the last few years the isotopic methodologies plaid a role more and more important both in interdisciplinary and applied researches, and in a growing number of analysis and control procedures in industrial, environmental, biomedical, archaeometry, agricultural and food domains. The conventional (stable) isotope mass spectrometry (IRMS) and the accelerator mass spectrometry (AMS) represent, then, a powerful search tool widely used in the field of sciences applied to the diagnostics of cultural heritage.
For example, the measurement of the isotopic composition of elements such as Carbon, Oxygen, Nitrogen, Strontium, Sulfur, in the different archaeological materials, such as charcoal, bone, teeth, seeds, organic remains in pottery ceramics, marble, mortar, are a powerful tool to quantify small variations of fluxes of such elements, often due to anthropogenic activities, and then get information on still unclear occupation sequences and "mode of life" in archaeological sites.
In this paper a review of use of stable and radioactive isotopic methodology on the cultural heritage, will be presented, focusing the attention both on methodological and applicative aspects. Furthermore some examples of Bayesian statistic applied at the analysis of isotopic measurements will be given.
Late Chalcolithic Population in Arslantepe Site: Stable Isotope Reconstruction of Diet and Subsistence Strategies

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Isotope analysis carried out on human and animal bones from Arslantepe site (Eastern Anatolia), and belonging to two Late Chalcolithic stages, VIII (6250-5900 BP) and VII (5900-5350 BP), have provided several information on diet and subsistence strategies of these ancient population. Adults human, exclusively female, had a diet mostly based on C\textsubscript{3} plants such as barley and emmer (as witnessed by the archaeobotanical evidence) and on animals having themselves a C\textsubscript{3} diet. Indeed, δ\textsuperscript{15}N values demonstrate that animal proteins were present in the diet, placing the humans on higher trophic level than animals. Only the diet of the child (6-7 years old) is not different from that of adults. Among adults, there is only a female, who is a young female, with all three isotope signals different in comparison to the others: Most likely, she had come from another place, warmer, with different dietary habits.

The children were breastfed and place at a higher trophic level than females, as evidenced by δ\textsuperscript{15}N and δ\textsuperscript{18}O values. The foetuses, instead, reflect the value of water ingested by mothers.

The faunal record provided many samples of sheep and goats, which are the most abundant species, followed by cattle and pigs. It follows that the breeding was widespread even if with different methods. The δ\textsuperscript{13}C and δ\textsuperscript{18}O values indicate that the Caprinae and Bos from period VIII were gathered in large herds grazing on vast area, while cattle from VII period and pigs were bred near the settlement and in confined areas. Something changed in the cattle breeding from VIII to VII period. The remains of wild fauna, are also indicative of C\textsubscript{3} ecosystem, and demonstrate that hunting was practiced areas around the site.
Stable Isotope Study of Shells and Fish Bones to Characterize Mesolithic Environmental Conditions

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Oxygen and carbon isotopic analyses of fish bone apatite and gastropod shells can provide valuable information about freshwater system [1] [2]. Here, we present an isotopic study performed to improve our knowledge of the paleo-environmental conditions of the wetlands along the White Nile during the Mesolithic period. The total sample consists of modern and fossil semi-aquatic gastropods that inhabit river-connected swamps, floodplains and other stagnant water and six fossil species of fish with their own ecological niches. Oxygen and carbon stable isotopes are analysed. The $\delta^{18}$O values clearly indicate a different hydrological budget of the river with less evaporation than today. The wetter climate would have supported seasonally flooded grassy plain and therefore more resources for animals and groups of people [3]. Finally, our results indicate that $\delta^{13}$C may be an indicator of ecosystem changes in wetlands and indicate a dominance of C4 plants.

References:
The investigation of animal ranging areas is a matter of interest in modern ecology, archaeological and palaeontology. Stable isotopes have proven to be a useful tool to study animal mobility, since they are proxies for the geographic, environmental and climatic features in which these animals lived and roamed. The isotopes of a certain element are transferred from the environment to the individual’s body tissues through ingested food and drink, for example, in the case of an herbivore, through consumed plants and water (both as drinking water and as water in leaves) in the roving area. Carbon isotope ratios in plants are related to the physiology and type of plant, and are indicative of climate and environments. They can change over time in the same area due to stressful conditions. Oxygen isotopes, besides being also related to the physiology of plants, can give information on the hydrological circulations and temperature in a certain area at the time these animals lived and drunk these water sources. Taken together, these two isotope systems are good indicators of the climate and environmental conditions, such as for example cold vs warm, and humid vs dry conditions. It follows that stable isotope ratios measured in animal’s soft and calcified tissues, such as those of carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$), can offer insights into the state and type of vegetation available to the animal.

Strontium isotopes $^{87}$Sr/$^{86}$Sr in animal tissues, though also routed from the environment though ingested food and drink, are instead strongly associated to the local geology. From the local bedrocks, strontium and its isotopes are taken up by plants with negligible isotopic fractionation, given the small relative mass difference between the two isotopes. From plants, these are in turn transferred almost unmodified to the primary (herbivores) and secondary (omnivores and carnivores) consumers. Strontium isotopes vary in the landscape mainly in relation to the type and age of the underlying rocks. Generally, young and igneous mafic rocks are the most $^{87}$Sr-depleted substrates, while Palaeozoic rocks, granite and shale are amongst the most $^{87}$Sr-enriched ones. Sedimentary rocks sit often in between.

Sr, C and O isotopes have been employed alone or in combination to study the mobility and origins of human and animal single individuals and populations from their skeletal remains. The premises are that local individuals should have isotopic compositions consistent with those expected for the retrieval site. If these compositions are in disagreement with the local expectations, then the individuals are generally considered non-locals to the place of recovery.

Such techniques have been successfully used in the past two decades from the bulk sampling of bones and tooth remains, but they become even more powerful when stable and radiogenic isotopes are measured sequentially in teeth. This paper presents the results of serial measurements of Sr, O and/or C isotopes in ungulate tooth enamel. Intra-tooth specimens were collected perpendicular to the growing direction of teeth, which generally develop more or less linearly in most ungulates. The chemical and isotopic composition of this tissue represents a snapshot in the individual’s life, such as that of tooth mineralization, which occurs in the juvenile stage. Ungulate teeth generally mineralize within a timeframe of a few months to a couple of years according to tooth type and species, thus they are able to record intra-annual seasonal changes. The composition acquired in this period does not remodel in subsequent periods of life. The serially measured isotope compositions are the expression of the seasonal climatic and/or environmental changes that these animals experienced during the period of tooth formation (enamel mineralization), including the movements they underwent in this period. Results from a few case studies will be shown.
You Are What You Eat Plus a Few Permil: Applications of Stable Isotopes of Carbon and Nitrogen to Ecological Studies

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In recent years, stable isotope analysis has been used to obtain interesting insights on ecosystem functioning, indicating the origin and fate of organic matter in terrestrial and aquatic ecosystems \cite{1}. In particular, contextual analysis of the carbon and nitrogen stable isotope composition ($\delta^{13}$C and $\delta^{15}$N) of primary producers and consumers has been used to follow the flow of organic matter into food webs, resolve food web structure and reveal trophic relationships between organisms, also in complex ecosystems such as marine coastal environments. The assumption underlying this method is that primary producers have distinct isotopic signatures and, where this is the case, carbon isotopes allow the identification of food sources for animals, with the $\delta^{13}$C of consumers reflecting that of the assimilated diet with a minor change. The nitrogen isotope ratio undergoes a greater increase (generally variable between 3 and 5\% as a function of animal group and diet) with each trophic level, defining the trophic position of organisms in systems where the trophic interactions are not known.

Since the 1970’s, a number of isotopic studies have been carried out in complex coastal ecosystems all over the world, such as mangroves, sea grass beds and salt-marshes, to assess the relative trophic role of each primary producer and the magnitude of their contribution to upper trophic levels. Stable isotope studies in the Mediterranean began in the late 1980’s and have increased consistently only in very recent years. This approach has been fundamental in clarifying a number of points and its use has been widened to address a number of applied issues such as the assessment of anthropogenic pressure on natural ecosystems, using the potential of SIA to provide food web-scale indicators for estimating cumulative anthropogenic pressures (e.g., chemical pollution, release of dissolved and particulate nutrients, climate change, invasive species) \cite{2}. In addition, the application of recently implemented metrics makes SIA highly effective in integrating information on the spatial-temporal dynamics of aquatic food webs. Here, we describe the main applications of stable isotope analysis to ecological studies and provide specific case studies showing their effectiveness and their contribution to the advancement in many ecological fields.

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\cite{2}

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Use of Stable Isotope Ratios to Investigate the Migratory Routes of Passerines

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For the conservation of migratory birds it is of paramount importance to determine migratory routes, in particular delineating the structure of flyways that link breeding and wintering areas [1]. However, this type of information is limited and innovative methods are required to outline these spatial links for effective conservation. Intrinsic markers such as stable isotope ratios have become a powerful tool for tracking the spatial movements of animals. This is due to the fact that the isotopic composition of bird tissues reflects the isotopic signatures of the environment in which the tissues were grown [2]. Furthermore, the combination of stable isotope methods with other approaches to tracking individuals (e.g. ringing data) can increase the precision of origin assignments.

The aim of this study was to evaluate, for the first time, the use of isotopic and ringing data to efficiently delineate the geographical origin of two of the most abundant passerines migrating through the Italian Alps during the post-breeding season [3]: the pied flycatcher (Ficedula hypoleuca) and the European robin (Erithacus rubecula). The stable isotope ratios of C, H, N and S in feathers were determined in about 200 pied flycatchers and European robins captured in the Italian Alps during autumn migration. The stable isotope ratios were determined with Isotope Ratio Mass Spectrometry, following preparation according to Bontempo et al. 2014 [4]. Furthermore, data from the EURING data bank, which includes the capture and recapture histories of individual adults and hatch-year flycatchers and robins until 2008, were used to determine the direction of autumnal movement of the birds recaptured in the Italian Alps.

$\delta^2$H and ringing data were used to develop a likelihood-based assignment method to assign a geographical origin to each sampled robin and flycatcher. Furthermore, generalised linear regression models were developed to determine the seasonal isotopic shift for the four stable isotopes, in order to compare the trends for the two species, which have a distinct migration phenology.

The results of this study show the effectiveness of combining stable isotopes with ringing data in defining migratory connectivity on a continental scale. Using this approach, the birthplace of two common species was determined during autumnal migration through the Italian Alps. In particular, the direction of movement by robins resulting from recapture data was shown to lie on a north-northeast to south-southwest line between the Baltic basin and the Alps. Furthermore, probabilistic geographical assignment suggests that the birds migrating through the Italian Alps originate predominantly in Central Europe, but with several individuals probably originating in the Baltic basin. On the other hand, the area of origin of flycatchers was shown to be more easterly, expanding the area of natal origin from the Baltic basin to western Russia [5]. As with robins, geographical assignment showed that most flycatchers were of central-European origin, with several individuals probably originating in the Baltic basin and south-western Russia.

This study highlights for the first time several advantages of using a multi-isotopic approach associated with classic ringing data to study two passerine species migrating along the Western Palearctic flyway across the Italian Alps. In particular, the use of carbon, nitrogen and sulphur stable isotope ratios to gain insight into spatial patterns with $\delta^2$H isoscapes was shown to considerably increase accuracy for probabilistic calculation of the geographical assignment of birds.
References:


A new approach for δ13C data evaluation of natural compounds is proposed. This method is based on Comprehensive Isotopic Data Evaluation (CIDE) which consists of the analysis of the entire volatile’s carbon isotope ratio, integrating the whole gas chromatogram (volatile bulk) determined by GC-C-IRMS and plotting this δ13C value vs. that of a selected pure compound. The results will allow to cluster the samples of identical nature based on their origins. In this presentation will be given few examples of how this analytical approach works to distinguish the geographic origin of food ingredients, the botanical or animal source of food, and eventually the differentiation of natural and synthetic flavorings. In particular the CIDE approach is here applied on coffee samples of different varieties and geographic origins (C. robusta and C. arabica), on milk and diary products obtained in different Italian regions from different mammals (cow, goat and buffalo) fed with different types of pastures (C3 vs C4 plants), and in sandalwood of different botanical and geographic origin. The results will provide a bidimensional separation on the plane based on the parameters selected by the CIDE approach. It is quite interesting to appreciate CIDE’s capability to unveil possible frauds and assess the origin of food ingredients.
Isotopic Metabolic Fractionation of Endogenous Steroids by 5α-Reductase Inhibitors

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5α-Reductase inhibitors are drugs commonly used in the treatment of benign prostatic hyperplasia, androgenic alopecia and hirsutism. The only two pharmaceutical product commercially available and FDA-approved for clinical purpose are Finasteride and Dutasteride \cite{1}. They are both synthetic 4-azasteroids that irreversibly inhibit the 5α-reductase, competing against the substrate, testosterone (T), in the linkage with enzyme-NADH complex and in its conversion into dihydrotestosterone (DHT). Dutasteride inhibits both 5α-reductase isozymes (type 1 and 2) with an high effectiveness, while Finasteride preferentially inhibits the isozyme type 2, located not only in prostate but also in hair follicles \cite{2}. In anti-doping analyses, the use of 5α-reductase inhibitors represents a serious issue because, even if they are not currently included in the WADA Prohibited List as performance-enhancing drugs, they are confounding factors affecting several urinary steroid profile parameters: androsterone/etiocholanolone (A/Etio) and 5α-androstane-3α,17β-diol/5β-androstan-3α,17β-diol (5αAdiol/5βAdiol) \cite{3,4}.

This work is a preliminary study of the remarkable effects that Finasteride and Dutasteride have not only over these ratios but also over the isotopic fractionation of endogenous steroid hormones observed after GC-c-IRMS analyses. For the longitudinal analysis of the steroid profile, urine samples were collected within a year and analysed by the routine steroid screening method by GC-MS/MS. Urine samples collected after the administration of Propecia 1 mg and Avodart 0.5 mg were, instead, analysed by the confirmation procedure for the detection of synthetic forms of endogenous anabolic androgenic steroids by GC-c-IRMS. Briefly, the method is based on enzymatic hydrolysis of urine samples (6 – 12 mL), liquid-liquid extraction with TBME, HPLC-purification and GC-c-IRMS analysis.

As defined by the WADA Technical Document-TD2016IRMS \cite{5}, the IRMS analysis is a confirmatory procedure to establish whether the alteration of steroid profile parameters, not supported by any physiological reasons, can be attributed to an exogenous administration of synthetic steroids, considering that the carbon isotopic ratio of endogenous steroids, $^{13}$C/$^{12}$C (δ\textsubscript{13}C), is different from that of synthetic ones \cite{6}. The typical δ\textsubscript{13}C values of endogenous steroids ranges from -18 to -25 ‰, whereas the range of steroids synthetically produced are from -27 to -33 ‰. This work wants to focus on another possible application of the IRMS analysis in the study of specific enzymatic processes in the steroid metabolic pathways. We proved that the inhibition of 5α-reductase, responsible for the conversion of T into 5α-DHT, alters not only the concentration ratios between the testosterone metabolic products (A/Etio and 5αAdiol/5βAdiol), but also their characteristic delta values. The longitudinal study performed on a male volunteer urine samples showed that the δ\textsubscript{A} (μ = -22.25 ‰) is more positive than δ\textsubscript{Etio} (μ = -23.17 ‰) with a Δδ of 0.90 ‰. Specular trend can be registered observing their direct precursors, 5αAdiol (μ = -23.81 ‰) and 5βAdiol (μ = -24.20 ‰) respectively \cite{7}. Despite the administration of both 5α-reductase inhibitors reduced the Δδ values between A and Etio and those between 5αAdiol and 5βAdiol, the effect of Finasteride, the isozyme type 2 selective inhibitor, was more significant than the effects observed after Dutasteride administration. IRMS is currently the WADA accredited method to confirm the exogenous origin of pseudo-endogenous steroids from human urine samples. The present work underlines how the evaluation of endogenous steroid metabolites delta values can be also useful to study enzymatic processes. Based on the preliminary outcomes, it could possible to assume the administration of 5α-reductase inhibitors by monitoring the variation of Δδ\textsubscript{A-Etio} and Δδ\textsubscript{5αAdiol-5βAdiol}. The relevance of this approach is also emphasized considering
that only the main Finasteride metabolite is detectable in urine by LC/MS; while no known metabolites of Dutasteride can be detected in urine samples.

References:
Flavourings are ingredients that bring taste to a variety of different food and beverage products. Most of the flavouring compounds are produced via chemical synthesis or by extraction from natural materials. The cost of natural flavours, however, is often a factor of 10 or higher compared to the price of synthetic copies. Owing to this price advantage and to the difficulty of differentiating natural flavours from the same molecules made by chemical synthesis, it has been reported that synthetic flavours are sometimes sold as natural ones [1]. At the moment, gas chromatography isotope ratio mass spectrometry (GC-C-IRMS) is perhaps one of the most specific and sophisticated methods for determining food authenticity [2].

The use of solid-phase microextraction (SPME) coupled with isotope ratio mass spectrometry (IRMS) for the $\delta^{13}$C analysis of aroma compounds from apple and strawberry recovery aromas has been evaluated using headspace sampling (HS). The method was used to differentiate between 16 natural and synthetic apple and strawberry aroma compounds. Our established research procedure contains several steps that need to be taken, including sample and standard selection, sample preparation, compound identification, $\delta^{13}$C measurements, data processing, database creation based on authentic aroma samples and authenticity assessment of commercial recovery aromas.

Our results show that gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis of key volatile compounds with the headspace solid phase microextraction (HS-SPME) as a sample preparation method is an appropriate tool for authenticity assessment of apple and strawberry aromas. One of the main objectives of the present research was to establish the database of $\delta^{13}$C values for several most common volatile compounds present in apple and strawberry recovery aromas. Analysis of commercial recovery aromas, labelled as natural, revealed that the $\delta^{13}$C value of the majority of the compounds present was within the expected authentic range. The data also revealed some possible falsifications, of mainly strawberry samples. Our data also reveals some differences in $\delta^{13}$C value between natural apple and strawberry recovery aromas, which requires further investigation.

Since many different compounds with different concentrations are present in one sample, the selection of reference material and appropriate data processing and interpretation of the results obtained is crucial. To gain more confidence in the interpretation of measured values, an extensive database, which is under construction, is required. Although the method was developed to differentiate between natural and synthetic apple and strawberry aroma compounds it can be easily transferred also to other commodities.

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Advanced Genuineness Assessment Capability in Flavor and Fragrance Field Exploiting a MDGC-qMS/IRMS Prototype

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Worldwide, each year, around three thousand tons of declared essence of bergamot are marketed, but the genuine essence of bergamot produced annually amounts to no more than one hundred tons. Bergamot essential oil is particularly subject to adulteration. It is produced in relatively small quantities due to the restricted growing area of the plant. Generally adulteration is to "cut" the oil, i.e. adding distilled essences of poor quality and low cost, for example of bitter orange and bergamot mint and/or mixtures of terpenes, natural or synthetic, or "reconstruct" the essence from synthetic chemicals, coloring it with chlorophyll. Genuineness evaluation is commonly achieved by enantioGC analysis. Recently the use of genetically modified plants, able to produce the typical enantiomeric ratio of key components, made more difficult to highlight this fraud. Conventional enantio-GC analysis showed co-elutions of some enantiomers, compromising the determination of their enantiomeric distribution or leading to a bogus enantiomeric ratio. Thus an enantio-Multi Dimensional Gas Chromatography (e-MDGC) technique was used to carry out an enantio selective separation. This research focuses on the genuineness assessment of Bergamot essential oils, by e-MDGC with simultaneous detection by quadrupole mass spectrometry (qMS) and isotopic ratio mass spectrometry (IRMS). Such an approach was able to determine the enantiomeric distribution and the isotopic ratios of key compounds evaluated simultaneously in the same GC run. The double detection provides more reliable capability to detect adulteration in Citrus essential oils. In fact, in some circumstance only one of the two techniques, allows to discriminate adulterated or contaminated oils. In cases where only small anomalies are detected by the two techniques due to subtle adulterations, their synergic use allows to express judgments. e-MDGC in synergy with qMS and IRMS detection can be considered the most sophisticated approach for accurate and precise analysis of chiral components.
HS-SPME MDGC-C-IRMS with Simultaneous Quadrupole MS Detection for the Analysis of Truffle and Truffle Aromatized Food Products

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Truffles are among the most expensive foods available in the market, usually used as flavouring additives for their distinctive aroma. The most valuable species is 

Tuber magnatum Pico, better known as “Alba white truffle”. In the present research, a high-efficiency HS-SPME MDGC-C-IRMS with simultaneous quadrupole MS detection, has been applied for the evaluation of the \textsuperscript{13}C ratio of the key aroma compound of white truffle, namely bis(methylthio)methane. This application demonstrates the capability of an MDGC-C-IRMS system to overcome some of the historical problems of IRMS, associated with the combustion and measurement of impure peaks. Extracolumn band broadening has been greatly reduced thanks to the optimization of the micro-combustion furnace and to the elimination of the heart-split valve, not necessary in a multidimensional configuration. Twenty-four genuine white truffles harvested in Italy were analyzed, attaining \( \delta^{13} \text{C} \) values between -42.6 \( \% \) and -33.9 \( \% \), with a maximum standard deviation lower than 0.7 \( \% \). Two commercial intact truffles and 14 commercial samples of pasta, sauce, olive oil, cream, honey and fresh cheese flavoured with truffle aroma were analysed. The results from \( \delta^{13} \text{C} \) measurement were evaluated in comparison with those of genuine “white truffle” range and commercial synthetic bis(methylthio)methane standard.
Relations Between the Isotopic Characteristics of Groundwater and Milk in the Production Area of Parmigiano Reggiano: Biogeochemical Overview

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The \(^{18}\text{O}/^{16}\text{O}, \text{H}/^{1}\text{H} \text{ and } ^{13}\text{C}/^{12}\text{C} \text{ isotope ratios of groundwater and local food are indicators of the area of the milk origin being affected by the climatic factors [1].\]

The main aim of the investigation is the evaluation of the dependence of the isotopic composition of milk on food (hay and fodder) and water ingested by the cow.

The stable isotopes of the elements such as O, H, C, N are monthly evaluated on groundwater, milk, hay and fodder samples from ten cattle-shed belonging to the “Consorzio del Parmigiano Reggiano” over a period of one year.

Overall, isotopic analysis has good potential for determining the geographic origin of dairy products [2]. The research presents a double interest, scientific and economic, because it can be used to verify the origin of milk, hay and fodder and also to reduce food fraud [3].

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Coupling of GC-IRMS with High-Resolution Mass Spectrometry for Final Confirmation in Food Authentication and Sports Drug Testing

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Compound-specific isotope analysis by online conversion of organic compounds separated by gas chromatography and subsequent isotope ratio mass spectrometry (GC-IRMS) is a well-established analytical approach to a variety of samples from routine and research areas, such as environmental forensics, food authentication, energy exploration, paleo-climate and metabolism research. Concomitant data are critical to qualify the true identity of a compound. This has become mandatory in confirmation of so-called adverse analytical findings. By coupling GC-IRMS with the Q Exactive GC system, the isotopic compositions and the comprehensive qualitative and quantitative sample information with high levels of selectivity, sensitivity, and confidence are accessible simultaneously from a single injection.

Here we present how hyphenated GC-MS-IRMS system can be used for confirmatory analysis of food and doping control samples.

Vanillin is one of the compounds which has been extensively investigated. Vanillin is costly extracted from the cured pods of tropical orchid of the genus Vanilla and the resources are limited. This is the reason why vanillin is often synthetically produced. Detecting dilution of a natural product with chemically created substance is of great importance for the food industry in order to prevent an opportunity of economically motivated fraud. Here we present vanillin analysis in ice cream samples by a GC-IRMS system online coupled with a Q Exactive GC.

In the light of emerging cases of drug abuse, GC-IRMS allows distinguishing between endogenous steroids from their synthetic analogs in urine by the determination of $^{13}\text{C}/^{12}\text{C}$ isotope ratios for doping control.

Here we present the results of an excretion study after testosterone administration. One male volunteer was administered 30 mg of testosterone orally. Urine samples collected were prepared according to the standard protocol of the doping control routine and analyzed by a GC-IRMS system online coupled with a Q Exactive GC. The data demonstrates that testosterone doping can be still approved after 24 hours by utilizing the IRMS results of testosterone and its main metabolites.

Combining the Thermo Scientific™ GC IsoLink™ IRMS System with the Q Exactive GC Orbitrap GC-MS/MS provides a refined methodology for food and doping control analysis. Simultaneous analysis by a GC/MS/IRMS system fulfills the demand for high resolution and accurate mass determination.
The present research deals with the investigation of the $^{13}$C ratio of vanillin present in a commercial vanilla aromatized ice-cream, by means of a MDGC-qMS/C-IRMS prototype characterized by the improved resolution capability of the heart-cut mode and the simultaneous qMS and IRMS detection of the 2D chromatographic bands. Vanilla is a complex blend of flavour and fragrance ingredients extracted from the seed pods of the vanilla orchid. The most important ingredient in this blend is vanillin. Most products that want to impart the aroma of vanilla do not use vanilla but rather synthetic vanillin (99% of all vanillin consumed worldwide) made primarily from petrochemicals or chemically derived from lignin. Based on the origins its commercial value greatly changes.

Vanillin and vanilla extracts have an estimated annual total volume of 16,000 metric tons, worth some USD 650 million in total. Natural vanilla extract represents less than 1% by volume, though it is more important in terms of value. Sales prices range from about USD 1,500 per Kg for natural vanilla extract to USD 10-20 per Kg for synthetic vanillin.

The coupling of IRMS to multidimensional GC has proved to be powerful and versatile. MDGC in synergy with qMS and IRMS detections can be considered the most advanced approach for accurate and precise analysis of $\delta^{13}$C for qualitative and origin evaluation of foods and other natural products. The IRMS software allows a multipoint calibration in a gas chromatographic environment by the use of externally calibrated substances that allow for the identical treatment of the standard and component of interest, thereby mitigating any possible influence of the separation and combustion steps in the analytical pathway.
Nowadays, organic agriculture is catching on all over Italy in order to reduce the impact on human health and environment, and prevent soil degradation. We are conducting a study on an apple orchard (Gala cv.) in Valsugana (Trentino Alto Adige, Northern East Italy), with the overall aim of comparing conventional and organic agronomic management. Mineral fertilizers and pesticides are used in conventional management, conversely, animal composted manure and substances admitted in organic farming are employed in the organic one. In this investigation, we aim to identify the nitrogen source of plants in both managements. Particularly, we are studying the uptake of nitrogen sources by plants and nitrogen translocation to fruits. Nitrogen stable isotope ratio ($\delta^{15}$N) is one of the strongest markers for organic production [1]. Indeed, mineral fertilizers have a low $\delta^{15}$N ($-6 - +6‰$) because it is synthetized by atmospheric N$_2$. Whereas, $\delta^{15}$N is higher in organic fertilizers than mineral ones, and varies between 1 and 37‰ [2], due to transformation mechanisms that enrich matrix in heavier nitrogen [3]. After plants uptake and metabolic processes, nitrogen preserves similar isotope ratio of absorbed products. Therefore, we can deduce the kind of nitrogen source absorbed and used by plants [4]. The experimental field has been managed with conventional agronomic practices until 2017. In April 2018 it has been divided into two plots, each subjected to one management. This experimental design includes sampling of soil and plant (leaves) every six weeks and sampling of apples on harvest for three years. Samples are being collected in ten replicates for management. We conducted a preliminary investigation of soil before fertilization ($T_0$), in order to assess the initial conditions. The field is quite heterogeneous in organic matter content, soil texture and carbonates. On average the soil has a good pool of organic matter and medium-high content of carbonates. Texture varies from silt loam to sandy loam. This experimentation will allow us to follow $\delta^{15}$N dynamics in conventional and organic farming, from ground to fruit. In this way we can extend our knowledge on nitrogen isotope ratio as marker of organic products in apple crops, which has been not extensively studied. Finally we will evaluate how $\delta^{15}$N is related with other parameters linked to fruits quality.

References:


Characterization of Slovenian Truffles Using Stable Isotope Approach and Elemental Composition

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Out of all underground fungi, several species from the genus \textit{Tuber} are especially famous. The fact that they can reach quite high prices on the market certainly helps to their popularity. The high cost consequently attracts more or less exquisite adulteration practices. The species in \textit{Tuber} genus, which is in Europe represented by around 20 species, can be roughly divided into white and black truffles. The most known are \textit{Tuber magnatum} Pico 1788 and \textit{Tuber melanosporum} Vittadini 1831, followed by other commercially interesting species such as \textit{Tuber aestivalv} Chatin 1887, \textit{Tuber macrosporum} Vittadini 1831, \textit{Tuber brunale} Vittadini 1831, \textit{Tuber borchii} Vittadini 1831, etc. Truffles are used in culinary as fresh fruiting bodies or as ingredients in the processed product. Since 2011 the truffle hunting is again legal in Slovenia. Since the commercial trufferies have not been yet fully established, the whole truffle production comes from hunting wild truffles. While there can be little less than 20 species found across Slovenia \cite{1}, the two most appreciated \textit{T. magnatum} and \textit{T. melanosporum} are mainly found in Slovenian Istria. The local forests, with their abundance of oaks and poplars, are the perfect terrain for those truffles. In 2017, six truffle samples were collected in Slovenian Istria. Two samples of \textit{T. magnatum}, one \textit{T. borchii}, one \textit{T. melanosporum}, and two \textit{T. aestivalv} were analyzed for elemental composition by X-ray fluorescence (XRF) and for stable isotope composition (H, O, C, N, and S) by isotope ratio mass spectrometry (IRMS). Stable isotope composition of carbon was also determined for the main volatile organic compounds by the use of headspace solid-phase microextraction (HS-SPME) coupled with IRMS. High variability of δ\textsubscript{2}H, δ\textsubscript{15}N, and δ\textsubscript{34}S was observed between different truffle species. The statistical evaluation of elemental and stable isotope compositions indicated that samples can be separated according to δ\textsubscript{15}N, Pb and P. δ\textsubscript{13}C values in VOC were also significantly different between different truffle species. It was also speculated that different VOC components can be produced not only by the truffle bur also or exclusively by the microbes such as yeasts or bacteria. Lowest δ\textsubscript{13}C values (< −44‰) which were observed in 1-propanol indicate its microbial origin. The results of this preliminary study show that elemental composition and stable isotope composition could be used for determination of species variety and trophic status of truffles. It was also indicated that truffles offer a unique opportunity to better understand the ecological function of microbes associated with fungi and their involvement in aroma formation.

The research was performed within ARIMnet REALMed project with the aim of “Pursuing authenticity and valorization of Mediterranean traditional products”.

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Characterization and Valorization of the Italian Walnut (Juglans regia L.): a First Application of Stable Isotope Ratio Analysis to Determine Walnut Geographical Origin

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Juglans regia (L.), commonly named Persian or English walnut, is cultivated for nut production and is one of the oldest food sources known. Native to the mountain valleys of Central Asia, J. regia are grown worldwide in temperate areas. Walnut world production keeps increasing, exceeding three million tons since 2011, with China, USA and Iran as leading producing countries [1]. In Italy, despite the constantly growing demand due to walnuts high nutritional value, walnut cultivation went through a strong decline in the last decades. This may be due to the low agronomic standard care, the shortage of producers co-operation and association, which would allow farmers to act jointly reducing production costs, the use of plants grown from seed, causing the absence of product homogeneity, and the lack of selected and locally adapted varieties. As a consequence, Italy is among the top five net importing countries of in-shell walnut since the mid 1970s [1]. However, the climate and environmental conditions of the Italian peninsula are well suited to walnut cultivation, and still few local unique Italian varieties and ecotypes have survived. This is the case of the ‘Bleggiana’ variety, present in a restricted area – Bleggio – of the mountainous region Trentino, and still propagated by grafting. Important Italian ecotypes are Feltrina, present only in the mountainous area of Feltre in the Veneto region, and Sorrento, which is represented by at least eight different ecotypes distributed across the Sorrento peninsula and Campania region [2]. Additionally, international commercial varieties, such as Lara, Franquette and Chandler, showed to adapt well to the Italian variegated landscape and have already been introduced also in regions previously not dedicated to the walnut culture. The current context is therefore favorable to reintroduce the walnut cultivation on the Italian territory heading towards a local, high quality production. In this respect, tools capable of tracing the origin of specific products can help this process, especially protecting the local origin of the product. The exploratory analysis here presented investigates the capability of H, O, C, N, and S stable isotope ratios to determine the geographical origin of walnut varieties locally cultivated in Italy. It is performed within a multidisciplinary project that aims to a complete characterization of the local Italian walnut accessions through the definition of a unique and typical profile by means of i) genetic profiling using microsatellite molecular markers; ii) H, O, C, N, and S stable isotope analysis; iii) metabolic compounds analysis, particularly lipids and phenols; and iv) sensory analysis. Dried samples of in-shell walnuts, from orchards located mainly in North-Eastern Italy, were provided by producers during the fruit harvest in 2017, each sample consisted of 10 to 15 walnuts from the same orchard. A total of 22 Italian samples were retrieved from four regions: Veneto (16), Trentino (3), Piedmont (1), Emilia (1) and Campania (1). As very preliminary test to discriminate between Italian and international samples, one sample from four foreign countries (Australia, California, Chile and France) was introduced in the analysis. All nuts of each sample were manually cracked and shelled, and the seed kernels were frozen at -80 °C; subsamples of 50 g on average were subsequently ball-milled using Mixer Mill MM400 homogenizer (Retsch GmbH, Haan, Germany) and stored at -20°C until lyophilisation. Around 4 g of grounded and lyophilised nut kernels were extracted with 25 mL mixture of petroleum ether: ether (2:1) homogenising with an Ultraturrax device (model X-620, Staufen, Germany; 11,500 rpm for 3 min) and using a centrifuge (ALC PK 131R, Thermo Electron Corporation, Germany; 4100 rpm for 5 min) to separate the ether from the residue. The residue was re-extracted twice. The ether was evaporated using a model R210- A rotary evaporator (Büchi, Flawil, Switzerland) at 37°C. The defatted kernel was washed with deionised water twice with 30 mL of water
using a centrifuge (4100 rpm for 3 min) to separate the water. The solid residue was lyophilised and conserved at room temperature up the analysis. The $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{34}\text{S}/^{32}\text{S}$ stable isotope ratios of defatted powder and $^{13}\text{C}/^{12}\text{C}$ of oil fraction were measured using an Isotope Ratio Mass Spectrometer (Isoprime, Manchester, UK) after combustion of the sample (Isotope Vario, Elementar, Bremen, Germany). Measurement of $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ of both the defatted powder and oil fraction was carried out with a Delta Plus XP IRMS (Thermo Fisher Scientific, Bremen, Germany) connected to a TC/EA pyrolyzer (Thermo Fisher Scientific). The preliminary investigation performed shows that H and O stable isotopes ratios seem to better discriminate different geographical origin (Fig. 1), with defatted kernel powder and walnut oil fraction providing similar trends. Specifically, the Italian samples cluster together, with the only exception of one sample from the Campania region. All the international samples fall out the Italian cluster; this is evident mainly for Chile and Australia. The sample from California and France, although not clustering with the Italian samples, are less distant. The analysis of stable isotope ratios for C, N and S, does not result in a clear distinction of samples according to their geographical origin. Distinctive values of $^{34}\text{S}$ were detected only in four samples: the one from Australia and three from Italy (10.1‰, sample from Piedmont; -5.9‰ sample from Veneto; 11.4, sample from Trentino). To the best of our knowledge these exploratory analyses are the first application of the isotope ratio mass spectrometry (IRMS) in assessing the geographical origin of walnuts. The preliminary results here presented, although revealing the need for an improvement in our data collection, in order to cover homogeneously the areas of walnut production, suggest that at least H and O stable isotope ratios could provide a successful approach to determine walnut origin and to detect potentially commercial frauds.

![Fig. 1. Score plot of $\delta^2\text{H}$ versus $\delta^{18}\text{O}$ in walnuts kernel (defatted powder) from different geographical origin (different colors represent different provenances, see legend)](image_url)

References:
2nd Isotope Ratio MS Day – June 27-29, 2018, Messina

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