Development and implementation of stable isotope ratio analysis in bulk products and sub-components to ensure food traceability

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GLOSSARY

Non-technical

CEN	European Committee for Standardization
DOC	Controlled Designation of Origin
DOCG	Controlled and Guaranteed Designation of Origin
EEC	European Economic Community
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organisation
IAEA	International Atomic Energy Association
IGP	Protected Geographic Indication
ISMEA	Indicatori del Sistema Agroalimentare Italiano
ISTAT	Istituto Nazionale di Statistica
OIV	The International Organization of Vine and Wine
PDO	Protected Designations of Origin
PGI	Protected Geographical Indication
SINAB	Sistema Informazione Nazionale Sviluppo Agricoltura Biologica
TSG	Traditional Speciality Guaranteed

Technical

AIR	AIR - atmospheric air used as an international standard for which the $^{15}N/^{14}N$ ratio is precisely known and is defined as 0‰ on the $\delta^{15}N$‰ scale.
ATP	Adenosine Triphosphate
$^{13}C/^{12}C$	The ratio of the isotope of carbon with atomic mass 13 to the isotope of carbon with atomic mass 12

$\delta^{13}C$‰	"Delta carbon-13 *per* mil". The $^{13}C/^{12}C$ ratio expressed relative to the international standard Vienna-Pee Dee Belemnite.
CAM	Crassulacean acid metabolism
CSIA	Compound-Specific Isotope Analysis
EA	Elemental Analyser
EVOO	Extra-virgin olive oil
FA	Fatty acid
FAME	Fatty acid methyl ester
GA3P	Glyceraldehyde 3-phosphate GA3P
GC	Gas Chromatography
GC-C-IRMS	Gas Chromatography-Combustion-IRMS
GC-Py-IRMS	Gas Chromatography-Pyrolysis-IRMS
$^{2}H/^{1}H$	The ratio of the isotope of hydrogen with atomic mass 2 to the isotope of hydrogen with atomic mass 1
$\delta^{2}H$‰	"Delta hydrogen-2 *per* mil". The $^{2}H/^{1}H$ ratio expressed relative to the international standard Vienna-Standard Mean Ocean Water.
HTC	High temperature conversion
IRMS	Isotope Ratio Mass Spectrometry
KIE	Kinetic Isotope Effect
MWL	Meteoric Water Line
$^{15}N/^{14}N$	The ratio of the isotope of nitrogen with atomic mass 15 to the isotope of nitrogen with atomic mass 14.
$\delta^{15}N$‰	"Delta nitrogen-15 *per* mil". The $^{15}N/^{14}N$ ratio expressed relative to the international standard AIR.
$NADP^{+}$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NMR	Nuclear Magnetic Resonance

$^{18}O/^{16}O$	The ratio of the isotope of oxygen with atomic mass 18 to the isotope of oxygen with atomic mass 16.
$\delta^{18}O$‰	"Delta oxygen-18 *per* mil". The $^{18}O/^{16}O$ ratio expressed relative to the international standard Vienna-Standard Mean Ocean Water.
OO	Olive oil
OOA	Oxaloacetate
PEP	Phosphoenolpyruvate
PGA	Phosphoglycerate
RuBP	Ribulose-1,5-bisphosphate
$^{34}S/^{32}S$	The ratio of the isotope of sulfur with atomic mass 34 to the isotope of sulfur with atomic mass 32.
$\delta^{34}S$‰	"Delta Sulfur-34 *per* mil". The $^{34}S/^{32}S$ ratio expressed relative to the international standard Vienna-Canyon Diablo Troilite
SI	International System
SIRA	Stable Isotope Ratio Analysis
SNIF-NMR	Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance
TMU	Tetramethylurea
V-CDT	Vienna-Canyon Diablo Troilite - AgS S-1 silver sulfide used as an international standard for which the $^{34}S/^{32}S$ ratio is precisely known and is defined as 0.3‰ on the $\delta^{34}S$‰ scale
V-PDB	Vienna-Pee Dee Belemnite - Calcium carbonate used as an international standard for which the $^{13}C/^{12}C$ ratio is precisely known and is defined as 0‰ on the $\delta^{13}C$‰ scale
V-SMOW	Vienna-Standard Mean Ocean Water - ocean water used as an international standard for which the $^{18}O/^{16}O$ ratio is precisely known and is defined as 0‰ on the $\delta^{18}O$‰ scale
‰	Parts *per* thousand (*per* mil)

ABSTRACT

Objective assessment of food authenticity has become of paramount importance today, with an increasing demand for analytical methods able to provide proof of origin and prevent food adulteration. In order to achieve this goal, the European Union has reinforced control activities and investigated new methods able to support food characterisation and geographical traceability.

Stable isotope ratio analysis of so-called bioelements such as hydrogen, carbon, oxygen, nitrogen and sulfur has been used since the 1990s for food authenticity control of different premium products. Application of isotopic composition is based on evidence that the isotopic signature is affected by various environmental factors, such as photosynthetic and nitrogen cycles, the pedological characteristics of soils, agricultural practices, botanical origin, geographical origin and climatic or hydrological conditions.

In this study, isotope ratio mass spectrometry methods were developed to characterise and determine the authenticity of cereal crops, pasta, wine, balsamic vinegar, oenological tannins and extra-virgin olive oils.

In particular, isotopic variability measured along the Italian pasta production chain allowed good discrimination in relation to geographical provenance. $\delta^{2}H$, $\delta^{18}O$ and $\delta^{13}C$ were shown to be significantly correlated to geographical factors (e.g. longitude), whereas $\delta^{15}N$ and $\delta^{34}S$ were affected by geology and fertilisation practices. Measurement of amino acid $\delta^{15}N$ and $\delta^{13}C$ values improved discrimination of conventional and organic wheat compared to stable isotope ratio analysis of bulk.

Variability in the $\delta^{15}N$ value was checked along the entire oenological chain for the first time. The study included evaluation of the effect of the fermentation process using different types of yeast, the addition of nitrogen adjuvants and ultrasound lysis simulating wine ageing. Despite nitrogen isotope fractionation observed from soil to wine through the plant, the $\delta^{15}N$ value of proline conserved the nitrogen isotopic fingerprint of the growing soil and can therefore be used as an additional isotopic marker to trace the geographical origin of wine.

The same analytical approach, when applied to commercial tannin samples, made it possible to characterise them based on botanical origin. The $\delta^{13}C$ values were shown to be significantly more negative in tannins from grapes, tea and acacia and less negative in tannins from oak.

Furthermore, the $^{2}H/^{1}H$ and $^{13}C/^{12}C$ stable isotope ratios of acetic acid and the $^{18}O/^{16}O$ of water were investigated in “aceto balsamico di Modena IGP” (ABM) samples. No isotopic variation was observed along the ABM production chain, providing experimental evidence that such analytical parameters, used routinely for wine, can also be used for ABM in authenticity studies.

Finally, the $^{2}H/^{1}H$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ of extra-virgin olive oils were analysed in bulk samples and compounds, specifically in fatty acids. The results showed that in some cases bulk analysis was able to discriminate between different countries on the basis of specific geo-climatic conditions. Moreover, both the $^{13}C/^{12}C$ and $^{2}H/^{1}H$ values of the main fatty acids allowed good discrimination between European and non-European extra-virgin olive oils.

To conclude, the methods developed for the specific commodities considered can be proposed as suitable tools for the detection of mislabelling and for consumer protection, demonstrating that isotopic analysis can effectively contribute towards distinguishing the authenticity of commercial food samples.

Keywords: IRMS, GC-C/Py-IRMS, food, origin, traceability, authenticity

CHAPTER 1 INTRODUCTION

1.1 FOOD AUTHENTICITY

Adulteration of food and beverages is an increasing global problem and new fraud cases are frequently reported. Fraud and adulteration of foodstuff can be related to the geographical origin, agricultural system used or processing method, or involve cases in which products have been diluted or in which chemical food components have been substituted by cheaper imitations (FPDI, https://foodprotection.umn.edu/). Nowadays, objective assessment of food authenticity has become of paramount importance, as consumers are increasingly demanding information and reassurance regarding the origin and content of their food. Traceability has thus become a cornerstone of the quality policy system of the European Union (EU), as it is an effective risk-management tool which enables the food industry or the authorities to withdraw or recall products identified as dangerous. According to EU law, "traceability" means the ability to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing and distribution (EC Reg. 178/2002). In 1992 and subsequently in 2006 (EEC Reg. 2081/1992 and EC Reg. 510/2006), the EU created the PDO (Protected Designations of Origin), PGI (Protected Geographical Indication) and TSG (Traditional Speciality Guaranteed) systems to provide a set of common rules across countries to register and protect geographical names used to identify food products and traditional production processes.

In the majority of cases, the standard traceability system adopting paper documentation can guarantee the geographical origin of foods. However, the increasing complexity and length of the food chain and recent food scares have underlined the need for tools ensuring that foods are of a high quality and safe to be eaten. Thus, the food industry urgently requires analytical methods to provide proof of origin and prevent undeclared deliberate or accidental admixture to food samples. Isotopic analysis has been used in official controls since the 1990s but in fact it has been applied and reported in the literature since the 1970s [Bricout, 1973]. Application of isotopic composition is based on evidence that the isotopic signature is affected by various environmental factors, such as botanical origin and climatic or geographical conditions.

1.2 STABLE ISOTOPES

1.2.1 Definition of stable isotopes

Isotopes are atoms of the same element that contain equal numbers of protons but different numbers of neutrons in their nuclei, and hence differ in atomic mass. The main elemental constituents (H, C, N, O, and S) of bio-organic material exist in nature as 2 or more stable isotopes (^{2}H, ^{1}H; ^{13}C, ^{12}C; ^{15}N, ^{14}N; ^{18}O, ^{17}O, ^{16}O; ^{36}S, ^{34}S, ^{33}S, and ^{32}S). The most abundant isotope found in nature is the lighter, as shown in Table 1, which reports their mean abundance. They are called stable because the time of decay for this kind of isotope is very long, in the order of billions of years, in contrast to radioactive isotopes which have a time of decay in the order of thousands of years.

Table 1. Mean natural abundance of some stable isotopes of the light bio-elements

Element	Stable isotope	Mean natural abundance (%)
Hydrogen	^{1}H	99.99
	^{2}H	0.01
Carbon	^{12}C	98.89
	^{13}C	1.11
Nitrogen	^{14}N	99.63
	^{15}N	0.37
Oxygen	^{16}O	99.76
	^{17}O	0.04
	^{18}O	0.20
Sulphur	^{32}S	95.00
	^{33}S	0.76
	^{34}S	4.22
	^{36}S	0.02

The isotopic composition of organic compounds shows fluctuations around these mean values, and variations can be measured precisely and accurately, even if in the order of ppm, using dedicated analytical techniques such as Isotope Ratio Mass Spectrometry (IRMS). Stable isotope compositions are normally expressed as delta values (δ) according to equation 1:

$$X = \frac{R_s - R_{std}}{R_{std}} \qquad (1)$$

and are subsequently presented in units of parts *per* thousand (‰) or in the International System (SI) unit urey (mUr, which is equivalent to ‰) [Brand and Coplen, 2012]. R_s and R_{std} are the molar ratios of the heavy isotope relative to the light isotope in the sample and a standard respectively. For example, X can be δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$, and R values are the corresponding isotope ratios: $^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ respectively. δ(‰) values are normally reported relative to the following internationally agreed standards (R_{std}): Vienna - Standard Mean Ocean Water (V-SMOW) for δ^2H and $\delta^{18}O$, Vienna - Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, air for $\delta^{15}N$, Vienna-Canyon Diablo Troilite (V-CDT) for $\delta^{34}S$ [Brand *et al.*, 2014].

A positive δ(‰) value indicates that the sample has relatively more of the heavy isotope than the standard, while a negative δ(‰) value indicates that the sample has relatively less of the heavy isotope compared to the standard [Sulzman, 2007; Carter and Barwick, 2011]. The biological, biochemical and climatic causes of these delta value differences are addressed in the following section.

1.2.2 Natural variation in stable isotope abundance

The isotopes of an element have the same chemical properties, but they nonetheless show different abundance. The distribution of isotopes varies in nature, depending on the physical and (bio)chemical reaction in which they are involved – a process known as isotope fractionation. Indeed, mass differences can interfere with both the rate of reaction (kinetic effect) and the energetic state of the system (thermodynamic effect), resulting in enrichment or depletion of an isotope away from its mean natural abundance [Galimov, 1985].

Kinetic isotope fractionation is a process that separates stable isotopes from each other by their mass during (bio)chemical processes. Due to their lower weight, lighter isotopes show greater mobility and smaller bound strength and consequently lower activation energy. The thermodynamic effect is due to the different free energy of isotopically different molecular species: heavier molecules have lower free energy, so they have greater inertia in reactions, and in the physical states tend to concentrate in the condensed phases. Isotopic fractionation can also be due to situations with an altered reaction equilibrium, such as an instantaneous change in temperature, removal of a reactant or reaction product. This kind of fractionation (of non equilibrium, such as enzymatic reactions) determines the enrichment of a particular isotopic species, but without pre-established rules.

Consequently, factors affecting the variability of the isotopic ratios were explored in more depth.

CARBON

As discussed earlier, carbon has two naturally occurring stable isotopes and the ratio of the heavier isotope, ^{13}C, to the light isotope, ^{12}C, can be determined using IRMS. Figure 1 shows the carbon isotopic composition of some natural substances.

The original source of carbon in plants comes from the CO_2 in air. Plants absorb CO_2 and combine it with water and light to make carbohydrates – the process known as photosynthesis. Formation of C-C bounds is promoted if carbon atoms are lighter and more movable, so photosynthetic products are enriched in ^{12}C and depleted in ^{13}C as compared to atmospheric CO_2 ($\delta^{13}C_{CO2}$ around -8‰). Furthermore, different photosynthetic pathways (C_3, C_4 and CAM) are reflected in the different carbon isotopic ratios of plants, because of the different isotopic discrimination capability of carboxylase enzymes involved in CO_2 fixation.

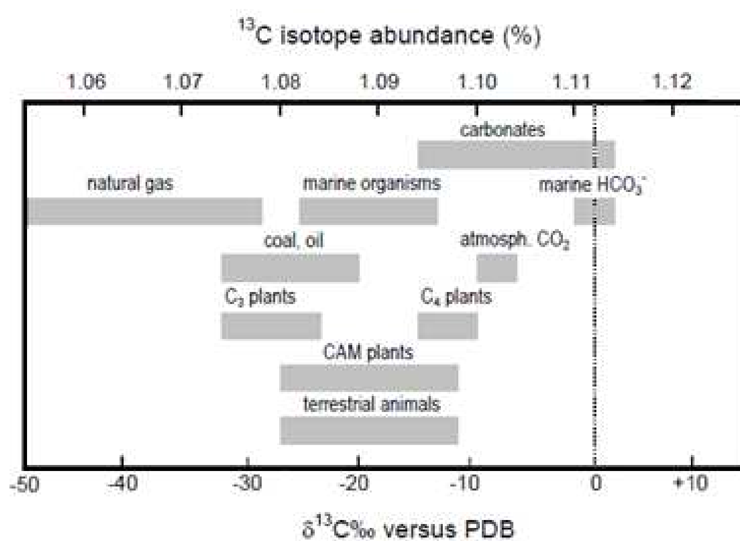

Figure 1. ^{13}C isotope variation ranges of carbon pools [Kelly, 2001]

Calvin cycle

The Calvin cycle is the metabolic process used for fixing carbon dioxide, characteristic of plants growing in cold-temperate areas (e.g. tomatoes, potatoes, beetroot, wheat, rice, oats, barley, rye, soybean, grapes, oranges, apples). Plants that use the Calvin cycle are known as C3 plants, because CO_2 is fixed in intermediate products with three atoms of carbon. Calvin cycle reactions can be divided into three main stages: carbon fixation, reduction and regeneration of the starting molecule (Figure 2).

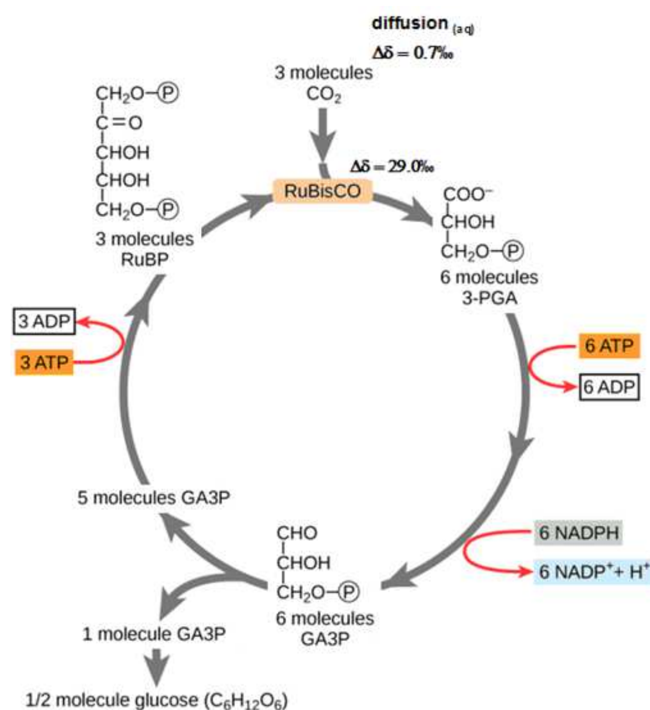

Figure 2. Synthesis of carbohydrate in the Calvin (C_3) cycle

Atmospheric CO_2 diffuses into the leaf through the stomata and dissolves in the cytoplasm [Hatch and Slack, 1966; Smith and Epstein, 1971]. There it combines with a five-carbon sugar, ribulose-1,5-bisphosphate (RuBP), producing two molecules of phosphoglycerate (PGA, a C3 molecule), which are subsequently phosphorylated by ATP and then reduced by NADPH to form Glyceraldehyde 3-phosphate (GA3P, a C3 sugar) [Taiz and Zeiger, 1998].

Five out of six GA3P molecules are used to synthesise more RuBP via a series of complex reactions driven by ATP. The sixth molecule of GA3P is used to synthesise glucose (usually regarded as the end product of photosynthesis) via combinations and rearrangements.

Although the initial reaction of CO_2 with RuBP produces the overriding ^{13}C isotope effect associated with these species, there are many other factors that contribute to the final $\delta^{13}C$‰ value of plant material, such as temperature, fertilisation, salinity, CO_2 concentration, light intensity and photorespiration [O'Leary, 1981]. The interplay of all of these factors results in $\delta^{13}C$‰ values between -22‰ and -34‰ for 80% to 90% of plants utilising the C_3 pathway [Krueger and Reesman, 1982].

Hatch-Slack cycle

The Hatch-Slack cycle is the metabolic process for fixing carbon dioxide used by plants able to utilise CO_2 concentrations as low as 0.1 ppm (while the Calvin mechanism does not operate at atmospheric concentrations of less than approximately 50 ppm). Plants which use the Hatch-Slack cycle are known as C4 plants (e.g. maize, sugar cane, corn, sorghum, millet and some types of pasture grasses), because CO_2 is fixed in intermediate products with four atoms of carbon. The Hatch-Slack cycle is characterised by two sequential carboxylation reactions as shown in Figure 3.

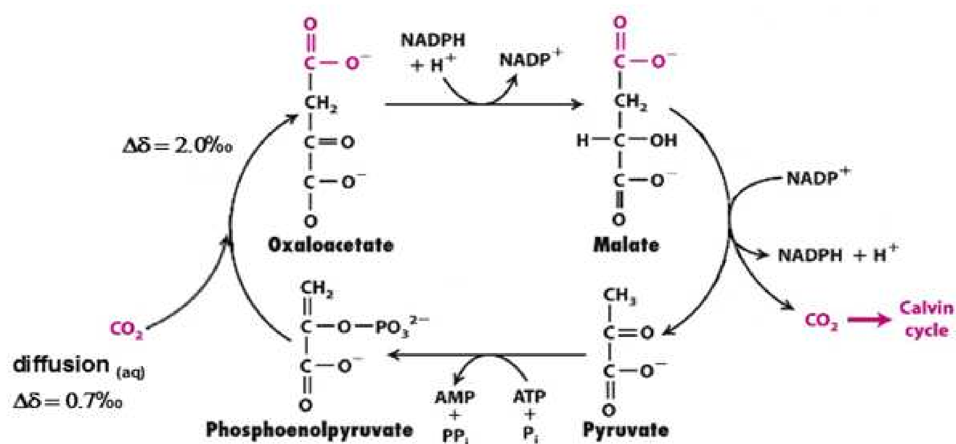

Figure 3. The Hatch-Slack pathway of C_4 photosynthesis

When atmospheric CO_2 enters the leaf through the stomata, it is fixed at a 3-carbon compound, phosphoenolpyruvate (PEP), to form oxaloacetate (OOA), a 4-carbon acid. This first stage proceeds with much smaller fractionation as compared to the Calvin cycle, $\Delta\delta \sim 2‰$ [O'Leary, 1981]. OAA is then rapidly reduced by NADPH to form malate (MAL, a C_4 acid). This acid is transported deeper into the C_4 plant leaves and oxidised by $NADP^+$ to form pyruvate (a C_3 compound) and CO_2. The C_3 compound is converted to PEP by the action of ATP, whereas the CO_2 feeds into the Calvin cycle, where it is used to synthesise glucose as shown in Figure 2. It is important to note that although the C_3 carboxylase enzyme shows extensive ^{13}C isotope fractionation, it is *not expressed* in the Hatch-Slack photosynthetic pathway. This is because the pre-fixation of CO_2 by carboxylation of PEP is an irreversible reaction. This results in relatively enriched $\delta^{13}C‰$ values for C_4 plants, between -10‰ and -14‰ [Winkler, 1984].

Crassulacean Acid Metabolism

Crassulacean acid metabolism, also known as CAM photosynthesis, is a carbon fixation pathway evolving in some plants (e.g. pineapple, vanilla, cacti) as an adaptation to arid conditions. In a plant using CAM, the stomata in the leaves remain shut during the day to prevent water loss through transpiration [Krueger and Reesman, 1982], but open at night, using C_4 metabolism to fix CO_2.
However, if the daytime temperature is relatively low, the stomata may open and the plant will adopt direct C_3 metabolism of CO_2. The metabolism adopted by CAM plants is therefore linked to local climatic conditions and in extreme cases may be predominantly C_3 or C_4. Consequently, the $\delta^{13}C$‰ value of CAM plant material varies widely between -30‰ and -12‰ [Winkler, 1984].

Secondary carbon metabolism

Many metabolic processes involving side reactions or branching processes cause further isotope fractionation. Generally, secondary metabolites tend to be relatively depleted in ^{13}C compared with the primary source, due to kinetic isotope effects (KIEs) [Schmidt and Kexel, 1999].
This is most notable in lipid fractions, which may differ from leaves by as much as 10‰. This ^{13}C depletion is caused by an isotope effect associated with the decarboxylation of pyruvic acid during fatty acid chain construction and by a non-statistical ^{13}C distribution in sugars precursors [DeNiro and Epstein, 1977; Monson and Hayes, 1982].

Climatic effect

$\delta^{13}C$ is also influenced by climatic factors such as local humidity and temperature, which influence leaf stomata opening and hence the efficiency of photosynthesis [Ferrio *et al.* 2003]. Dry conditions cause a restriction of the stoma with limitation of atmospheric CO_2 admission to the leaf, causing an increase in $\delta^{13}C$.

NITROGEN

Two nitrogen stable isotopes exist in nature, namely ^{14}N and ^{15}N. The range of ^{15}N variation in the terrestrial environment is shown in Figure 4.

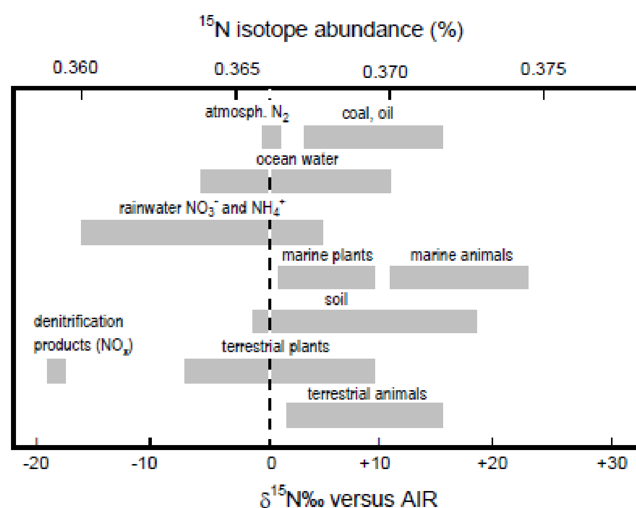

Figure 4. ^{15}N isotope variation ranges of nitrogen pools [Kelly, 2001]

The atmosphere is the principal nitrogen reservoir and through physical processes and the activity of microorganisms it is transformed into inorganic forms (nitrates, ammonia) and organic forms (amino acids, proteins) present and available in soil. The natural cycle of nitrogen encompasses five main mechanisms, shown in Table 2: nitrogen fixation, nitrogen assimilation\dissimilation, nitrogen mineralisation, nitrification and denitrification [Meier-Augenstein, 2010]. Depending on which of these mechanisms occurs, the $\delta^{15}N$ values of soils can vary considerably, generally between -10 and +15‰.

In the context of plant materials, enrichment or depletion of ^{15}N is mainly influenced by the type of fertiliser applied. Synthetic fertilisers, produced from atmospheric nitrogen, show $\delta^{15}N$ values between -4 and +4‰, whereas manure and organic fertilisers are enriched in ^{15}N, with values ranging between +0.6 and +36.7‰ [Bateman *et al.*, 2007].

Plants display $\delta^{15}N$ values that are linked to ammonia and nitrates in soils and are thus affected by the same variability factors described above, but also by the isotopic fractionation involved in uptake and organic compound assimilation processes [Werner and Schmidt, 2002]. Factors such as proximity to the sea and water stress can induce enrichment in ^{15}N [Heaton, 1987]. Leguminous and nitrogen-fixing plants are a separate case, as they can fix nitrogen directly from the air, showing $\delta^{15}N$ values around 0‰ [Yoneyama, 1995].

Table 2. Processes in the nitrogen cycle that give rise to isotopic fractionation

Process	Description of the process	Fractionation
Fixation	Natural process, either biological or abiotic, by which nitrogen (N_2) in the atmosphere is converted into ammonia: - through bacteria (*e.g.* through nitrogenise enzyme in legumes); - through physical processes producing high temperatures (*e.g.* lightening, fire); - through human activities (*e.g.* production of energy or fertilisers).	–3/+1‰ (data concerning legumes) (Fogel and Cifuentes, 1993)
Assimilation	Process of incorporation of nitrogenous compounds (NO_x, NH_3) by microorganisms or plants. At the beginning nitrogen oxides are reduced to ammonia and subsequently integrated within organic matter.	Assimilation favors incorporation of ^{14}N compared to ^{15}N, with a mean fractionation of -0.5‰ which is negligible in plants.
Dissimilation	Metabolic reactions that use the assimilated nitrogen.	
Mineralisation	Transformation of organic nitrogen in soil into ammonia.	±1‰
Nitrification	Biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates.	-12/-29‰
Volatilisation	Volatilisation reaction of ammonia as a gas from soil to atmosphere (very marked in alkaline soil).	+20‰
Denitrification	Nitrate reduction that may ultimately produce molecular nitrogen.	Enrichment in ^{15}N.

OXYGEN

Oxygen exists in nature in three stable isotopic species: ^{16}O, ^{17}O and ^{18}O. Of the two minor isotopes, only ^{18}O has been employed in isotopic studies and the natural variation range of $\delta^{18}O$ is shown in Figure 5.

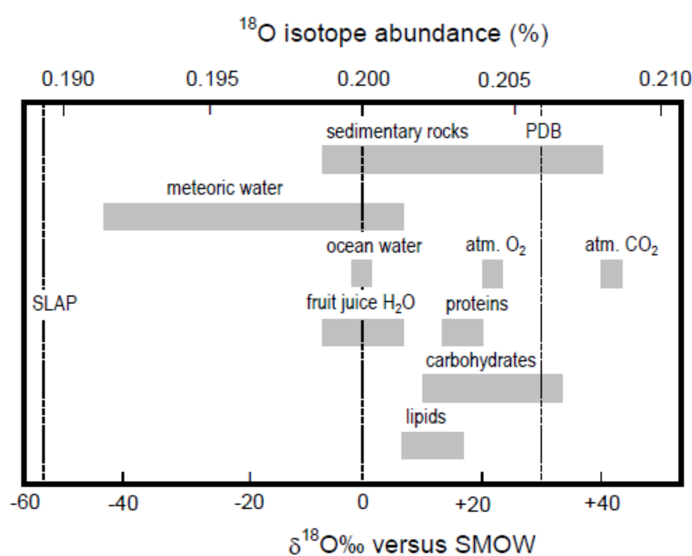

Figure 5. ^{18}O isotope variation ranges of oxygen pools [Kelly, 2001]

The $\delta^{18}O$ variability of meteoric water is based on hydrological cycles of evaporation from oceans, atmospheric vapour transport, precipitation and the subsequent return of freshwater to the ocean (directly via precipitation and via runoff/iceberg melting).

The oxygen isotopic composition of oceanic water is close to 0‰, with values ranging between -1 and +0.7‰ [Clark and Fritz, 1997]. During evaporation there is enrichment of the lighter isotope in vapour, calculated in a hypothetical situation of equilibrium as:

$\delta^{18}O$ = -10.0‰ for oceans at 20°C
$\delta^{18}O$ = -11.6‰ for oceans at 10°C

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

$\delta^{18}O$ = 1.5‰ for precipitation at 15°C
$\delta^{18}O$ = 2.2‰ for precipitation at 5°C

These values do not agree with the mean isotopic composition of world precipitation ($\delta^{18}O$ = -4‰), proving that from an isotopical point of view evaporation and condensation are non-equilibrium processes (mainly determined by low humidity level, temperature, wind and degree of salinity).
After evaporation, water vapour moves from subtropical zones toward the poles, where it condenses in the form of precipitation due to cold temperatures, becoming depleted in heavy isotopes, which are concentrated in initial rainfall (Figure 6).

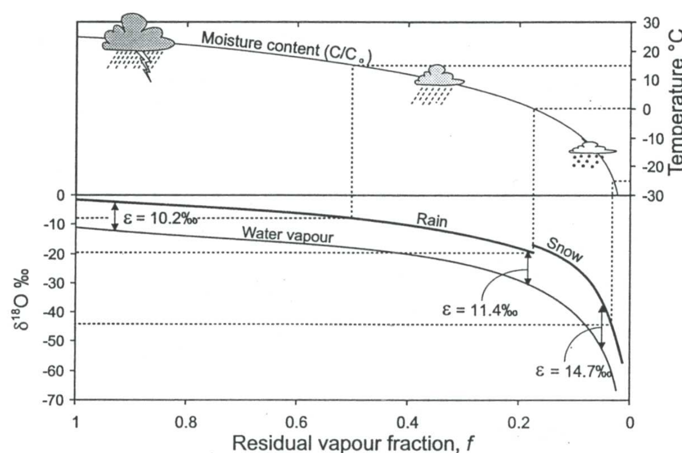

Figure 6. Isotopic fractionation of vapour mass in relation to temperature [Clark and Fritz, 1997]

Thus, equatorial precipitations and vapour are richer in heavier isotopes than water at the poles, with an intermediate situation according to latitude. Consequently, latitude is a discriminating factor

in oxygen isotopic fractionation. However, in Figure 7 considerable deviations can be noted (*e.g.* on the east coast of South America or on the Atlantic Ocean between Mexico and Scandinavia), due to a "continental effect". This latter is related to the vapour masses moving over continents, causing precipitation along coasts which is isotopically richer than that in continental areas (mean depletion of -2.8‰/1000 km from the coast) (Förstel and Hutzen, 1984). Moreover, different altitudes inland lead to ^{18}O depletion of about -0.15‰ to -0.5‰ *per* 100 metres elevation (Förstel and Hutzen, 1984), because at higher altitude there is lighter vapour. Finally, another deviation is due to seasonal trends; during summer there is enrichment in ^{18}O, especially inland.

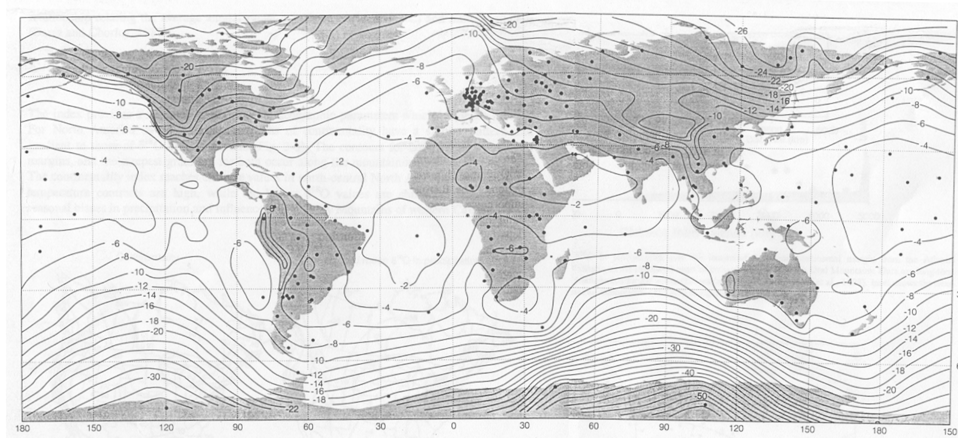

Figure 7. Distribution of mean $\delta^{18}O$ values for precipitation in 1992 and 1993 [Rozanski *et al.*, 1993]

Ground water has an isotopic composition related to the mean annual isotopic composition of precipitation water, and its $\delta^{18}O$ depends only on geographical factors (altitude, latitude and distance from the sea) but not on the season.

The isotopic composition of vegetal water in plants is related to the water absorbed from the soil. Furthermore, vegetal water in the leaf undergoes isotopic fractionation during evapotranspiration processes, which are affected by temperature and relative humidity and lead to an enrichment in heavier isotopes.

Oxygen in vegetal compounds derives from vegetal water but also from atmospheric CO_2 and O_2, with $\delta^{18}O$ values that are constant around +40.3/+42.5‰ and +23.5/+23.8‰ respectively. Moreover, oxygen integration through metabolic processes induces considerable additional isotopic fractionation. For example, the $\delta^{18}O$ of cellulose is correlated with the $\delta^{18}O$ of leaf water, with enrichment of around 27‰, caused by isotopic fractionation occurring during exchanges between the carbonylic group and water [Schmidt *et al.*, 2001; Barbour, 2007].

HYDROGEN

The primary source of hydrogen in nature is found in the hydrosphere. Hydrogen has two stable isotopes (^{1}H and ^{2}H) and examples of typical ^{2}H isotope ranges in various materials are shown in Figure 8.

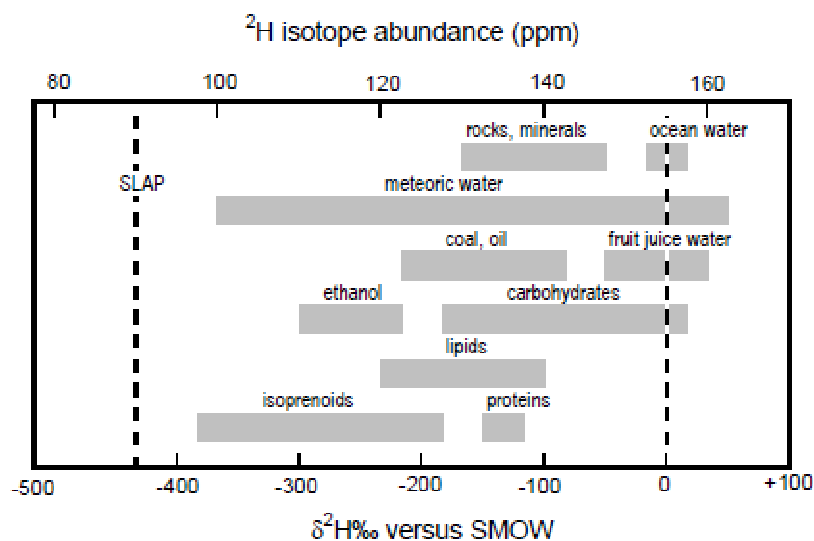

Figure 8. ^{2}H isotope variation ranges of hydrogen pools [Kelly, 2001]

The double mass of the heavier isotope results in significantly different physicochemical properties, particularly as regards the rate of reaction. This means that physical processes such as evaporation, condensation and biological processes can lead to significantly different levels in relation to natural ^{2}H abundance levels [White, 1988], as shown in Figure 9. The variation in ^{2}H in the hydrosphere follows an analogous pattern to the ^{18}O variation discussed above [Craig 1961].

The mean annual isotope ratios for hydrogen and oxygen in precipitation from regions as different as the Arctic, Antarctic, tropics and European and American continents all fall on the meteoric water line (MWL) [Dansgaard, 1964] described by the following equation:

$$\delta^{2}H‰ = 8\delta^{18}O + 10 \qquad (2)$$

So, as for oxygen, meteoric water that has passed through the meteorological cycle of evaporation, condensation and precipitation shows a systematic geographical isotope variation [Yurtsever and Gat, 1981].

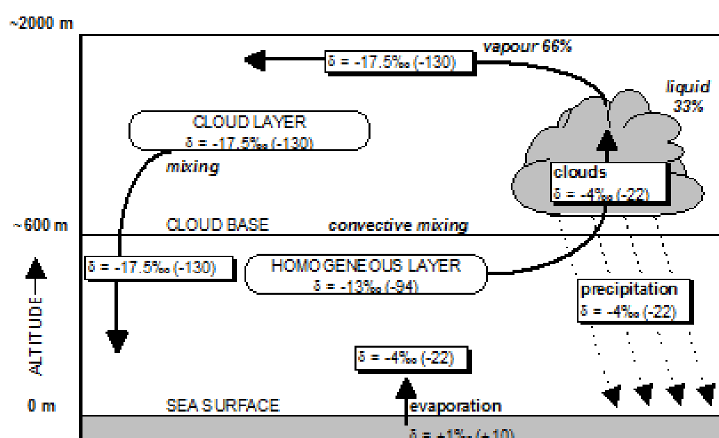

Figure 9. Evaporation/condensation model with typical delta values for atmospheric water masses.
The first δ-value refers to $\delta^{18}O$‰ and the second (in brackets) refers to $\delta^{2}H$‰ [Yurtsever and Gat, 1981]

Evaporation of water from the oceans is a fractionation process that causes a progressive depletion in heavy water ($H^{2}H^{16}O$, $HH^{18}O$) in the clouds as compared to the sea. Consequently, ground water reflects the isotopic gradient from the coast to inland areas [Dansgaard, 1964].

The hydrogen present in plant material originates from the water taken in by the roots [Ziegler *et al.*, 1976]. Water is transported through the xylem system, and the isotopic composition of water xylem is the same as that of ground water, while the water is then taken in by the leaves without any change in isotopic composition.

Evapotranspiration of water through the leaf stomata enriches the remaining water with heavier isotopomers. There are no apparent differences in the degree of enrichment of ^{2}H in the leaf water of plants utilising the Calvin (C_3), Hatch-Slack (C_4) or Crassulacean acid metabolism (CAM) photosynthetic pathways [Bricout, 1982]. Crops growing in regions with low humidity therefore show relatively enriched $\delta^{2}H$‰ values, due to a higher rate of evapotranspiration from leaves [Martin *et al.*, 1986].

Others factors affecting the extent of ^{2}H enrichment in plant products growing in similar temperate climates are the varietal origin and timing of maturation. Typically, leaf and fruit water enrichment is around 20 to 40‰.

Generally, primary metabolites (e.g. carbohydrates) tend to be relatively depleted in ^{2}H, resulting in $\delta^{2}H$‰ values of -90 to -180‰. Secondary metabolites exhibit additional depletion due to KIEs. The $\delta^{2}H$‰ value of protein does not differ significantly from carbohydrate [Winkler, 1984], however other carbohydrate reduction products such as ethanol, cholesterol and lipids are relatively depleted in deuterium.

The depletion in lipids can be further divided into two groups [Estep and Hoering, 1980]. The δ^2H‰ of triglycerides generated by two-carbon precursors in the fatty acid biosynthetic pathway is approximately –30 to –60‰, whereas nonsaponifiable lipids synthesised *via* the five-carbon isoprenoid pathway are depleted by a further 80‰, resulting in–110 to –140‰ depletion relative to carbohydrate.

SULFUR

In nature there are several inorganic compounds containing sulfur, the most common being elemental sulfur (S^0), sulphates (SO_4^{2-}) and hydrogen sulphide gas (H_2S).

Sulfur exists in four stable isotopes (^{32}S, ^{33}S, ^{34}S, and ^{36}S), and the isotopic abundance in a chemical substance depends on its source, mass fractionation and the KIE effect during the formation process. Of the three minor isotopes, only ^{34}S has been employed in isotopic studies and the natural $\delta^{34}S$ variation range is shown in Figure 10.

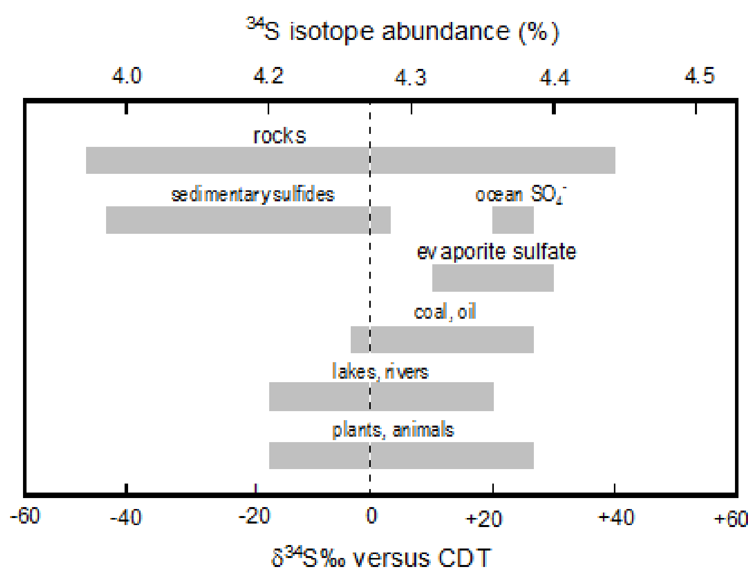

Figure 10. ^{34}S isotope variation ranges of sulfur pools [Kelly, 2001]

The average value of the sulfur isotope ratio on Earth reflects that of the solar system, which has value of around 22.22‰ [Thode *et al.*, 1961]. However, distribution of the different sulfur isotopes in global ecosystems is conditioned by biological activity, according to the kind of reducing bacteria, electron donors and the enzymatic reaction rate [Detmers *et al.*, 2001; Schidlowski, 1982]. Sulphates dissolving in seawater enter many of the redox processes occurring in the sea, leading to a basically uniform $\delta^{34}S$ value of around +21‰ [Rees *et al.*, 1978]. Sea spray, deriving from

dissolved oceanic sulphate, is one of the most important sources of atmospheric sulfur and causes an enrichment of $\delta^{34}S$ values in coastal regions [Lott *et al.* 2003, Hobson 2005, Zazzo *et al.* 2011]. Moreover, the sulfur cycle may also be influenced by anthropogenic input [Wynn *et al.* 2014]. The combustion of fossil fuels containing sulfur and some industrial processes involving sulfur compounds represent the main anthropogenic sources of primary SO_2 in the atmosphere. The $\delta^{34}S$ values of anthropogenic emissions generally show a wide range, depending on the nature of the source (*e.g.* coal – from -35 to +30‰, petroleum natural gas – from -20 to +30‰) [Nielsen, 1978]. The most common natural processes that can cause input of sulfur into the atmosphere, with typically depleted $\delta^{34}S$ values, are volcanic activities and forest fires [Camin *et al.* 2007]. Another important source of atmospheric sulfur is biogenic sulfur. This latter is released from soils and wetlands and characterised by ^{34}S-depleted values [Wadleigh and Blake, 1999; Mast *et al.*, 2001]. Natural factors that influence $\delta^{34}S$ values in terrestrial plants are the abundance of heavy sulphides in the soil, but also aerobic or anaerobic growing conditions [Rubenstein and Hobson, 2004], underlying local bedrock, microbial processes active in soil, fertilisation procedures and atmospheric deposition [Krouse and Mayer, 2000], such as the sea-spray effect over forage in coastal areas [Attendorn & Bowen, 1997].

The S source in any animal tissue is the sulfur contained in plant material. The $\delta^{34}S$ values of animal samples basically reflect diet, but show some ^{34}S enrichment depending on the state of nutrition, trophic level and individual tissue [Tanz and Schmidt, 2010].

1.3 STABLE ISOTOPE RATIO ANALYSIS

1.3.1 Bulk analysis

For analysis of stable isotope ratios, the sample was weighed into tin capsules to determine $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ and silver capsules for quantification of $^{18}O/^{16}O$ and $^{2}H/^{1}H$. The weighted amount depended on the % m/m of the elements in the sample. In each analytical sequence, a working in-house standards calibrated against international reference materials were analysed after every ten samples and used to calculate the isotope ratios of the samples. Sample analysis was carried out in duplicate, then calculating the mean values.

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values were measured after combustion of the sample, using a DELTAplus XP IRMS (ThermoFinnigan, Bremen, Germany) and visION IRMS (Isoprime Ltd, UK). The DELTAplus XP IRMS was equipped with a Flash EA 1112 elemental analyser (ThermoFinnigan) operating at 900°C in the presence of O_2 and CuO (Figure 11). The developed gases (CO_2, N_2 and SO_2) were then separated in a Porapak QS 80-100 mesh (3 m) GC column at 45°C and transferred into the ion source of an IRMS with helium as carrier gas (130 mL/min).

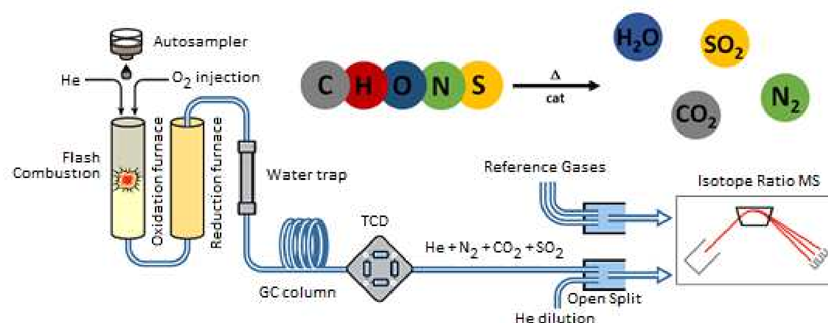

Figure 11. EA-IRMS schematic diagram and principle of combustion\oxidation (ThermoFinnigan)

The visION IRMS was equipped with a Vario Isotope Cube elemental analyser (Elementar Analysensysteme GmbH, Germany) operating at 850°C in the presence of O_2 and WO_3 (Figure 12). The developed gases (CO_2, N_2 and SO_2) were then separated with three molecular sieve traps and transferred into the ion source of an IRMS with helium as carrier gas (230 mL/min).

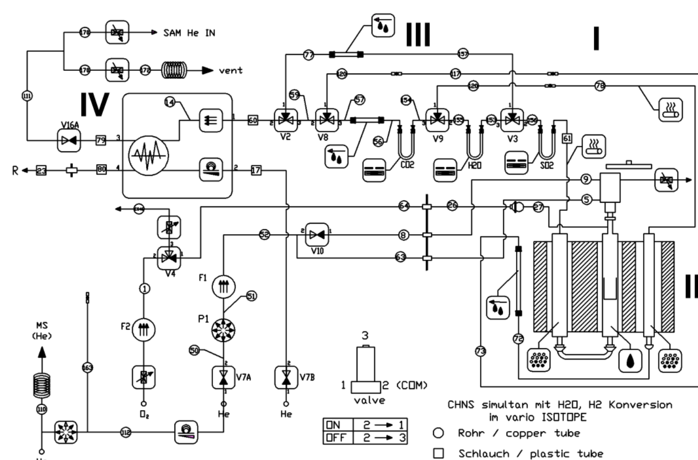

Figure 12. EA-IRMS schematic diagram (Isoprime)

For measurement of $\delta^{18}O$ and $\delta^{2}H$ values, the sample was instead subjected to high temperature conversion (HTC), using a Delta Plus XP IRMS (ThermoFinnigan, Bremen, Germany) equipped with a Finnigan™ TC/EA pyrolyser (ThermoFinnigan) (Figure 13). The developed gases (H_2 and CO) were separated in a Molecular Sieve 5A (3 m) GC column at 100°C and then transferred into the ion source of an IRMS with helium as carrier gas (100 mL/min).

A high furnace temperature (1450°C), low instrumental $[H_3]^+$ factor (<8, for correction of the contribution of $[H_3]^+$ to the m/z 3 signal) and dry conditions during analysis were ensured, in order to obtain reproducible results. Weighed samples were put in the carousel of the autosampler and stored in a desiccator above phosphorus pentoxide (P_2O_5) for at least 24 h until analysis. The carousel was then inserted into the autosampler and equipped with a suitable cover. During measurement, dryness was guaranteed by flushing dry nitrogen continuously over the samples.

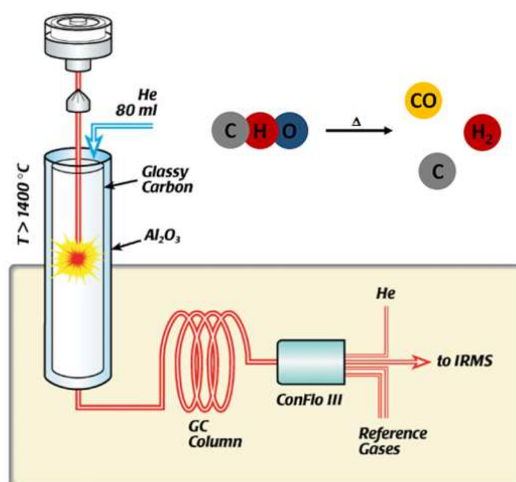

Figure 13. TC/EA-IRMS schematic diagram and principle of high temperature conversion (ThermoFinnigan)

Analysis of $\delta^{18}O$ in water was performed directly on the fresh sample, according to the UNI ENV 12141 method (1997). Analysis was carried out using an IRMS (VG Isogas, Middlewich, UK) interfaced with a CO_2-water equilibrator system (Isoprep18, VG Isotech, Middlewich, UK) allowing equilibration of oxygen exchange between sample water and a reference CO_2 (Figure 14). The CO_2 gas obtained from the sample and the same reference CO_2 were introduced alternately, via a dual-inlet system, to an IRMS for $\delta^{18}O$ measurement. In each analytical sequence of 24 samples, 4 working in-house standards were analysed and used to calculate the isotope ratio values of the samples.

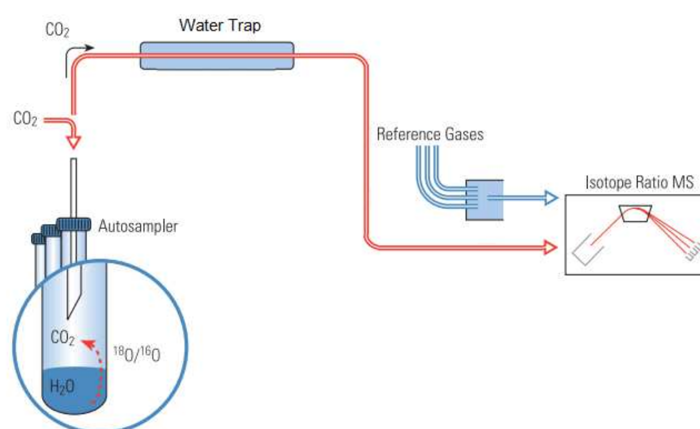

Figure 14. CO_2 equilibration - IRMS schematic diagram (adapted from ThermoFinnigan)

In IRMS, the gases (H_2, N_2, CO_2, and SO_2) enters the ion source through a narrow capillary where it is then ionised. The electrons needed for ionisation are produced by a hot filament usually made of rhenoim or thoriated tungsten [Scrimgeour and Robinson, 2004]. The sample ions generated are then accelerated by a series of electrode "lenses" before entering the mass analyser [Kelly, 2003]. In the mass analyser, the ions are deflected by either a permanent magnet or an electromagnet [Kelly, 2003]. The radius of deflection depends on the mass-to-charge-ratio, where ions with the same ratio experience the same deflection and heavy ions are less deflected than light ions. This deflection focuses the ions into several beams that finally enter the ion detector (Table 3).
In the ion detector, each of these beams is detected separately using a Faraday-cup [Kelly, 2003; Scrimgeour and Robinson, 2004]. The voltage produced via the discharge of the ion in the cup is then amplified and transformed into digital output. Different resistors in the ion detector can be adjusted for different beam intensities, so that all beams can be detected in the same voltage range [Scrimgeour and Robinson, 2004].

Table 3. Masses measured using IRMS to determine stable isotope ratios

Isotope	Molecular specie determined	Mass
H	H_2	2
^{2}H	$^{1}H^{2}H$	3
^{12}C	$^{12}CO_2$	44
^{13}C	$^{13}CO_2$	45
^{14}N	$^{14}N_2$	28
^{15}N	$^{14}N^{15}N$	29
^{16}O	$C^{16}O$	28
^{18}O	$C^{18}O$	30
	$^{12}C^{18}O^{16}O$	46
^{32}S	$^{32}SO_2$	64
^{34}S	$^{34}SO_2$	66

1.3.2 Compound-specific analysis

The role of EA and TC/EA is to quantitatively convert the target element present in a sample to the appropriate gas for IRMS analysis, regardless of the number of individual chemical species present. Techniques broadly described as Compound-Specific Isotope Analysis (CSIA) comprise an additional initial stage, in which the individual compounds present in a sample are separated on the basis of time. Each compound eluting from the GC column is then converted into simple gases when traversing a capillary micro-reactor. Accordingly, all compound specific isotope ratios can be analysed using IRMS. CSIA was carried out using a Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific), interfaced with an IRMS (DELTA V, Thermo Scientific), through an open split interface and with a single-quadrupole GC-MS (ISQ Thermo Scientific) to identify the compounds (Figure 15).

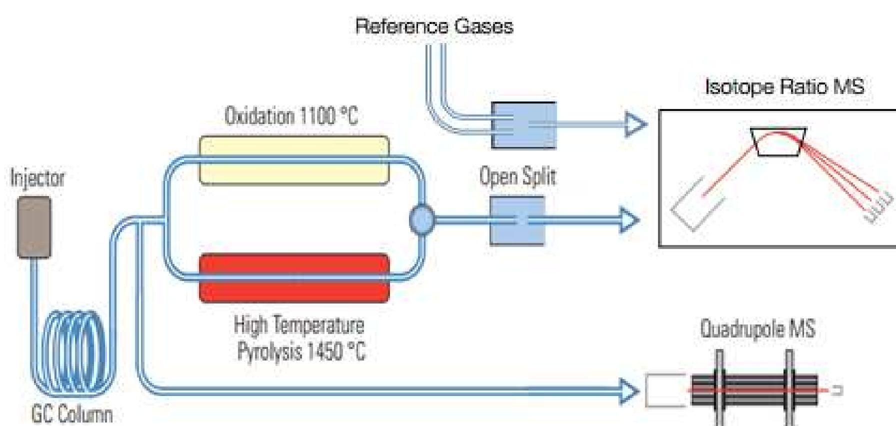

Figure 15. GC-C/Py-IRMS schematic diagram (ThermoFinnigan)

To determine $\delta^{13}C$ and $\delta^{15}N$, the compounds were oxidised in a capillary reactor (Ni/Cu/Pt) to CO_2, N_2 and H_2O at 1100°C. The H_2O forming in the oxidation process was removed with an online, maintenance-free water removal system. For analysis of $\delta^{15}N$, all CO_2 was retained in a liquid nitrogen trap before transfer to the IRMS. For measurement of δ^2H, each compound was passed through a high temperature reactor, operating at 1450°C, where it was subjected to high HTC, with development of hydrogen gas.

1.3.3 Site-specific analysis

Special Nuclear Magnetic Resonance (NMR) systems, such as Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR) can be used to measure the relative isotopic ratios of a given element (e.g. ^2H) for each molecular site. In the ^2H-NMR spectrum, the signal intensities are directly proportional to the number of moles of ^2H nuclei in resonance. Consequently, the site-specific isotope ratios ($^2H/^1H$) can be determined through quantitative measurement at each site and calculated by comparison with an internal standard (e.g. tetramethylurea TMU) having a certified isotopic value [Martin and Martin, 1990].^2H-NMR spectra were recorded using a SNIF-NMR spectrometer, equipped with a probe tuned to the characteristic resonance frequency of ^2H for the corresponding field. The specific ^2H probes had a proton decoupling channel and also a field-frequency stabilisation channel tuned to the fluorine frequency (lock).
The $^2H/^1H$ ratios obtained using SNIF-NMR are expressed in parts per million (ppm), it is possible to report the values on the δ-scale using the following equation:

$$\delta^2 H_i ‰ = \left(\frac{(^2H/^1H)_i}{(^2H/^1H)_{SMOW}} - 1 \right) \cdot 1000$$

where $(^2H/^1H)_i$ and $(^2H/^1H)_{SMOW}$ are the hydrogen isotopic values expressed in ppm of a specific intramolecular site (i) and standard V-SMOW (155.8 ppm) respectively.

1.4 STABLE ISOTOPES IN FOOD AUTHENTICATION

One of the most powerful techniques used in food authenticity studies is IRMS. IRMS has been used for the authentication of a wide range of foodstuffs, and recently this technique has gained wider acceptance in food control laboratories. Fraud and adulteration of foodstuff can be related to the geographical origin, agricultural system used or processing method, or involve cases in which products have been diluted or in which chemical food components have been substituted by cheaper imitations.

The isotopic fractionation processes described above often cause unique isotopic signatures for food products. These signatures are often sufficiently strong to verify the authenticity of a product. A selection of the major fractionation processes and the available associated information of relevance to food authenticity testing is presented in Table 4.

Table 4. Selected elements, corresponding delta notation and fractionation processes relevant for authenticity testing [Kelly *et al.*, 2005; Sulzman, 2007; Berglund and Wieser, 2011; Brand *et al.*, 2014]

Element	Delta notation	Fractionation processes	Information
Hydrogen	$\delta^{2}H$	Evaporation, condensation, precipitation, transpiration	Geography, water usage, fertilisation rate
Carbon	$\delta^{13}C$	CO_2 assimilation, stomatal conductance	Water use efficiency, fertilisation rate
Nitrogen	$\delta^{15}N$	N transformation processes, atmospheric loss	Fertiliser type and rate
Oxygen	$\delta^{18}O$	Evaporation, condensation, precipitation, transpiration	Geography, water usage, fertilisation rate
Sulfur	$\delta^{34}S$	S transformation processes, sea spray	Geography, fertiliser type

The relevant literature is summarised in Table 5, which shows the various commodity groups investigated, the parameters measured and the corresponding references.

Table 5. Summary of the relevant literature relating to the use of multi-isotopic analysis for food authenticity testing [Kelly *et al.*, 2005; Gonzalvez *et al.*, 2009; Laursen *et al.*, 2016]

Food commodity	**Authemticy case**	**Analytical technique**	**Parameters measured**	**Reference**
Meet	Geographical origin	IRMS	H, C, N, S	Schmidt *et al.*, 2005, Camin *et al.*, 2007, Heaton *et al.*, 2008, Perini *et al.*, 2009, Bong *et al.*, 2010, Osorio *et al.*, 2011
	Agricultural origin	IRMS	C, N, S	Bahar *et al.*, 2008
Butter	Geographical origin	IRMS	C, N, O, S	Rossmann *et al.*, 2000, Balling and Rossmann, 2004
Cheese	Geographical origin	IRMS	H, C, N, O, S	Pillonel *et al.*, 2004, Manca *et al.*, 2006, Bontempo *et al.*, 2012, Camin *et al.*, 2012, Camin *et al.*, 2015, EU Reg. 584/2011
Milk	Geographical origin	IRMS, GC-Py-IRMS	H, C, N, O, S	Rossmann *et al.*, 1998, Crittenden *et al.*, 2007, Camin *et al.*, 2008, Molkentin and Giesemann, 2010, Ehtesham *et al.*, 2013a, Ehtesham *et al.*, 2013b,
	Agricultural origin	IRMS	C, N	Molkentin and Giesemann, 2010, Chung *et al.*, 2014
Wine and must	Geographical origin	IRMS	H, C, O	Breas *et al.*, 1994, Rossmann *et al.*, 1999, Ogrinc *et al.*, 2001, Cristoph *et al.*, 2004, Gremaud *et al.*, 2004
	Sugar/water addition	IRMS, SNIF-NMR	H, C, O	EU Reg 555/2008
Vegetable oil	Geographical/botanical origin	IRMS, GC-C-IRMS GC-Py-IRMS	H, C, O	Kelly *et al.*, 1997, Breas *et al.*, 1998, Angerosa *et al.*, 1999, Spangenberg and Ogrinc, 2001, Aramendia *et al.*, 2007, Iacumin *et al.*, 2009, Bontempo *et al.*, 2009, Camin *et al.*, 2010a, Camin *et al.*, 2010b, Guo *et al.*, 2010, Faberi *et al.*, 2014, Banerjee *et al.*, 2015, Jeon *et al.*, 2015, Portarena *et al.*, 2015, Mihailova *et al.*, 2015, Horacek *et al.*, 2015
	Adulteration	IRMS GC-C-IRMS	H, C, O	Woodbury *et al.*, 1995, Angerosa *et al.*, 1997, Spangenberg *et al.*, 1998, Hrastar *et al.*, 2009, Seo *et al.*, 2010, Richter *et al.*, 2010, Kim *et al.*, 2015
Vinegar	Sugar/water addition, synthetic acetic acid	IRMS, SNIF-NMR	H, C, O	Thomas and Jamin, 2009, Camin *et al.*, 2013

Fruit and vegetables	Geographical origin	IRMS	H, C, N, O, S	Perez *et al.*, 2006, Meylahn *et al.*, 2006, Horita, 2008, Longobardi *et al.*, 2015, Mimmo *et al.*, 2015
	Agricultural origin	IRMS	H, C, N, O, S	Camin *et al.*, 2011, Sturm *et al.*, 2011, Bat *et al.*, 2012, Flores *et al.*, 2013, Laursen *et al.*, 2013, Mihailova *et al.*, 2014
Honey	Geographical origin	IRMS	H, C, N, S	Kropf *et al.*, 2010a, Kropf *et al.*, 2010b, Schellenberg *et al.*, 2010, Bontempo *et al.*, 2015
	Sugar addition	IRMS	C	White *et al.*, 1998
Cereal crops	Geographical origin	IRMS	C, N, O, S	Goitom-Asfaha *et al.*, 2011, Kelly *et al.*, 2002, Brescia *et al.*, 2002
	Agricultural origin	IRMS	H, C, N, O, S	Laursen *et al.*, 2013
Tomato passata	Water addition	IRMS	O	Bontempo *et al.*, 2014a
Fruit juice	Geographical origin	IRMS	C	Kornexl *et al.*, 1996, Martin *et al.*, 1996
	Sugar/water addition	IRMS, SNIF-NMR	H, C, O	Rossmann *et al.*, 1997, Simpkins *et al.*, 2000, Rummel *et al.*, 2010, Bontempo *et al.*, 2014b
Eggs	Agricultural origin	IRMS	C, N	Rogers *et al.*, 2015
Seafood	Geographical origin	IRMS	H, C, N, O	Carter *et al.*, 2015, Ortea and Gallardo 2015
Coffee and caffeine	Geographical origin	IRMS	H, C, N	Weckerle *et al.*, 2002
	Synthetic caffeine addition	GC-C-IRMS	C	Zhang *et al.*, 2012
Vanillin	Geographical origin	GC-C-IRMS GC-Py-IRMS	H, C	Hansen *et al.*, 2014
	Synthetic vanillin addition	GC-C-IRMS GC-Py-IRMS	H, C, O	Hener *et al.*, 1998, Greule *et al.* 2010
Tea	Geographical origin	IRMS	H, C, N	Pilgrim *et al.*, 2010

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CHAPTER 2 AIMS OF THE THESIS

2.1 AIMS OF THE THESIS

The general aim of this thesis was to verify the applicability of analysis of multi-element stable isotope ratios to food authenticity. In particular, analytical approaches based on bulk isotope analysis and compound-specific isotope analysis were developed for the traceability of different agricultural products and derivatives.

In detail, the research focused on:

– study of multi-element stable isotopes along the pasta production chain, to evaluate if and how the farming system and geographical origin affect the isotopic signature;

– development of an analytical approach to measure the isotopic fingerprint of individual amino acids of wheat samples in order to discriminate between organically and conventionally grown plants;

– investigation of the feasibility of using $\delta^{15}N$ as an additional isotopic marker able to link wine to the area of origin;

– evaluation of the efficacy of IRMS as a tool for tracing the botanical origin of oenological tannins;

– characterisation of "aceto balsamico di Modena": application of stable isotope ratio analysis for authentication of origin;

– geographical traceability of extra-virgin olive oils, by combining both stable isotope ratio analysis and NMR profiling approaches;

– geographical traceability of European and non-European extra-virgin olive oils: creation of a dataset with isotopic values measured in bulk samples and fatty acids.

CHAPTER 3 WHEAT AND DERIVATIVES

3.1 INTRODUCTION

Cereals (rice, wheat, corn and soybean) are the most important staple foodstuffs and play a vital role as the main source of energy, protein and fat intake for almost all the world's population. Wheat is a major food and important commodity in the grain market, with global production currently standing at around 730 million tonnes (2014 data provided by FAO, http://faostat.fao.org/). The largest wheat producer is China, accounting for 17% of world production, while the major wheat-growing countries in Western Europe are France, Germany, the United Kingdom, Italy, Spain and Portugal, in order of production.

Wheat is the most important arable crop in Italy in terms of area cultivated and economic significance, due to the production of pasta, which is the main constituent of the Mediterranean diet (ISTAT, http://agri.istat.it). Italy is the principal global producer of pasta, which represents about 7% of total agrofood exports, therefore having a primary role in the Italian economy and representing one of the main Italian products worldwide.

Nowadays, consumers tend to prefer organically produced food, which is generally considered to be healthier than conventional products. In Italy, cereals are some of the most widespread organic crops (SINAB, http://sinab.it) and the market for organic pasta has shown a growing trend in the last few years (ISMEA, http://ismea.it). It is therefore clear that there is an economic basis for developing analytical methods able to verify if labelling claims regarding the farming system and geographical origin are correct.

In the last few years, stable isotope ratio analysis (SIRA) has become a promising approach for cereal grain traceability, with a growing number of research papers published on this subject, suggesting that geographical discrimination of wheat grains is possible, due to the impact of microclimate, geology and pedology on isotopic composition [Longobardi *et al.*, 2015; Li *et al.*, 2016].

Wheat samples of different origin were successfully differentiated using the natural abundance of the isotopic ratios of C, N, O and H [Brescia *et al.*, 2002; Luo *et al.*, 2015; Wu *et al.*, 2015; Liu *et al.*, 2015]. Moreover, satisfying results were also obtained by combining the stable isotope ratios of the heavy mass element strontium ($^{87}Sr/^{86}Sr$) with light mass elements C, N, O and S [Goitom-Asfaha *et al.*, 2011; Podio *et al.*, 2013].

The use of SIRA for geographical authentication of cereals is more highly developed than verification of farming systems (organic or conventional). Considering different agricultural practices, some works have applied stable isotopic analysis of H, C, N, O and S in order to discriminate between organic and conventional wheat [Schmidt *et al.* 2005; Laursen *et al.* 2013].

SECTION 3.3

Multi-isotopic signatures of organic and conventional Italian pasta along the production chain

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Statement of the author: My personal contribution to this work mainly concerned the stable isotope ratio analysis ($\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$) of durum wheat, flour and pasta samples.

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Multi-isotopic signatures of organic and conventional Italian pasta along the production chain

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The variability of stable isotope ratios (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$) along the production chain of pasta (durum wheat, flour and pasta) produced by using both conventional and organic farming systems in four Italian regions in 2 years was investigated. The aim was to evaluate if and how the farming system and geographical origin affect stable isotope ratios determined along the production chain. Irrespective of the processing technology, 65% of the samples were correctly classified according to the farming system and 98% were correctly classified regarding the geographical region. When considering both farming system and geographical region simultaneously, 80% of the samples were correctly classified. The measured isotope parameters were thus primarily affected by the geographical origin. In conclusion, it is expected that the use of these parameters will allow the development of analytical control procedures that can be used to check the geographical origin of Italian organic and conventional pasta and its raw materials. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: durum wheat; geographical origin; IRMS; organic food; stable isotopes

Introduction

The awareness of food quality and safety has steadily increased amongst consumers during the past decades.[1] Consumers and European policy makers have an increased focus on the traceability of food products (Regulation EC No. 178/2002). Under the EU law, 'traceability' means the ability to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing and distribution. This includes both the geographical origin of a food product or its raw materials, as well as the farming system or processing method (e.g. organic *vs* conventional). The legal requirements regarding indication of the geographical origin of a product by labelling depend on the specific food considered but could be compulsory (e.g. honey, milk, milk used as an ingredient in dairy products, swine, sheep, goat and poultry meat) or optional (e.g. cereals).[2] On the other hand, EU regulations regarding labelling of organic products are very clear, and organic farmers, processors and traders must comply with specific requirements if they want to use the EU organic logo and label their products as organic (Council Regulation EC No. 834/2007 and the following amendment Regulation EC No. 967/2008). Although the EU regulation for organic farming system and geographical origin of food sets a legal frame and some general principles, clear operational principles, methods and related criteria are still missing in order to evaluate if the declarations on food labels are true or not.

Pasta is one of the basic and major food products of the Mediterranean diet. Italy is the principal global producer of pasta, and in 2014, Italy exported more than 2 million tons of it worldwide (Istituto di Servizi per il Mercato Agricolo Alimentare data, April 2015, http://www.ismeaservizi.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/5560). Pasta constitutes about 7% of the total agro-food exports of Italy, thereby having a primary role in the Italian economy and representing one of the principal 'Made in Italy' products worldwide. The Italian organic pasta sales were more than 25,000 tons in 2011 with a growing trend the past years.[3] Italian organic cereal products including pasta are potential candidates for future frauds and adulterations as no routine analytical methods are available for verifying if labelling claims regarding farming system and geographical origin are correct. Numerous studies have been conducted on pasta and its raw materials (more than 4302 when typing 'pasta' in the Scopus database, search on 8th July 2016), but these have mainly focused on its quality and/or the associated health impacts when consuming it in its 'original' form, when produced with specific cereal species and varieties or when diverse 'supplements' have been added.[4] Some studies have focused on verification of the geographical origin of wheat by using different approaches: e.g. multi-elemental analysis and/or stable isotope analysis of several light or heavy mass elements including H, C, N,

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O, S and Sr,[5–9] ^{1}H-NMR profiling[10] and near-infrared reflectance spectroscopy[11–13]. Only a limited number of studies have focused on discriminating organic and conventional wheat (both bread and durum) and cereals in general.[14–17]

In the past decade, the ability of stable isotope analysis to determine the agricultural and geographical origin of food has increasingly been investigated. In particular, ratios of stable nitrogen isotopes (δ^{15}N) in the bulk tissue or in isolated chemical compounds of the foodstuff (e.g. nitrate) have been investigated mainly in fruits and vegetables to detect differences between organic and conventional products.[15,18–23] This approach is based on the fact that the nitrogen isotopic composition of synthetic fertilizers, widely used in conventional farming systems, is generally causing lower δ^{15}N values compared with animal manures and composts often used in organic systems.[24] However, different conclusions have been reached in these studies as a consequence of highly different experimental conditions including variation caused by plant species, food products, growth conditions, fertilizer types and application rates and experimental designs. In addition, the use of δ^{15}N for organic authentication is challenged by the fact that N_2-fixing plants (the *Leguminosae family*) have δ^{15}N values overlapping with the range of synthetic fertilizers and are frequently used as cover crops or green manures in organic crop rotations. Several studies have thus demonstrated that δ^{15}N as a single parameter does not enable authenticity testing of organic plant products in all real-life situations.[15,17] To eliminate this problem, oxygen isotope analysis of nitrate has been utilized in a few studies.[15,21] This has generated additional information regarding the fertilizer source and has enabled discrimination of plants grown with synthetic fertilizers and legume-based green manures. In addition, gas chromatography coupled to isotope ratio mass spectrometry (GC-c-IRMS) for the analysis of δ^{15}N and δ^{13}C of amino acids was recently successfully applied to Danish bread wheat and Italian durum wheat.[25] Another solution is to combine the isotopic ratio of N with those of other bio-elements, such as H, C, O and S, as it has been performed for some organic fruits.[19] This approach may deliver information about both the agricultural and geographical origins and thus improve the authenticity evaluations of a food product.

The use of stable isotope analysis for geographical authentication of cereals is generally more developed than the verification of farming systems (e.g. organic/conventional). Asfaha *et al.*[5] combined stable isotope ratios of the heavy mass element strontium (^{87}Sr/^{86}Sr) and the light mass elements C, N, O and S (δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S) with the multi-elemental composition for provenance testing of European cereal samples with satisfying results individualizing 15 out of 17 European sampling sites. Wu and colleagues[6] and Luo *et al.*[7] found differences in wheat samples from China, Australia, USA and Canada, related to the values of δ^{13}C. Liu and colleagues[26] verified that δ^{2}H, δ^{13}C and δ^{15}N determined in wheat collected in different Chinese regions were significantly affected by the region. Podio *et al.*[27] were successfully able to differentiate wheat from three Argentinian sites by using δ^{13}C and δ^{15}N coupled with ^{87}Sr/^{86}Sr and the elemental composition. These differentiations were suggested to be possible due to the impact of microclimate, geology and pedology on the isotopic composition of wheat grains.

In the present study, we aim to discriminate the geographical and agricultural origins of durum wheat by using stable isotope analysis of the elements H, C, N, O and S. In addition, we go steps further to investigate the stability of these multi-isotopic signatures along the production chain of pasta by analysing the finished products and associated raw materials. Our previous studies have already documented that such consistency in the isotopic signatures are present along the production chain of tomato passata[28] and in cheese[29]. To our knowledge, similar studies have not been conducted on pasta but Bostic and colleagues[30] found that baking and fermentation do not affect δ^{13}C and δ^{15}N signatures of grain-based foods. For the first time, the δ^{2}H, δ^{13}C, δ^{18}O, δ^{15}N and δ^{34}S signatures along the production chain of pasta (wheat–flour–pasta) were investigated. The samples were produced either organically or conventionally in Italy in different regions and years.

Materials and methods

Plant materials and sampling

Samples of durum wheat (*Triticum turgidum* ssp. durum) were collected from commercial farms in 2012 and 2013 in four Italian wheat-producing regions (Basilicata, Molise, Emilia-Romagna and Tuscany) (Figure 1 and Table S1 in the Supporting Information). Wheat was grown in each location in either a conventional system (C, with mineral fertilizer) or an organic system (O, with organic fertilizer, or without any fertilizer, or green manure – after 5 years of legumes) in triplicate (from here onwards called 'repetitions'), which summed up to a total of 90 durum wheat samples (four locations × 2 years × two systems × two varieties × three repetitions = 96 samples; 6 samples were lost due to an earthquake in Emilia-Romagna in 2012). Varieties (*Khorasan*, *Senatore Cappelli*, *Simeto*, *Taganrog*, *Levante*, *Etrusco*, *Creso*, *Iride*, *Appio* and *Duilio)* and systems varied between locations according to common agricultural practice in each region. The organic systems were managed in full compliance with the rules for organic farming (European Community Council Regulation EC 834/2007 and following amendments) for at least 8 years prior to the sampling (Table S1). A batch of the durum wheat samples (for initial bulk tissue stable isotope analysis) was washed with double deionized water containing a few drops of detergent (Tween 20, Sigma Aldrich, MO, USA) followed by a wash in double-deionized water and a wash in Milli-Q water

Figure 1. Sampling plan of wheat, flour and pasta ($N = 150$) collected in four Italian regions (Basilicata, Emilia Romagna, Molise and Tuscany) both organically and conventionally produced.

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(Millipore, Billerica, MA, USA) to remove surface contamination. Samples were subsequently freeze-dried 72 h (ScanVac, CoolSafe, Labogene, Lynge, DK) and ground to a fine powder by using a centrifugal mill equipped with a titanium rotor (ZM200, Retsch GmbH, Haan Germany). Another batch of the durum wheat samples was delivered to one local mill, where wheat grains from all the regions were processed into flour in a stone mill at low temperature. Flour samples (500 g) were packed and stored at room temperature until analysis. The samples from the mill were processed into dry pasta simply by adding water to flour prior to air-drying. Dry pasta was packed in 500 g packages and stored at room temperature. For the flour and pasta production, the three replicates within each year, location, system and variety were combined which resulted in 30 flour and 30 pasta samples in total. Pasta samples were freeze-dried and ground into a fine powder prior to analysis as described earlier.

Stable isotope ratio analysis

Samples were weighed (~1.6 mg) and placed in tin capsules to measure $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in one run by an IRMS (Vario Isotope Cube, Elementar Analysensysteme GmbH, Germany coupled to an Isoprime100, IRMS, Isoprime Ltd, UK). Around 0.5 mg of the sample was weighed in silver capsules to measure $\delta^{18}O$ and $\delta^{2}H$ (Delta Plus XP – ThermoFinnigan, Bremen, Germany, IRMS equipped with a thermal conversion elemental analyzer pyrolysis unit). The isotope ratios were expressed in δ ‰ against Vienna-Pee Dee Belemnite for $\delta^{13}C$, Vienna Standard Mean Ocean Water for $\delta^{18}O$ and $\delta^{2}H$, air for $\delta^{15}N$ and Vienna Canyon Diablo Troilite for $\delta^{34}S$ according to the following formula:

$$\delta = \left(R_{\text{sample}} - R_{\text{standard}}\right)/R_{\text{standard}}$$

where R_{sample} is the isotope ratio measured for the sample and R_{standard} is the isotope ratio of the international standard. Sample analyses were carried out in duplicate. The isotopic values were calculated against working in-house standards (casein and/or wheat for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$; keratin for $\delta^{2}H$ and $\delta^{18}O$), which were themselves calibrated against international reference materials: fuel oil NBS-22 [International Atomic Energy Agency (IAEA), Vienna, Austria] and sugar IAEA-CH-6 for $^{13}C/^{12}C$; L-glutamic acid US Geological Survey (USGS) 40 (IAEA), hair USGS 42 and USGS 43 for $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ and benzoic acid IAEA-601 for $^{18}O/^{16}O$. For $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, the values were calculated against two working standards through the creation of a linear equation. Stable sulfur isotopic compositions were expressed relative to Vienna Canyon Diablo Troilite on a scale normalized such that the $\delta^{34}S$ value of IAEA-S-1 silver sulfide is −0.3‰. The $\delta^{13}C$ values were reported relative to Vienna-Pee Dee Belemnite on a scale normalized by assigning a value of −46.6‰ to LSVEC lithium carbonate (IAEA). A commercial wheat and a casein sample were analysed as independent controls for each batch of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ determinations. The control samples had to respect the control charts limits otherwise the whole list was canceled and the analysis repeated. The $\delta^{2}H$ and $\delta^{18}O$ values were calculated against Caribou Hoof Standard ($\delta^{2}H = -197 \pm 2$‰ and $\delta^{18}O = +3.8 \pm 0.1$‰) and Kudu Horn Standard ($\delta^{2}H = -54 \pm 1$‰ and $\delta^{18}O = +20.3 \pm 0.2$ ‰) through the creation of a linear equation. [31] We used these two keratinous standards because of the absence of any international organic reference material with a similar matrix of ours. A commercial keratin and a casein sample were analysed as independent controls for each batch for $\delta^{2}H$ and $\delta^{18}O$ determinations. The control samples had to respect the control charts limits otherwise the whole list was canceled and the analysis repeated. Before the analysis, the weighed samples and standards were left at laboratory air moisture for at least 96 h, then placed in a desiccator with P_2O_5 under vacuum for other 96 h. Samples were then loaded onto the autosampler tray, put on the carousel, sealed with a cover and purged with argon. All results reported for non-exchangeable H were normalized relative to the Vienna Standard Mean Ocean Water–Standard Light Antarctic Precipitation standard scale. The uncertainty of methods (calculated as 1 standard deviations when analysing the same sample at least ten times in reproducibility conditions) was 0.1‰ for $\delta^{13}C$, 0.2‰ for $\delta^{15}N$, 0.3‰ for $\delta^{18}O$ and $\delta^{34}S$ and 1‰ for $\delta^{2}H$. Every precaution recommended to avoid instrumental drift problems and memory effects was applied. For example, along the sequence, standards and quality control samples were inserted for every 10 sample, and the instrumental drift was corrected using these samples as suggested by Carter and colleagues.[32] To avoid the overlapping of the N2 peak with the subsequent CO peak during the determination of $\delta^{18}O$, a GC column long 120cm has been used.

Statistical analysis

The data were analysed with the Statistica version 9 (StatSoft Inc., Tulsa, OK, USA) package. Analysis of variance (ANOVA) and honestly significantly different Tukey's test for unequal N analysis were applied to verify differences according to production year, matrix, region and farming system; the level of significance was accepted at $p \leq 0.001$. It was not possible to verify the influence of wheat variety as varieties were not homogeneously distributed between regions and farming systems. To verify differences between sample types (grain, flour and pasta) within the production chains, a paired *t*-test was used. A multivariate canonical discriminant analysis (CDA) was applied to determine the differences between geographical origin, farming system and farming system within the production region. To validate the model, a segmented cross validation was applied three times, with 10% of the analysed samples used as unknowns to validate the model built on the basis of the remaining samples.

Results and discussion

In Table 1, the mean values, the relative standard deviations and the minimum and maximum values of $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ of wheat, flour and pasta samples are displayed according to the geographical origin and the farming system. No significant effects ($p > 0.05$) of growth season were observed for the two harvest years, which may be explained by the comparable dry and warm climates of Central–South Italy during the two growth seasons.

The durum wheat data of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ covered a wider range compared with e.g. the data reported by Schmidt *et al.* [16] in wheat produced in different regions of Germany. In particular, $\delta^{13}C$ values ranged between −27.4 and −22.6‰, thereby representing more positive values compared with the German samples (−28.0 to −26.2‰). This is probably related to the significantly warmer Italian climate typical of the regions of central and southern Italy that influence water availability, stomatal conductance and the water use efficiency, thus affecting $\delta^{13}C$ of plants.[33] In addition, $\delta^{13}C$ values increased systematically when going from the northern Emilia-Romagna region to the southern Basilicata region, indicating a higher degree of stomatal closure and thereby a lower discrimination against ^{13}C in the southern regions. In this study, the $\delta^{15}N$ values ranged from −0.4 to +10.6‰, whereas German samples from the Schmidt study[16] were from around 0 to 5‰. The higher

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Table 1. Stable isotope ratio values (mean, standard deviation, minimum and maximum values) of durum wheat samples, flour and pasta across the two growth seasons ($N = 90$, 30 and 30, for wheat, flour and pasta, respectively)

Product	Region	System	δ^2H				$\delta^{13}C$				$\delta^{15}N$				$\delta^{18}O$				$\delta^{34}S$			
			Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	Mean	Std. Dev.	Min.	Max.	Mean	SD	Min.	Max.
Wheat	Basilicata	Conventional	−48	8	−61	−37	−23.3	0.4	−23.8	−22.6	3.7	1.1	2.8	5.5	29.6	1.1	27.9	31.5	2.7	2.4	−1.0	5.3
		organic	−55	8	−66	−43	−23.9	0.5	−24.6	−23.4	4.9	2.0	2.4	8.8	30.2	1.5	28.5	32.6	−5.5	3.0	−9.8	−0.3
	Molise	Conventional	−54	6	−63	−45	−26.3	0.4	−26.8	−25.5	1.6	1.1	−0.4	4.0	29.7	0.6	28.8	31.0	5.2	2.8	0.8	8.9
		organic	−68	6	−79	−59	−26.1	0.8	−26.9	−25.1	2.3	1.2	0.5	3.8	29.3	1.1	26.2	30.4	4.9	3.7	0.3	8.9
	Tuscany	Conventional	−58	3	−64	−54	−25.4	0.4	−25.8	−24.8	1.5	1.1	0.1	3.6	28.7	0.8	27.9	30.0	−22.8	2.0	−25.0	−19.6
		organic	−61	3	−66	−57	−25.7	0.8	−26.4	−24.6	1.4	1.0	0.2	3.0	29.1	1.6	27.0	31.5	−20.4	4.4	−25.2	−14.9
	Emilia-Romagna	Conventional	−58	4	−66	−54	−27.2	0.1	−27.3	−27.0	4.7	1.8	0.8	7.0	28.1	0.5	27.6	29.1	−1.6	1.2	−2.5	0.4
		organic	−60	3	−65	−55	−27.2	0.1	−27.4	−27.0	4.0	2.0	0.7	6.8	28.2	0.5	27.6	29.0	−1.8	0.8	−2.5	−0.4
Flour	Basilicata	Conventional	−49	10	−60	−36	−23.3	0.5	−23.6	−22.6	4.4	1.5	2.9	5.8	30.1	0.6	29.3	30.7	1.4	3.7	−2.7	4.7
		organic	−54	5	−57	−46	−23.8	0.3	−24.3	−23.5	5.8	3.4	2.9	10.6	30.3	0.9	29.6	31.3	−4.4	2.8	−6.8	−1.1
	Molise	Conventional	−55	9	−68	−48	−25.9	0.6	−26.3	−25.0	2.3	1.4	0.8	4.2	29.2	0.6	28.6	30.0	3.7	2.0	1.1	5.3
		organic	−65	4	−69	−60	−25.6	1.1	−26.7	−24.6	2.5	1.6	0.9	4.3	29.4	0.8	28.2	30.2	4.6	3.9	−0.1	8.1
	Tuscany	Conventional	−53	7	−62	−47	−25.2	0.5	−25.6	−24.4	1.9	1.9	0.1	4.5	29.5	0.8	28.8	30.6	−22.3	1.9	−23.8	−19.8
		organic	−58	5	−63	−52	−25.5	0.9	−26.3	−24.5	2.7	1.2	1.5	3.8	29.7	0.9	28.4	30.5	−20.2	3.0	−23.8	−17.2
	Emilia-Romagna	Conventional	−56	5	−59	−50	−27.2	0.2	−27.3	−27.0	5.4	1.5	4.5	7.1	27.6	0.5	27.2	28.2	−1.7	1.7	−3.1	0.2
		organic	−62	7	−70	−57	−27.2	0.1	−27.2	−27.1	5.2	1.2	4.4	6.5	28.2	0.1	28.2	28.3	−1.9	2.2	−3.2	0.7
Pasta	Basilicata	Conventional	−49	6	−57	−43	−23.4	0.4	−23.7	−22.8	4.5	1.6	3.0	6.1	30.6	0.4	30.0	31.0	0.8	3.3	−2.5	3.9
		organic	−55	4	−60	−51	−24.0	0.3	−24.5	−23.9	5.5	3.4	2.4	10.3	31.4	1.2	30.2	32.9	−4.2	2.9	−6.8	−0.5
	Molise	Conventional	−56	9	−65	−45	−26.3	0.7	−26.8	−25.3	1.4	0.5	0.9	1.9	29.4	0.3	29.1	29.7	4.2	2.1	1.8	6.6
		organic	−67	5	−74	−61	−26.0	0.7	−26.6	−25.4	2.3	1.3	1.0	3.7	29.9	0.8	29.0	31.0	5.8	4.3	−0.7	8.2
	Tuscany	Conventional	−61	2	−63	−58	−25.4	0.5	−25.8	−24.8	1.7	1.5	0.3	3.7	29.7	0.9	28.9	30.8	−22.4	1.6	−23.5	−20.1
		organic	−57	2	−60	−55	−25.7	1.1	−27.1	−24.5	3.3	1.4	1.5	4.7	29.3	0.7	28.5	30.1	−15.8	8.9	−24.2	−3.3
	Emilia-Romagna	Conventional	−60	1	−61	−59	−27.2	0.1	−27.4	−27.1	5.3	1.4	4.4	6.9	28.6	0.1	28.5	28.7	−3.2	0.3	−3.5	−2.9
		organic	−61	5	−65	−56	−26.9	0.8	−27.4	−25.9	4.4	2.9	1.6	7.3	29.2	0.7	28.7	30.0	−9.5	11.1	−22.3	−2.9

Values are reported in δ ‰ against the internationally recognized reference materials (see the *Materials and methods* section).

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$\delta^{15}N$ values for the Italian samples could be an indication of a greater application rate of animal manure.[34] Regarding $\delta^{34}S$, Italian wheat samples ranged from −25.2 to +8.9‰, whereas German samples ranged from +3 to +6‰.[16] As asserted by Schmidt and colleagues, $\delta^{34}S$ is highly affected by the local geology and soil conditions. The lowest values found within the Italian samples were those of Tuscany in compliance with $\delta^{34}S$ values of other foodstuffs.[35,36] These extreme values are suggested to be caused by the high amounts of volcanic sulfur present in this region.[37] The $\delta^{13}C$ and $\delta^{15}N$ data found in Chinese, Canadian, American and Argentinian samples by other authors[6,26,27] were similar to those found for samples in this study even if their ranges of values

Table 2. $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ data (mean values) of wheat, flour and pasta collected along 16 pasta production chains in Italy in 2012 and 2013 organically and conventionally produced ($N = 80$)

Production chain	Region and system	Matrix	$\delta^{2}H$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{18}O$	$\delta^{34}S$
1	Basilicata Organic	Wheat	-56	-23.7	4.7	29.0	-2.6
		Flour	-57	-23.8	3.8	30.8	-1.1
		Pasta	-57	-23.9	4.0	30.7	-0.5
2	Basilicata Conventional	Wheat	-42	-23.7	5.4	28.6	-0.5
		Flour	-47	-23.6	5.7	30.3	-0.8
		Pasta	-43	-23.7	5.5	31.0	-1.5
3	Tuscany Organic	Wheat	-61	-24.6	1.2	27.2	-15.0
		Flour	-52	-24.5	1.5	29.9	-17.2
		Pasta	-55	-24.5	1.5	29.7	-17.5
4	Tuscany Conventional	Wheat	-58	-24.9	0.2	28.1	-22.6
		Flour	-62	-24.4	0.1	29.4	-22.0
		Pasta	-60	-24.8	0.3	30.8	-23.5
5	Molise Organic	Wheat	-74	-25.5	1.3	28.2	8.6
		Flour	-69	-24.9	1.4	30.2	8.1
		Pasta	-74	-25.4	1.4	29.7	7.6
6	Molise Organic	Wheat	-61	-25.2	1.3	29.4	2.1
		Flour	-66	-24.6	0.9	29.4	2.7
		Pasta	-61	-25.4	1.0	29.0	2.8
7	Molise Conventional	Wheat	-46	-25.7	1.3	30.2	2.7
		Flour	-48	-25.0	0.8	30.0	3.1
		Pasta	-45	-25.3	0.9	29.5	1.8
8	Molise Conventional	Wheat	-55	-26.4	1.4	29.6	2.9
		Flour	-53	-26.3	1.8	28.8	5.3
		Pasta	-53	-26.6	1.7	29.1	3.2
9	Basilicata Organic	Wheat	-65	-24.6	4.9	30.2	-3.8
		Flour	-56	-24.3	5.8	29.6	-3.2
		Pasta	-60	-24.5	5.2	30.2	-3.3
10	Basilicata Conventional	Wheat	-60	-23.4	2.8	29.7	4.9
		Flour	-52	-23.5	2.9	29.3	4.2
		Pasta	-57	-23.6	3.0	30.0	3.9
11	Tuscany Organic	Wheat	-63	-26.4	3.0	29.5	-25.1
		Flour	-61	-26.1	3.6	28.4	-23.8
		Pasta	-60	-26.1	3.1	28.8	-24.2
12	Molise Organic	Wheat	-71	-26.8	2.9	30.2	8.1
		Flour	-64	-26.4	3.3	29.7	7.5
		Pasta	-65	-26.6	3.1	31.0	7.9
13	Molise Organic	Wheat	-67	-26.8	3.7	29.2	0.6
		Flour	-60	-26.7	4.3	28.2	-0.1
		Pasta	-67	-26.6	3.7	29.7	-0.7
14	Molise Conventional	Wheat	-62	-26.3	2.2	30.1	6.1
		Flour	-68	-26.3	2.3	29.4	5.3
		Pasta	-65	-26.4	1.9	29.7	5.2
15	Emilia Romagna Organic	Wheat	-57	-27.2	4.6	28.2	-2.5
		Flour	-60	-27.2	4.4	28.2	-3.2
		Pasta	-62	-27.4	4.4	28.7	-3.4
16	Emilia Romagna Conventional	Wheat	-54	-27.2	4.6	27.9	-2.3
		Flour	-59	-27.2	4.5	27.5	-3.1
		Pasta	-61	-27.4	4.4	28.6	-3.3

Data are reported in δ ‰ against the internationally recognized reference materials (see the *Materials and methods* section).

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Table 3. Mean values of wheat, flour and pasta samples considered together collected in Italy during 2012 and 2013 harvests ($N = 150$) grouped according to the farming system across all regions (organic *vs* conventional) or the farming system of cultivation within the provenance regions (Basilicata, Emilia Romagna, Molise and Tuscany)

Region	System	δ^2H	δ^{13}C	δ^{15}N	δ^{18}O	δ^{34}S
All	Conventional	-58^a	-27.2^a	4.9^a	28.1^a	-1.9^a
	Organic	-61^b	-27.1^a	4.3^a	28.4^a	-3.4^a
Basilicata	Conventional	-49	-23.3^a	4.0	29.9	2.0^a
	Organic	-55	-23.9^b	5.2	30.5	-5.0^b
Emilia Romagna	Conventional	-58	-27.2	4.9	28.1	-1.9
	Organic	-61	-27.1	4.3	28.4	-3.4
Molise	Conventional	-55^a	-26.2	1.7	29.5	4.7
	Organic	-67^b	-26.0	2.3	29.4	5.0
Tuscany	Conventional	-58	-25.4	1.6	29.1	-22.6
	Organic	-59	-25.7	2.1	29.3	-19.4

Significantly different mean values, when found (Tukey's honestly significantly different test, $p < 0.001$), are indicated with different letters.

were narrower. The δ^2H has only been determined in a few previous studies[15,26] and resulted in a range of values between −75 and −56‰ and −110 and −81‰ for Chinese and Danish wheat samples, respectively. However, a direct comparison with these data is not possible as procedures for data correction were different. This situation is due to the lack of internationally recognized organic reference materials for wheat having exchangeable hydrogen.[38]

Isotopic trends along the production chain

Sixteen samples were strictly followed along the production chain from durum wheat samples to milled flour and finally pasta (Table 2).

The mean difference for δ^2H, δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S between wheat, flour and pasta were lower than 1‰, 0.1‰, 0.1‰, 0.2‰ and 0.3‰, respectively, calculated as the average of the differences between the three sample types within each specific chain. No differences were found in the trend between the two farming systems (organic/conventional). The differences between the three sample types (wheat, flour and pasta) were not statistically significant different according to a paired *t*-test either ($p > 0.05$). It was thus documented that the pasta production processes did not affect any of the five isotope parameters. These results were expected for δ^{13}C, δ^{15}N and δ^{34}S but were less predictable for δ^2H and δ^{18}O because of the addition of tap water during pasta production and the loss of water during the drying of pasta. From these results, it is evident that the drying of pasta by warm air (no more than 40 °C to avoid the alteration of nutritional quality) for 24–72 h did not alter the isotopic content of oxygen and hydrogen nor the other ratios and the addition of tap water of the same production area of the wheat.

As the growing season and the type of samples (wheat, flour or pasta) did not result in statistically significant differences for any of the isotope ratios, in the subsequent sections, wheat, flour and pasta across the two growth years will be considered together.

Effects of the agricultural farming system

Considering all the regions together, ANOVA and Tukey tests (Table 3) found statistically significant differences between conventional and organic agricultural practices for δ^2H ($p < 0.001$). The δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S values did not result in any statistically significant differences ($p > 0.001$; Table 3). The results for δ^{13}C, δ^{15}N and δ^{34}S were in agreement with what was found for wheat by Schmidt *et al.* (2005)[16] with no statistical differences between organic and conventional products. The δ^2H mean values found for conventional products resulted in higher values compared with organic ones (−58‰ *vs* −61‰, respectively). This finding is in compliance with the study of Laursen *et al.* (2013)[15], in which statistically higher δ^2H values were found for conventional wheat and barley compared with organic ones. It was suggested that these differences were associated with a higher transpiration and evaporative loss of ^1H_2O from conventionally grown cereals. Georgi and colleagues (2005)[39] hypothesized that differences in cultivation practices such as plant density and growth rates can influence respiration, water uptake and evapotranspiration with an effect on δ^{18}O of leaf water. However, in this study, no statistical significant differences between δ^{18}O of organic and conventional farming systems were found. The δ^2H and δ^{18}O seemed to behave differently, which is probably because, as already reported, hydrogen and oxygen were derived from different sources (hydrogen solely from the source water, oxygen also from atmospheric CO_2 and O_2), and in generative plant tissues, they are subjected to different fractionation processes during carbohydrate biosynthesis, the main constituents of wheat grains.[15]

Considering the differences between agricultural practices across durum wheat grains, flour and pasta within the regions, the situation was slightly different. The δ^2H values resulted in differences between organic and conventional systems only in Molise ($p < 0.001$) but not within Tuscany, Basilicata and Emilia Romagna (Table 3). However, the tendency of higher δ^2H values in conventional plant products was confirmed within all the four regions. The δ^{13}C values resulted in highly statistically significant differences between organic and conventional products only in the Basilicata

Table 4. Mean values of wheat, flour and pasta samples considered together, collected in Italy during 2012 and 2013 harvests ($N = 150$) grouped according to the provenance regions (Basilicata, Emilia Romagna, Molise and Tuscany)

	δ^2H	δ^{13}C	δ^{15}N	δ^{18}O	δ^{34}S
Basilicata	-52^a	-23.6^a	4.6^a	30.2^a	-1.5^b
Emilia Romagna	-59^b	-27.2^d	4.6^a	28.3^c	-2.7^b
Molise	-61^b	-26.1^c	2.0^b	29.5^{ab}	4.8^a
Tuscany	-58^b	-25.5^b	1.8^b	29.2^b	-21.0^c

Significantly different mean values (Tukey's honestly significantly different test, $p < 0.001$) are indicated with different letters.

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region ($p < 0.001$). In particular, the $\delta^{13}C$ values showed that organic products were ^{13}C depleted compared with conventional ones confirming results of previous studies.[19,20,39] The $\delta^{34}S$ values resulted in highly statistically significant differences between organic and conventional products only in the Basilicata region ($p < 0.001$). It is worth noting that all the Basilicata $\delta^{34}S$ values of organic products were below 0‰, whereas the values of the conventional products were above 0‰ (−5.0‰ *vs* +2.0‰). The explanation could be the type of fertilizers used as well as the inherent isotopic signature of the soil. In conventional agricultural systems, synthetic fertilizers are allowed. The $\delta^{34}S$ values of such fertilizers typically range from −6.5 to +21.6‰ depending on the origin of the raw material (e.g. pyrite around 1‰, sulfide deposits in Japan or Australia with values near 7‰ and marine evaporite deposits with values near 20‰).[40] In organic systems, only the use of 'naturally derived products' are allowed (e.g. $CaSO_4$–chalk, $MgSO_4$, elemental sulfur and marine weed). These also have a wide range of $\delta^{34}S$ values sometimes overlapping with those of the synthetic fertilizers. The $\delta^{15}N$ and $\delta^{18}O$ values did not result in statistically significant differences between farming systems within the regions. The $\delta^{15}N$ values were slightly higher in organic products in Basilicata, Tuscany and Molise but not in Emilia Romagna where farmyard manure was used in the conventional system and thus reduced the differences between organic and conventional concerning fertilization in this region. Consequently, $\delta^{15}N$ in this study was not systematically affected by the agricultural practice, caused by the large variation in fertilization strategies between the different geographical regions and in particular the use of N_2 fixing plants (the *Leguminosae family*) in organic agriculture with $\delta^{15}N$ values overlapping with the range of synthetic fertilizers (-Table S1). This corresponds with findings in previous studies[15] and highlights the challenges associated with using $\delta^{15}N$ as a single parameter for authenticity testing of organic plant products.

Geographical effect

When conducting ANOVA tests, all the five isotope ratio parameters resulted in highly statistically significant effects of the geographical origin. At Tukey test (Table 4), $\delta^{2}H$ resulted in statistically higher values in Basilicata (mean of −52‰) in comparison with the other regions (mean values between −58 and −61‰). This was expected as Basilicata is at the south of Italy at the lowest latitude with the warmest climate. It is worth noting that Molise is also located at a lower latitude than Tuscany and Emilia but does not have higher $\delta^{2}H$ values. This deviation could be related to the fact that Molise is along the Adriatic coast of Italy that in a previous study on olive oils resulted in more ^{2}H depletion than the Tyrrhenian coast, due to a different source and isotopic composition of rainfall as well as the different climatic conditions on the two coasts.[41] The $\delta^{18}O$ values were significantly lower in Emilia Romagna (mean value of 28.3‰) compared with the other regions ($p < 0.001$), and Basilicata had $\delta^{18}O$ (30.2‰) higher values than Tuscany ($p < 0.001$) and Molise ($p < 0.01$) (for $p < 0.001$; Table 3). The $\delta^{18}O$ thereby followed a 'perfect' latitudinal scale. The $\delta^{13}C$ values were statistically different between all the regions with increasing mean values from −27.2‰ in Emilia Romagna to −26.1‰ in Molise, −25.5‰ in Tuscany and −23.6‰ in Basilicata. This corresponds with the linkage of $\delta^{13}C$ to different climatic characteristics (especially temperature) of each geographical location that could influence, in particular, the degree of stomatal closure at leaf level. The $\delta^{15}N$ values were highly statistically different ($p < 0.001$) between the group Basilicata and Emilia Romagna compared with the group Molise and Tuscany. In

Table 5. Reclassification discriminant analysis of $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ of wheat, flour and pasta from four Italian regions (Basilicata, Emilia Romagna, Molise and Tuscany) both organically and conventionally produced

a

	% correct classification	CONV	ORG
CONV	65	49	26
ORG	64	27	48
Total	65	76	74

b

	% correct classification	BAS	ER	MOL	TUS
BAS	100	40	0	0	0
ER	97	0	29	0	1
MOL	98	0	1	39	0
TUS	98	0	1	0	39
Total	98	40	31	39	40

c

	% correct classification	BAS ORG	BAS CONV	TUS ORG	TUS CONV	MOL ORG	MOL CONV	ER ORG	ER CONV
BAS ORG	95	19	1	0	0	0	0	0	0
BAS CONV	90	2	18	0	0	0	0	0	0
TUS ORG	75	0	0	15	4	0	0	0	1
TUS CONV	85	0	0	3	17	0	0	0	0
MOL ORG	80	0	0	0	0	16	0	3	1
MOL CONV	85	0	0	0	0	3	17	0	0
ER ORG	47	0	0	1	0	0	0	7	7
ER CONV	73	0	0	0	0	0	0	4	11
Total	80	21	19	19	21	19	17	14	20

Results of classification matrix considering (a) only the farming system, (b) only the geographical origin and (c) both the geographical origin and the farming system.

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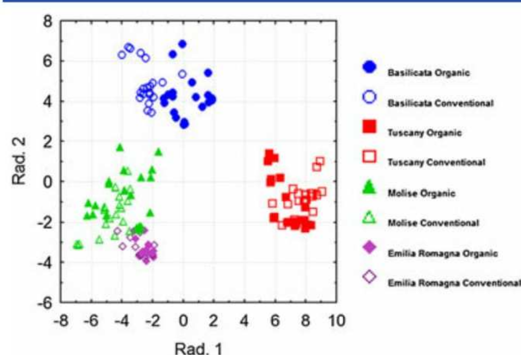

Figure 2. Canonical discriminant analysis of δ^2H, δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S of wheat, flour and pasta ($N = 150$) collected in four Italian regions (Basilicata, Emilia Romagna, Molise and Tuscany) both organically and conventionally produced: scatterplot of the first two canonical variables.

particular, Basilicata and Emilia Romagna presented higher values (mean 4.6‰) than the Molise–Tuscany group (around 2.0‰), primarily as a consequence of a different fertilization practices (Table S1). The δ^{34}S value was statistically different in Tuscany and in Molise compared with the other regions ($p < 0.001$). Molise showed mean positive values (4.8‰), whereas Tuscany was characterized by particularly low values (−21.0‰) as mentioned earlier. Emilia Romagna and Basilicata were different from Tuscany and Molise but not between each other (mean values of −2.7 and −1.5‰).

Multivariate analysis

Canonical discriminant analysis was carried out to investigate the possibility of discriminating between geographical origins and the agricultural systems using all the five isotope parameters. The developed models were validated through a segmented cross validation, with 10% of the analysed samples used as unknowns to validate the model built on the basis of the remaining samples.

Considering the farming system, the model led to overall 65% of the samples being correctly classified (Table 5a). The first canonical variable (Rad. 1) was mainly loaded by δ^2H (−0.8), confirming that in this study, this parameter and not δ^{15}N was primarily affected by the agricultural practice.

Regarding the geographical origin the CDA led to a model that explained almost 100% of the total variability with the first three canonical variables. Overall, 98% of the samples were correctly classified (Table 5b) with a reclassification percentage of 100% in the case of Basilicata. Rad. 1 was loaded negatively with δ^{34}S (−0.6) and δ^{13}C (−0.5). Rad. 2 was mainly loaded positively with δ^{13}C (+0.7) and negatively with δ^{34}S (−0.6), whereas Rad. 3 was mainly negatively loaded by δ^{15}N (−0.9).

When considering the geographical origin and the farming system, together, CDA correctly reclassified 80% of the samples and the first three canonical variables explained 97% of total variability (Figure 2; Table 5c). Rad. 1 was loaded negatively with δ^{34}S (−0.8), and along it, the Tuscany region was separated from the other origins. Rad. 2 was mainly loaded positively with δ^{34}S (+0.5) and negatively with δ^{13}C (−0.8), whereas Rad. 3 was loaded negatively by δ^{15}N (−0.9). Along the second variable, it was possible to separate Basilicata from the other regions due to the higher values of δ^{13}C. To test the predictive discrimination power and the stability of this model, a segmented cross validation was used. In detail, three different sets of 16 samples (about 10% of the original data were selected: two organic and two conventional samples from each of the four locations were removed from the data, and each time the model was calculated on the remaining 134 samples and was validated with all 150 samples (including the excluded samples). In all analyses, around 98% of the samples were correctly classified in the right geographical region, 65% according to the right farming system and 80% in the right combination of geographical origin and farming system.

In conclusion, in this study, the isotopic signature (δ^2H, δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S) of wheat samples collected in four Italian regions, produced organically or conventionally, was investigated along all steps of the production chain of pasta. It was shown that the production processes along the pasta production chain did not significantly affect any of the five isotope parameters nor did the production year. The factors influencing the isotopic signatures of pasta and related raw products were primarily the geographical origin, which significantly affected all the five considered parameters. In particular, δ^2H, δ^{18}O and δ^{13}C showed a latitudinal gradient with higher values in samples from the southern regions and lower values in those from the northern ones. In contrast, δ^{15}N and δ^{34}S were primarily affected by geology and fertilization practices. Differences between conventional and organic agricultural practices were primarily detected for δ^2H, which was suggested to be caused by a higher transpiration of conventional plants compared with organic ones.

In this study, it was demonstrated that multi-isotopic analysis can be used for determining the geographical origin of Italian organic and conventional pasta and its raw materials. The isotope parameters investigated in this study were less effective in discriminating farming system (conventional *vs* organic). However, when focusing on a single geographical region, several stable isotope parameters proved to be useful for discriminating organic and conventional samples, which is to be further explored in future studies, maybe in combination with other analytical techniques and parameters (e.g. multi-elemental or metabolomic fingerprinting[42]).

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Supporting information

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SECTION 3.4

Compound-specific $\delta^{15}N$ and $\delta^{13}C$ analyses of amino acids for potential discrimination between organically and conventionally grown wheat

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Statement of the author: My personal involvement in this research started with the development of experimental design to measure the carbon and nitrogen isotopic values of amino acids extracted from wheat: protein hydrolysis, derivatisation and analysis by GC-C-IRMS. As regards method development, I personally performed all the experiments and data analysis presented in the manuscript. Moreover, I measured the carbon and nitrogen isotopic values of wheat in bulk samples. As first author I was responsible for writing the manuscript and managing the comments and improvements to the text by the other co-authors.

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Compound-Specific δ^{15}N and δ^{13}C Analyses of Amino Acids for Potential Discrimination between Organically and Conventionally Grown Wheat

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Supporting Information

ABSTRACT: We present a study deploying compound-specific nitrogen and carbon isotope analysis of amino acids to discriminate between organically and conventionally grown plants. We focused on grain samples of common wheat and durum wheat grown using synthetic nitrogen fertilizers, animal manures, or green manures from nitrogen-fixing legumes. The measurement of amino acid δ^{15}N and δ^{13}C values, after protein hydrolysis and derivatization, was carried out using gas chromatography−combustion−isotope ratio mass spectrometry (GC-C-IRMS). Our results demonstrated that δ^{13}C of glutamic acid and glutamine in particular, but also the combination of δ^{15}N and δ^{13}C of 10 amino acids, can improve the discrimination between conventional and organic wheat compared to stable isotope bulk tissue analysis. We concluded that compound-specific stable isotope analysis of amino acids represents a novel analytical tool with the potential to support and improve the certification and control procedures in the organic sector.

KEYWORDS: *amino acids, authentication, compound-specific, GC-C-IRMS, stable isotopes, wheat*

■ INTRODUCTION

Adulteration of food products is an increasing global problem. This is also the case for organic products as they are sold at premium prices compared to their conventional counterparts. Consequently, several studies have aimed to develop analytical methods to verify if organic food has been produced according to the organic legislation.

The application of stable isotope ratio analysis for authenticity testing of organic products has been largely investigated and has recently been reviewed.[1−3] It has been stated that the nitrogen stable isotope ratio ^{15}N/^{14}N (expressed as δ^{15}N) is one of the strongest markers for organic production. This is based on the fact that synthetic nitrogen fertilizers, commonly used in conventional agriculture, have δ^{15}N values significantly lower (from −6 to 6‰) than most manures and organic fertilizers allowed in organic agriculture (from 1 to 37‰).[4] For most crops the applied fertilizer represents the main source of nitrogen for plant growth. Thus, the application of synthetic nitrogen fertilizers in conventional plant production tends to reduce δ^{15}N values in plant tissue.[4] In contrast, organically grown plants, if supplied with animal manure or compost, will have higher δ^{15}N values.[5] The ^{15}N contents (δ^{15}N) of composted manures are higher than those of synthetic fertilizer due to different N transformation mechanisms (e.g., ammonia volatilization, denitrification, nitrification) of the lighter N isotope (^{14}N) than of ^{15}N during the composting process prior to plant uptake.[6,7] This has led to the general conclusion that δ^{15}N analysis constitutes a useful tool to discriminate between organically and conventionally produced plants, especially if combined with isotopic ratios of elements other than nitrogen and other analytical markers using chemometric approaches.[8−10]

The use of δ^{15}N for organic authentication is challenged by the fact that N_2-fixing plants (the Leguminosae family) are frequently used as cover crops or green manures in organic crop rotations. Leguminous green manures contribute carbon (C) and nitrogen (N) inputs to the soil, thus improving soil quality and fertility. Legumes can increase N availability to subsequent crops and improve soil physical properties. They can contain large quantities of N, most of which is released during the first year after soil incorporation.[11] Thus, the use of legumes represents a common and valuable strategy for improving soil fertility in organic agriculture. However, legumes have a nitrogen isotopic composition similar to that of synthetic fertilizers.[1] N_2-fixing plants can obtain their N from the atmosphere, whereas non-N_2-fixing plants take up N from the soil. Because the δ^{15}N value of air is defined as 0‰ by convention, N_2-fixing plants tend to have δ^{15}N values close to 0‰,[12] which is overlapping with the range of synthetic fertilizers. This represents one of the major challenges of utilizing δ^{15}N for determining the fertilization

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history of organic plants. This has partly been addressed by compound-specific isotope analysis of N and O of plant-derived nitrate, which has proven promising for organic authentication of vegetables.[1,13] However, due to the inherently low nitrate contents of most generative plant tissue, it remains unclear if stable isotope analysis of nitrate from cereal grains is feasible. Consequently, this calls for the development of novel analytical methods for authenticity testing of organic crops.

The stable isotope ratio of carbon $^{13}C/^{12}C$ (expressed as $\delta^{13}C$) has also been investigated as well for organic authentication. In C3 plants, $\delta^{13}C$ is affected by water availability and drought stress, but also shows significant interactions with nutrient availability and fertilization,[14,15] which frequently can be systematically different between organic and conventional systems. Additionally, $\delta^{13}C$ values can be related to a different contribution and isotopic ratio of CO_2 from soil respiration or from other sources (e.g., a CO_2-enriched atmosphere in the greenhouse) to plant photosynthesis.[16−18] The soil management practices used in organic systems and the use of green manure may lead to higher microbial activity in organic soils compared to conventionally grown.

Traditionally, stable isotope analysis has relied on measurements of bulk plant tissue, but emerging methods aimed at individual chemical compounds provide a means of obtaining a more in-depth understanding.[19] GC-C-IRMS has been utilized for examining the effects of land use and fertilizer practices on $\delta^{15}N$ value of individual amino acids in soil.[20] Previous studies[21] also showed that the $\delta^{15}N$ signatures of individual amino acids (e.g., histidine and phenylalanine) can be used to discriminate between different plant species in relation to the acquisition of available N sources. Amino acids are indeed the dominant low molecular weight nitrogen-bearing biomolecules in plants, and the pattern of isotopic fractionation during synthesis of these compounds records a range of information about the growth environment, such as the form of plant-available nitrogen in the soil.

New and more sensitive markers are needed to verify the authenticity of organically grown plants, and this study is thus timely and of practical relevance. We investigated the feasibility of using compound-specific nitrogen and carbon isotope analysis of plant-derived amino acids for differentiation between organically and conventionally grown wheat. The study was conducted on common wheat from Danish field trials and on durum wheat from Italian on-farm studies.

■ MATERIALS AND METHODS

Reagents and Reference Materials. L-Amino acid standards at ≥98% purity (alanine, aspartic acid, glutamic acid, glycine, isoleucine, norleucine, leucine, phenylalanine, proline, and valine) and analytical grade cation-exchange resin (Amberlite IR120 hydrogen form) were purchased from Sigma-Aldrich. All other solvents (dichloromethane, isopropanol, acetone, and ethyl acetate) and reagents (triethylamine and acetic anhydride) used were of analytical grade and purchased from Sigma-Aldrich and VWR (Milan, Italy).

Plant Materials and Sampling. Eighteen samples of winter wheat (*Triticum aestivum* L. cv. Tommi) were grown at three different Danish geographical locations: Flakkebjerg (FL), Foulum (FO), and Jyndevad (JY). The plants were grown in three systems (one conventional and two organic) under controlled conditions in a long-term field trial and were sampled in 2007 and 2008. The conventional system (CS) relied on the use of pesticides and synthetic fertilizers, whereas the organic systems (OSA and OSB) were managed in full compliance with the guidelines for organic farming (European Community Council Regulation EEC 2091/91 and EC 834/2007). The two organic systems OSA and OSB differed with regard to nutrient input. In the OSA system pig manure was imported from conventional farms, whereas the OSB system relied on nutrient input from green manures originating from cover crops being mixtures of grasses and N_2-fixing legumes. Fertilizer application rates as well as the percentage content of C and N in wheat samples are shown in Table 1. A map of the locations, soil characteristics, further details regarding field trials, climatic conditions, sampling of wheat grains and sample preparation are described elsewhere.[22]

Table 1. Danish Wheat: Sample Location, Agricultural System, Production Year, Fertilizer Application Rate, and Content of C and N in Wheat Samples in the Three Cropping Systems in Two Years

location	system[a]	year	fertilizer (nitrogen source) (kg/ha)	C (%)	N (%)
Flakkebjerg	Org (OSA)	2007	115	41.8	1.5
Flakkebjerg	Org (OSA)	2008	100	41.6	1.4
Foulum	Org (OSA)	2007	105	42.2	1.5
Foulum	Org (OSA)	2008	110	41.4	1.5
Jyndevad	Org (OSA)	2007	105	42.0	1.5
Jyndevad	Org (OSA)	2008	65	40.9	1.3
Flakkebjerg	Org (OSB)	2007	0	42.3	1.5
Flakkebjerg	Org (OSB)	2008	5	41.8	1.5
Foulum	Org (OSB)	2007	0	42.2	1.6
Foulum	Org (OSB)	2008	0	41.4	1.4
Jyndevad	Org (OSB)	2007	0	42.3	1.7
Jyndevad	Org (OSB)	2008	0	41.2	1.5
Flakkebjerg	Conv (CS)	2007	165	42.5	2.3
Flakkebjerg	Conv (CS)	2008	170	41.7	2.1
Foulum	Conv (CS)	2007	165	42.3	2.0
Foulum	Conv (CS)	2008	165	41.8	2.0
Jyndevad	Conv (CS)	2007	160	42.1	1.9
Jyndevad	Conv (CS)	2008	155	41.2	1.7

[a]CS, conventional; OSA, organic with animal manure; OSB, organic with green manures.

In addition to the 18 samples from Denmark, 17 samples of durum wheat (*Triticum turgidum* ssp. durum cv. Khorasan, Senatore Cappelli, Simeto, or Levante) were collected from commercial farms in 2012 in the main wheat-producing regions of Italy (Basilicata, n = 7; Molise, n = 7; and Emilia-Romagna, n = 3). Wheat was grown in each location in either a conventional system (CS, with mineral fertilizer) or an organic system (with organic fertilizer, OSA; or after 5 years of legumes, OSB; or without any fertilizer). The organic systems were managed in full compliance with the guidelines for organic farming (European Community Council Regulation EC 834/2007) for at least 8 years prior to the sample production. The systems were all based on stockless crop production but were managed differently depending on soil characteristics, climate, and farm structure. In the conventional system, pesticides and inorganic fertilizers were used according to usual farming practice. Nutrient supply in the organic system was based on the crop rotation built fertility and in some cases addition of commercial pelleted organic fertilizers. No irrigation was used for any of the organic and conventional fields. Information about location, cropping system, variety, and fertilizers used as well as the percentage content of C and N in wheat samples is reported in Table 2. All durum wheat samples were harvested at maturity and consequently on different days in the different regions (from mid-June until mid-July).

EA-IRMS Analysis. The $\delta^{15}N$ and $\delta^{13}C$ values of pure non-derivatized single amino acids and of bulk samples were measured using an elemental analyzer (Flash EA 1112, Thermo Scientific, Bremen, Germany), equipped with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced through a ConFlo IV dilutor device (Thermo Finnigan, Bremen, Germany) with a DELTA V isotope ratio mass spectrometer (Thermo Scientific). The isotopic values were calculated against L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA), and sugar IAEA-CH-6

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Table 2. Italian Durum Wheat: Sampling Location, Agricultural System, Variety, Fertilizer Application Rate and Type, and Content of C and N in Durum Wheat Samples in the Italian Cropping Systems

location	system[a]	variety	fertilizer (nitrogen source)	C (%)	N (%)
Basilicata	Org (OSA)	Khorasan	32 kg N/ha (organic fertilizer)	42.3	1.8
Basilicata	Org (OSA)	Khorasan	32 kg N/ha (organic fertilizer)	42.8	1.9
Basilicata	Org (OSA)	Senatore Cappelli	32 kg N/ha (organic fertilizer)	43.2	2.3
Basilicata	Org (OSA)	Senatore Cappelli	32 kg N/ha (organic fertilizer)	43.1	2.3
Molise	Org (OSA)	Simeto	36 kg N/ha (organic fertilizer)	43.3	1.8
Molise	Org (OSA)	Simeto	36 kg N/ha (organic fertilizer)	43.0	2.1
Emilia-Romagna	Org (OSB)	Levante	5 years of Lucerne (*Medicago sativa*) cultivation	42.6	2.3
Emilia-Romagna	Org (OSB)	Levante	5 years of Lucerne (*Medicago sativa*) cultivation	43.2	2.4
Emilia-Romagna	Org (OSB)	Levante	5 years of Lucerne (*Medicago sativa*) cultivation	43.2	2.4
Molise	Org	Senatore Cappelli	nothing	42.8	1.8
Molise	Org	Senatore Cappelli	nothing	42.6	1.7
Molise	Conv (CS)	Simeto (R)	75 kg N/ha (mineral fertilizer)	42.3	2.6
Molise	Conv (CS)	Simeto (R)	75 kg N/ha (mineral fertilizer)	43.2	2.5
Molise	Conv (CS)	Simeto (A)	120 kg N/ha (mineral fertilizer)	42.9	2.2
Basilicata	Conv (CS)	Khorasan	100 kg N/ha (mineral nitrate)	43.1	2.5
Basilicata	Conv (CS)	Khorasan	100 kg N/ha (mineral nitrate)	43.1	2.4
Basilicata	Conv (CS)	Senatore Cappelli	100 kg N/ha (mineral nitrate)	43.8	2.9

[a]Conv, conventional; Org, organic.

for $^{13}C/^{12}C$ and against L-glutamic acid USGS 40 and potassium nitrate IAEA-NO3 for $^{15}N/^{14}N$.

The content of N and C was measured using IRMS (ANCA-SL elemental analyzer coupled to a 20-20 Tracermass mass spectrometer, Sercon Ltd., Crewe, UK). Quality assurance was performed by duplicate measurements of all samples as well as analysis of Certified Reference Materials (NIST 1515, NIST 8436, and 141d acetanilide $CH_3CONHC_6H_5$, National Institute of Standards and Technology) as previously described.[22]

Hydrolysis of Protein and Purification. Wheat samples were defatted three times with petroleum ether/ethyl ether (2:1 v/v, 30 mL) homogenizing with an Ultraturrax device (model X-620, Staufen, Germany; 11500 rpm for 3 min) and using a centrifuge (ALC PK 131R, Thermo Electron Corp., Germany; 4100 rpm for 6 min) to separate the ether from the residue. The defatted powdered samples were left open to air-dry and then stored at room temperature until analysis.

Acid hydrolysis was conducted to obtain individual amino acids from protein: 250 mg of lipid-extracted sample was digested with 2 mL of 6 M HCl at 110 °C for 24 h in a PTFE-capped Pyrex vial. After cooling, the hydrolyzed solution was filtered on glass wool, and the solution was blown to dryness under N_2 and redissolved in 2 mL of 0.1 M HCl. A known quantity of norleucine (8 mg mL^{-1} in 0.1 M HCl) was added as internal standard, and the solution was stored at −20 °C.

Amino acids were purified through ion-exchange chromatography on an Amberlite IR120 cation-exchange resin, previously saturated with H^+ on all exchange sites. This was accomplished by soaking the resin overnight in 3 M NaOH, followed by washing in distilled water and soaking overnight in 6 M HCl, after washing with distilled water. The H^+ saturated resin was pipetted into a glass pipet column fitted with a quartz wool plug, and a fraction of the hydrolyzed sample (500 μL) was added to the column. Salts were removed with distilled water and the amino acids eluted with NH_4OH (10 wt %) and then dried under N_2 before derivatization. Under those conditions, the resin does not cause any isotopic fractionation.[23,24]

Derivatization. Amino acids were analyzed after *N*-acetylisopropyl derivatization based on the method reported elsewhere.[25] Samples were first esterified in 1 mL of acidified isopropanol (1:4 acetyl chloride/isopropanol) at 100 °C for 1 h. Esterifying reagents were evaporated under a gentle stream of nitrogen at 40 °C, and dried esters were rinsed with two sequential 250 μL aliquots of dichloromethane to remove reagents in excess. Amino acid esters were acylated with 1 mL of acetic anhydride/triethylamine/acetone solution (1:2:5 v/v) at 60 °C for 10 min. The reagents were evaporated under a N_2 stream at room temperature, and dried derivatives were dissolved in 1 mL of saturated NaCl−water solution and 1 mL of ethyl acetate and mixed vigorously. The organic phase containing the amino acids was collected and dried under nitrogen at room temperature. Residual water was removed with two sequential 250 μL aliquots of dichloromethane. Finally, samples were dissolved in 200 μL of ethyl acetate and stored at −20 °C until analysis.

GC-C-IRMS Analysis. The isotopic values of 10 amino acids, alanine (Ala), aspartate (Asx), glutamate (Glx), glycine (Gly), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), proline (Pro), threonine (Thr), and valine (Val), were determined by GC-C-IRMS (Figure 1).

Due to the acid hydrolysis, asparagine (Asn) and glutamine (Gln), present in wheat, are converted into aspartic acid (Asp) and glutamic acid (Glu), respectively. Therefore, the $\delta^{15}N$ and $\delta^{13}C$ values determined of Asx and Glx represent the nitrogen and carbon isotopic value of both aspartate + asparagine and glutamate + glutamine, respectively.[24]

Individual amino acid isotopic analysis was carried out using a Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific) interfaced with an IRMS (DELTA V, Thermo Scientific) through an open split interface and with a single-quadrupole GC-MS (ISQ Thermo Scientific) to identify the compounds. For $\delta^{15}N$ analysis 0.8−1.0 μL of each sample was injected in splitless mode by an autosampler (Triplus, Thermo Scientific). A HP-INNOWAX capillary column (60 m × 0.32 mm i.d. × 0.25 μm film thickness; Agilent) with He as carrier gas (at a flow of 1.4 mL/min) was used. The injector temperature was set at 250 °C, and the oven temperature of the GC started at 40 °C, at which it was held for 2 min before heating at 40 °C/min to 140 °C, at 2.5 °C/min to 180 °C, at 6 °C/min to 220 °C, and finally at 40 °C/min to 250 °C and held for 15 min. For $\delta^{13}C$ analysis 1.0 μL of each sample was injected in split mode and a stronger polar column was used to obtain higher and sharper peaks (ZB-FFAP capillary column, 30 m × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex). The oven temperature of the GC started at 40 °C, at which it was held for 1 min before heating at 15 °C/min to 120 °C, at 3 °C/min to 190 °C, and finally at 5 °C/min to 250 °C and held for 7 min.

The eluted compound was combusted into N_2, CO_2, and H_2O in a combustion furnace reactor operated at 1030 °C and composed of a nonporous alumina tube (320 mm length) containing three wires

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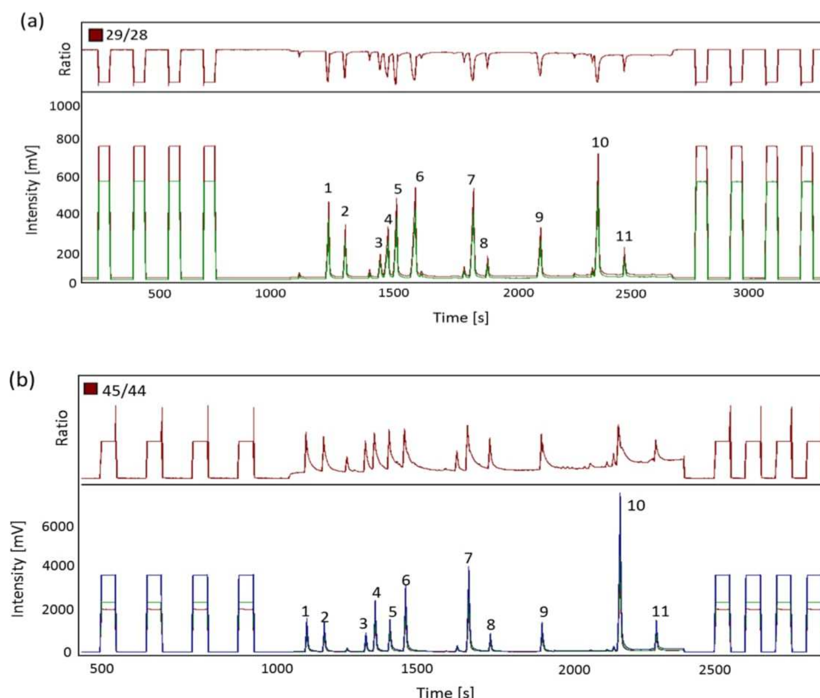

Figure 1. GC-C-IRMS chromatogram of *N*-acetyl-isopropyl derivatives of amino acids in a wheat sample (nitrogen isotopic ratio (a) and carbon isotopic ratio (b)). Peaks: 1, Ala; 2, Val; 3, Ile; 4, Leu; 5, Gly; 6, Nleu (internal standard); 7, Pro; 8, Thr; 9, Asx; 10, Glx; 11, Phe. The four first and last peaks of each panel are reference gas signals.

(Ni/Cu/Pt, 0.125 mm diameter, 240 mm identical length) braided and centered end-to-end within the tube. Water vapor was removed by a water-removing trap, consisting of a Nafion membrane. During $\delta^{15}N$ analysis a liquid nitrogen trap was added after the combustion oxidation reactor to remove CO_2 from the oxidized and reduced analyte.

To monitor instrumental performance a mixture of L-amino acid standards was derivatized and the $\delta^{15}N$ and $\delta^{13}C$ values were measured by GC-C-IRMS before each analytical run. Moreover, the isotopic value of the internal standard norleucine added to each sample was checked. Norleucine was chosen as internal standard because it is not naturally present in wheat. The $\delta^{15}N$ and $\delta^{13}C$ values of pure norleucine (+14.0 and −27.6‰, respectively) and of the pure L-amino acids (see below) were determined by EA-IRMS. The analytical run was accepted when the differences between GC-C-IRMS and EA-IRMS values were, at most, ± 1.0 and ±1.5‰, respectively, for $\delta^{15}N$ and $\delta^{13}C$.

Data Analysis and Corrections. All of the $\delta^{15}N$ and $\delta^{13}C$ values are reported relative to reference N_2 and CO_2 of known nitrogen and carbon isotopic composition, respectively, introduced directly into the ion source at the beginning and end of each run (Figure 1). All samples were measured in triplicate, and the isotope ratios were expressed in δ‰ versus atmospheric nitrogen for $\delta^{15}N$ and V-PDB (Vienna − Pee Dee Belemnite) for $\delta^{13}C$ according to eq 1

$$\delta = \left[\frac{(R_s - R_{std})}{R_{std}}\right] \tag{1}$$

where R_s is the isotope ratio measured for the sample and R_{std} is the isotope ratio of the internationally accepted standard.

Measured $\delta^{13}C$ values for derivatized amino acids are the product of both the carbon native to the molecule and the contribution from the reagents used for derivatization. The $\delta^{13}C$ values of the mixture of standard amino acids measured by EA-IRMS and their respective *N*-acetylisopropyl esters obtained with GC-C-IRMS are presented in the Supporting Information (SI 1). Therefore, an empirical correction[26] was applied to determine the effective carbon isotope value. Correction factors were calculated by determining the $\delta^{13}C$ values of the underivatized amino acid standards (EA-IRMS) and the derivatized amino acid standards (GC-C-IRMS):

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \tag{2}$$

n is the number of moles of carbon, and the subscripts c, d, and cd represent the compounds of interest, the derivative group, and the derivatized compound, respectively.

When calculating the uncertainty of $\delta^{13}C$ measurement (see below), we considered measurement errors as well as the total analytical error, which arises from the different derivatization steps:

$$\sigma_c^2 = \sigma_s^2\left(\frac{n_s}{n_c}\right)^2 + \sigma_{sd}^2\left(\frac{n_s + n_d}{n_c}\right)^2 + \sigma_{cd}^2\left(\frac{n_c + n_d}{n_c}\right)^2 \tag{3}$$

n is the number of moles of carbon, and the subscripts c, cd, d, s, and sd, represent the underivatized compound, the derivatized compound, the derivatizing reagent, the underivatized standard, and the derivatized standard, respectively.

Accuracy and Precision of GC-C-IRMS. To test the accuracy of the determination of amino acid isotopic values, we compared the $\delta^{15}N$ and $\delta^{13}C$ values of the mixture of standard amino acids measured by GC-C-IRMS with the isotopic values of pure nonderivatized single amino acids obtained with EA-IRMS. The $\delta^{15}N$ and $\delta^{13}C$ values determined by EA-IRMS were the mean of two measurements, whereas the $\delta^{15}N$ and $\delta^{13}C$ values determined by GC-C-IRMS were the average of three runs. Both

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$\delta^{15}N$ and $\delta^{13}C$ values obtained from EA-IRMS were linearly correlated with those from GC-C-IRMS after derivatization, as shown in the Supporting Information (SI 2 and SI 3), and the difference between the values measured by EA-IRMS and by GC-C-IRMS after empirical correction was not higher than ±0.5 and ±1.6‰ for $\delta^{15}N$ and $\delta^{13}C$, respectively (SI 1).

To evaluate the precision, 10 replicates of the reference amino acid mixture were derivatized, and each of them was analyzed by GC-C-IRMS in triplicate. The precision (1σ) of GC-C-IRMS determinations was on average ±0.4‰ for $\delta^{15}N$ and ±0.8‰ for $\delta^{13}C$.

To evaluate the uncertainty of measurements for all of the processes, a wheat sample was hydrolyzed and derivatized 10 times, and each of the samples was analyzed by GC-C-IRMS. The standard deviation obtained (1σ) was on average ±0.3‰ for $\delta^{15}N$ and ±1.0‰ for $\delta^{13}C$ (Supporting Information (SI 4).

Statistical Analysis. The data were statistically evaluated using Statistica v 9 (StatSoft Italia srl, Padua, Italy).

■ RESULTS AND DISCUSSION

Wheat Amino Acid $\delta^{15}N$ and $\delta^{13}C$ Values. The $\delta^{15}N$ and $\delta^{13}C$ values of the amino acids Ala, Val, Ile, Leu, Gly, Pro, Thr, Asx, Glx, and Phe from the 18 Danish and from the 17 Italian wheat grain samples are shown in the Supporting Information (SI 5 and SI 6), whereas the mean and standard deviation (SD) values across agricultural systems are reported in Tables 3 and 4.

From the $\delta^{15}N$ values it is evident that the ^{15}N content between different amino acids can vary significantly due to isotopic fractionation occurring during N metabolism within the plant. Ammonium (NH_4^+) from soil is assimilated directly into plant amino acid metabolism via the enzyme glutamine synthetase, whereas nitrate (NO_3^-) undergoes sequential reduction to nitrite and then subsequently to ammonia via the nitrate and nitrite reductase enzymes.[27] The first amino acid produced by ammonia assimilation is glutamine, which therefore plays a central role in the amino acid metabolism. Glutamate and then other amino acids are produced by the transfer of the amide of glutamine to keto acids and by transamination.[28] It follows that in our case the $\delta^{15}N$ of glutamic acid derived from acid hydrolysates of glutamic acid and glutamine (Glx) represents the main source of imported nitrogen being a product of the net flux of N entering and leaving the Gln and Glu pools.

Figure 2a shows mean values of differences between the $\delta^{15}N$ values of Ala, Val, Ileu, Leu, Gly, Pro, Thr, Asx, and Phe and that of Glx across all 35 wheat samples. This allows comparison of the $\delta^{15}N$ value of single amino acids normalizing the possible variation caused by the external nitrogen source (e.g., fertilizer practices, soil composition, location). The $\delta^{15}N$ relative trend among amino acids is in agreement with the results reported in other studies.[29] As compared to the $\delta^{15}N$ value of Glx (Figure 2a), the $\delta^{15}N$ values of Phe (+4.4 ± 1.4‰) and Pro (+1.0 ± 0.8‰) are higher. The relative ^{15}N enrichment of Phe and Pro compared to the other amino acids in cereal grains is in agreement with the scientific literature on the subject, concluding that there is an isotopic fractionation associated with this enzymatic reaction.[29] Phenylalanine ammonia-lyase catalyzes the deamination of Phe, and the kinetic isotope effect associated with this reaction leaves the residual Phe enriched in ^{15}N, whereas the ^{15}N enrichment of Pro is caused by a kinetic isotope effect associated with the enzymes (proline dehydrogenase and Δ^1-pyrrolidine-5-carboxylate dehydrogenase) involved in its catabolism.[29,30]

The $\delta^{15}N$ values of Ala (−3.1 ± 1.5‰), Val (−1.4 ± 1.4‰), Ileu (−4.1 ± 1.8‰), Leu (−5.7 ± 1.3‰), Gly (−5.0 ± 1.8‰), Thr (−9.8 ± 2.5‰), and Asx (−2.9 ± 1.6‰) are lower than the

Table 3. Amino Acid $\delta^{15}N$ and $\delta^{13}C$ Values of Danish Wheat Samples[a]

		wheat amino acid $\delta^{15}N$ and $\delta^{13}C$ values (‰)																			
		Ala		Val		Ile		Leu		Gly		Pro		Thr		Asx		Glx		Phe	
system		N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
CS	mean	1.1b	−24.9b	2.7b	−30.8	0.1b	−26.9	−1.2b	−34.0	−1.2b	−14.7	5.9b	−28.1	−6.4b	−14.6	0.8b	−25.2	5.3b	−26.1b	8.6b	−26.5
	SD	1.0	0.7	0.9	0.7	0.9	0.9	1.2	0.8	1.9	1.4	0.7	0.8	1.8	0.5	1.1	1.0	1.1	0.3	0.7	1.4
OSB	mean	1.7b	−23.7a	3.3b	−30.3	0.3b	−26.1	−1.0b	−33.7	−1.5b	−13.4	6.1b	−26.8	−5.4b	−12.5	1.1b	−24.1	5.3b	−24.6a	9.4b	−26.4
	SD	1.3	0.5	1.6	0.9	1.7	0.7	1.7	0.5	2.5	1.0	2.1	0.7	1.3	1.1	1.8	0.9	2.3	0.6	1.7	1.6
OSA	mean	6.2a	−24.1b	8.4a	−30.2	5.1a	−26.1	3.6a	−33.8	3.2a	−13.4	10.4a	−27.3	−0.2a	−12.8	6.0a	−24.4	9.3a	−24.0a	14.5a	−27.2
	SD	1.7	1.0	1.3	1.4	1.4	1.6	1.5	0.7	1.3	1.6	0.8	1.4	2.7	3.1	1.0	2.0	0.9	1.0	0.8	1.7

[a]Values are the means of three analytical replicates. CS, conventional; OSB, organic with green manures; OSA, organic with animal manure; SD, standard deviation. Significantly different mean values (HSD Tukey's, $p < 0.05$) of wheat samples from the three systems across locations are indicated with letters "a" and "b".

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Table 4. Amino Acid $\delta^{15}N$ and $\delta^{13}C$ Values of Italian Wheat Durum Samples[a]

		wheat amino acid $\delta^{15}N$ and $\delta^{13}C$ values (‰)																			
		Ala		Val		Ile		Leu		Gly		Pro		Thr		Asx		Glx		Phe	
system		N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
CS	mean	1.2	−22.5a	3.1	−30.9	0.4	−27.0	−1.4	−34.9a	0.8	−15.8ab	5.2	−26.2a	−4.5	−13.1ab	2.4	−24.3	4.1	−27.0b	8.4	−26.2a
	SD	1.9	1.9	2.8	1.5	2.6	1.3	2.2	1.0	2.2	2.1	2.2	1.5	1.8	1.9	2.3	3.0	2.3	0.9	2.2	1.2
OSB	mean	1.2	−27.0b	3.6	−32.7	1.4	−28.7	−1.7	−37.1b	1.1	−21.1b	5.2	−29.4b	−3.4	−17.0b	2.7	−25.8	3.9	−22.8a	9.8	−33.4b
	SD	3.9	0.1	2.9	0.3	4.2	0.2	2.7	1.1	2.1	2.5	3.1	0.8	5.3	2.5	3.4	0.8	2.2	0.6	2.8	3.5
OSA	mean	4.1	−22.8a	4.9	−31.5	2.9	−26.6	1.0	−34.5a	2.0	−15.0a	7.6	−25.3a	−3.2	−11.5a	4.4	−24.5	6.2	−23.8a	10.5	−27.1ab
	SD	2.9	1.9	3.3	1.2	3.4	1.7	3.4	0.6	3.6	3.5	3.0	1.0	4.0	2.0	3.3	1.5	2.6	1.4	2.7	2.9
nothing	mean	3.0	−23.9ab	4.6	−31.9	1.9	−28.5	1.6	−35.5ab	3.4	−17.3ab	7.2	−26.9ab	−4.7	−13.1ab	3.2	−25.6	6.6	−24.4a	10.5	−32.5ab
	SD	2.5	0.1	2.6	0.4	3.9	0.2	0.9	1.0	4.1	1.1	2.5	0.2	4.2	0.6	3.7	2.5	3.0	0.8	1.3	7.9

[a]Values are the means of three analytical replicates. CS, conventional; OSB, organic with green manure; OSA, organic with animal manure; nothing, organic without fertilizer treatment; SD, standard deviation. Significantly different mean values (HSD Tukey's, $p < 0.05$) between groups are indicated with letters "a" and "b".

$\delta^{15}N_{Glx}$ value. These amino acids are principally produced during transamination of precursors, amino acids (e.g., glutamic acid), and keto acids. Previous studies[31] have reported that enzymatic reactions of transamination involved in plant metabolism induced a kinetic isotope effect leading to the lower $\delta^{15}N$ values in NH_2 of product amino acid and that the extent of isotopic fractionation may vary depending on the reaction rates.

The mean $\delta^{13}C$ value of plant material is fixed by that of the primary carbon source (CO_2) and by the kinetic isotope effect of the CO_2-binding reaction (RuBP-carboxylase and PEP-carboxylase, respectively). The pattern of aliphatic amino acids and aromatic amino acids derived from glucose and shikimic acid, respectively, reflects the resulting ^{13}C distribution of these precursors, and it is supplemented by the introduction of additional C atoms depleted by the kinetic isotope effect on the PEP−carboxylase reaction.[32] Figure 2b shows mean values of differences between the $\delta^{13}C$ values of Ala, Val, Ileu, Leu, Gly, Pro, Thr, Asx, and Phe and that of Glx across all 35 wheat samples from all three agricultural systems and locations. $\delta^{13}C$ values of Gly (+9.6 ± 3.3‰) and Thr (+11.6 ± 2.7‰) were higher than the $\delta^{13}C_{Glx}$ value, whereas the $\delta^{13}C$ values of Val (−6.1 ± 2.2‰), Ileu (−2.0 ± 2.1‰), Leu (−9.6 ± 2.1‰), Pro (−2.1 ± 2.2‰), and Phe (−2.7 ± 4.0‰) were lower. The measured $\delta^{13}C$ values of Ala and Asx were very similar to that of Glx (within ±2.5 and ±2.4‰, respectively) in all of the wheat samples.

$\delta^{15}N$ and $\delta^{13}C$ Amino Acid Values for Organic Authentication. In Tables 3 and 4 the mean and standard deviation of the data grouped according to agricultural system across locations in Denmark and Italy, respectively, as well as the results of the honestly significantly difference (HSD) for unequal N Tukey's test are reported.

Grouping the Danish samples according to the agricultural systems, Tukey's test results (Table 3) showed that conventionally grown wheat had significantly lower $\delta^{15}N$ values than the organic wheat OSA, whereas it was not distinguishable from the organic wheat fertilized with green manure (OSB). This corresponds with results from bulk tissue analysis in previous studies.[1] This finding clearly highlights the limitation of $\delta^{15}N$ as a marker of organic production when plant fertilization is based on N_2-fixing green manures. On the other hand, the $\delta^{13}C$ value of Glx was significantly ($p < 0.05$) lower in the conventional wheat compared to both organic systems (OSA and OSB). $\delta^{15}N$ values of plant amino acids are clearly affected by the nitrogen source, which in this case results in similar $\delta^{15}N$ values for CS and OSB samples. In contrast, $\delta^{13}C$ is more linked to the different characteristics of the field under conventional and organic regimens in terms of water availability, drought stress, and nutrient availability, which influence the photosynthetic activity and stomatal conductance of leaves.[14−18] Here we found lower $\delta^{13}C$ values in the conventional wheat, which is expected to be due to a higher stomatal conductance in these plants due to the higher nitrogen content (as reported in Tables 1 and 2). Indeed, previous studies[33] showed that the maximum CO_2 assimilation rates and maximum stomatal conductance, when measured under higher N availability conditions, as typically observed in conventional crops, did lead to a higher discrimination of RuBisCo against $^{13}CO_2$. In contrast, N deficiency, as in the case of organic crops, can induce stomatal closure, which is caused by a lower ratio of the N-regulated plant hormones cytokinin and abscisic acid. This leads to a reduced discrimination against $^{13}CO_2$ and thereby higher $\delta^{13}C$ values. Lower $\delta^{13}C$ values were also observed in proteins extracted from conventional apples,[34]

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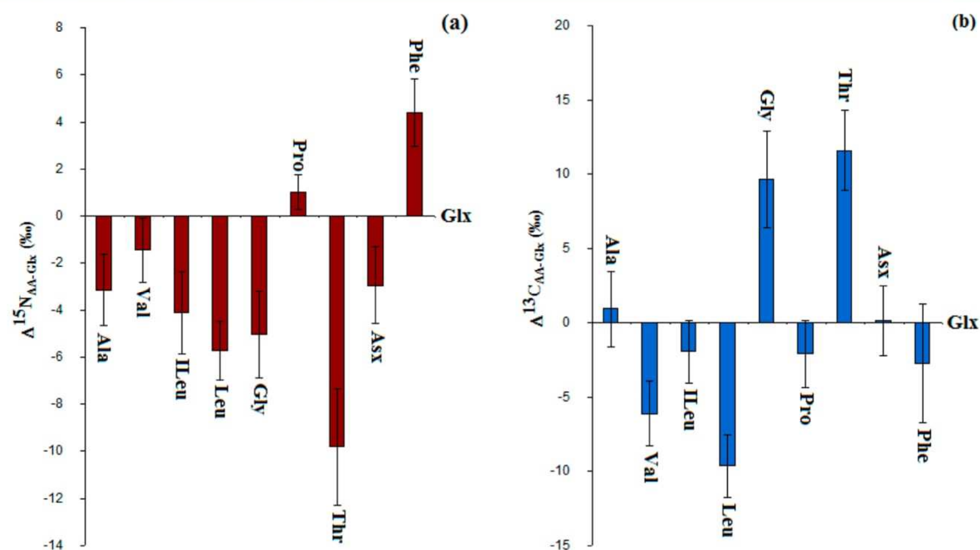

Figure 2. Differences between the $\delta^{15}N$ (a) and $\delta^{13}C$ (b) values of Ala, Val, Ileu, Leu, Gly, Pro, Thr, Asx, and Phe and the $\delta^{15}N$ and $\delta^{13}C$ of Glx for all of the Danish and Italian wheat samples. Bars represent mean values ± SD ($n = 35$).

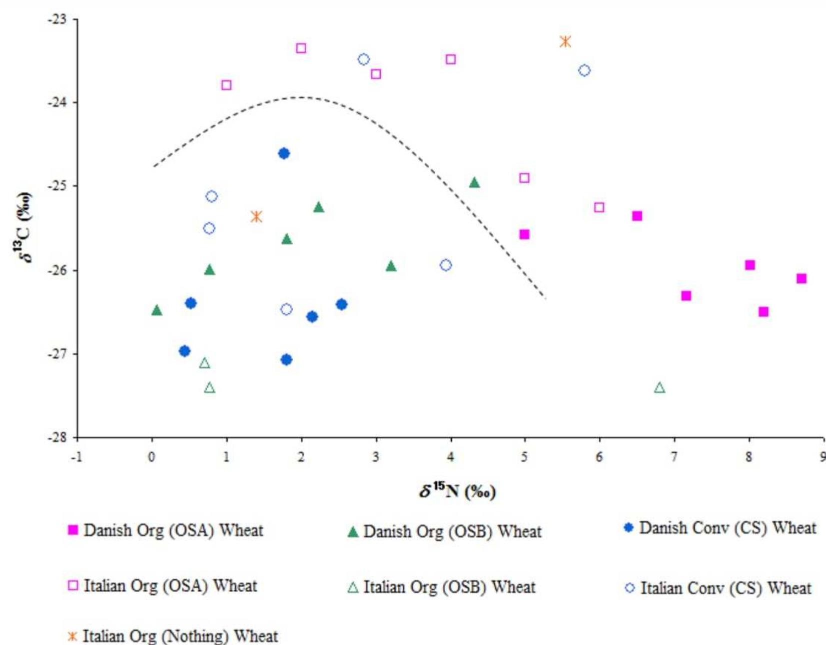

Figure 3. $\delta^{15}N$ plotted against $\delta^{13}C$ in bulk tissue samples of Danish and Italian wheat samples. Circles are CS (conventional), squares are OSA (organic with animal manure), triangles are OSB (organic with green manure), and stars are nothing (organic without fertilizer treatment). Solid and open symbols indicate Danish and Italian samples, respectively. Please note that the dashed line is only for illustrative purposes and does not represent statistical analysis.

but not in conventional onions and cabbages[15] or in soil of conventional grassland.[35]

Applying Tukey's test on $\delta^{15}N$ amino acid values of the Italian durum wheat samples grouped according to the agricultural

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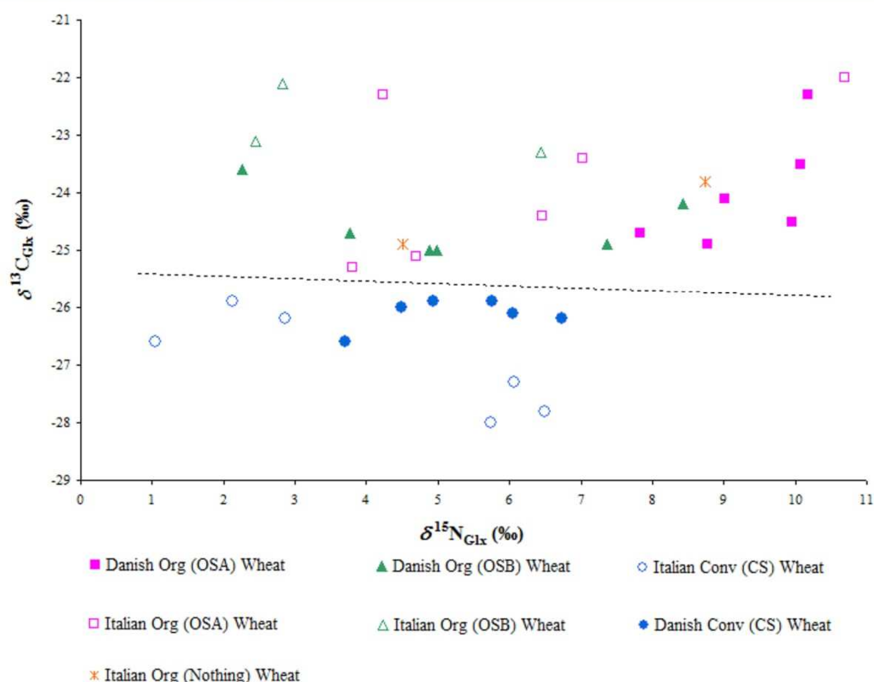

Figure 4. $\delta^{15}N$ in Glx plotted against $\delta^{13}C$ in Glx of Danish and Italian wheat samples. Circles are CS (conventional), squares are OSA (organic with organic fertilizer), triangles are OSB (organic with green manure), and stars are nothing (organic without fertilizer treatment). Solid and open symbols indicate Danish and Italian samples, respectively. Please note that the dashed line is only for illustrative purposes and does not represent statistical analysis.

regimen, no significant differences between the four groups were observed (Table 4). Conventionally cultivated durum wheat was not distinguishable from either the organic wheat OSA (fertilized with organic fertilizer) or OSB (fertilized with green manure) or organic wheat grown without fertilization. The main reason for this finding is that we considered samples produced in different Italian regions (Emilia Romagna, Basilicata, Molise) potentially characterized by different $\delta^{15}N$ values of the soil.

As observed for Danish wheat and also for Italian durum, the $\delta^{13}C$ values of some amino acids were significantly different between agricultural regimens. In particular the $\delta^{13}C$ of Glx was significantly lower ($p < 0.05$) in the conventional samples compared to organic systems (OSA, OSB, nothing) as for Danish samples.

On the basis of Tukey's test results we decided to consider all Danish and Italian samples together focusing on $\delta^{15}N$ and $\delta^{13}C$ values of Glx for discriminating between the agricultural systems. Moreover, we considered $\delta^{15}N$ and $\delta^{13}C$ values of bulk tissue wheat grain samples, taking the data from the literature for Denmark[1] and measuring the samples from Italy to conduct a direct comparison with the compound-specific data. Figure 3 shows the distribution of $\delta^{15}N$ and $\delta^{13}C$ values of Danish and Italian bulk tissue samples, whereas Figure 4 shows the same distribution for $\delta^{15}N$ and $\delta^{13}C$ values of Glx. The bulk tissue analysis allowed separation of wheat samples grown with the use of animal manure (OSA) from samples grown using synthetic fertilizers (CS), but it was impossible to distinguish the agricultural system based on legumes as green manures (OSB) from the conventional one, as discussed above.

However, the $\delta^{15}N$ and $\delta^{13}C$ values of Glx (Figure 4) improved the discrimination between the conventional and organic agricultural systems. In particular, the $\delta^{13}C$ value of Glx was able to distinguish organic samples from those grown in the conventional agricultural system—even when including the variations caused by wheat species, variety, harvest period, geographical location, etc.

Previous studies[36] have shown that multivariate statistical analysis can be a tool to improve the discrimination between different foods (e.g., organic and conventional). Linear discriminant analysis (LDA) is probably the most frequently used supervised pattern recognition method, which is based on maximization of the ratio of between-class variance and minimization of the ratio of within-class variance. LDA was performed using the data set composed by all wheat samples (35 organic and conventional samples) and 20 variables ($\delta^{15}N$ and $\delta^{13}C$ amino acid values) to establish if it is possible to enhance the separation between agricultural regimens. After application of LDA, three discriminant functions were calculated, and Figure 5 shows the score plot of the wheat samples using as axes the first two functions that explain 91.2% of total variance.

The three categories of wheat (OSA, OSB, and CS) were well separated across the principal component Rad 1. The latter correlates negatively with $\delta^{15}N$ Pro (−2.849) and $\delta^{13}C$ Val (−2.158) and positively with $\delta^{15}N$ Phe (3.436) and $\delta^{15}N$ Glx (2.622). Rad 2 improves the separation between OSA and the

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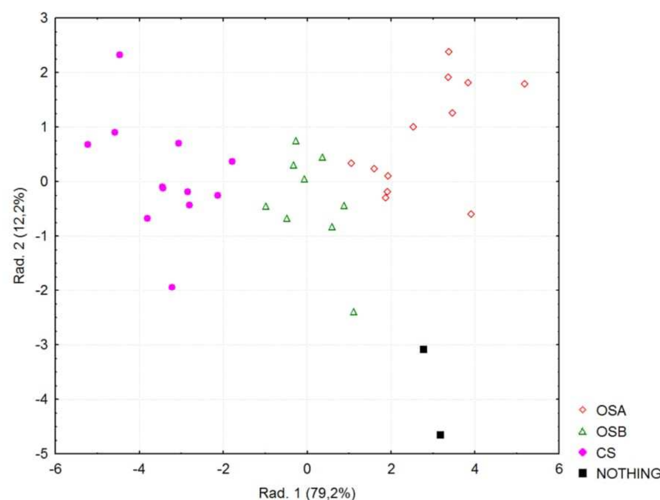

Figure 5. LDA scores plot of nitrogen and carbon isotope analysis of amino acids for organic and conventional wheat samples.

"nothing" group and correlates negatively with δ^{15}N Leu (−1.339), δ^{15}N ILeu (−1.036), and δ^{15}N Glx (−1.018), whereas it correlates positively with δ^{15}N Ala (1.314). δ^{13}C Glx did not correlate significantly with Rad 1 and Rad 2 because it is capable of separating organic from conventional samples but not the three types of organic agricultural systems.

We can conclude that compound-specific δ^{15}N and δ^{13}C analysis of plant-derived amino acids represents a promising tool for verification of the fertilization history of crops. δ^{13}C of glutamic acid and glutamine, in particular, but also the combination of δ^{15}N and δ^{13}C of the 10 amino acids Ala, Val, Ile, Leu, Gly, Pro, Thr, Asx, Glx, and Phe did improve the separation between organic and conventional wheat, even when organic wheat production was based on fertilization and crop rotation using N_2-fixing plants. It is recommended that the novel method is tested on a larger set of samples and across different plant species and locations to further evaluate the applicability of compound-specific stable isotope analysis of amino acids for organic authentication.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental tables and figure. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b00662.

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Notes

The authors declare no competing financial interest.

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CHAPTER 4 WINE, ADJUVANTS AND RELATED PRODUCTS

4.1 INTRODUCTION

Wine is the most famous and appreciated alcoholic beverage worldwide, obtained from the fermentation of fresh grapes or grape must. Approximately 38 billion bottles of wine are produced around the world each year, with a wholesale value of US$98 billion.

The International Organization of Vine and Wine (OIV) has estimated that Italy's wine production reached 48.8 million hectoliters in 2016, making Italy the world leader in terms of wine production, followed by France, Spain and the USA. With more than 2400 different styles, Italy offers the largest and most diverse array of wines in the world, including at least 300 DOC (Controlled Designation of Origin) and DOCG (Controlled and Guaranteed Designation of Origin) wines. The value of these premium drinks is determined almost exclusively by the brand/origin and the age/vintage.

In the last few years, total wine production has dropped by about 6%, while world consumption is rising. This shortage inevitably leads unscrupulous vendors to fill the gap in the market with bogus products.

SIRA of wine has been applied since 1991, becoming the official method for identifying the authenticity of wine in terms of watering down, sugar addition and mislabelling (OIV methods MA-AS311-05, MA-AS312-07 and MA-AS2-12, EU Reg. 555/2000). The addition of exogenous sugar and water in wine can be detected by analysing the isotopic ratios of hydrogen ($^{2}H/^{1}H$) and carbon ($^{13}C/^{12}C$) in ethanol and oxygen ($^{18}O/^{16}O$) in water. Additions and counterfeiting are detected by comparing the results against an official databank, set up by the EU for all wine-producing countries within its territory. So far, the isotopic ratio of N in wine has not yet been investigated as well as the isotopic ratios of tannin.

"Aceto balsamico di Modena IGP" is another Italian premium product which has obtained the IGP (Protected Geographic Indication) recognition, renowned throughout the world due to its specific characteristics. This unique balsamic vinegar can only be produced and matured in the province of Modena and its production method is specified in European regulations (EU Reg. 583/2009). Its success, particularly at international level, has contributed to the emergence and diffusion of fraud, represented by products that imitate or simply claim the title "balsamic", harming producers and increasing consumer confusion.

Very recently, isotopic methods have been officially recognised by OIV and the European Committee for Standardization (CEN) as a means of verifying the authenticity of wine vinegar (CEN methods EN 16466-1, EN 16466-2, EN 16466-3 and OIV 510/2013). $^{13}C/^{12}C$ and $^{2}H/^{1}H$ values in acetic acid and the $^{18}O/^{16}O$ value in water have provided a powerful tool for detecting the addition of exogenous acetic acid and tap water to wine vinegar. Moreover, a recent study [Camin *et al.*, 2013] showed that legal limits set on the basis of the wine isotope databank can be used as a reference for $\delta^{18}O$ analysis to detect the authenticity of wine vinegar.

SECTION 4.3

From soil to grape and wine: Variation of light and heavy elements isotope ratios

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Statement of the author: My personal contribution to this work mainly concerned the setting up of instrumental conditions, analysis and validation of the method to measure the nitrogen isotopic value in grape juice and wine. Moreover, I perfomed the nitrogen isotopic analysis of soil and branches samples.

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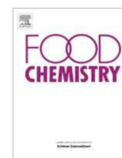

From soil to grape and wine: Variation of light and heavy elements isotope ratios

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ABSTRACT

In the development of a geographical traceability model, it is necessary to understand if the value of the monitored indicators in a food is correlated to its origin or if it is also influenced by 'external factors' such as those coming from its production. In this study, a deeper investigation of the trend of direct geographical traceability indicators along the winemaking process of two traditional oenological products was carried out. Different processes were monitored, sampling each step of their production (grape juice, intermediate products and wine). The results related to the determinations of $\delta^{18}O$, $(D/H)_I$, $(D/H)_{II}$, $\delta^{13}C$, $\delta^{15}N$ and $^{87}Sr/^{86}Sr$ have been reported. Furthermore, correspondence with the isotopic values coming from the respective soil and vine-branch samples have been investigated as well, showing the optimal traceability power of the monitored geographical tracers.

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1. Introduction

The geographical origin or production area of food is acquiring more and more importance for consumers that associate food quality with a well defined or recognizable origin. As a consequence, the production of a food in a particular region or using traditional methods is considered as an added value for the product itself.

Moreover, with the introduction of the product designations, namely Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG), the different labelling systems and the EC 178/2002 Regulation (Regulation EC 178/2002, 2002) the EU has set out the basis for a new control method by defining the terms of traceability and production chain traceability. Unfortunately, many of the actual traceability systems are generally not referred to objective criteria but mostly based on certifications supported by papery declarations. Thus, in a context of globalized market and international food trade, with high risk of imitation, counterfeiting and adulteration, the objective possibility to trace the provenance of food on the basis of objective analytical criteria could be certainly a valuable support for the traditional papery declarations. Therefore, the definition of criteria for geographical traceability of food, with a particular attention to those awarded with quality marks, such as the PDO, represents a real and challenging task. Generally speaking, from an analytical point of view, there are two approaches that can be adopted in this context: the former is based on a discriminating approach which uses direct and indirect indicators with the aims to differentiate 'homologous' food products but of different geographical origin (Bontempo et al., 2011; Trincherini, Baffi, Barbero, Pizzoglio, & Spalla, 2014); while the latter consists on a more systematic approach, mainly addressed to objectively link the food product to its area of origin and afterwards to discriminate it from the others on the basis of its provenance. The first approach relies on the use of the properties related to the food while the second one considers those related to its territory. In the latter case, it is unavoidable the analysis of matrices such as soils, water and plants (Durante et al., 2013; Marchionni et al., 2013). Starting from these evidences, it is possible to assume that the "territory" contribute to shape the fingerprint of the different products. Therefore, an *a priori* systematic study of the performance of the investigated indicators within the steps that characterise the 'production cycle' of a food, *soil-water-plant-fruit-raw materials-finished food*, represents the holistic approach to develop a model for the geographical origin of food. Only in this case, it is possible to establish a unique link between the final product and its territory of origin.

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This study represents an improvement of the knowledge of the trend of several geographical traceability indicators along the whole winemaking process of two traditional oenological products from soil to grape till the final wine. In particular, the data herein reported are part of a long term research project (http://www.progettoager.it) focused on the development of geographical traceability models of oenological products, *Lambrusco* PDO and *Trentodoc*, by means of primary indicators such as elements concentration and isotope ratios of radiogenic and light elements and secondary ones such as NMR spectra polyphenols fingerprint (Papotti et al., 2013) and the aromatic profile respectively. The project is characterised by an innovative analytical methodology due to the use of a systematic approach (Bertacchini et al., 2013; Totaro et al., 2013) which required a deep knowledge of the whole matrices that characterised the investigated systems, namely soils, branches, grape juices, intermediate products and wines.

As far as wine chain is concerned, winemaking process could encompass several steps, which may introduce changes in the element pattern, due to both natural and anthropogenic/extraneous sources. Several studies have been carried out on the variation of some element concentrations during the cellar practices (Cheng & Liang, 2012). In particular, storage tanks, pipes and other wine cellar equipment (brass and stainless steel based tools) increase the content of Cd, Cr and Pb (Kristl, Veber, & Slekovec, 2002) or Fe, Al and Cr (Kment et al., 2005). Variations in the element content occur also as a consequence of the fermentation process (Castiñeira Gómez, Brandt, Jakubowski, & Andersson, 2004). As a consequence, an accurate study to verify whether the strontium and the light isotope ratio are also influenced is necessary in order to finally achieve trustworthy results on the basis of this indicator.

As regard $^{87}Sr/^{86}Sr$, if no sources of "external" strontium are added (i.e. the use of additives to stabilise the process and eventually to clarify the final product), an increase or decrease in the strontium concentration does not directly affect the isotope ratio. In any case, it is worth to investigate whether and which strontium contaminations occur during the vinification. Generally, an increase of strontium is found in the first steps of the winemaking process, probably released by seeds and skins, whilst a slight decrease during the ageing period is a consequence of strontium precipitation with colloidal particles (Almeida & Vasconcelos, 2004). Therefore, these two phenomena are natural, not being due to external sources, and should not affect the $^{87}Sr/^{86}Sr$ isotope ratio. On the contrary, the addition of bentonites, used for wine clarification, or calcium carbonate, to deacidify, could be the main extraneous sources of strontium (Horn, Schaaf, Holbach, Hiilzl, & Eschnauer, 1993).

In literature, it has been demonstrated that the winemaking process generally does not affect the strontium isotope ratio values. Nevertheless, the rationale of these results relies on the fact that neither additives were used nor relevant difference (around the fourth figure) were present in $^{87}Sr/^{86}Sr$ value. In fact, the few attempts to investigate the influence of the winemaking process on the strontium isotope ratio (Almeida & Vasconcelos, 2001; Marchionni et al., 2016 and references herein reported) reveal no differences in the values measured in the different phases. However, for the former investigation a quadrupole-based ICP-MS was used for the evaluation of the strontium isotope ratio, while in the latter, an internal correction method has been used (Durante et al., 2013 and references herein reported). Indeed, starting from the recent observation that mass discrimination may also occur for heavy elements isotopic systems (Johnson, Beard, & Albarède, 2004) and that these experimental evidences are attained thanking the high sensitivity of the MC-ICP-MS instruments, a detailed study on this topic should be performed.

With regard to isotope ratios of light elements, the variability factors including the oenological ones, of $\delta^{18}O$ of water and the D/H and $\delta^{13}C$ of ethanol have been studied since the 1990th in wine, being thenceforth the official analyses for identifying the authenticity of wine in terms of watering down, sugar addition and mislabelling (OIV methods MA-AS311-05, MA-AS312-07 and MA-AS2-12, EU reg 555/2000). Their source are the water, oxygen and the carbon dioxide adsorbed by the plant and their variability is mainly influenced by the geographic and climatic characteristics of the production area (Camin et al., 2015). $\delta^{13}C$ of ethanol originates from that of sugar, $(D/H)_I$ from D/H of the nonexchangeable sites of glucose, whereas $(D/H)_{II}$ from the water medium (Martin, Zhang, Naulet, & Martin, 1986). If the yield of fermentation is more than 70%, type of yeast, temperature of fermentation, concentration of sugar, variation in the reaction rates induced by the medium, stopping of fermentation and type of nutrients have not a significant effect on $\delta^{13}C$ and $(D/H)_I$ of ethanol, whereas $(D/H)_{II}$ may exhibit significant variations (Fauhl & Wittkowski, 2000; Perini et al., 2014). The must concentration through reverse osmosis was found not effecting the isotopic values of water and ethanol, whereas the concentration through high-vacuum evaporation modifies the $\delta^{18}O$ of water and the D/H of ethanol (Guyon, Douet, Colas, Salagoity, & Median, 2006). Very recently it has been studied the effect of addition of Arabic gum as wine additive on the H, O, C and N isotope ratios of colloids. They found that only the $\delta^{18}O$ of colloid is shifted, whereas the isotope ratios of ethanol and water are not affected (Sprenger, Meylahn, Zaar, Dietrich, & Will, 2015). Moreover it was shown that wine dealcoholisation has an impact on the $\delta^{18}O$ of water and on $\delta^{13}C$ of ethanol but not on D/H (Ferrarini, Ciman, Camin, Bandini, & Gostoli, 2016).

The $^{15}N/^{14}N$ ratio has been measured in several foods to trace geographical origin and agricultural systems (Kelly, Heaton, & Hoogewerff, 2005), but not in wine so far. Nitrogen of wine derives not only from soil through plants and grapes, but can derive also from exogenous sources. For example, many winemakers add nitrogen rich substances to the fermenting wine to ensure that the yeast has sufficient nourishment to carry out the conversion of the sugars.

In this study, the results related to the determinations of some of the investigated direct indicators, namely $\delta^{18}O$ in water, $(D/H)_I$, $(D/H)_{II}$, $\delta^{13}C$ in ethanol and $\delta^{13}C$ and $\delta^{15}N$ in lyophilised sample and $^{87}Sr/^{86}Sr$ in different oenological production chains have been reported. One of the main aims of this study has been to test the real performance of the studied tracer indicators in the development of geographical traceability model, starting from a deeper analysis of their trend within the production cycle of *Lambrusco* PDO and *Trentodoc*.

Different wine-making processes of several producers of *Lambrusco* PDO and *Trentodoc* wines were monitored. Samples for each step of the process (grape juice, intermediate products, wine) were tracked. Furthermore, for some of the investigated producers, it has been also possible to assess a correspondence with the isotopic values coming from the respective soil and vine-branch samples as well.

2. Materials and methods

2.1. Reagents and materials

All the sample preparation procedures were carried out under horizontal laminar flow hood, in order to prevent the occurrence of any ambient contamination. Solutions were prepared by using high-purity deionized water TYPE1 (physical and chemical parameters for TYPE1 water comply with ASTM TYPE I and ISO3696 GRADE I purity specifications) obtained from a Milli-Q system (Millipore, Bedford MD) with a resistivity better than 18 MΩ cm.

Ultrapure HNO_3 65% w/w was obtained from analytical grade nitric acid (Carlo Erba, Milan, Italy) after sub-boiling distillation performed with a sub-boiler SAVILLEX DST 1000 (Savillex Corp. USA) apparatus. All the solutions were gravimetrically prepared and all the samples were accurately weighted by using a Mettler AE200 analytical balance (Mettler Toledo AG, Greifensee, Switzerland) with ±0.0001 g sensitivity. All PFA devices (bottles, tubes, vessels, etc.) were cleaned with solutions of heated HNO_3 10 M, deionized water and finally rinsed with ultrapure Milli-Q water before use.

NIST SRM 987 $SrCO_3$, certified for its Sr isotope composition with a $^{87}Sr/^{86}Sr$ certified value of 0.71034 ± 0.00026 and a "generally accepted" one of 0.71026 ± 0.00002 (Stein et al., 1997 and references herein reported) (the uncertainty is expressed as twice the standard deviation, 2 s), has been used for bracketing procedure as well as for the evaluation of the accuracy and precision of the obtained values.

The standard solution of $SrCO_3$, for bracketing sequences, was gravimetrically prepared using the NIST SRM 987. All working solutions were stored in PFA vessels (Nalgene).

The Eichrom Sr resin SR-B100-S (50–100 μm) was used for Sr/Rb matrix separation according to previous studies (Durante et al., 2013 and references herein reported). In particular, 10 g of resin were conditioned in a 100 mL PFA bottle with approximately 50 mL of HNO_3 1% w/w. After overnight soaking, the supernatant was removed and the bottle was refilled with fresh HNO_3 1% w/w until the final content of the solution was at least 100 mL. The suspension ready for the use was stored at room temperature. Before each analysis, the bottle was shaken with an automatic end-over-end agitator (Rotator SB3, Stuart) for at least 30 min. Finally, 2 mL and 1 mL of the resin, for soils and vine branches/juices, respectively, were loaded in homemade SPE columns, namely polypropylene (Alltech, Milan, Italy) SPE extract clean TM reservoirs equipped with 20 μm polyethene frits (Alltech, Milan, Italy), by using a positive displacement micropipette (Gilson M1000) equipped with polyethylene piston and tips. The used resin was not recycled. Final Sr fraction solutions were collected in PFA tubes and analysed in few days.

NH_4NO_3 1 M, Suprapur® (Merck, Milan, Italy), was used for the Sr bio-available fraction extraction in soil.

IV-ICP-MS-71A standards solution (Inorganic Ventures, New Jersey, USA) was used for the determination of the Sr and Rb concentrations in the analysed samples.

Reagent and materials for light isotopes are indicated in the relevant OIV methods (MA-AS311-05, MA-AS312-07 and MA-AS2-12).

2.2. Sampling and samples pre-treatment

Lambrusco wine is one of the main typical products of the Modena district and it is classified as a red sparkling product obtained by the fermentation of grapes of *Lambrusco* varietals (*Lambrusco di Sorbara*, *Lambrusco Salamino*, *Lambrusco Grasparossa* and *Lambrusco di Modena*) coming from well-defined areas of the whole district of Modena. According to their rule of production, the cultivation of grapes is fixed in three distinct and well-defined areas of the district of Modena. During the winemaking process, different modifications can occur, namely the addition of organic additives to stabilise the winemaking procedure as well as of selected yeast to support fermentation or bentonite (clay mineral) to eventually clarify the final product.

Trentodoc is one of the main typical products of Trento district, which obtained the designation of controlled origin in 1993. In particular, the designation *Trento* is reserved to the white and rosé sparkling wines obtained only by grape coming from Trento district by using the method of second fermentation in the bottle according to the conditions and requirements reported in the respective rule of production. The following different grape varietals can be used: *Chardonnay* and/or *Pinot Bianco* and/or *Pinot Noir* and/or *Meunier*. Finally, the product able to the commercialization has to be aged from a minimum of 15 months up to 10 years for obtaining the Reserve denomination.

2.2.1. Lambrusco winemaking sampling

In order to obtain a representative sample set, collaboration with two local *Lambrusco* wine producers (PB and MO) and with two wineries (Winery 1 and Winery 2) has been established. Four *Lambrusco* winemaking processes coming from wineries/producers were monitored in the period September 2011–April 2012. For each of the investigated process, a representative sampling of (i) grape juices, obtained from two different *Lambrusco* grape varieties, namely *Sorbara* and *Grasparossa* grapes, (ii) intermediate products of each winemaking production step and (iii) final commercial *Lambrusco* wines were obtained. As regards the winemaking processes, an aliquot of sample was systematically collected after each variation in the product chain, i.e. filtration, use of additives, mixing of the content of different tanks, etc. The additives, used in the Lambrusco productions to make the wine sparkling, namely rectified concentrated must (MCR), red concentrate, etc., were also sampled, when available. All the collected samples were stored at 0 °C until analysis. Given the peculiarities of each wine production, detailed information about the investigated winemaking processes as well as the collected samples have been reported in Table 1.

Briefly, in the case of PB producer, the starting must is obtained from *Lambrusco Sorbara* grapes and is initially stored in two silos of 300 hL capacity. After the mechanical harvesting, the grape crushing is directly performed on field. Grapes are put into a destemming machine, conveying must and grape skins in the collecting tank. A small amount of SO_2 is added to delay fermentation processes. In the winery, the must is stored into two silos, added with enzymes and tannins, for oenological use, to facilitate skins separation and clarification, and finally roughly filtered (samples PB1a and PB1b). The content of the two silos is then mixed in a fermentation tank, 500 hL capacity, and added with yeasts. After one week, the fermentation process stops owing to the complete transformation of the sugar substrate (sample PB2ab). The fermented sample is added with SO_2 and transferred in an autoclave (sample PB3ab). Different products, i.e. MCR or red concentrate, may be added to produce the sparkling wine. Finally the wine is bottled (sample PB4ab).

The wine production of MO producer starts from *Lambrusco Grasparossa* grapes. The collected grapes are brought to the winery and threated by a mechanical crusher/destemmer. The grape must is stored with its skins (sample MO1). Later, skins are removed and yeasts are added to start the fermentation. After about one month, the fermentation process ends (sample MO2) and clarifying substances are added to the wine, i.e. bentonites, polyvinylpolypyrrolidone (pvpp) and gelatin (sample MO3). The wine ages for a period and then is bottled (sample MO4).

As regard Winery 1, the wine is made from Lambrusco Grasparossa grapes. After the harvest, the grapes are crushed and the must is transferred in a 1500 hL wine tank (sample CR1). The must is filtered with the aid of perlites (sample CR2), and then the fermentation starts by using selected yeasts (sample CR3). At the end of the fermentation process, must is centrifuged and added with rectified concentrated must. The product is stabilized at low temperature (sample CR4), subjected to tangential filtration (sample CR5) and finally bottled (sample CR6).

Finally, in Winery 2, the raw materials come from two starting silos filled with *Lambrusco Sorbara* grape must (samples CM1a and CM1b), mixed together at different steps of the production procedure. The grapes are crushed in the winery and stored in silos, with

Table 1
Collected samples for the *Lambrusco* and TrentoDOC winemaking chains.

Lambrusco						TrentoDOC					
PB and MO producers		Winery 1 (CR) and 2 (CM)				A (AN), B (FP and FC), C (MS), D (MR), E (SC/SG/SP), F (PI) producers					
PB1a	Grape juice coming from the 1st tank after filtration	CR1	Grape juice coming from the 1st tank after filtration	CM1a	1st tank of Lambrusco Sorbara grape juice, added with SO_2	AN1	Grape juice (Chardonnay)	PI1	Grape juice (Chardonnay)	SC1	Drained must (Chardonnay)
PB1b	Grape juice coming from the 2nd tank after filtration	CR2	Grape juice after perlite filtration	CM1b	2nd tank of Lambrusco Sorbara grape juice, added with SO_2	AN2	Must with addition of SO_2	PI2	Must with addition of SO_2	SC2	Clarified must
PB2ab	Mixing of products PB1a and PB1b at the end of the fermentation process	CR3	Grape juice waiting for the fermentation process to start	CM2ab	Mixing of CM1a and CM1b samples, after flotation process and starting fermentation	AN3	Filtered must	PI3	Filtered must	SC3	Wine before ageing
PB3ab	Fermented sample after SO_2 addition	CR4	Fermented juice after low temperature stabilization	CM3ab	Fermented juice	AN4	Wine before ageing	PI4	Wine before ageing		
PB4ab	*Lambrusco Sorbara* wine	CR5	Product after tangential filtration	CM4ab	Fermented juice after centrifugation						
		CR6	*Lambrusco Grasparossa* wine	CM5ab	Sample after low temperature stabilization, with addition of PAG_CM1*	FP1	Drained must (Pinot Nero)	MS1	Grape juice (Chardonnay)	SG1	Drained and pressed must (Chardonnay)
MO1	Grape juice			CM6ab	*Lambrusco Sorbara* wine	FP2	Pressed must	MS2	Clarified must	SG2	Clarified must
MO2	Fermented grape juice					FP3	Wine before ageing	MS3	Wine before ageing	SG3	Wine before ageing
MO3	Fermented juice added with bentonites, PVPP, gelatin										
MO4	*Lambrusco Grasparossa* wine					FC1	Grape juice (Chardonnay)	MR1	Must after settling (Pinot Nero)	SP1	Drained and pressed must (Pinot Nero)
						FC2	Pressed must	MR2	Must with yeasts	SP2	Clarified must
						FC3	Wine before ageing	MR3	Wine before ageing	SP3	Wine before ageing

* PAG_CM1: concentrated must obtained from Sorbara grapes.

addition of SO_2, pectolytic enzymes, gelatins and N_2 to facilitate the separation of skins and other waste material from the must. Then, the must is transferred in a fermentation tank (sample CM2ab) for about a week till the end of the alcoholic fermentation process (sample CM3ab). The fermented liquid is centrifuged to remove yeasts and particulates (sample CM4ab). The product is stabilised at low temperature till the next stage of the wine production (sample CM5ab). The final product is obtained by a passage in autoclave and the addition of concentrated must (sample CM6ab).

2.2.2. Trentodoc winemaking sampling

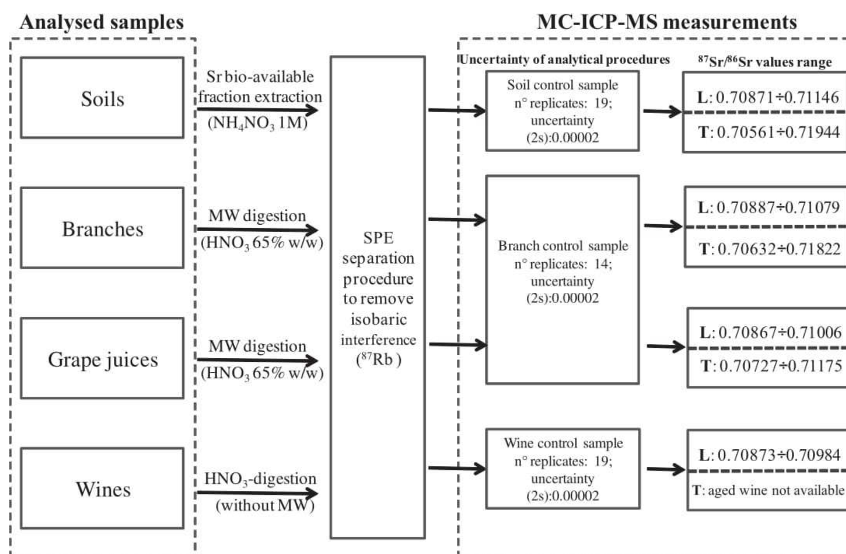
As regards the *Trentodoc* wine, thanks to the collaboration with the Istituto Trentodoc, it was possible to monitor nine winemaking chains (AN, FC, FP, MS, MR, SC, SG, SP,PI) of six producers (A, B, C, D, E and F) characterised by a long chain production (soil-vineyard-juice-intermediate product). For each one of the investigated processes, a representative sampling of grape juices, obtained from two different grape varieties, namely Chardonnay and Pinot grapes and intermediate products of each winemaking production step was sampled. No final commercial wine was available for these productions since the bottled wine have to be aged for at least fifteen months before being sold. For these winemaking chains, detailed information on each sample and the used grape varieties are reported in Table 1. Also for the *Trentodoc*, the winemaking processes were monitored in the period from September 2011 to April 2012 and an aliquot of sample was systematically collected after every variation occurred during the process. All the collected samples were stored at 0 °C until analysis.

2.2.3. Soils and branches sampling

Since two of the *Lambrusco* farms, PB and MO, and all the *Trentodoc* ones are also wine producers, it has been possible to investigate the relationships between the winemaking production and the soil of the vineyards. Soils and branches were collected as well. The sampling procedures for Lambrusco have been carefully detailed in a previous work (Durante et al., 2013). Moreover, to better elucidate the strategy adopted for $^{87}Sr/^{86}Sr$ determination, a flow diagram of the analytical protocol has been reported in Scheme 1. The number of the investigated vineyard for each producer and of soil holes has been reported in Table S1 as Supplementary Material.

As regards *Lambrusco* wines, strontium isotopic ratios values determined in samples coming from winemaking procedure were compared with the ones of the respective soils and vine-branches obtained in previous studies (Durante et al., 2013). For *Trentodoc*, all the measurements have been performed in this study.

From each farm, vine branches were collected from the grapevines roughly grown in proximity of the sampled holes in soil. Furthermore, in the case of the *Lambrusco* area, vine branches were picked up during two different periods of the years (spring and summer) in order to investigate the influence of the weather conditions and/or of the vineyard treatments on the monitored indicators.



Scheme 1. Overall sampling and analytical protocol strategy adopted for $^{87}Sr/^{86}Sr$ determination in *TRENTODOC* (T) and *Lambrusco* (L) winemaking chain.

2.2.4. Samples pre-treatments

Soils samples were pre-processed as detailed in a previous study (Durante et al., 2013).

The digestion of vine branches, grape juices and concentrated musts was performed by means of an Ultrawave microwave autoclave instrument supplied by Milestone. The autoclave apparatus consists of a single reaction chamber where samples are placed simultaneously for microwave digestion under high temperature and pressure conditions. Sample mineralization was carried out on batch consisting of five samples and on a total sample aliquot ranging from 10 to 12 g of organic matter. Samples were accurately weighted into quartz vessels (20 mL) and then added with 4 mL concentrated HNO_3 (65% w/w). This mixture was left to react for at least 30 min before the microwave digestion starts. Finally, an appropriate control sample (as next explained) was repeated several times, to monitor the procedure performances. At the end of the digestion process, all samples were transferred in PFA bottles and diluted with 65% HNO_3 to obtain a solution with a final HNO_3 concentration close to 8 mol L^{-1}.

The microwave instrumental parameters are reported in Tables S2a and S2b as Supplementary Material.

Wine samples were pre-treated according to a suitable procedure optimized in a previous study (Durante et al., 2014). The obtained solution, after correcting the HNO_3 concentration to 8 mol L^{-1}, was ready for the Sr/Rb separation procedure.

For $^{15}N/^{14}N$ analysis, vine branches were dried at 100 ± 2 °C in an oven for 24 h and crushed using a electric grinder, whereas from grape juice and wines the samples were dealcoholized (for wine) and dehydrated using freeze-drying devices in order to remove as much water and/or ethanol as possible.

For the D/H and $^{13}C/^{12}C$ analysis of ethanol, the alcohol was obtained by distillation using Cadiot columns avoiding isotope fractionation (OIV MA-aS-311-05, MA-aS-312-06).

2.3. $^{87}Sr/^{86}Sr$ analytical determination

For each sample, the strontium concentration was determined as well, in order to dilute the solution to an optimal Sr concentration to gain the best instrumental conditions in terms of accuracy and precision of the measurements. In particular, an atomic absorption spectrometer, AAS, (Varian, Spectra AA 220FS) was used equipped with an introduction/dilution system, SIPS 10, provided by the same company. All the instrumental parameters and experimental conditions are reported in a previous manuscript (Durante et al., 2013).

After that, an *a priori* Sr/Rb SPE separation procedure is performed to remove the isobaric interference given by the presence of ^{87}Rb on the ^{87}Sr determination. This interference is usually minimized through a matrix simplification procedure, which generally involves a SPE (Solid Phase Extraction) separation whose specificity and selectivity for strontium are nitric acid concentration dependent.

The setting parameters of the separation procedures were optimized by means of Design of Experiment (DoE) approach (Durante et al., 2013).

All the procedure were carried out in order to obtain a final Sr concentrations close to 200 μg kg^{-1}; furthermore, at the end of the separation procedure, a proper aliquot of 65% HNO_3 was added to all the water eluted fractions to obtain 4% HNO_3 final solutions.

Strontium isotope ratio measurements were accomplished with an MC-ICP-MS spectrometer (Neptune, ThermoFinnigan, Bremen, Germany). The instrument consists of a double focusing, multicollector mass spectrometer with a forward Nier-Johnson geometry. The spectrometer is equipped with nine Faraday collectors, eight movable and a central fixed one. Data acquisition, performed in low resolution mode, was simultaneous for all the measured ion masses, m/z: 82 (L4), 83 (L3), 84 (L2), 85 (L1) 86 (C), 87 (H1), 88 (H2). The instrumental parameters are reported in previous study (Durante et al., 2013). For each session of measurements, a gain calibration was performed for the multicollector system. Ion-lens setting was daily tuned to obtain the better compromise among the maximum sensitivity, instrumental stability and optimal flat-top peak shape. Owing to the low Sr concentration in some of the *Trentodoc* winemaking samples (<100 μg kg^{-1}, after the Rb/Sr separation procedure) the measurements of $^{87}Sr/^{86}Sr$ have been performed with the aid of a desolvating

apparatus (APEX-IR, manufactured by Elemental Scientific Inc.) in order to enhance the instrumental sensitivity.

The method for the analytical determination of $^{87}Sr/^{87}Sr$, such as sequence characteristics, calculation procedure and mathematical corrections, is described in previous works (Durante et al., 2013, 2014).

2.4. Light isotope determination

The $^{15}N/^{14}N$ of soil, vine-branches and grape juices samples were measured using an elemental analyser (Flash EA 1112, Thermo Scientific, Bremen, Germany), equipped with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced through a ConFlo IV dilutor device (Thermo Finnigan, Bremen, Germany) with a DELTA V isotope ratio mass spectrometer Thermo Scientific. The isotopic values were calculated against potassium nitrate IAEA-NO3 (IAEA-International Atomic Energy Agency, Vienna, Austria) and L-glutamic acid USGS 40.

The D/H and $^{13}C/^{12}C$ of the alcohol were determined following the official methods established by the International Organization of Vine (OIV MA-aS-311-05, MA-aS-312-06) and using SNIF-NMR (Site-specific Natural Isotope Fractionation-Nuclear Magnetic Resonance) (FT-NMR AVANCE III 400, Bruker BioSpin GmbH, Karlsruhe, Germany) and IRMS (Isotope Ratio Mass Spectrometry) (SIRA II-VG ISOGAS, FISIONS, Rodano, Milano, Italy) interfaced with an Elemental Analyser (Flash 1112, Carlo Erba, Milano, Italy). The D/H values were measured site-specifically in the methyl and methylene positions of ethanol [$(D/H)_I$ and $(D/H)_{II}$] and are expressed in ppm.

$^{18}O/^{16}O$ ratio analysis of wine and must water was performed using a SIRA II, (VG Fisons, Middlewich, United Kingdom) connected to a water/CO_2 equilibration system Isoprep 1, (VG Fisons, Middlewich, United Kingdom). The procedure is described in the OIV-MA-AS2-12 method (2009).

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ values are denoted in delta in relation to the international V-PDB (Vienna-Pee Dee Belemnite), AIR and V-SMOW (Vienna-Standard Mean Ocean Water) standards according to the following general equation (Brand, Coplen, Vogl, & Prohaska, 2014):

$$\delta^{i}E = \frac{(^{i}RSA - {}^{i}RREF)}{^{i}RREF}$$

where *i* is the mass number of the heavier isotope of element E (for example, ^{13}C).

RSA is the respective isotope number ratio of a sample (such as for C: number of ^{13}C atoms/number of ^{12}C atoms or as $^{13}C/^{12}C$ approximation).

RREF is that of internationally recognised reference materials (see above).

The delta values are multiplied by 1000 and are expressed in units "per mil" (‰).

The analytical uncertainty (estimated by combining within-laboratory reproducibility standard deviation with estimates of method and laboratory bias using proficiency test data, Magnusson, Näykk, Hovind, & Krysell, 2012) of the measurements was 0.3‰, 0.6‰, 0.8 ppm and 1.2 ppm for $\delta^{13}C$, $\delta^{18}O$, $(D/H)_I$ and $(D/H)_{II}$.

3. Result and discussion

3.1. Uncertainty of the analytical methodologies

In this study a total of 206 samples were processed and measured: 68 and 34 soil and branch samples coming from Trento district; 29 and 23 oenological samples coming from *Trentodoc* and *Lambrusco* winemaking processes in addition to 52 control samples for the evaluation of the $^{87}Sr/^{86}Sr$ precision of the analytical procedures.

For $^{87}Sr/^{87}Sr$ analysis the reproducibility of the whole analytical procedure has been already shown in previous works (Durante et al., 2013, 2014). Accuracy and reproducibility of the instrumental measurements were evaluated by using the data obtained by NIST SRM 987 coming from bracketing measurements (n: 300) obtaining a $^{87}Sr/^{86}Sr$ value of 0.71027 ± 0.00002 and the uncertainty (u) is expressed as twice the standard deviations (2 s).

Furthermore, three different control samples for soils, branches and wines were analysed several times (n: 19, 14 and 19, respectively), according to the entire analytical procedure, namely, pre-treatment of samples, Rb/Sr separation procedure and experimental measurements. An uncertainty (u) of 0.00002 was obtained for all the cases and applied to the several obtained values, since all samples were generally once prepared and measured. Finally, the absence of influence given to the Rb/Sr separation procedure and to the process blanks was shown in a previous work (Durante et al., 2013, 2014).

Repeatability and reproducibility are reported in the relevant OIV methods for $\delta^{18}O$ of water, $(D/H)_I$, $(D/H)_{II}$ and $\delta^{13}C$ of ethanol. For the innovative parameter $\delta^{15}N$, we developed a method for the analysis of wine and checked accuracy and uncertainty. A pre-treatment of wine to remove as much as possible water and ethanol from samples must be performed. The dehydration and dealcoholization was obtained using two different methods (freeze-drying and evaporation under vacuum) to test the impact of the process on $\delta^{15}N$ of wine. We considered 10 samples and each of them was analysed 10 times for method in order to determine the repeatability. The results of $\delta^{15}N$ are reported in Table S3 as Supplementary Material.

Both methods show a good repeatability and the comparison of the mean $\delta^{15}N$ values reveals a good agreement between the two techniques and confirms their reliability.

The analytical uncertainty of $\delta^{15}N$ for all the considered matrices, estimated by combining within-laboratory reproducibility standard deviation with estimates of method and laboratory bias using proficiency test data, (Magnusson et al., 2012) is 0.3‰.

3.2. $^{87}Sr/^{86}Sr$ in Lambrusco winemaking and its temporal variability

$^{87}Sr/^{86}Sr$ isotope ratio was determined on grape juices, intermediate products and commercial wines for all the investigated winemaking processes. A measurement uncertainty equal to ±0.00002, expressed as twice the standard deviation of the respective control sample, was associated to each value. The data, obtained for the four producers, are reported in graphical form in Fig. 1.

From this graph, some general consideration can be pointed out. Firstly, the $^{87}Sr/^{86}Sr$ values range between 0.70867 ÷ 0.71006 and the values *related* to *Lambrusco Grasparossa* variety (MO, and Winery1 producers) are, on average, higher than those of the *Lambrusco Sorbara* (PB and Winery 2 producers). Indeed, the cultivation area of *Lambrusco Grasparossa* grapes is located in the southern part of the Modena district, which comprises hill and in-plain territories and presents an higher strontium isotope ratio owing its geological features (Durante et al., 2013); whilst *Sorbara* variety is mainly cultivated in in-plain regions with more homogeneity characteristics owing the influence of the alluvial sediments of the Po river basin. Generally, a rather constant $^{87}Sr/^{86}Sr$ value is found, but some exceptions and peculiarities in the winemaking chains may require a more detailed discussion.

Indeed, as regards the PB winemaking process, the data interpretation is quite simple; the production comprises two starting silos (each of 300 hL) of grape juices (PB1a and PB1b samples, respectively), which present different strontium isotope ratios

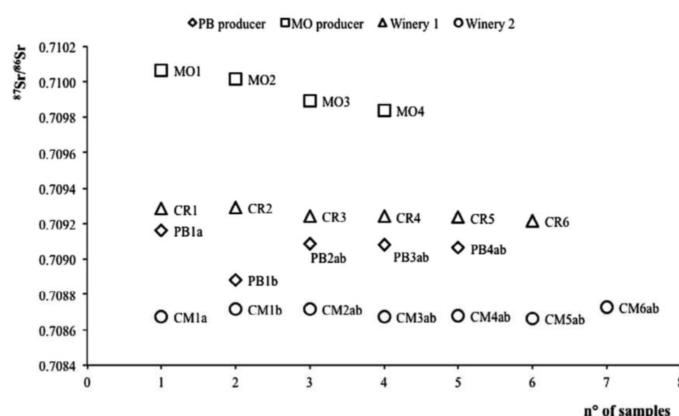


Fig. 1. Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices coming from the four investigated *Lambrusco* winemaking producers from Modena district.

values, since grapes come from various fields (vineyards). In the second step, these samples were mixed, obtaining a value, which is perfectly in the range of the two starting juices according to the mixing rule (Hosono et al., 2007). The following samples, from this intermediate to the final commercial wine, present similar (differences are below the measurement uncertainty) $^{87}Sr/^{86}Sr$ values.

Different consideration can be stressed out for the strontium isotope ratio values obtained for MO winemaking procedure. In this case, the standard deviation of the four collected samples is slightly higher with respect the data uncertainty. On one hand, the $^{87}Sr/^{86}Sr$ values measured for the samples coming from the first two steps, MO1 and MO2, present no significant differences. On the other side, a decrease in the $^{87}Sr/^{86}Sr$ ratio is found for the fermented must, MO3 sample, whilst, the value obtained for the commercial wine, MO4, is perfectly in agreement with the one obtained from the previous step.

Now, a meaningful variation of the isotope ratio value along the chain process, as the case of MO3 sample, is always difficult to justify since many factors could take place, such as fractionation phenomena and/or contamination process. However, the former are quite difficult to occur and many studies have already demonstrated that the isotopic signature is transferred along the process. On the other side, the latter, the contamination, must follow the mixing rule and, in this case, it is difficult to suppose a strontium release capable of modifying the isotopic ratio of a huge wine mass. Furthermore, taking as face value the measured data, these anomalies could probably due to a not perfect sampling procedure or to a mismatch in the producer declarations.

As far as the results obtained for Winery 1 are concerned, it is worth to note that it is characterised by a significant production of wine, around 1500 hL for batch, with respect the previous ones. Notwithstanding, the strontium isotope ratio value is perfectly transferred from grape juice to the intermediate products and then to the final product, demonstrating that the use of perlite (CR2) or the tangential filtration of the last step (CR5) do not affect the value observed in the commercial wine (CR6).

Finally, as regard the production of commercial wines of Winery 2 (around 1000 hL), the two starting grape juices (CM1a and CM1b) present quite similar isotopic ratio values. During the winemaking procedure, no significant variability in the strontium isotope ratio is found (s: 0.00003). The addition of filtered must of *Sorbara* grapes (PAG_CM1) does not introduce changes. As a matter of fact, the grapes used to obtain PAG_CM1 probably come from approximately the same area of those used for the produced wine.

Now, considering geographical traceability issue, in a previous work, the results relating the $^{87}Sr/^{86}Sr$ values obtained for soils and branches coming from PB and MO vineyards have been deeply discussed and it emerged that when the geological composition of soil is complex (as in MO cases), the bio-available fraction extracted with NH_4NO_3 could be not capable to perfectly reproduce the plant uptake and therefore the isotopic signature of the food.

Hence, the experimental evidences confirmed that plants are optimal tools to trace the territory of provenance. Therefore, in this study, the branch values have been compared with the data obtained for the matrices coming from the winemaking procedure for PB and MO producers. The trend of the measured data is depicted in Figs. S1a and S1b, (both the figures are reported as Supplementary Material).

From Fig. S1a, it emerges that for the wine production, PB producer has used juices coming from both its fields; in particular, there is a good agreement between the used raw materials, PB1a and PB1b, and the strontium isotopic range characteristic of the V1 and V2 vineyards, respectively.

Considering the strontium isotope range of variability of MO vineyard, Fig. S1b, data obtained for the grape juice used in the winemaking chain of *Lambrusco Grasparossa* are in perfect agreement with the values of the strontium isotope ratio of the vineyard of origin, i.e. falls within the respective range of variability.

Finally, Fig. S2 (reported as Supplementary Material), shows the $^{87}Sr/^{86}Sr$ values obtained for branches coming from two sampling periods (spring and summer 2012) have been shown. As it can be pointed out, there is not any temporary dependence of the monitored indicator, since the same values (differences are generally not statistically significant) are obtained for most of all the investigated samples. However, peculiar situation occurs for the branches of point 2 and 5 of the producer MO, which present differences among the values obtained for the two sampling period. Probably, these discrepancies are caused by small differences in the repositioning procedure introduced by the GPS system used for the geo-location of the samples.

3.3. $^{87}Sr/^{86}Sr$ in Trentodoc winemaking

$^{87}Sr/^{86}Sr$ ratio was determined on the grape juices and intermediate products of the investigated *Trentodoc* winemaking chains as well. As for *Lambrusco* case, a measurement uncertainty of ±0.00002 has been associated to each value. The obtained data

for the nine investigated batches were graphically reported in Fig. 2.

From these results, it is evident a great variability of the values obtained for the different winemaking chains, which range from 0.70727 to 0.71175. In particular, the investigated indicator differentiates the winemaking in three different group namely, MR winemaking with the lowest values, PI, SG, SC, MS, FP and AN with medium ones and FC and SP with high values.

Looking at the $^{87}Sr/^{86}Sr$ trend within each winemaking chain, it is possible to notice two opposite situations. In the former, the production process does not seem to influence the $^{87}Sr/^{86}Sr$ value, i.e. in the case of the three batches (PI, SG and MR), which present standard deviations below the measurement uncertainty, while for the latter, the internal variation of the isotope ratio, on the fourth decimal figure, is grater than the measurement uncertainty. Usually, it can be observed an isotopic decreasing trend from the grape-juices to the final products (the situation is the opposite for MS producer, where the grape juice presents an isotopic value lower with respect to its grape juice). This trend could be due to different modifications that can occur during the winemaking process, namely the addition of external additives, as bentonite (clay mineral), to eventually clarify the final product and capable to contaminate the original value of ^{87}Sr of grapes. Unfortunately, information about the winemaking procedure is not available.

Furthermore, as in the case of Modena district, the $^{87}Sr/^{86}Sr$ isotope ratio has been determined on other matrices coming from the investigated area namely soils and branches. Results have been reported in Fig. 3.

Generally speaking, there is a presence of a reasonable heterogeneity within the soil samples of the same farm (intra-site variability), and along the vertical profile of each sampled hole. In particular, it is possible to note an increase in the isotope ratio from depth up (10–30 cm) to down (30–60 cm). This trend is quite peculiar and probably due to a stratification of soils that result to be constituted by fragments of different origins. The same trend, at a firstly glance, can be also highlighted considering the $^{87}Sr/^{86}Sr$ obtained for the branches sampled in the proximity of the different soil holes. In particular, the trend of the data point out a good match between the matrices belonging to the same chain/sampling site for most of all the producers. In fact, in several cases, the $^{87}Sr/^{86}Sr$ values of branches perfectly overlap with the ones of the respective soils. However, it is worth to note that there are some minor correspondences between the obtained values for the sites coming from producers PI and MR. In particular, these differences could be due to several factors, such as (i) the performed sampling (branches were sampled from different plants grown as close as possible to the site of respective soils sample), (ii) the extracted element composition, which does not perfectly mimic the bio-available fraction (plant uptake) and (iii) the practices of fertilisation/irrigation used by the producers, which, as it has been shown (Hosono et al., 2007), could affect at least 25% of the final $^{86}Sr/^{87}Sr$ value. However, in all the cases (with the only exception of AN producer), the strontium isotope values obtained for all the investigated grape juices (used in the several winemaking procedure) perfectly lie within the range of variability found for the respective soils and branches.

3.4. Light elements isotope ratios in Lambrusco and Trentodoc winemaking: from soil to wine

Among stable isotope ratios of light elements, the only one in grapes and wine deriving from soil through vines is that of nitrogen. The other elements derive from soil water (H and O), from air molecular oxygen (O) or from atmospheric CO_2 (C and O).

$^{15}N/^{14}N$ isotope ratio was determined in soil (different depths and seasons), vine-branches and wine-making intermediates for all the investigated oenological chains.

In soil $\delta^{15}N$ didn't statistically change according to the depth ($p < 0.01$, paired t test) however in few cases $\delta^{15}N$ presented lower values in the higher soil layer than in the corresponding lower layer. In the literature it was found that $\delta^{15}N$ values increase according to soil depth (Billy, Billen, Sebilo, Birgand, & Tournebize, 2010; Yang, Siegwolf, & Körner, 2015). The less variation detected in this study could be related to the fact that was performed in an agricultural environment with fertilization and ploughing practices that could interfere with the settle of 'real' layers as occurs e.g. in forests, as well as to the depth size considered for the layers. The increasing $\delta^{15}N$ values according to the soil depth have been previously explained as mainly due to the increasing action of denitrification that cause a ^{15}N enrichment of the residual nitrate (Billy et al., 2010). A robust correlation was found between $\delta^{15}N$ determined in the upper layer of soil and in the lower layer ($p < 0.001$, $R^2 = 0.54$) (Fig. S3 reported as Supplementary Material). A good correlation between the $^{15}N/^{14}N$ ratios measured in spring and summer was also found in soils (Fig. S4a) as well as in branches (Fig. S4b) (both figures are reported as Supplementary Material). A mean difference of 0.2‰ between the soils collected in the two seasons and of 0.4‰

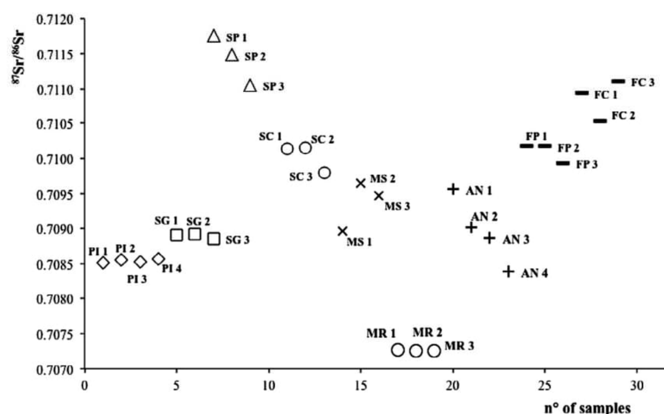


Fig. 2. Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices coming from the nine investigated *Trentodoc* winemaking producers form Trento district.

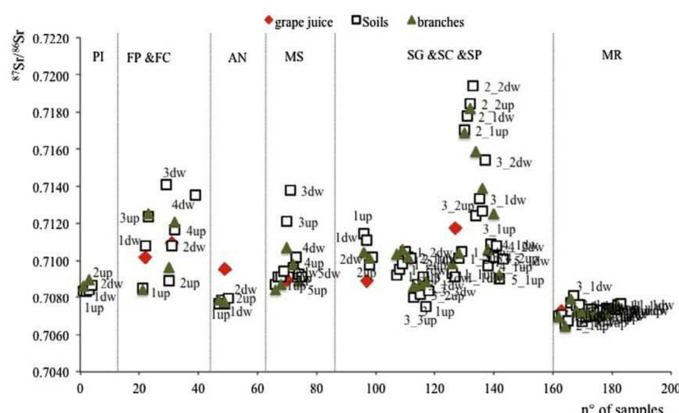


Fig. 3. Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices, namely soils, vine branches and grape juices, belonging to vineyards of the investigated *Trentodoc* producers.

between branches was detected and both soils and branches resulted not statistically different between the two seasons according to a paired t test ($p < 0.01$). It could be concluded that the different weather conditions occurring within spring and summer don't affect the nitrogen signature of vineyard soils and, as a consequence, also of vine branches. In Fig. 4, $\delta^{15}N$ values of soil (merging different depths and seasons), vine-branches and grape juices for all the chains are shown for the samples coming from the Modena and Trento districts, respectively (the intermediates are discussed afterwards). As previously reported for other plant species grew in different environments (Yang et al., 2015), $\delta^{15}N$ values of branches resulted more negative than that of the corresponding soil. In general plant material is depleted in ^{15}N compared to bulk soils, as the bulk $\delta^{15}N$ of plant depends not only on that of the inorganic primary nitrogen sources, but also on isotope fractionation during their uptake or assimilation (Werner & Schmidt, 2002). In the specific, plants can discriminate against ^{15}N during nitrogen acquisition, either directly or indirectly via associations with mycorrhizal fungi (Evans, Bloom, Sukrapanna, & Ehleringer 1996; Hobbie, Jumpponen, & Trappe, 2005; Michelsen, Schmidt, Jonasson, Quarmby, & Sleep 1996).

The results obtained by analysing grape juices demonstrate that the $\delta^{15}N$ values of must were always depleted compared to the growth soil and slightly enriched compared to the branches. These results were recently observed also in another study (Verdenal et al., 2015) that reported higher $\delta^{15}N$ values in musts respect to other parts of Vitis Vinifera plant (roots, trunk and cane, leaves and shoots), even if not statistically significant. This behaviour could be maybe related to the role of predominant sink of the bunches (Verdenal et al., 2015). Up to now and to our knowledge, no studies investigated how $\delta^{15}N$ varies along the entire 'chain' soil – plant – grape juice. Data showed in both the districts that the highest $\delta^{15}N$ values of soil are memorised by the corresponding grape juice that showed in turn the highest values. This is the confirmation that the majority of the total organic nitrogen in vine material is 'recovered' by the soil nitrogen from root absorption or plant reserves as previously hypothesized by Verdenal et al. (2015) for grapes and that the nitrogen isotopic signature is inherited from the soil by the plant material. Lower values were found in MO soil and particularly in the corresponding branches and were memorised in grape juice. This behaviour can be justified by the fact that MO comprises also hill territory. Indeed, starting from 1980, some studies, (e.g. Amundson et al., 2003; Liu & Wang, 2010; Mariotti, Pierre, Vedy, Bruckert, & Guillemot, 1980) examined the variation of $\delta^{15}N$ along an elevation gradient in different plants and soils and found that both the values declined with increasing elevation. Hilton, Galy, West, Hovius, and Roberts (2013), hypothesized that these negative correlations can be explained by an increase in nitrogen loss on steeper slopes, with particulate nitrogen removal. The found results mean that $\delta^{15}N$ in grape memorise the variability of soil and can be used as further marker for geographical traceability of wine.

The variation of stable isotope ratio of O in water, of H and C in ethanol and of N in the freeze dried sample from must to wine is showed in Table S4, Supplementary Material.

As observed for $^{87}Sr/^{86}Sr$ for *Lambrusco* chains, PB2ab has isotopic values which correspond to a mixture of PB1a and PB1b. MO chain is the less homogeneous, whereas CR and CM the most homogeneous. Of the *Trentodoc* chains, the less homogeneous are MS, AN and SP, as observed also with $^{87}Sr/^{86}Sr$, and this is probably due to mixture of grape juices of the chain with other batches for the following intermediates. Considering the most conservative chains, we do not observe in general significant variations in the isotopic values, with mean difference between the final and the starting values of −0.5‰, 0.0 ppm, −0.2‰, 0.1‰, −0.4‰ for $\delta^{18}O$ of water, $(D/H)_I$ and $\delta^{13}C$ of ethanol, $\delta^{15}N$ and $\delta^{13}C$ of bulk sample. This means that wine making processes such as filtration, addition of clarifying agents or of must of the same origin do not change the isotopic ratios of must. Indeed $\delta^{18}O$ of wine water memorizes that of must, if dilution or concentration does not take place, whereas $\delta^{13}C$ and $(D/H)_I$ do not change during regular fermentation from sugar to ethanol (Fauhl & Wittkowski, 2000).

On the other hand, $(D/H)_{II}$ which is linked to the fermentation conditions (Perini et al., 2014), has the highest variation (mean difference −2.4 ppm) and considering the values of the different winemaking intermediates, also the innovative parameter $\delta^{15}N$. This is due to the fact that $\delta^{15}N$ of wine is influenced also by the addition of N-based additives, such as the fermentation activators used to ensure that the yeast has sufficient nourishment to carry out the conversion of the sugars in ethanol. In depth studies on the influence of different N-based additives on the $\delta^{15}N$ are needed to confirm these assumption.

4. Conclusion

The use of $^{87}Sr/^{86}Sr$ isotope ratio as geographical tracer of food origin is related to the constancy of its value in the transfer from

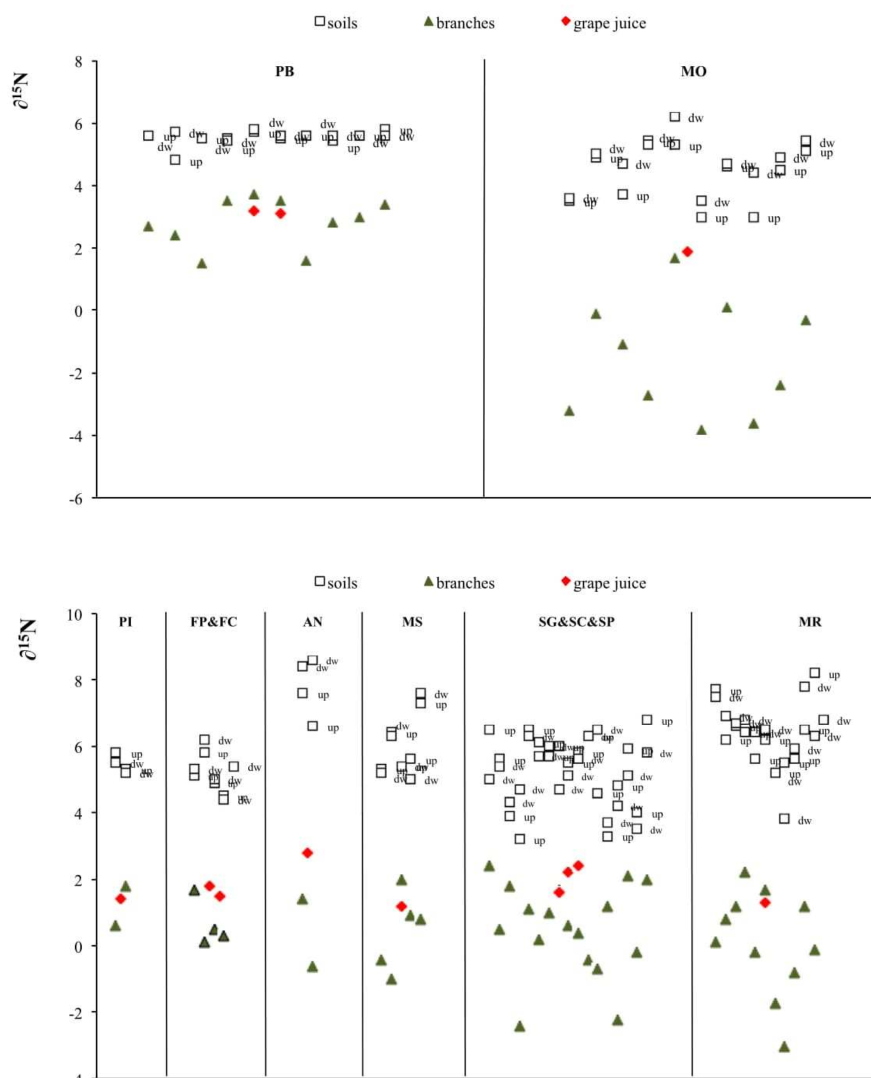


Fig. 4. $\delta^{15}N$ in soil, branches and grape juices for Modena (up) and Trento (down) districts.

the soil to the plant and then into the final product. The absence of fractionation phenomena implies that all the transformations and biological processes, which occur along the winemaking chain should have not effects on the $^{87}Sr/^{86}Sr$ values. However, the cellar practices in some cases comprise the use/addition of different additives, such as clarification or deacidification agents as well as concentrated musts, which could modify the strontium concentration. Thus, the $^{87}Sr/^{86}Sr$ ratio found in the final product may be influenced by these winemaking processes.

Several winemaking processes for the two case studies, namely *Lambrusco PDO* and *Trentodoc* have been deeply investigated.

For most of the investigated winemaking chains, the strontium isotope values obtained for all the samples are in excellent agreement, showing no changes during the production and thus the possibility to directly relate the final product to its raw materials and geographical area.

On the other hand, some productions could present variations in the value of the isotope ratio of the final product (i.e. for MO producer). These variabilities highlight the need to perform tailored investigation in order to identify the peculiarities of each process.

All things considered, the $^{87}Sr/^{86}Sr$ isotope ratio is certainly a powerful geographical traceability marker, owing to its peculiarities which allow discriminating samples with different origins and characteristics. However, the great variability of the indicator in soils characterised by a complex geology could be also considered as a drawback, since it contributes to data dispersion, leading to a more difficult distinction and interpretation of samples coming from different geographical areas. In this case, it again emerged

that the use of the plant as sampling device is it utmost of importance for several aspects as reported also in previous works. Furthermore, by considering the strontium isotope branches values that show also a time independence it will be possible to overcome the problems associated with soil sampling as well as those related to the evaluation of the element bio-available fraction.

As regards the ratios of the lighter elements, for the first time, the variation of $^{15}N/^{14}N$ isotope ratio was checked along the entire production chain from soil to vine-branches and wine-making intermediates. The results obtained demonstrated that $\delta^{15}N$ values of must were always depleted compared to the growth soil and slightly enriched compared to the branches. Although this isotopic fractionation occurring along the production chain $\delta^{15}N$ of must demonstrated to memorise the isotopic variability of the provenance soil, and can be therefore proposed as further isotopic marker for the geographical characterisation of grape products. In depth studies on the influence of different N-based additives on the $\delta^{15}N$ of wine are needed to understand if also the $\delta^{15}N$ of wine memorise the isotopic variability of the soil.

Finally, the already officially recognised isotopic ratios of H, C of ethanol and O of water do not vary significantly from grape to wine during wine making process, except for $(D/H)_{II}$ which is the parameter more linked to the fermentation conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.108.

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SECTION 4.4

δ^{15}N from soil to wine in bulk samples and proline

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Statement of the author: My personal involvement in this research started with the experimental design to measure the nitrogen isotopic value through the entire oenological chain (soil - leaves - grape - wine). Moreover, I developed the analytical method to determine the $\delta^{15}N$ value of proline in grape juice and wine: extraction, derivatisation and analysis by GC-C-IRMS. As regards method development, I personally performed all the experiments and data analysis presented in the manuscript. As first author I was responsible for writing the manuscript and managing the comments and improvements to the text by the other co-authors.

Research article

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δ^{15}N from soil to wine in bulk samples and proline

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The feasibility of using δ^{15}N as an additional isotopic marker able to link wine to its area of origin was investigated. The whole production chain (soil–leaves–grape–wine) was considered. Moreover, the research included evaluation of the effect of the fermentation process, the use of different types of yeast and white and red vinification, the addition of nitrogen adjuvants and ultrasound lysis simulating wine ageing. The δ^{15}N of grapes and wine was measured in bulk samples and compounds, specifically in proline, for the first time.

Despite isotopic fractionation from soil to wine, the δ^{15}N values of leaves, grapes, wine and particularly must and wine proline conserved the variability of δ^{15}N in the growing soil. Fermentation and ultrasound treatment did not affect the δ^{15}N values of grape must, which was therefore conserved in wine. The addition of inorganic or organic adjuvants was able to influence the δ^{15}N of bulk wine, depending on the amount and the difference between the δ^{15}N of must and that of the adjuvant. The δ^{15}N of wine proline was not influenced by adjuvant addition and is therefore the best marker for tracing the geographical origin of wine. Copyright © 2016 John Wiley & Sons, Ltd.

Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords: IRMS; GC-c-IRMS; soil-wine chain; proline; geographical marker

Introduction

Stable isotope ratio analysis of so-called bioelements (H, C, N, O and S) has been applied since the 1990s for food authenticity control and to assign geographical origin.[1] One of the most well-known fields of application is analysis of the ^{2}H/^{1}H, ^{13}C/^{12}C and ^{18}O/^{16}O ratios of wine ethanol and wine water to check for adulteration (e.g. addition of cane and beet sugar and watering down) and to verify the origin declared on the label,[2,3] whereas the ^{15}N/^{14}N stable isotope ratio (expressed as δ^{15}N) has not been extensively investigated in wine to date, to our knowledge.

Nitrogen stable isotope ratio analysis has been shown to be a useful tool for characterizing the geographical origin of food products in combination with other isotopic parameters[1] and is commonly used to verify the management system applied in agriculture.[4,5]

It could also be a useful marker for tracing the origin of wine, because in contrast with H, O and C, nitrogen arrives directly in the vine from the soil, and therefore the factors affecting its isotopic variability are different from those of other elements.[6]

Grapes contain various nitrogenous compounds such as ammonium, amino acids, peptides and proteins. The nitrogen content of the grape varies with variety, climate, soil, fertilization and other cultural practices and increases during the maturation period.[7] Nitrogen-containing compounds are used in biosynthesis, ammonium and amino acids being the preferred sources of nitrogen. Nitrogen influences biomass fermentation, the rate of fermentation and production of various by-products, which in turn affects the sensory attributes of wine.[8,9]

The isotopic ratio of nitrogen in grape juice is influenced by the nitrogen isotopic value of the grapevine, which is related to that of its N sources in soil, and is an average of the δ^{15}N values of all potential N sources, weighted according to their availability.[10]

During the fermentation of grape juice, the yeast metabolism could cause some isotopic effects, specifically the addition of nitrogen-rich substances, added to ensure that the yeast has sufficient nourishment to carry out conversion of fermentable sugars, could modify the δ^{15}N of wine. Another possible source of interference is the nitrogen compounds released by yeast autolysis during wine processing, for example, in the case of sparkling wines ageing, due to the presence of hydrolytic enzymes[11,12] or in the case of wines processed by using traditional practices 'sur lies' through contact between the wine and lees during ageing.[13,14] These compounds can be also released by yeast autolysis during wine storage.[13,15] Other studies[16] have shown that the application of ultrasound in winemaking entails a concrete enrichment of wine in different fractions (e.g. glycoproteins and polysaccharides) as a result of the cavitation effect on yeast cells in order to accelerate the process of ageing on the lees.

Traditionally, stable isotope analysis has relied on measurement of bulk products or specific components after proper extraction, but emerging methods aimed at direct analysis of individual chemical compounds provide a means of obtaining a more in-depth understanding.[17] Recently, gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS) has been used to

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examine the effects of fertilization practices on the δ^{15}N and δ^{13}C values of individual amino acids in wheat and durum wheat to discriminate between organically and conventionally grown plants.[5] Amino acids in grapes and wines have various origins, and their nature and concentration influence the growth of yeast during fermentation, the fermentation rate and the production of aromatic compounds.[18] Of the amino acid pool, proline (Pro) is among the most abundant in grapes and wine (30–85% of total amino acid content), and its utilization pathway requires the presence of oxygen. In anaerobic fermentation conditions, Pro is not used by yeast as a nitrogen source and is therefore maintained in wine. Pro is considered as a potentially interesting parameter for distinguishing wines according to grape variety and specific growth conditions (temperature, sun exposure time, amount of rainfall and grape maturity) and is thus related to identification of the origin, possible adulteration and quality of wines.[19]

In this study, we investigated the feasibility of using nitrogen isotopic ratio as an analytical indicator able to link wine to its area of origin. δ^{15}N was measured along the wine production chain (soil–leaves–grapes–wine) of different vineyards located in the province of Trento in north-eastern Italy, in order to examine its variability within the different matrices. We also evaluated the influence of the fermentation process by using different types of yeast and both white and red vinification (experiment 1).

Moreover, we investigated the effect of adding nitrogen-rich substances and of ultrasound lysis on yeast cell structures, with liberation of the nitrogen fraction (experiment 2).

The δ^{15}N of grapes and wine was measured not only in bulk samples but also in Pro for the first time.

Experimental

Reagents and reference materials

L-amino acid standard at ≥98% purity (Pro) and analytical grade cation-exchange resin (Amberlite® IR120 hydrogen form) were purchased from Sigma Aldrich (Milan, Italy). All other solvents (ethanol, dichloromethane, isopropanol, acetone and ethyl acetate) and reagents (triethylamine and acetic anhydride) used were of analytical grade and purchased from Sigma Aldrich (Milan, Italy) and VWR (Milan, Italy). Inorganic nitrogen adjuvant (IA) and organic nitrogen adjuvant (OA) were purchased from Collis (Veneto Wine Group, Italy).

Experiment 1: δ^{15}N from soil to wine

Samples

Soils, leaves and grape berries (*Vitis vinifera* L. cv. Chardonnay) were collected from seven different vineyards located in Trentino, in north-eastern Italy (labelled from A to G; Table S1).

In each vineyard, two soil samples were collected: top soil (TS, 5–30 cm) and sub-soil (SS, 30–60 cm). Grape and leaf samples were collected from grapevines grown in proximity to the soil sampling sites. Specifically, in each vineyard, 40 leaves were collected from 40 different vines, picking the fourth leaf after the second bunch, and 100 undamaged and sound berries were picked from 20 bunches on different vines at random, cutting the pedicel just above the base.

Microvinification

Grape samples were collected in sufficient amounts for microvinification. Each of the seven musts was separately inoculated with ten different selected dry yeasts (Table S2) according to the manufacturer's specifications and processed as for white wines. Moreover, each must was also inoculated with AW1503 dry yeast according to the manufacturer's specifications, applying the protocol for red wine.[20] Overall, 77 wines were produced.

Experiment 2: impact of N adjuvants on δ^{15}N

Samples

Crushed samples of three different grapevine varieties (Pinot blanc, Chardonnay and Pinot gris) collected in Trentino, with a different yeast assimilable nitrogen (YAN) content (very low, low and medium concentration respectively), were considered. YAN comprises ammonia and *alpha*-amino acids and is, apart from sugar, by far the most important nutritional requirement in wine fermentation. To determine the YAN value, the free *alpha*-amino groups of the primary amino acids were measured, and this value was then added to the ammonia (NH_4^+) concentration value by using a FTIR interferometer (WineScan™ SO_2, Foss, Denmark). The chemical composition of the three grape musts is summarized in Table 1.

As the N adjuvant, we collected 32 commercial samples available on the market (Table S3). 12 were inorganic matrices (ammonium, IA), 18 were organic (combining amino acids and ammonium nitrogen, OA), and 2 were mixtures (organic/inorganic). To carry out the experiment, we chose an IA and an OA with a different YAN and δ^{15}N value.

The IA had an ammonium concentration of 291.6 g/Kg (YAN content of 227 g N/Kg) and a δ^{15}N value of 1.3 ‰.

The concentration of nitrogen compounds in the OA was as follows (expressed as g/Kg): ammonium 1.8, alanine 54.1, aminobutyric acid 15.6, arginine 20.8, asparagine 18.6, aspartic acid 26.8, citrulline 0.8, ethanolamine 0.2, glutamic acid 85.2, glutamine 5.0, glycine 13.1, histidine 5.9, isoleucine 23.1, leucine 35.0, lysine 21.6, ornithine 8.1, phenylalanine 25.8, proline 8.56, serine 21.9, threonine 19.3, tryptophan + methionine 13.3, tyrosine 16.4 and valine 36.6 (YAN content of 53 g N/Kg). The OA had a δ^{15}N value of −2.3‰.

The chemical composition was obtained through ionic chromatography measurement.[21]

Microvinification

The three must samples were inoculated with the commercial EC1118 dry yeast (*Saccharomyces cerevisiae*), following the supplier's suggestions as regard the quantity (30 g/hl) and rehydration protocol. The grape must was fermented with four different protocols, without (A) and with inorganic (B) and organic (C1 and C2) nitrogen supplementation, according to the fermentation plan shown in Fig. 1. IA was added respecting the legal limit (100 g/hl) reported in European Commission Regulation no. 606/2009, whereas OA was added at two different concentrations: 50 g/hl (C1; max amount recommended by the producer) and 428 g/hl (C2; to reach the same YAN as in fermentation trial B). All the

Table 1. YAN of the three grape musts used in the fermentation plan

Variety	YAN (mg/l)	N α-amino (mg/l)	N NH_4^+ (mg/l)
PB	42	42	0
CH	82	79	3
PN	133	118	15

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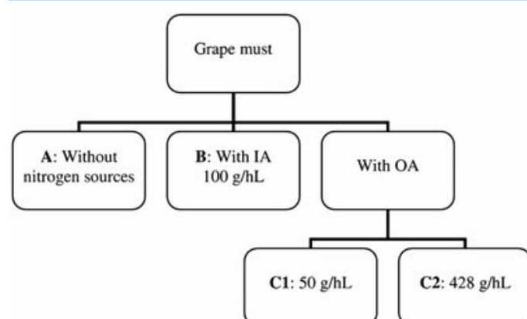

Figure 1. Fermentation plan.

microvinification trials were carried out in duplicate, obtaining 24 wines (1 l for each trial).

Ultrasound treatment

At the end of fermentation, an aliquot of the 24 wines was treated with ultrasound and centrifuged. Sonication was carried out by using an ultrasonic processor (Branson Digital Sonifier®, Branson, USA) with a titanium sonotrode probe (12.7 mm). 100 ml of wine with a yeast suspension was placed in an ice-water bath, and the ultrasound probe was immersed in the sample to a depth of 5 cm. Disruption included six cycles, consisting of a 5 min sonication and a 2 min break at the amplitude level of 70% (20 kHz frequency and 400 W working power).[22]

The efficiency of the method in releasing intercellular yeast compound was evaluated by reading the absorbance value of the supernatant solution at wavelengths $\lambda = 260$ and 280 nm. The absorbance peaks at these wavelengths corresponded to absorption by nucleic acid and proteins respectively, these being the main intracellular constituents of *S. cerevisiae*.[23]

EA-IRMS analysis

Sample pre-treatment

The $\delta^{15}N$ value of the bulk samples was measured in all the samples of soil, leaves, grapes and wine in experiments 1 and 2. The soil was air-dried (at room temperature) and sieved <2 mm, while the leaves were washed with 0.2% citric acid to remove possible residues from the surface (e.g. pesticides, dust and soil), rinsed with water, dried at 35 °C and ground. The grape samples were processed within 3 h of picking. The berries were washed with a 1% (v/v) HNO_3 solution, rinsed with Milli-Q water, blotted with clean paper, frozen (−20 °C) and homogenized by using an Ultraturrax T25 (dispersion tool: Ø 15 mm; IKA-WERKE, Staufen, Germany).

Pre-treatment of grape and wines was performed to remove as much water or/and ethanol as possible from the samples: 5 ml of sample was dehydrated or/and dealcoholized by using the freeze-drying method.[24]

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

The $\delta^{15}N$ value of the bulk samples was measured by using an elemental analyser (Flash EA 1112, Thermo Scientific, Bremen, Germany), equipped with an autosampler (Finningan AS 200, Thermo Scientific) and interfaced through a ConFlo IV dilutor device (Thermo Finningan, Bremen, Germany) with a DELTA V Thermo Scientific isotope ratio mass spectrometer.

GC-c-IRMS analysis

Isolation and derivatization of proline

The $\delta^{15}N$ value of Pro was measured in all the grape samples from the seven oenological chains investigated and in four wine samples (three white and one red) from each oenological chain in experiment 1. Moreover, it was measured in the wine samples in experiment 2 before ultrasound treatment.

Three grams of freeze-dried grape samples was extracted with 60 ml of water–ethanol solution (25% ethanol in water) as solvent, by means of ultrasound at 70 °C.[25] The solution was blown to dryness under N_2 and redissolved in 2 ml of 0.1 M HCl. The wine samples were adjusted to pH 2.3 through the addition of 0.01 M HCl.

A known quantity of norleucine (NLeu; 8 mg ml^{-1} in 0.1 M HCl) was added as internal standard to all the samples (must extract solution and acidified wine), and the amino acids were isolated through ion-exchange chromatography on an Amberlite IR120 cation-exchange resin, previously saturated with H^+ at all exchange sites. This was accomplished by soaking the resin overnight in 3 M NaOH, washing in distilled water, soaking overnight in 6 M HCl and washing with distilled water. The H^+-saturated resin was pipetted into a glass pipette column fitted with a quartz wool plug, and a fraction of the sample (5 ml) was added to the column. The resin was washed with water, and the amino acids were eluted with NH_4OH (10 wt%) and then dried under N_2 before derivatization. Working in these conditions, the resin does not cause any isotopic fractionation.[26,27]

Amino acids analysed after *N*-acetylisopropyl derivatization, based on the method reported elsewhere.[28] The samples were first esterified in 1 ml of acidified isopropanol (1 : 4 acetyl chloride : isopropanol) at 100 °C for 1 h. Esterifying reagents were evaporated under a gentle stream of nitrogen at 40 °C, and dried esters were rinsed with two sequential 250 μl aliquots of dichloromethane to remove excess reagents. Amino acid esters were acylated with 1 ml of acetic anhydride–triethylamine–acetone solution (1 : 2 : 5 v/v) at 60 °C for 10 min. The reagents were evaporated under a N_2 stream at room temperature, and dried derivatives were dissolved in 1 ml of saturated NaCl–water solution and 1 ml of ethyl acetate and mixed vigorously. The organic phase containing the amino acids was collected and dried under nitrogen at room temperature. Residual water was removed with two sequential 250 μl aliquots of dichloromethane. Finally, the samples were dissolved in 200 μl of ethyl acetate and stored at −20 °C until analysis.

GC-c-IRMS analysis of Pro

The $\delta^{15}N$ value of Pro was determined by using a Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific) interfaced with an IRMS (DELTA V, Thermo Scientific) through an open split interface and with a single quadrupole GC-MS (ISQ Thermo Scientific) to identify the compounds. A 0.5 μl of each sample was injected in splitless mode using an auto-sampler (Triplus, Thermo Scientific), and a HP-INNOWAX capillary column was used (60 × 0.32 mm ID × 0.25 μm film thickness; Agilent) with He as carrier gas (at a flow of 1.4 ml/min). The injector temperature was set at 250 °C, and the oven temperature of the GC started at 40 °C, where it was held for 2 min before heating at 40 °C/min to 140 °C, 2.5 °C/min to 180 °C, 6 °C/min to 220 °C and finally at 40 °C/min to 250 °C, where it was held for 15 min.

The eluted compound was combusted into N_2, CO_2 and H_2O in a combustion furnace reactor operated at 1030 °C and made up of a

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non-porous alumina tube (320 mm length) containing three wires (Ni/Cu/Pt, 0.125 mm diameter and 240 mm identical length) braided and centred end-to-end within the tube. Water vapour was removed with a water-removing trap, consisting of a Nafion® membrane. During δ^{15}N analysis, a liquid nitrogen trap was added after the combustion oxidation reactor to remove CO_2 from the oxidized and reduced analyte.

In order to monitor instrumental performance, the Pro standard was derivatized and the δ^{15}N value (+1.1‰) was measured by using GC-c-IRMS before and after each analytical run. Moreover, the isotopic value of the internal standard NLeu added to each sample was checked. NLeu was chosen as internal standard because it is not naturally present in wine. The δ^{15}N value of pure NLeu (+14.0‰) was determined by using EA-IRMS, and we found a mean difference of 0.2‰ between the GC-c-IRMS and EA-IRMS δ^{15}N. The analytical run was accepted when the δ^{15}N value of NLeu was between 13.5 and 14.5‰.

Data expression

All the δ^{15}N values are reported relative to reference N_2, of known isotopic composition, introduced directly into the ion source at the beginning and end of each EA and GC run. All the samples were measured in triplicate, and the isotope ratio was expressed in δ‰ *versus* atmospheric nitrogen according to Eqn (1).

$$\delta = \left[\frac{R_s - R_{std}}{R_{std}}\right] \quad (1)$$

where R_s is the isotope ratio measured for the sample and R_{std} is the isotope ratio of the internationally accepted standard (AIR).

The δ^{15}N values determined with EA-IRMS were calculated against the working in-house standard (commercial protein), calibrated against international references materials: L-glutamic acid USGS 40 (International Atomic Energy Agency, Vienna, Austria) and potassium nitrate IAEA-NO3. The δ^{15}N value of Pro in wine was calculated against the Pro standard, analysed before and after each sample. The instrumental data were corrected on the basis of the difference existing between the δ^{15}N values of the Pro amino acid standard in GC-c-IRMS (mean of six results, three before and three after the samples) and in EA-IRMS, which was in any case always lower than 0.5‰.

Accuracy and precision

The analytical uncertainty of the δ^{15}N of bulk analysis for all the matrices, expressed as 2 * SR (within-laboratory reproducibility standard deviation), was 0.4‰,[24] whereas the reproducibility limit expressed as 2 * rad 2 * SR was 0.6‰.

For the most innovative analysis of Pro by using GC-c-IRMS, we checked both accuracy and precision. To test the accuracy, we compared the δ^{15}N value of the derivatized standard measured by using GC-c-IRMS with the isotopic values of pure non-derivatized amino acid obtained with EA-IRMS. The δ^{15}N value determined by using EA-IRMS was the mean of the two measurements, whereas the δ^{15}N value determined with GC-c-IRMS was the average of three runs. The difference between the δ^{15}N value measured with EA-IRMS and GC-c-IRMS after derivatization was no higher than ±0.5‰.

To evaluate precision, ten replicates of the reference amino acid were derivatized, and each of them was analysed by using GC-c-IRMS in triplicate. The precision (1σ) of GC-c-IRMS determination was on average ±0.2‰.

To evaluate the uncertainty of measurements for all the processes, a wine sample was treated and derivatized ten times, and each of the samples was analysed by using GC-c-IRMS. The standard deviation obtained (1σ) was on average ±0.3‰.

Statistical analysis

The data were analysed with the Statistica version 9 (StatSoft Inc., Tulsa, OK, USA) package. To verify differences between matrices within the production chains as well as between treatments, a paired *t*-test was used. Statistically significant correlations were checked by using the Pearson correlation test.

Results and discussion

Experiment 1: δ^{15}N from soil to wine

Table 2 shows the δ^{15}N values of soil (different depths), leaves, grapes and microvinified wine for the seven oenological chains investigated (A–G) and by using ten different types of yeast, following white or red vinification.

The difference between the δ^{15}N measured in the upper layer of soil (TS) and in the lower layer (SS) was no more than 0.3‰ at each site, and therefore much lower than the reproducibility limit of the analysis (0.6‰). Moreover, a strong correlation was found between the δ^{15}N determined in TS and SS ($p < 0.001$, $R^2 = 0.99$). This means that the δ^{15}N data did not change according to depth at each sampling site.

An increase in soil δ^{15}N with depth has been observed in forest,[29–32] grasslands,[33–35] tundra[36] and pastures[33,37,38] due to the increasing action of denitrification, which causes ^{15}N enrichment of the residual nitrate, as reported in the scientific literature. In these environments with no antropogenic 'intervention', the

Table 2. δ^{15}N value (‰) of soil, vine leaves, grape berries and wines produced using different yeast strains

	Soils		Leaves	Grapes	White wine										Red wine
Site	TS	SS			FR95	FAP	FR-WP	VP5	EM2	FR-SN6	CKS102	DV10	VL1	AW1503	AW1503
A	4.1	4.1	1.3	2.1	1.9	1.6	2.1	1.8	1.5	1.6	1.5	1.9	1.5	2.0	2.1
B	4.3	4.4	0.5	1.4	1.5	1.5	1.6	1.5	1.7	1.6	1.4	1.6	1.7	1.3	1.6
C	7.5	7.4	2.3	3.7	2.8	2.7	3.2	3.1	2.9	2.7	2.6	3.2	2.7	3.1	3.2
D	6.2	6.5	1.7	2.6	3.0	2.7	2.7	2.5	2.5	2.4	2.6	2.4	2.4	2.8	2.6
E	4.4	4.5	0.5	1.9	1.5	1.5	1.6	1.5	1.4	1.6	1.6	1.6	1.5	1.4	1.7
F	4.4	4.3	0.1	1.1	0.5	0.3	0.6	0.3	0.4	0.5	0.3	0.5	0.6	0.6	0.7
G	5.5	5.5	1.1	2.1	1.2	1.5	1.3	1.4	1.1	1.5	1.4	1.3	1.4	1.2	1.6

TS, top soil; SS, sub-soil.

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increase in $\delta^{15}N$ through the soil profile with depth can arrive at differences of 20‰ within a 30 cm change in depth. On the other hand, a previous study on vineyards[39] reported much more limited differences and with a trend that was not always clear. So it seems that in agricultural environments, human activities (e.g. harrowing, fertilization, pesticide application, etc.) can make the $\delta^{15}N$ trend through the soil profile less linear and well-defined, as observed here.

As shown in Table 2, the $\delta^{15}N$ of leaves was on average 4.1‰ lower than that of the corresponding soil (calculated as the mean of TS and SS), and the difference was highly significant ($p < 0.001$ paired *t*-test). Plant $\delta^{15}N$ reflects the $\delta^{15}N$ of external N sources and its subsequent fractionation in soil during and after N uptake by the plant. Known N fractionation in the soil relates to that occurring during ammonia volatilization, nitrification, denitrification and leaching. Plant-associated fractionation that may distort the $\delta^{15}N$ signal of the source is linked to that occurring during assimilation, transformation, translocation and loss.[40] The relation between plant $\delta^{15}N$ and the N source can be further complicated because the available N sources vary in amount and ^{15}N composition.[41] Globally, plant $\delta^{15}N$ values are more negative than soils,[42] and the difference ($\delta^{15}N_{plant}$–$\delta^{15}N_{soil}$) increases with decreasing mean annual temperature and secondarily increasing mean annual precipitation, suggesting a systematic change in the source of plant-available nitrogen (organic/NH_4^+ *vs* NO_3^-) with climate.[43]

The $\delta^{15}N$ values of grapes were significantly ($p < 0.001$) lower (on average −3.1‰) than those of the growing soil (Table 2) and higher (+1.1‰) than those of leaves, according to the paired *t*-test. These results were recently observed in another study,[44] which reported higher $\delta^{15}N$ values in must as compared with other parts of the plant (roots, trunk and cane, leaves and shoots), even if not statistically significant. This behaviour could perhaps be related to the role of the predominant sink for the bunches.[44]

To evaluate the effect of yeast type and the wine-making protocol (red or white), we compared the difference between values with the reproducibility limit of the analysis (0.6‰). We found that fermentation carried out with ten different *Saccharomyces* yeast strains did not change the final $\delta^{15}N$ value of wine (the largest deviation was 0.6‰) and that the different wine-making protocols applied (white or red wine) did not have an impact either. The absence of any effect of the yeast type and wine-making protocol was also confirmed by applying the paired *t*-test ($p < 0.001$).

As yeast and vinification protocol had no influence, we computed the mean value of the 11 wine samples for each chain and compared this value with that of the grapes. $\delta^{15}N$ was on average lower (−0.4‰) in wine, but not significantly ($p < 0.001$).

The $\delta^{15}N$ values of Pro measured in grapes and wine samples (three white and one red) from the seven different oenological chains are reported in Table 3.

Fermentation carried out with different yeast strains did not change the final $\delta^{15}N$ value of Pro (the largest deviation was 0.2‰), and the different wine-making protocols applied (white or red wine) did not have any significant impact on values. By calculating the mean value for wine for each chain and comparing these values with those of grape, the $\delta^{15}N$ value of Pro in wine was on average 0.1‰ lower, with a maximum difference of 0.2‰. On applying the paired *t*-test, the difference was not significant ($p < 0.001$). The $\delta^{15}N$ value of Pro in wine therefore always reflected the value in grapes, as expected, because this amino acid is not used by yeasts as a nutrient and hence it is not metabolized during the fermentation process, avoiding modification of its isotopic signature. Moreover, the $\delta^{15}N$ values of Pro in grapes and wine were very close to the $\delta^{15}N$ values of the soil of origin, with an average deviation of −0.3‰. Statistical treatment of the data (paired *t*-test, $p < 0.001$) indicated that there was no significant difference between the $\delta^{15}N$ values of Pro in grapes and wine and those of the growing soil.

Table 3. $\delta^{15}N$ value (‰) of Pro in grapes and wines obtained with different yeast strains

	Grapes	White wine			Red wine
Site		FR95	EM2	AW1503	AW1503
A	3.6	3.6	3.5	3.4	3.5
B	4.1	3.9	4.0	4.0	4.2
C	7.1	6.8	7.0	6.9	7.1
D	6.3	6.2	6.4	6.2	6.1
E	4.3	4.2	4.4	4.3	4.7
F	4.1	4.1	3.9	4.0	3.7
G	5.5	5.3	5.2	5.4	5.3

Considering the correlations between soil (mean of TS and SS), grapes, wine (mean value of the 11 wines), grape Pro and wine Pro (mean value of the four wines; Table 4), we found that the $\delta^{15}N$ values were significantly correlated within the different matrices. The most significant correlations were those between the $\delta^{15}N$ of leaves and grapes and between the $\delta^{15}N$ of grapes and wine Pro and growing soil.

We can therefore conclude that despite nitrogen isotope fractionation from the soil to the wine, the $\delta^{15}N$ values of leaves, grapes, wine and in particular of Pro in must and wine conserve the variability of $\delta^{15}N$ in the growing soil and can therefore be used as additional isotopic markers to trace the geographical origin of wine.

Experiment 2: impact of N adjuvants on $\delta^{15}N$

Grape must often contains an inadequate concentration of assimilable nitrogen,[45] and supplementation with nitrogen is useful to prevent problems in fermentation and to optimize yeast performance. The most common source of nitrogen for YAN supplementation is IA, but OA can be a very good source of nutrients for fermenting yeast because it contains a high concentration of organic nitrogen (amino acids), vitamins and minerals.[46] To evaluate the effect of addition on the $\delta^{15}N$ value, three grape must samples with a different YAN content (very low, low and medium concentration) were fermented with and without an exogenous nitrogen source by using both IA and OA (Fig. 1).

Table 4. Matrix of correlation between the $\delta^{15}N$ values of soil (mean of TS and SS), grapes, wine (mean value of the 11 wines), grape Pro and wine Pro (mean value of the four wines)

	Soil	Leaves	Grapes	Wine	Grape Pro
Leaves	*				
Grapes	**	***			
Wine	*	***	**		
Grape Pro	***	*	*		
Wine Pro	***	*	*	*	***

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

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A survey of the δ^{15}N of nitrogen-rich fermentation adjuvants available on the market showed a δ^{15}N range between −2.3‰ and +1.7‰ (Table S3).

To carry out the experiment, we chose the OA with the lowest δ^{15}N value (−2.3‰) and the IA with the highest (+1.3‰) in order to cover the full range of δ^{15}N values. As the two adjuvants have a different YAN, they were employed in different amounts, as detailed in the section on Materials and Methods. After fermentation, in order to release the nitrogen compounds incorporated within the yeast cells, the wine samples were subjected to ultrasound sonication.

The δ^{15}N was measured in bulk grape must, with and without IA and OA, in the corresponding bulk wine samples before and after ultrasound treatment and in the Pro of wine samples before ultrasound treatment. The values are shown in Table 5.

Considering the δ^{15}N variation along the rows, we can see the effect of fermentation and ultrasound treatment on the nitrogen isotopic signature. The δ^{15}N values of bulk wine, also in presence of IA or OA, were on average slightly higher (+0.3‰) than those of bulk must, but the difference was not significant ($p < 0.001$). After ultrasound treatment, the δ^{15}N value of wine become even closer (on average −0.1‰) to that of the grape must of origin. During fermentation, yeast transports NH_4^+ and amino acids from the grape must to the yeast cell, where they are metabolized. On the basis of the results obtained, we can surmise that transport and metabolization can lead to slight discrimination against ^{14}N and therefore a higher final δ^{15}N value in wine. Ultrasound treatment, which simulates wine ageing, entails the release of nitrogen fractions, incorporated within yeast, as a result of the cavitation effect on yeast cells.

Considering the δ^{15}N variation along the columns in Table 5, we can see the adjuvant's effect on the nitrogen isotopic signature.

Taking into account first of all the values of bulk must and wine, we can see that the addition of IA slightly decreases (on average −0.1‰) the values of both grape must and wine before and after ultrasound treatment, but the decrease is not significant ($p < 0.01$). Indeed, the δ^{15}N value of IA was 1.3‰, therefore not far from the original values for the three must samples. On the other hand, the presence of OA significantly influences ($p < 0.01$) the δ^{15}N values of grape juice and wine, because OA has a δ^{15}N value of −2.3‰. The decrease was on average 1‰ with the addition of the amount suggested by the producer (0.5 g/l) and was around 3.5‰ when the addition was large scale (4.3 g/l). The observed deviation can be considered the largest achievable deviation, because the use of other adjuvants with a higher δ^{15}N (Table S3) would have less impact on the N isotope ratio of wine.

Taking into account the δ^{15}N of Pro, we can see first of all that the value is much higher than those of bulk wine and must, as observed in the chains in experiment 1. Moreover, it is evident that addition of both IA (1 g/l) and OA (0.5 g/l) did not have any impact on the δ^{15}N of Pro. The addition of OA in massive quantities (4.3 g/l) significantly influenced ($p < 0.01$) the δ^{15}N value of Pro, but the decrease was 0.5‰, therefore much less significant than that observed in bulk wine. OA contained 0.86% (w/w) of Pro with a δ^{15}N value of −3.0‰.

We can therefore conclude that neither fermentation, as observed in experiment 1, nor ultrasound treatment, simulating wine ageing, affect the δ^{15}N values of grape must, which are therefore conserved in wine. The addition of IA, with the conditions used here, did not influence δ^{15}N, whereas the addition of OAs was able to substantially change the δ^{15}N of bulk wine, with decreases of up to 4.5‰. This variation depends on the amount added and on the δ^{15}N value of both must and OA, because the larger the difference between the δ^{15}N of must and that of the adjuvant, the larger the δ^{15}N difference. The δ^{15}N of wine Pro did not change substantially, even in the presence of massive amounts of OA.

The δ^{15}N of wine Pro is therefore the best candidate for tracing the geographical origin of wine.

Table 5. δ^{15}N of bulk grape must with and without IA and OA, bulk wine samples before and after ultrasound treatment (US) and δ^{15}N of proline in wine before US

Variety	Fermentation conditions	YAN (mg/l)	δ^{15}N (‰)			
			Grape must	Wine	Wine after US	Pro in wine
PB	No adjuvant	42	3.3	3.5	3.2	11.5
	1 g/l IA	72	3.1	3.4	3.0	11.5
	0.5 g/l OA	58	2.2	2.9	2.0	11.4
	4.3 g/l OA	75	−0.6	−0.4	−0.8	11.0
CH	No adjuvant	82	2.6	2.8	2.5	7.7
	1 g/l IA	118	2.5	2.7	2.4	7.6
	0.5 g/l OA	95	1.7	2.0	1.5	7.6
	4.3 g/l OA	120	−1.2	−0.4	−1.3	7.1
PG	No adjuvant	133	4.8	5.1	4.8	10.1
	1 g/l IA	165	4.6	4.9	4.5	10.2
	0.5 g/l OA	152	3.9	4.3	3.8	10.1
	4.3 g/l OA	170	1.1	1.3	1.2	9.6

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SECTION 4.5

Botanical traceability of commercial tannins using the mineral profile and stable isotopes

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Statement of the author: My personal contribution to this work mainly concerned the developing of a methodology to measure the $\delta^{13}C$ value of tannin samples. Subsequently, I was involved in the carbon isotopic analysis of all tannin samples collected in this study.

Research article

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Botanical traceability of commercial tannins using the mineral profile and stable isotopes†

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Commercial tannins are natural polyphenolic compounds extracted from different plant tissues such as gall, the wood of different species and fruit. In the food industry they are mainly used as flavourings and food ingredients, whereas in winemaking they are classified as clarification agents for wine protein stabilisation, although colour stabilisation, metal removal, unpleasant thiol removal and rheological correction are also well-known and desired effects.

Due to their particular technical properties and very different costs, the possibility of correct identification of the real botanical origin of tannins can be considered a primary target in oenology research and in fulfilling the technical and economic requirements of the wine industry. For some categories of tannins encouraging results have already been achieved by considering sugar or polyphenolic composition. For the first time this work verifies the possibility of determining the botanical origin of tannins on the basis of the mineral element profile and analysis of the $^{13}C/^{12}C$ isotopic ratio.

One hundred two commercial tannins originating from 10 different botanical sources (grapes, oak, gall, chestnut, fruit trees, quebracho, tea, acacia, officinal plants and tara) were analysed to determine 57 elements and the $^{13}C/^{12}C$ isotopic ratio, using inductively coupled plasma mass spectrometry and isotope-ratio mass spectrometry, respectively.

Forward stepwise discriminant analysis provided good discrimination between the 8 most abundant groups, with 100% correct re-classification. The model was then validated five times on subsets of 10% of the overall samples, randomly extracted, achieving satisfactory results. With a similar approach it was also possible to distinguish toasted and untoasted oak tannins as well as tannins from grape skin and grape seeds. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: commercial tannins; mineral elements; $^{13}C/^{12}C$; botanical origin; traceability

Introduction

Tannins are polyphenolic compounds of botanical origin, with a molecular weight ranging from 500 to 20 000, able to bind and precipitate proteins.[1] These products can basically be classified into two groups: hydrolysable tannins (such as gallo- and ellagitannins), mainly extracted from gall, tara, myrabolan, oak and chestnut, and condensed tannins (or proanthocyanidins) present in the skin and seeds of grapes, tea, quebracho and mimosa.[2,3] Known since ancient times, oak wood and other plant extracts were traditionally used for tanning animal hides in leather production. Nowadays they are still widely used in many industrial fields, from the leather industry to the floatation process in mining activities. Nevertheless their use as an adjuvant in food production is probably the most crucial and complex in technical and health terms. The European Union recognises tannins as flavourings and food ingredients (EC No 1334/2008, EU Regulation No. 872/12), while the International Organisation of Vine and Wine (OIV) restricts their use to clarification agents for protein stabilisation in must and wine. According to OIV (COEI-1-TANINS: 2009), oenological tannins can be produced by extraction from different botanical sources: nutgalls (of *Quercus* and tara), wood rich in tannin, such as chestnut (*Castanea sativa*), oak (*Quercus sp.*) or exotic wood (e.g. quebracho: *Schinopsis balansae*) or from grape seeds and skin (*Vitis vinifera*). Other plants and plant tissues, although not expressly mentioned, have similar characteristics: for example the bark, stems or leaves of plants such as gambier (*Uncaria gambir*), mimosa (*Acacia sp.*), tara (*Caesalpina spinosa*) and myrobolan fruit (*Terminalia chebula*).

Sometimes these polyphenolic compounds can also have significant effects on wine rheological perception, colour and flavour and act as antioxidant and antimicrobial agents.[3–5] In the mouth, tannins form complexes with the proline-rich salivary proteins and mucopolysaccharides, causing precipitation and/or aggregation of the proteins with the loss of their lubricating properties, and inducing the typical feeling of dryness, bitterness and astringency.[6,7] Due to their much smaller degree of polymerisation, catechins from grape seeds (and stems) are sensed as bitter and more astringent than those extracted from grape skins.

In light of the high number of possible plant sources, the huge differences in chemical characteristics, the specific oenological properties[3] and last but not least, the considerable differences in the commercial value of products, the ability to correctly recognise the origin of tannins must be considered to be a key tool in effectively fulfilling the technical and economic requirements of the wine industry. Tannins of different origin can show characteristic spectra and chromatographic fingerprints, as highlighted using UV–vis spectroscopy, Fourier-transform mid-infrared spectroscopy,

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Table 1. Statistical distribution of mineral element content and $\delta^{13}C$ value in tannins of grape, oak and gall. Macro elements (Na, Mg, P, K, Ca) expressed in g/kg; micro elements (B, Al, Ti, Mn, Fe, Ni, Cu, Zn, Rb, Sr, Mo, Ba) expressed in mg/kg and other trace elements expressed in μg/kg. DL = detection limit; min = minimum content; m = median content; max = maximum content

		Grape (N = 43)					Oak (N = 21)					Gall (N = 10)				
	DL	Min	25th	m	75th	Max	Min	25th	m	75th	Max	Min	25th	m	75th	Max
$\delta^{13}C$		−30.8	−29.6	−29.1	−28.6	−27.8	−25.5	−25.0	−24.9	−24.6	−24.2	−27.2	−26.5	−26.2	−26.1	−25.1
Na	0.01	0.05	0.34	1.57	2.86	14.8	0.41	0.75	1.00	1.38	2.67	0.02	0.03	0.08	0.40	0.64
Mg	0.01	0.02	0.19	0.41	0.66	2.97	0.10	0.30	0.41	0.54	1.25	<DL	0.00	0.02	0.42	0.66
P	0.01	0.03	0.86	1.39	4.82	29.1	0.06	0.16	0.27	1.24	3.56	0.01	0.04	0.15	0.72	1.14
K	0.01	0.06	1.10	4.06	13.3	45.3	1.94	4.51	4.96	7.07	18.8	0.02	0.13	0.92	2.13	2.66
Ca	0.07	0.08	0.51	0.73	1.10	9.83	0.12	1.00	1.25	1.79	3.96	<DL	<DL	0.05	0.76	1.10
B	0.05	1.39	6.96	16.3	30.3	119	17.0	29.2	30.8	33.1	55.7	0.72	2.70	11.7	20.0	31.5
Al	0.24	0.34	3.54	7.42	11.2	31.8	0.39	1.70	2.30	11.3	37.6	0.70	2.97	8.53	37.4	66.7
Ti	0.11	<DL	0.39	0.55	1.03	2.68	<DL	<DL	0.16	0.32	0.92	<DL	<DL	0.06	0.51	0.99
Mn	0.01	0.20	2.04	4.55	10.2	37.4	12.7	68.6	87.3	129	209	<DL	0.24	1.12	73.3	111
Fe	0.26	1.61	11.6	37.9	109	409	1.84	2.83	5.02	18.0	38.2	0.54	1.94	4.93	32.8	77.8
Ni	0.01	0.02	0.23	0.42	1.52	10.8	0.54	0.72	0.97	1.14	2.26	<DL	<DL	0.04	0.96	1.42
Cu	0.02	0.33	1.99	2.84	6.96	66.9	0.24	0.40	0.56	1.33	4.22	<DL	0.05	0.25	0.52	0.81
Zn	0.36	<DL	2.32	3.99	6.30	23.8	0.66	0.80	1.43	2.86	10.2	<DL	<DL	1.25	2.53	3.79
Rb	0.05	0.05	0.82	2.90	6.63	51.2	3.76	11.2	12.6	16.5	24.9	<DL	0.10	0.98	1.89	3.10
Sr	0.04	0.43	3.71	5.66	11.2	104	0.45	8.02	10.3	12.7	25.8	0.07	0.12	0.20	4.93	10.3
Mo	0.03	0.04	0.12	0.18	0.50	1.29	<DL	0.04	0.04	0.08	0.11	<DL	<DL	0.03	0.05	0.06
Ba	0.01	0.16	0.58	0.83	1.90	33.6	1.23	2.25	2.84	8.38	35.4	0.03	0.22	0.42	25.9	38.4
Li	1.8	27	187	242	470	6261	263	760	998	1502	3399	3.3	15	36	104	167
Be	0.65	<DL	<DL	0.99	2.03	12.4	1.54	18.1	21.1	39.2	53.6	<DL	<DL	1.36	6.94	13.2
V	19	<DL	22	75	292	3122	<DL	<DL	<DL	<DL	113	<DL	<DL	<DL	38	96
Cr	3	16	189	341	682	4310	16	43	71	157	708	<DL	3	40	187	419
Co	0.2	9.0	28	45	72	674	40	51	65	115	191	2.7	7.1	8.8	110	218
Ga	0.07	0.50	3.49	6.90	11.3	69.5	<DL	<DL	0.63	1.14	5.43	<DL	0.10	1.51	5.23	10.5
Ge	0.50	<DL	2.01	4.36	6.89	20.5	<DL	<DL	0.60	0.99	2.50	<DL	<DL	<DL	0.77	1.77
As	1.4	8.3	64	124	213	1275	56	102	121	154	273	<DL	7.9	31	44	418
Se	0.6	2.2	18	39	56	161	5.6	11	21	25	55	<DL	2.0	2.3	6.0	8.1
Y	0.3	0.4	3.1	5.2	11.1	40.5	<DL	0.6	0.9	2.1	8.0	2.6	3.0	4.6	6.3	23.1
Pd	0.30	<DL	<DL	0.37	0.59	5.60	<DL	0.31	0.49	0.61	0.80	<DL	<DL	<DL	0.35	0.58
Ag	0.20	<DL	<DL	0.27	1.48	8.58	<DL	<DL	<DL	0.35	0.60	<DL	<DL	<DL	0.47	0.62
Cd	0.25	<DL	1.51	2.67	4.97	7.90	2.47	10.9	15.5	24.5	34.9	<DL	<DL	1.69	12.4	22.8
In	0.14	<DL	<DL	0.25	1.03	12.0	<DL	<DL	<DL	0.97	1.22	<DL	<DL	0.10	0.80	1.18
Sn	0.6	1.4	10.6	26.1	66.0	1805	2.2	5.0	7.0	17.4	143	<DL	3.0	9.3	13.9	20.4
Sb	0.34	4.12	20.0	26.1	53.7	136	10.8	15.3	16.4	18.9	35.7	1.29	2.59	3.51	11.2	42.9
Te	0.45	<DL	<DL	0.50	0.85	1.98	<DL	<DL	<DL	<DL	0.91	<DL	<DL	<DL	0.72	0.83
Cs	0.4	<DL	<DL	<DL	6.8	150	<DL	0.6	60.6	123	215	<DL	<DL	<DL	5.1	38.5
La	0.9	<DL	1.9	3.7	9.0	20.6	<DL	<DL	1.0	1.6	8.3	2.7	3.5	5.2	8.8	26.5
Ce	0.3	0.5	3.5	7.8	18.2	50.8	<DL	0.5	1.3	1.9	21.1	5.2	8.7	10.7	14.6	35.6
Pr	0.19	<DL	0.54	1.09	2.08	7.12	0.09	0.21	0.35	0.48	2.02	0.64	1.00	1.34	1.74	4.60

(Continues)

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Table 1. *(Continued)*

	DL	Grape (N = 43)					Oak (N = 21)					Gall (N = 10)				
		Min	25th	m	75th	Max	Min	25th	m	75th	Max	Min	25th	m	75th	Max
Nd	0.2	0.3	1.8	3.9	9.3	30.1	<DL	0.4	0.8	1.1	7.0	2.3	3.7	4.4	6.9	18.6
Sm	0.14	0.16	0.43	1.15	2.05	7.37	<DL	0.29	0.40	0.63	2.15	0.27	0.98	1.13	1.63	3.45
Eu	0.05	0.15	0.78	1.05	1.19	2.95	0.26	0.49	0.75	1.05	2.46	0.39	0.53	0.84	1.90	2.94
Gd	0.18	<DL	0.61	1.38	2.28	8.50	<DL	0.37	0.67	1.03	2.03	0.47	0.90	1.45	1.73	4.59
Dy	0.03	0.32	0.57	1.15	2.03	8.26	0.08	0.37	0.46	0.74	1.79	0.49	0.83	1.13	1.45	2.97
Ho	0.053	0.083	0.223	0.308	0.457	1.88	<DL	0.125	0.336	0.401	0.623	0.118	0.250	0.440	0.545	0.657
Er	0.093	0.147	0.412	0.794	1.17	5.84	<DL	0.255	0.364	0.413	1.03	0.301	0.474	0.631	0.908	1.75
Tm	0.083	<DL	0.152	0.197	0.268	0.979	<DL	0.106	0.207	0.283	0.426	<DL	0.086	0.239	0.293	0.404
Yb	0.098	0.123	0.444	0.766	1.26	6.70	<DL	0.240	0.336	0.535	1.24	0.289	0.451	0.565	0.833	1.24
Re	0.068	0.335	0.731	1.24	2.19	13.2	0.162	0.465	0.643	0.951	1.33	0.122	0.195	0.626	0.977	8.33
Hg	0.44	<DL	0.60	1.08	2.90	47.0	<DL	0.48	0.53	0.63	0.92	<DL	<DL	<DL	0.61	0.70
Tl	3.9	<DL	6.8	8.9	14.1	39.3	14.8	26.2	33.3	46.3	69.9	<DL	<DL	7.5	10.3	13.8
Pb	2.6	5.9	43.7	104	155	730	6.2	16.9	36.9	63.0	249	<DL	13.5	32.6	62.1	93.3
Th	8	<DL	<DL	<DL	<DL	38	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	10
U	0.23	1.11	6.54	14.0	44.8	448	3.85	9.16	11.5	14.5	21.1	0.75	1.30	2.72	5.83	6.49

nuclear magnetic resonance, size-exclusion chromatography and liquid chromatography–tandem mass spectrometry (MS/MS).[8] The ultraviolet absorption spectrum, phenolic content (e.g. flavanol, proanthocyanidines, digallic acid, scopoletine, eugenol, 2-phenylethanol, vanillin and syringaldehyde) and the polyalcohol and monosaccharide profile (e.g. quercitol, pinitol, myo-inositol, arabitol, muco-inositol, chiro-inositol, bornesitol) were investigated separately to characterise the botanical origin of oenological tannins.[9–12] In a similar way, cyclic polyalcohol composition was shown to be useful in identifying the botanical origin of wood and chips used in the wine-ageing process.[13]

Recently, a new approach based on the combined use of simple sugars and free phenols was shown to correctly reclassify more than 90% of tannins of 10 different botanical origins.[14]

In this work we investigated the ability of the mineral profile and stable isotope ratio of C to trace the origin of 102 oenological tannins of various botanical origins. These techniques have indeed already been used to distinguish the botanical origin of several products (i.e. industrial ethanol, brandy, resins and amber, apple, tartaric acid, honey, amaranth seed and coffee).[15–24] The final aim of the study was to build a statistical model for verifying the declared botanical origin of commercial tannins. To the best of our knowledge the mineral profile and isotopic ratios of C have never previously been used to trace the botanical origin of tannins.

Experimental

Materials

One hundred two commercial oenological tannins of confirmed botanical origin (OIV method)[14] were collected from Italian commercial distributors and wineries: 43 were from grapes (19 from skin, 16 from seeds and 8 generically classified as grape tannins), 21 oak (12 from toasted wood and 9 from untoasted wood), 10 gall, 7 fruit trees, 5 chestnut, 5 quebracho, 5 tea, 4 acacia, 1 tara and 1 from an officinal plant. The samples were stored in the original packaging in a dry place until analysis.

As regards elemental analysis, concentrated nitric acid, provided by Carlo Erba (Milan, Italy; ultrapure 67–69%), and ultrapure Milli-Q® water (18.2 MΩ, Millipore, Bedford, MA, USA) were used for standard solutions and sample preparation. Copper, Mg, Na, Sr (all 1 g/l) standard solutions and inductively coupled plasma (ICP) multi-element standard solution VI were purchased from Merck (Darmstadt, Germany); Al, Ca, Fe, K, P (all 10 g/l) and Cs and Rb (1 g/l) standard solutions were from CPI International (Santa Rosa, CA); Hg standard-2A solution (10 mg/l) and multi-element calibration standard-1 and -3 solutions were from Agilent Technologies (Santa Clara, USA) while Sc, Rh and Tb (1 g/l, used as internal standards) standard solutions and the ICP-MS calibration standard 4 solution were from Aristar BDH (Poole, UK).

The reference material—'Peach Leaves' (SRM 1547)—was from NIST, National Institute of Standards & Technology (Gaithersburg, MD, USA).

For isotopic analysis, samples were weighed into tin capsules (Säntis analytical AG, Teufen, Switzerland). To compute the δ-values (see below), we used a working in-house protein standard, calibrated against the following international reference materials: NBS-22 fuel oil (IAEA-International Atomic Energy Agency, Vienna, Austria), IAEA-CH-6 sugar

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Table 2. Statistical distribution of mineral element content and $\delta^{13}C$ value in tannins of fruit tree, chestnut and quebracho. See Table 1 for unit of measurement, limit of detection and abbreviation

	Fruit tree (*N* = 7)					Chestnut (*N* = 5)					Quebracho (*N* = 5)				
	Min	25th	m	75th	Max	Min	25th	m	75th	Max	Min	25th	m	75th	Max
$\delta^{13}C$	−28.9	−27.1	−26.0	−25.9	−24.2	−25.6	−25.5	−25.4	−25.4	−25.4	−28.7	−26.2	−26.0	−25.8	−25.7
Na	0.09	0.67	1.13	1.43	1.66	1.28	1.30	1.86	1.89	2.11	0.45	5.29	5.53	5.63	5.74
Mg	0.01	0.41	0.55	1.21	1.32	0.91	0.93	0.93	1.01	1.39	0.09	0.68	1.26	1.30	1.49
P	0.01	0.23	0.36	1.58	2.66	0.14	0.18	0.33	1.33	1.67	0.01	0.09	0.20	0.22	1.12
K	0.09	5.09	6.95	8.55	10.6	1.12	1.24	1.31	1.34	2.69	0.25	0.53	0.59	1.78	5.61
Ca	<DL	0.56	0.85	1.75	3.85	1.46	1.49	1.56	1.57	1.80	0.12	0.36	0.93	1.95	4.60
B	0.88	6.62	20.0	28.0	36.1	15.8	15.8	19.0	19.9	24.5	4.34	21.4	28.9	29.1	35.1
Al	0.70	9.49	13.2	50.0	77.4	79.9	89.6	96.3	106	113	5.77	7.75	16.6	16.9	45.1
Ti	<DL	0.17	0.33	0.67	1.26	1.32	1.37	1.93	2.04	2.28	0.27	0.41	0.54	0.58	0.83
Mn	1.13	12.5	70.4	125	165	187	205	213	227	247	1.13	3.66	13.5	33.6	67.8
Fe	0.79	10.2	20.2	40.1	64.9	61.5	71.1	74.6	91.7	97.1	9.30	9.73	43.1	69.7	123
Ni	0.02	0.79	1.18	1.72	2.16	2.05	2.35	2.44	2.68	2.77	0.12	0.23	1.09	5.30	16.1
Cu	0.03	0.53	1.17	2.31	3.81	1.28	1.31	1.39	1.39	1.66	0.14	0.36	0.39	0.53	4.50
Zn	<DL	2.47	2.76	5.04	7.08	4.63	4.80	4.87	5.81	6.39	<DL	0.70	1.58	2.10	2.73
Rb	0.15	5.47	6.68	22.1	26.1	2.83	2.98	3.34	3.63	4.52	0.13	0.48	0.53	0.86	14.0
Sr	0.22	5.97	9.20	16.3	17.1	9.42	10.8	11.3	11.6	19.0	1.36	3.43	7.92	35.9	108
Mo	<DL	0.07	0.09	0.17	0.26	0.05	0.07	0.07	0.07	0.09	0.04	0.22	0.24	0.34	1.96
Ba	0.41	3.63	35.3	41.6	48.0	36.4	53.6	56.3	58.2	61.7	0.75	8.34	28.6	30.5	39.1
Li	6.3	150	159	232	366	194	208	213	237	260	626	1209	1679	1892	3724
Be	<DL	1.91	11.9	15.1	62.7	18.4	19.5	21.9	22.0	27.4	1.58	1.99	2.12	3.17	9.79
V	<DL	27	82	151	188	107	145	156	170	197	<DL	<DL	57	62	71
Cr	18	183	207	293	442	186	239	361	417	582	240	392	647	2143	12 350
Co	2.8	53	103	187	328	347	360	458	462	479	4.2	33	105	176	181
Ga	<DL	0.41	3.19	4.77	11.0	9.14	11.6	12.2	16.4	16.4	1.42	3.54	3.81	6.19	6.79
Ge	<DL	<DL	<DL	0.89	1.65	0.78	1.43	1.48	1.58	1.79	<DL	<DL	<DL	<DL	2.58
As	<DL	45	52	55	96	45	63	71	77	83	29	30	39	41	42
Se	<DL	4.1	6.9	10	15	2.3	2.3	4.2	4.6	7.1	4.8	6.9	9.0	12	16
Y	<DL	4.4	9.3	17.8	48.1	15.5	19.6	24.4	26.2	31.4	1.9	2.5	7.0	9.0	17.9
Pd	<DL	<DL	<DL	0.67	0.83	<DL	0.81	0.89	0.90	0.92	<DL	<DL	0.48	0.62	0.71
Ag	<DL	<DL	0.20	1.25	3.90	1.16	1.81	1.97	2.17	4.06	<DL	<DL	0.32	0.75	1.02
Cd	<DL	2.39	4.73	18.8	28.0	32.4	34.6	35.8	37.3	38.0	<DL	1.93	6.70	7.99	14.7
In	<DL	<DL	<DL	0.62	1.22	<DL	0.16	0.20	0.26	0.95	<DL	<DL	0.49	1.21	1.81
Sn	<DL	6.2	12.6	16.1	26.5	9.9	10.8	11.6	18.2	26.4	5.6	6.0	13.1	27.6	135
Sb	0.59	9.69	11.9	25.0	33.7	9.90	11.0	12.6	13.9	19.0	3.63	4.98	12.6	19.7	26.3
Te	<DL	<DL	0.45	0.68	0.84	<DL	<DL	0.47	0.80	0.84	<DL	<DL	<DL	<DL	<DL
Cs	<DL	<DL	20.9	112	250	<DL	<DL	26.0	29.8	34.0	<DL	1.5	13.0	17.0	109
La	<DL	5.5	10.6	36.3	69.5	24.9	31.4	37.5	43.6	57.5	2.8	3.4	10.6	14.1	23.9
Ce	0.6	7.1	15.1	25.5	77.1	27.9	30.2	53.9	57.4	75.4	3.2	4.9	21.4	25.9	33.8
Pr	0.23	1.16	2.21	4.86	11.09	4.25	5.64	7.34	7.47	10.5	0.66	0.70	2.44	2.52	4.87
Nd	0.3	4.1	7.7	16.3	40.2	16.6	20.3	27.3	30.4	38.6	2.2	2.2	7.8	8.6	17.0

(Continues)

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Table 2. *(Continued)*

	Fruit tree ($N=7$)					Chestnut ($N=5$)					Quebracho ($N=5$)				
	Min	25th	m	75th	Max	Min	25th	m	75th	Max	Min	25th	m	75th	Max
Sm	0.32	0.91	1.66	3.19	9.29	3.44	4.64	5.85	6.39	8.44	0.65	0.99	1.93	2.18	4.24
Eu	0.36	0.91	1.88	3.02	4.49	2.84	4.03	4.30	4.58	5.09	0.93	1.37	1.79	2.45	2.45
Gd	0.23	1.36	1.97	3.82	10.62	4.33	5.18	6.89	7.00	9.46	0.60	0.97	2.29	2.39	5.32
Dy	0.38	0.87	1.37	2.81	7.86	2.34	3.52	3.91	4.54	6.44	0.49	0.77	1.72	1.97	3.78
Ho	0.273	0.302	0.454	0.894	1.76	0.425	0.742	0.856	1.11	1.38	0.112	0.422	0.593	0.703	1.06
Er	0.262	0.519	0.729	1.66	4.26	1.17	1.81	1.93	2.47	2.98	0.235	0.523	0.979	1.23	1.88
Tm	0.103	0.170	0.323	0.476	0.760	0.169	0.265	0.436	0.441	0.592	0.101	0.236	0.304	0.423	0.536
Yb	0.174	0.567	0.781	1.77	3.24	0.853	1.35	1.49	1.70	2.22	0.185	0.394	0.639	1.15	1.64
Re	0.099	0.195	0.228	1.51	2.20	0.135	0.170	0.170	0.494	0.498	0.174	0.196	0.198	0.249	1.08
Hg	<DL	<DL	0.65	1.03	1.44	<DL	0.64	0.89	1.30	1.40	0.84	1.83	2.61	4.81	7.06
Tl	<DL	5.5	22.8	48.9	66.9	18.3	18.9	20.8	20.8	26.0	<DL	<DL	7.8	9.4	35.7
Pb	<DL	19.5	36.2	122	259	108	116	154	209	219	13.8	22.0	36.3	126	143
Th	<DL	<DL	<DL	10	18	11	14	18	18	20	<DL	<DL	<DL	<DL	<DL
U	0.26	3.22	7.42	25.1	41.9	4.21	5.95	6.25	7.76	8.18	0.75	1.71	3.03	3.17	6.41

(IAEA) and USGS 40 L-glutamic acid (IAEA), with $\delta^{13}C$ values in turn calibrated against the internationally recognised standard Vienna-Pee Dee Belemnite (V-PDB).

Elemental analysis

For acid digestion of samples, an aliquot of about 300 mg of tannin was weighed into a polytetrafluoroethylene tube (UltraWAVE Milestone, Shelton, CT, USA) and 4 ml of HNO_3 was added. One blank sample for each batch of digestion was prepared by substituting tannin with ultrapure water. Samples were mineralised using a microwave oven (UltraWAVE, Milestone), controlled by a three-step temperature programme. The temperature was first linearly increased from room temperature to 120 °C in 11 min, then to 230 °C in 9 min and subsequently maintained for 5 min. After thermal treatment the homogeneous solution was transferred into a 50-ml polypropylene vial and diluted to a final volume of 26 ml with Milli-Q® water.

The elemental profile (Li, Be, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Mo, Pd, Ag, Cd, In, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Er, Tm, Yb, Re, Ir, Pt, Au, Hg, Tl, Pb, Bi, Th and U) was quantified with an inductively coupled plasma mass spectrometer (ICP-MS; Agilent 7500ce Agilent Technologies, Tokyo, Japan) equipped with an Octopole Reaction System for removal of the principal polyatomic interferences. Helium was used as the collision gas for analysis of Na, Mg, V, Cr, Fe, Ni, Cu, Zn and Eu, whereas H_2 was used to determine Ca, Ga and Se.

An on-line internal standard solution of Sc, Rh and Tb, all 1 mg/l, was used during analysis.

Accuracy was verified by analysing the certified material SRM 1547 in each analytical batch. Considering both certified ($N=22$) and determined but not certified ($N=11$) elements, recovery was higher than 75%, except for V (73%) and Yb (not certified, 65%), and was considered acceptable for the purposes of this research. The detection limit (DL) of each element was calculated as 3 times the standard deviation of the signal of the blank sample, analysed 10 times (Table 1).

Carbon stable isotope analysis

Aliquots of 0.5 mg of the sample, weighed into tin capsules, were subjected to analysis of $^{13}C/^{12}C$ using an isotope ratio mass spectrometer (DELTA V, Thermo Scientific, Germany) following total combustion in an elemental analyser (EA Flash 1112, Thermo Scientific). The analysis was carried out twice, obtaining a mean value. The values are expressed as $\delta^{13}C‰ = [(R_{sample} - R_{standard}) / R_{standard}] \times 1000$, where R is $^{13}C/^{12}C$, against the international standard V-PDB.

The uncertainty (2σ) of measurements was <0.3‰.

Statistical analysis

For the purpose of statistical data processing, all content measured as under the DL was fixed at a nominal value of half the DL. The normality of the distribution of data for each mineral element was checked using the Kolmogorov–Smirnov test ($p < 0.05$), and, when necessary, data were normalised by applying Box–Cox transformation. Elements not showing a Gaussian distribution even after transformation (Ti, V, Ge, Ag, In, Te, Cs, Pd and Hg), as well as those highly correlated with others (Y, La, Ce, Pr, Nd, Gd, Dy, Ho Er, Tm, Yb and Al; all correlated with Sm as checked by applying Pearson's test,

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Table 3. Statistical distribution of mineral element content and $\delta^{13}C$ value in tannins of tea and acacia and content found in tannin from officinal plant and tara. See Table 1 for unit of measurement, limit of detection and abbreviation

	Tea (*N* = 5)					Acacia (*N* = 4)					Officinal plant (*N* = 1)	Tara (*N* = 1)
	Min	25th	m	75th	Max	Min	25th	m	75th	Max		
$\delta^{13}C$	−31.1	−29.7	−29.7	−29.3	−28.4	−32.2	−32.1	−30.6	−29.0	−28.9	−27.0	−26.6
Na	0.01	0.07	0.10	0.19	0.39	0.64	0.64	1.25	2.43	4.17	<DL	1.39
Mg	0.03	0.14	0.32	1.19	1.72	1.44	1.49	1.56	1.64	1.75	<DL	0.62
P	0.07	0.18	2.12	4.58	6.45	0.31	0.78	1.86	3.77	6.73	<DL	0.80
K	0.70	2.36	4.44	16.9	25.4	10.2	10.7	11.0	11.4	12.1	0.01	16.3
Ca	<DL	<DL	0.09	0.10	0.13	1.87	1.87	2.82	3.95	4.51	<DL	2.44
B	0.96	2.18	2.23	6.66	28.1	13.3	15.8	17.9	19.9	21.9	0.65	14.4
Al	11.6	109	134	404	597	5.56	8.68	55.1	103	111	0.30	12.4
Ti	<DL	0.14	0.16	0.30	0.36	0.13	0.29	1.20	2.27	2.92	<DL	0.27
Mn	12.5	18.0	44.8	91.4	314	14.6	19.1	22.4	24.2	24.4	0.04	8.52
Fe	8.55	12.1	12.9	13.1	14.8	9.79	10.4	51.0	99.2	122	<DL	18.3
Ni	0.47	1.07	1.75	2.79	7.57	0.19	0.19	1.11	2.04	2.10	<DL	0.19
Cu	0.19	0.73	3.70	9.50	12.7	0.46	0.47	0.63	0.99	1.58	<DL	1.36
Zn	0.62	0.99	3.96	10.6	16.1	3.22	3.32	4.19	5.16	5.52	<DL	4.90
Rb	1.67	4.70	14.2	24.2	86.3	34.0	35.7	42.9	52.9	62.9	<DL	11.9
Sr	0.07	0.32	0.69	0.80	1.60	12.5	15.8	17.4	19.4	23.6	<DL	8.12
Mo	0.03	0.04	0.10	0.12	0.14	<DL	0.03	0.11	0.20	0.24	<DL	0.31
Ba	0.09	0.60	0.61	0.74	0.76	3.86	4.16	36.9	73.7	86.1	0.08	1.90
Li	11	47	87	133	169	53	60	142	226	236	3.0	33
Be	<DL	2.13	2.53	4.55	6.33	2.66	2.82	2.89	2.94	3.05	<DL	2.65
V	<DL	<DL	<DL	<DL	59	<DL	<DL	64	124	140	<DL	<DL
Cr	776	780	1148	1870	9476	61	67	115	289	670	<DL	120
Co	24	103	114	315	510	39	39	76	120	141	0.2	46
Ga	<DL	<DL	1.79	1.89	2.14	<DL	1.11	9.05	17.3	19.1	0.20	2.57
Ge	<DL	<DL	1.05	1.08	2.51	<DL	0.66	1.63	2.60	3.02	<DL	<DL
As	20	23	28	29	125	10	14	44	84	115	<DL	15
Se	4.8	8.2	11	13	22	4.6	6.1	24	44	54	<DL	8.8
Y	1.2	1.9	34.5	80.5	128	16.0	16.0	21.6	32.0	46.8	<DL	34.3
Pd	<DL	<DL	<DL	0.59	0.60	<DL	0.32	0.39	0.43	0.53	<DL	<DL
Ag	<DL	<DL	<DL	0.30	0.84	<DL	<DL	0.29	0.73	1.42	<DL	0.22
Cd	<DL	0.84	1.81	2.90	7.15	3.02	5.34	7.02	8.13	8.70	<DL	4.62
In	<DL	<DL	0.16	0.23	0.91	<DL	<DL	0.23	0.47	0.94	0.90	<DL
Sn	3.4	6.3	6.7	29.1	33.9	2.3	3.7	27.7	54.4	64.2	<DL	15.4
Sb	3.46	4.04	4.16	5.29	7.52	1.60	2.52	9.46	61.2	197	0.82	4.48
Te	<DL	<DL	0.71	0.81	0.86	<DL	<DL	0.48	0.93	1.52	<DL	<DL
Cs	<DL	4.0	38.4	61.5	1003	1.9	7.9	109	369	852	<DL	174
La	<DL	2.0	9.7	16.6	71.5	28.7	29.0	30.8	32.9	34.3	<DL	20.6
Ce	1.2	3.8	7.6	11.2	21.9	44.0	46.1	51.2	57.0	60.9	0.4	50.3
Pr	0.09	0.54	1.72	3.35	6.06	4.78	5.34	5.95	6.60	7.27	<DL	6.77

(*Continues*)

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Table 3. (*Continued*)

	Tea ($N=5$)					Acacia ($N=4$)					Officinal plant ($N=1$)	Tara ($N=1$)
	Min	25th	m	75th	Max	Min	25th	m	75th	Max		
Nd	0.6	1.6	6.9	16.5	29.8	19.5	20.6	23.0	25.4	26.4	0.2	26.8
Sm	<DL	0.57	2.31	5.49	9.26	3.45	4.31	4.62	4.97	5.95	0.30	7.30
Eu	0.35	0.63	0.89	2.54	3.87	1.58	1.86	3.49	5.07	5.18	0.30	1.83
Gd	0.19	0.67	3.26	8.20	13.8	4.11	4.45	4.85	5.60	7.03	0.20	8.01
Dy	0.20	0.60	3.96	11.3	18.0	2.53	2.86	3.43	4.36	5.75	0.28	7.34
Ho	0.061	0.338	1.09	2.62	4.07	0.479	0.734	0.829	0.961	1.33	0.271	1.63
Er	0.130	0.453	2.80	7.77	12.5	1.32	1.53	1.98	2.67	3.59	0.228	3.73
Tm	<DL	0.202	0.635	1.26	1.94	0.191	0.294	0.340	0.418	0.618	0.162	0.810
Yb	0.173	0.410	2.92	8.61	13.5	0.925	1.08	1.57	2.25	2.98	0.159	3.26
Re	0.175	0.203	0.659	0.706	0.863	0.149	0.284	0.410	0.601	0.933	0.082	0.717
Hg	<DL	<DL	0.87	1.33	14.6	<DL	0.59	0.80	2.10	5.74	<DL	1.68
Tl	<DL	16.9	25.9	83.3	124	4.5	8.9	38.7	68.5	73.3	<DL	4.2
Pb	3.7	12.3	17.5	17.9	30.2	18.5	18.5	138	510	1269	<DL	61.1
Th	<DL	<DL	<DL	<DL	<DL	<DL	<DL	10	17	19	<DL	<DL
U	0.50	2.32	5.53	9.95	11.9	1.01	3.78	16.2	29.8	36.0	<DL	3.94

$p < 0.001$), or those present only in a small number of samples (Ir, Pt, Au, Th and Bi; quantifiable in less than 20% of samples) were not considered in the traceability modelling. In order to assess efficiency, in terms of the ability to discriminate the botanical origin of tannins, canonical standard and forward stepwise discriminant analysis were performed, considering 31 parameters ($\delta^{13}C$, Li, Be, B, Na, Mg, P, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Cd, Sn, Sb, Ba, Sm, Re, Tl, Pb and U).

Tukey's honestly significant difference test ($p < 0.05$) for an unequal number of samples was applied to check for significant differences in the content of tannins of different botanical origin. All statistical analysis was carried out using Statistica 9.1.[25]

Results and discussion

Tables 1, 2 and 3 show the statistical distribution of the 54 elements analysed and the $\delta^{13}C$ values of the 102 tannins, grouped according to the 10 different botanical origins. Only Li, B, Al, K, Co, Sb, Ba, Eu, Dy and Re were quantifiable in all the samples, Ti, Hg and Ge in about 75% of samples, Pd, Cs, In and V in around 60%,Te in 45% and Th, Ir, Bi and Au in less than 20% of samples, whereas Pt (DL = 0.5 μg/kg) was always not detectable. In particular, Bi was quantifiable in trace amounts only in some grape and gall tannins (DL = 8 μg/kg), while Au (DL = 1.8 μg/kg) and Ir (DL = 0.1 μg/kg) were only quantifiable in some grape and oak tannins.

Considering only the 8 botanical groups with at least 4 samples, the Li, Na, Mg, P, K, Mn, Ni, Cu, As, Se, Rb, Sr, Mo and U content was statistically lowest in tannins from gall. These tannins were also characterised by a low Be, B, Cr, Co, Cd, Sb, Ba and Tl content.

The Be, Mn, Co, Ga, Cd, Ba and Pb content was statistically highest in tannins from chestnut. This group also showed a high content of Na, Mg, Ca, Ni, Sr and rare earth elements. Tannins from grapes were the richest in P, Fe, Cu, Se, Mo, Sb, Re and U, whereas tannins from oak showed the highest B and Tl content and the lowest content of Fe, Ga and rare earths.

Statistically, the highest Li content was observed in tannins from quebracho, also characterised by a high Na, Mg, Cr and Sr content and a low Co content. Tannins from tea showed the statistically lowest Ca and Pb content. They also had a low B, Cd, Sb and Ba content and a high Cr and Ni content. Statistically, the highest content was observed in the acacia samples, which also had a high content of Mg, K, Ca, Sr and rare earths.

The overall mean content was of the same order of magnitude as the data reported by Nicolini *et al.* (2004)[26] except for Sr (mean = 10.5 mg/kg) and Cu (4.6 mg/kg) content, which was higher in this work in comparison with the values reported in the literature (2 mg/kg and 1.8 mg/kg, respectively), and for Ga (6.54 μg/kg), Pd (0.50 μg/kg), Cd (8.90 μg/kg) Sn (64 μg/kg) and Te (0.49 μg/kg) content, which was decidedly lower (being 1203 μg/kg, 318 μg/kg, 48 μg/kg, 2226 μg/kg and 13 μg/kg, respectively in the literature).

The International Oenological Codex (COEI-1-TANINS: 2009) establishes the maximum concentration for certain toxic elements in tannins: As must be under 3 mg/kg, Fe under 50 mg/kg, Pb under 5 mg/kg and Hg under 1 mg/kg. The As, Pb and Hg content was always well below these limits, while in 31 samples (about 30% of samples: 19 samples from grapes, all 5 samples from chestnut, 2 each from fruit trees, quebracho and acacia and 1 sample from gall) the limit set for Fe was exceeded, with a maximum content of 409 mg/kg in a grape tannin.

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The $\delta^{13}C$ values were significantly more negative in tannins from grapes, tea and acacia and less negative in tannins from oak (Fig. 1). The $\delta^{13}C$ of plants is mainly affected by the photosynthetic cycle of the plant (discrimination between C3 and C4 plants).[27] Trees and shrubs show generally similar $\delta^{13}C$ values, whereas herbages have significantly lower values.[28] When plants belong to the same photosynthetic cycle, as in this case, several environmental and physiological factors, such as relative humidity, temperature, amount of precipitation, drought stress and water use efficiency (WUE), plant age and maturation, have an impact on the $\delta^{13}C$ of vegetal compounds, as they influence stomatal conductance and the intercellular and ambient CO_2 concentration.[29–32] On this basis, the differences observed here can be attributed to the different geographical origins of the plants, linked to different climatic conditions or a different physiology, namely a different WUE.

Forward stepwise discriminant analysis was performed to evaluate the possibility of distinguishing the 8 most numerous botanical groups on the basis of the 31 compositional parameters selected. Stepwise analysis excluded 4 parameters: Sr, Sn, Sb and Pb. The most discriminant parameters were $\delta^{13}C$, Mn and Ba. The combination of the first 3 canonical variables accounted for 86% of the variability observed, assuring a good ability to discriminate the origin of tannins (Fig. 2). The least homogeneous and identifiable group was made up of tannins from fruit trees, probably because of the inhomogeneity of this broad commercial category, which included different plant species, such as cherry, citrus and other trees not easily botanically identifiable from the labels. The substantial variation observed within groups could also be due to the different geographical origin of samples belonging to the same botanical category. Despite this, all the tannins were correctly classified in accordance with their botanical origin (Table 4).

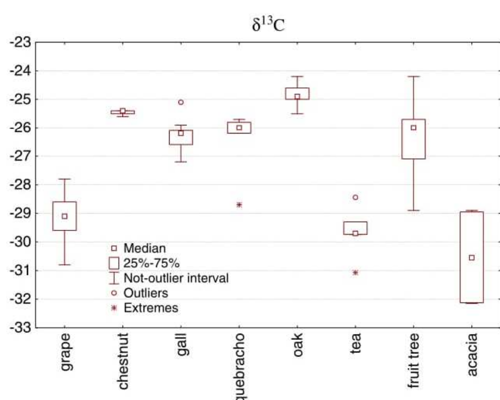

Figure 1. Box plot of $\delta^{13}C$ values in the 8 principal groups of tannins.

To verify the ability and effectiveness of this model in predicting discrimination, canonical standard discriminant analysis was carried out with the 27 parameters selected using the previous statistical processing, with a random subset made up of 90% of the complete dataset in order to build the predictive model, while the remaining 10% of samples was used as a validation subset. This procedure was repeated 5 times with different randomly selected subsets, representative of the different botanical groups. Each validation subset always included 4 tannins from grapes, 2 from oak, 1 from gall and rotating samples from the other minor groups, for a total of 10 samples. The first and second subsets included 1 chestnut, 1 quebracho and 1 fruit tree; the third subset included 1 chestnut, 1 tea and 1 fruit tree; the forth 1 quebracho and 1 tea (this subset having 5 tannins from grapes); the fifth 1 chestnut, 1 quebracho and 1 tea.

In 4 out of 5 models, all the samples were correctly classified. Only one model provided 98% correct classification, with 2 wrong assignments: 1 oak (commercially classified as 'Ellagic acid from oak') was grouped as gall, whereas one tea was classified as grape.

Considering all the 11 groups of tannins (toasted oak, untoasted oak, grape, grape skin, grape seeds, gall, fruit trees, chestnut, quebracho, tea and acacia) the model provided 91% correct classification. In detail, misassignment was observed only within the same botanical groups. Four out of 8 grape samples were assigned to grape seed ($N = 3$) and grape skin ($N = 1$) tannins; 3 out of 16 grape seed tannins were classified as grape skin ($N = 2$) and grape ($N = 1$) tannins; 1 of the 9 untoasted oak samples was classified as a toasted oak tannin whereas 1 of the 12 toasted oak samples was grouped with untoasted oak tannins.

The attempt to classify the tara ($N = 1$) and officinal plant ($N = 1$) tannin samples using the aforementioned model, built without considering these different botanical groups due to the insufficient number of samples, gave interesting results: the tara sample (Root1 = −1.47; Root2 = −3.47; Root3 = −2.52 considering Fig. 2) was classified as acacia, whereas the officinal plant tannin (Root1 =

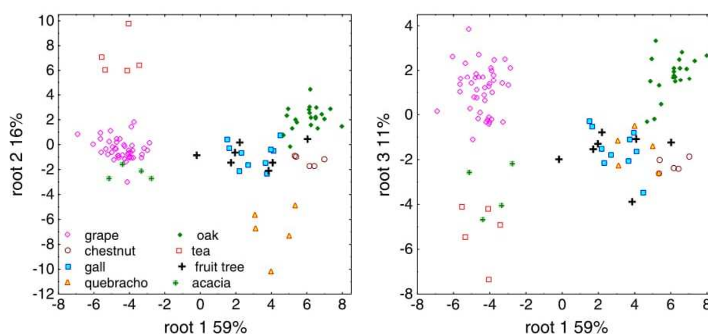

Figure 2. Scatter plot of the first three canonical variables in stepwise discriminant analysis based on the elemental and isotopic composition of 100 tannins of 8 botanical origins.

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Table 4. Classification matrix of 100 tannins in 8 botanical groups

	%	Grape	Chestnut	Gall	Quebracho	Oak	Tea	Fruit tree	Acacia
Grape	100	43	0	0	0	0	0	0	0
Chestnut	100	0	5	0	0	0	0	0	0
Gall	100	0	0	10	0	0	0	0	0
Quebracho	100	0	0	0	5	0	0	0	0
Oak	100	0	0	0	0	21	0	0	0
Tea	100	0	0	0	0	0	5	0	0
Fruit tree	100	0	0	0	0	0	0	7	0
Acacia	100	0	0	0	0	0	0	0	4
Total	100	43	5	10	5	21	5	7	4

4.13; Root2 = −3.66; Root3 = −4.10 considering Fig. 2) was classified as gall. Nevertheless, graphically, these 2 samples were placed in distinct areas, slightly aside from or at the limit of the group of formal assignation, giving a further 'visual' tool for classifying unknown commercial samples classified in this ways as questionable.

In order to achieve better differentiation between toasted and untoasted oak wood tannins ($N = 21$), forward stepwise discriminant analysis was performed. The new model produced selected Cd, Co, Cr, Cu, Fe, Ni, Mn and Sm as the most predictive parameters, and was capable of effectively distinguishing the 2 categories, correctly grouping all but one of the samples (Fig. 3). Interestingly, toasted oak tannins were characterised by a higher Mn and Cd content, the latter being a toxic metal, probably deriving from the wood preparation procedure in the ignition process. On the other hand, the Co, Cr, Cu, Fe, Ni and Sm content in toasted samples was lower.

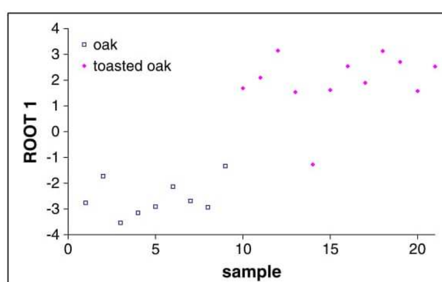

Figure 3. Discrimination between tannins originating from oak ($N = 9$) and toasted oak ($N = 12$) on the basis of Cd, Co, Cr, Cu, Fe, Ni, Mn and Sm content. Scatter plot of ROOT 1 variable (forward stepwise discriminant analysis).

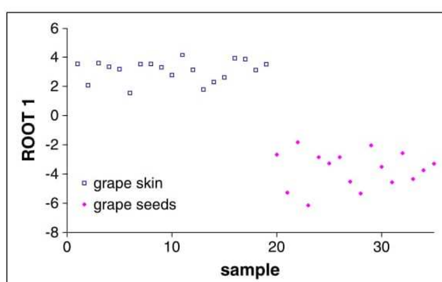

Figure 4. Discrimination between tannins originating from grape skin ($N = 19$) and grape seeds ($N = 16$). Scatter plot of ROOT 1 variable (forward stepwise discriminant analysis).

A similar statistical process was used to try to distinguish tannins from different grape tissues (skin, $N = 19$ and seeds, $N = 16$). This model was shown to be perfectly capable of discriminating between these 2 groups of grape tannins (Fig. 4), with 100% correct reclassification.

The parameters with higher standardised coefficient for the created variable were Re (4.9), As (2.6), Cu (2.1), P (−3.0), Se (−2.9) and Na (−2.4). The Cr, Fe, Na, Ni, Sb, Sn, Re and U content was statistically higher in tannins from grape skin whereas tannins from grape seeds were characterised by a high content of K, P and Se.

Conclusions

This work presents the composition of macro-, micro- and trace elements, in addition to the $^{13}C/^{12}C$ ratio values for different types of commercial tannins, about which little information is available in the literature. Moreover, it also shows the effectiveness and reliability of the joint use of the mineral element profile and ^{13}C isotopic ratio in assessing the botanical origin of commercial tannins, widely used in the food industry and winemaking as adjuvants.

This approach, achieving 100% correct classification of 100 tannins belonging to 8 different botanical groups, provided effective differentiation of tannins of different origin and could be a different or complementary tool to the approaches based on sugar or polyphenolic composition suggested by OIV.

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SECTION 4.6

Stable isotope ratio analysis for verifying the authenticity of balsamic and wine vinegar

Perini M., Paolini M., Simoni M., Bontempo L., Vrhovsek U., Sacco M.,
Thomas F., Jamin E., Hermann A., Camin F.

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Statement of the author: My personal contribution to this work mainly concerned the setting up of instrumental conditions, analysis and validation of the method to measure the carbon isotopic value of acetic acid extracted from balsamic and wine vinegard using GC-C-IRMS.

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Stable Isotope Ratio Analysis for Verifying the Authenticity of Balsamic and Wine Vinegar

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S *Supporting Information*

ABSTRACT: In this paper, we investigate whether the analysis of stable isotope ratios D/H and $^{13}C/^{12}C$ in ethanol and acetic acid and of $^{18}O/^{16}O$ in water can be applied to the ingredients of "aceto balsamico di Modena IGP" (ABM) to evaluate their authenticity. We found that impurities in the extraction solution do not affect the $^{13}C/^{12}C$ of acetic acid and the D/H values of acetic acid are not affected under a composite NMR experiment. The standard deviation of repeatability and standard deviation of reproducibility are comparable in wine vinegar and ABM and generally lower than those quoted in the official methods. This means that the validation parameters quoted in the official methods can also be applied to the ingredients of ABM. In addition, we found no changes in the isotopic values from wine to vinegar and to ABM, and from the original must to the ABM must, providing experimental evidence that reference data from wine databanks can also be used to evaluate the authenticity of vinegar and ABM.

KEYWORDS: *D/H, $\delta^{13}C$, $\delta^{18}O$, isotope ratio mass spectrometry, SNIF-NMR, vinegar authenticity*

INTRODUCTION

Wine vinegar is defined by EC Regulations 479/2008 (Annex IV, sections 1 and 17) as a product obtained from the acetous fermentation of wine, which is in turn defined as a product obtained exclusively from the alcoholic fermentation of fresh grapes, whether crushed or not, or from grape must. According to this definition, wine vinegar cannot contain acetic acids obtained from either petroleum derivatives or pyrolysis of wood (synthetic acetic acid) or from the fermentation of nongrape sugars (e.g., from beet or cane). Moreover, wine and wine vinegar cannot be produced from dried grapes diluted with water; therefore, the so-called "raisin vinegar", commonly produced in some Mediterranean countries by fermenting dried grapes and rehydrating with tap water, cannot be considered wine vinegar.[1]

This also applies to "aceto balsamico di Modena IGP" (ABM), a PGI (Protected Geographical Indication) vinegar now renowned throughout the world, obtained from cooked and/or concentrated grape must (at least 20% of the volume), with the addition of at least 10% of wine vinegar and a maximum 2% of caramel for color stability (EU Reg. 583/2009). The geographical origin of ABM ingredients is not specified.

In the case of must and wine, since 1991 the addition of water and exogenous sugars has been detected by analyzing the isotopic ratios of hydrogen (D/H) and carbon ($^{13}C/^{12}C$) in ethanol and of oxygen ($^{18}O/^{16}O$) in water. OIV (International Organization of Vine and Wine) methods are currently adopted: OIV-MA-AS311-05 for site-specific analysis of the D/H ratio using ^{2}H-site-specific natural isotope fractionation NMR (^{2}H-SNIF-NMR), OIV-MA-AS312-06 for analysis of the $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$ ‰) using isotope ratio mass spectrometry (IRMS), OIV-MA-AS2-12 for analysis of the $^{18}O/^{16}O$ ratio (expressed as $\delta^{18}O$ ‰) using IRMS. Addition is detected by comparing the results against an appropriate databank, such as the official databank set up in 1991 (EU Regulations 2347 and 2348/91) by the European Union for all wine-producing countries within its territory. According to current regulations (EU Regulation 555/2008), a number of samples representing the wine production of each member state are officially collected every year and analyzed using the above-mentioned OIV methods. The isotope data bank makes available reference data on a yearly basis, thus allowing legal limits to be defined on the basis of isotopic data for each country, each subarea (e.g., region), and each protected denomination (PDO-IGP), as well as general limits[2] when origin and year of production are not declared.

Very recently, isotopic methods have been recognized by the European Committee for Standardization (CEN) and in part by OIV as a means of detecting the presence of exogenous acetic acid and tap water in wine vinegar. The methods are EN 16466-1 for D/H in the methyl site of acetic acid using ^{2}H-SNIF-NMR, EN 16466-2 and OIV 510/2013 for analysis of $^{13}C/^{12}C$ in acetic acid using IRMS, and EN 16466-3 and OIV 511/2013 for analysis of $^{18}O/^{16}O$ in water using IRMS.

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Moreover, a recent paper[1] showed that the legal limits set on the basis of the wine isotope databank can be used as reference for $\delta^{18}O$ analysis to detect the authenticity of wine vinegar.

As yet, no studies have investigated application of these techniques to the ingredients of ABM (wine vinegar and must) or the possibility of using the wine databank as reference for vinegar and ABM for the isotopic ratios D/H and $\delta^{13}C$.

In this work, we aim to fill these gaps by investigating whether official isotopic methods recognized by OIV and CEN for must and wine vinegar can be used to analyze ABM. To do this, we verified and quantified the presence of impurities in solutions extracted from wine vinegar and ABM, determined the validation parameters of the methods applied to the ingredients of AMB, and compared them with those quoted in the CEN and OIV standards. We also evaluated the variation of D/H and $^{13}C/^{12}C$ between wine ethanol and acetic acid in wine vinegar and ABM in order to determine whether the wine isotope databank can also be used as reference for D/H and $\delta^{13}C$ of acetic acid in order to monitor the authenticity of vinegar and ABM.

MATERIALS AND METHODS

Samples. To assess variation in isotopic parameters along the production chain (Table 1), 13 production chains were considered (Table 1). Nine of them consisted of wine, the corresponding wine vinegar, grape must (100% concentrated must or 100% cooked must or mixtures of the two types), and the corresponding ABM. The other four were obtained directly from vinegar rather than starting from wine. Cane sugar acetic acid was added to two of these vinegar samples (chains 11 and 12).

Table 1. Summary of the Production Chain Samples[a]

chain	wine	vinegar	cooked must	concentrated must	ABM
1	×	×	× (100%)		×
2	×	×	× (52%)	× (48%)	×
3	×	×	× (20%)	× (80%)	×
4	×	×	× (20%)	× (80%)	×
5	×	×	× (50%)	× (50%)	×
6	×	×	× (50%)	× (50%)	×
7	×	×	× (50%)	× (50%)	×
8	×	×		× (100%)	×
9	×	×		× (100%)	×
10		×	× (54%)	× (46%)	×
11		×	× (50%)	× (50%)	×
12		×	× (50%)	× (50%)	×
13		×		× (100%)	×

[a]Crosses indicate samples from each chain. The first nine chains were started from wine, the other four from vinegar. Cooked grape must or concentrated must or a mixture of the two in different percentages (in parentheses) were used to produce the ABM.

To evaluate the effects of adulteration with exogenous sugars, one sample of wine was divided into seven parts to which were added increasing percentages of alcohol from cane sugar (from 0% to 42%) before being fermented into vinegar. Two of these vinegars (with 10% and 42% cane alcohol added) were used to make ABMs. In addition, two samples of ABM were adulterated with different percentages of synthetic vinegar (20% and 40%).

In the production of ABM, a maximum 2% of caramel can be added for color stability, tangential filtering is usual, and the product may be concentrated to increase its density. To evaluate the effects of these processes, one sample of vinegar was divided into three parts: one was used to produce ABM without filtration, another underwent tangential filtration but no caramel was added, while the third underwent filtration and addition of caramel (1.79%). Another sample of vinegar was divided into three parts, and ABM was produced without concentration from one subsample, and at two different degrees of concentration (45°Brix and 57°Brix) from the other subsamples.

Pilot Production of Vinegar and ABM. Wines were transformed into vinegars using a couple of fully automated pilot fermenters (each with a capacity of 8 L) and a continuous fermentation process (at least 10 days per sample) identical to the industrial process with respect to time, temperature, and concentration of alcohol and acetic acid (40 h to entirely transform the alcohol into acetic acid, 34−36 °C, 10.0−10.4% of alcohol in wine, transformation efficiency 95−97%). Oxidation of ethanol was performed by means of *Acetobacter* spp. bacteria.

The ABM was produced according to EU Reg. 583/2009.

When evaluating the effects of adulteration with exogenous sugars, in order to correctly measure the percentages of the different raw materials in each fermentation process, lithium (30 ppm) was added to the wine as a marker; the concentration of each new raw material was calculated on the basis of the lithium concentration in the product (measured using atomic absorption spectrometry).

Tangential filtration was carried out by passing the vinegar through a polysulphone membrane with a porosity of 0.6 μm; the maximum temperature throughout the process was below 37 °C.

ABM concentration was carried out in batches using pilot vacuum equipment normally used for jam production. The samples were collected at different times during a single concentration process.

Methods of Isotopic Analysis. Ethanol was distilled from wine samples following the official OIV method (MA-AS-311-05). Acetic acid was extracted from the vinegars and ABMs according to OIV method 510/2013. Briefly, acetic acid was extracted with diethyl ether or *tert*-butyl methyl ether by liquid−liquid extraction for at least 5 h, and the solvent was then eliminated by distillation using a column that prevents isotopic fractionation of acetic acid. The samples of must and of ABM residue after acetic acid extraction underwent alcoholic fermentation. Before fermentation, the ABM residue was heated to 40° for at least 6 h to remove residual ether. Each sample was inoculated with a fresh culture of *Saccharomyces cerevisiae* (strain ATCC 9763) without added water. Fermentation was carried out in glass bottles with a bubbler valve in the cap; each bottle contained 0.7 L of sample, and the temperature was adjusted to 25 °C. At the end of fermentation, the ethanol was extracted by distillation, according to the procedure described in the OIV MA-AS-311-05 method. The Brix degree of must was measured following the OIV-MA-AS2−02 R2009 method.

SNIF-NMR Analysis. The D/H of ethanol from wine and must fermentation (cooked and concentrated as well as the ABM residue after acetic acid extraction) and of acetic acid from vinegar and ABM was determined by SNIF-NMR (FT-NMR AVANCE III 400, Bruker BioSpin GmbH, Karlsruhe, Germany). An NMR spectrometer fitted with a selective 10 mm "deuterium" probe tuned to a frequency Vo characteristic of channel Bo (e.g., Bo = 9.4 T, νo = 61.4 MHz) with a decoupling channel (B2) and a field-frequency stabilization channel (lock) at the fluorine frequency. The second channel B2 was also used in the ^{1}H NMR experiment for acetic acid analysis. The resolution measured on the ^{2}H-SNIF-NMR spectrum, transformed without exponential multiplication (i.e., LB = 0) and expressed by the width at the half-height of the methyl signal of acetic acid and the methyl signal of TMU (see below for definition) was less than 0.5 Hz. Sensitivity (signal-to-noise ratio) measured when the FID was exponentially weighted by a factor equal to 2 Hz line B broadening (LB), was greater than or equal to 150 for the methyl signal of acetic acid containing less than 25% of water.

Site-specific D/H values were measured in the methyl and methylene sites of ethanol ($(D/H)_I$ or $(D/H)_{CH3}$ and $(D/H)_{II}$) and in the methyl site of acetic acid ($(D/H)_{CH3}$) and expressed in parts per million (ppm). The method described in OIV MA-AS-311-05 was used for wine and must, while for vinegar, we followed the method described in EN 16466-1 with certain modifications that took into account the fact that impurities can be extracted at the organic phase together with acetic acid (see the Results and Discussion section).

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3.25 gr of the extract containing acetic acid after filtration was placed in a 10 mm NMR tube together with 1.1 g of the internal standard tetramethylurea (TMU, certified reference material, STA-003m, EC-JRC, Geel, Belgium), as described in EN 16466-1, and a sufficient quantity of lock substance (for example: 50 μL of hexafluorobenzene or 150 μL of a homogenized mixture of hexafluorobenzene (C_6F_6)/trifluoroacetic acid (TFA) 90/10 v/v). This sample was then measured in the NMR tube by ^{1}H NMR and ^{2}H-SNIF-NMR by means of a composite NMR experiment.[3,4]

Typical conditions for obtaining ^{2}H-SNIF-NMR spectra are as follows: constant probe temperature (e.g., 303 K); rotation of the sample (e.g., 15−20 Hz); acquisition time of at least 5.5 s for 1200 Hz spectral width (i.e., about 20 × 10^{-6} at 46.1 MHz); 90° pulse; quadrature detection, fix the offset O1 between the OD and CH_2D reference signals for acetic acid; and determination of the value of the decoupling offset O2 from the ^{1}H NMR spectrum measured by the decoupling coil on the same tube. Good decoupling is obtained when O2 is located in the middle of the frequency interval lying between the CH_3 and TMU groups. Use of broad band decoupling sequence WALTZ-16.

Typical conditions for obtaining ^{1}H NMR spectra are as follows: constant probe temperature (e.g., 303 K); rotation of the sample (e.g., 15−20 Hz); acquisition time of at least 4.1 s for 8000 Hz spectral width (i.e., about 20 × 10−6 at 400 MHz); 30° pulse or less; D1 = 7s at least; parabolic detection, fix the offset O1 between the OH and CH_3 reference signals for acetic acid; and no decoupling.

For each spectrum, a number of accumulations sufficient to obtain the signal-to-noise ratio given above were carried out.

Appropriate software based on a complex least-squares curve fitting algorithm should be used to determine the signal area (first-order phasing and baseline correction are sensitive parameters for the proper evaluation of signal areas and have to be correctly adjusted). When larger errors in phasing and baseline distortions can safely be excluded (e.g., by applying suitable spectrometer routines for phasing and baseline correction) software based on the real part of the complex NMR data can be used instead.

For each ^{1}H NMR spectrum, we calculated the ratio $S_{TMU}/S_{acetic\ acid}$, where S is the ^{1}H NMR signal area, integrated by data processing software after Fourier transform of the free induction decay, with an appropriate line-broadening factor of 0.5 Hz.

For each ^{2}H-SNIF-NMR spectrum, we calculated the ratio $S'_{acetic\ acid}/S'_{TMU}$, where S' is the ^{2}H NMR signal area, integrated by data processing software after Fourier transform of the free induction decay, with a line-broadening factor of 2 Hz.

The ratio $(D/H)_{CH3}$ is calculated as follows:

$$(D/H)_{CH3} = S'_{acetic\ acid}/S'_{TMU} \times S_{TMU}/S_{acetic\ acid} \times (D/H)_{TMU}$$

where $(D/H)_{TMU}$ is the certified deuterium content in parts per million of the TMU provided by the supplier of the reference product.

Appropriate software enabled these three successive calculations to be made.

The standard deviation of repeatability (sr) of $(D/H)_{CH3}$ quoted in the methods (OIV AS311-05, EN 16466-1) is 0.3 for wine and must and 0.6 ppm for vinegar, whereas the standard deviation of reproducibility (sR) is 0.4 for wine and must and 0.6 ppm for vinegar.

IRMS Analysis. The $^{13}C/^{12}C$ of ethanol and acetic acid was measured using IRMS (SIRA II-VG ISOGAS, FISIONS, Rodano, Milano, Italy, or DELTA V, Thermo Scientific, Germany) interfaced with an elemental analyzer (Flash 1112, Carlo Erba, Milano, Italy, or EA Flash 1112, Thermo Scientific), according to the procedures described in OIV-MA-AS312-06, OIV 510/2013 and EN 16466-2.

To evaluate the impact of the impurities present in the extraction solution of acetic acid, we measured the $^{13}C/^{12}C$ in pure acetic acid using gas chromatography combustion (GC-C; Trace GC Ultra, Thermo Scientific) interfaced with an IRMS (DELTA V, Thermo Scientific) through an open split interface. A ZB-FFAP capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex) with He as carrier gas (at a flow rate of 2.7 mL/min) was used. A volume of 1.5 μL of a solution of acetic acid/diethyl ether (1:5) was injected in split mode by an autosampler (Triplus, Thermo Scientific). The injector temperature was set at 250 °C, and the oven temperature of the GC set at an initial 50 °C where it was held for 2.5 min before heating at 70 °C/min to 150 °C and held for 20 min. The eluted compound was combusted into CO_2 and H_2O in a combustion furnace reactor at 1000 °C and composed of a ceramic tube (320 mm in length) containing an NiO tube in combination with NiO and CuO wires (0.125 mm diameter, 240 mm identical length) braided and centered end-to-end within the tube. Water vapor was removed with a water-removing trap consisting of a Nafion membrane.

The $^{18}O/^{16}O$ analyses of wine, must, vinegar, and ABM were performed using an isotope ratio mass spectrometer (SIRA II, VG Fisons, Middlewich, UK, or Isoprime, Cheadle, UK) connected to a water/CO_2 equilibration system (Isoprep 18, VG Fisons or Isoprime Multiflow) according to the procedures described in the OIV-MA-AS2-12, OIV 511/2013, and EN 16466-3 methods for wine, must, and vinegar.

The $^{13}C/^{12}C$ and $^{18}O/^{16}O$ values are expressed on the δ ‰ scale against international standards V-PDB (Vienna-Pee Dee Belemnite, IAEA, Vienna) and V-SMOW (Vienna-Standard Mean Ocean Water) normalized to the VSMOW−SLAP (Standard Light Antarctic Precipitation) scale, respectively, according to the equation: $\delta‰ = ((R_{sample} - R_{standard})/(R_{standard}))\cdot 1000$ where R is the ratio of the heavy to light stable isotope in the sample (R_{sample}) and the international reference material ($R_{standard}$).

The sr of $\delta^{18}O$ quoted in the OIV and EN methods is 0.1 ‰ for wine, must, and vinegar, while the sr of $\delta^{13}C$ is 0.1 ‰ for wine and must and 0.2 ‰ for vinegar. The sR of $\delta^{18}O$ is 0.2 ‰ for wine, must, and vinegar, while the sR of $\delta^{13}C$ is 0.2 ‰ for wine and must and 0.3 ‰ for vinegar.

GC-MS/MS Analysis. The solutions after vinegar and ABM vinegar extraction were filtrated with a 0.22 μm filter and then diluted 5 times with 80% acetic acid (LC/MS grade), and for analysis of acetic acid, 2000 times with water (Milli-Q).

GC analysis was performed using a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum tandem mass spectrometer upgraded to the XLS configuration. A DuraBrite IRIS ion source with prefilter was installed to improve performance of the spectrometer. The system was fitted with a Triplus autosampler (Thermo Electron Corporation, Waltham, MA, USA). The injection volume was 1 μL, postinjection dwell time was 4 s, and tray temperature was 5 °C. GC separation was performed on a 30 m VF-WAXms capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (Varian, Inc., Palo Alto, CA, USA). The temperature program was as follows: 40 °C hold for 2 min after injection, 10 °C/min up to 50 °C, 1.4 °C/min up to 60 °C, hold for 2 min, 1.6 °C/min up to 70 °C, hold for 1 min, 2.2 °C/min up to 100 °C, hold for 0.5 min, 3.1 °C/min up to 140 °C, 4.4 °C/min up to 200 °C, 12 °C/min up to 250 °C, hold for 6 min. Injection parameters were as follows: split injection, split ratio 150 (split flow 120 mL/min), inlet temperature 250 °C, carrier gas was helium 5.5, programmed flow: 0.8 mL/min hold for 62.50 min, 0.8 mL/min up to 1.2 mL/min in 0.5 min, hold for 7 min. The mass spectrometer was operated in electron impact (EI) ionization mode at 70 eV. The temperature of the transfer line was 220 °C. Mass spectrometer parameters were EI ionization mode at 70 eV. Full scan acquisition from m/z 40 to m/z 400 using the third quadripole with a scan time of 0.200 s. Source temperature was 250 °C, and filament emission current was 50 μA. The mass spectrometer was tuned and calibrated using FC-43 (perfluorotributylamine (PFTBA)). Data acquisition and analyses were performed using Xcalibur Workstation software supplied by the manufacturer. Quantification was performed using Trace finder EFS 3.0 software (Thermo Scientific).

Statistical Analysis. The data were analyzed using Statistica v.9 software (StatSoft Italia srl, Padua, Italy). A paired t-test was carried out on the data set to identify differences between samples within the production chains.

ANOVA, honestly significant difference (HSD) Tukey, Kruskall−Wallis, and U-Mann−Whitney tests were carried out on the data set to identify differences between the various groups.

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Table 2. Mean % of Volatile Compounds in the Extraction Solutions of Vinegar and ABM

		vinegar (n = 10)				ABM (n = 11)			
compd	confirmed by[a]	mean	std dev	min	max	media	std dev	min	max
ethyl ether	STD MS	0.459	0.543	0.010	1.560	0.274	0.353	0.024	0.921
methyl acetate	RI MS	0.006	0.007	0.000	0.024	0.006	0.005	0.002	0.015
ethyl acetate	STD MS	0.843	0.756	0.087	2.677	1.631	1.829	0.072	5.867
2,3-butanedione	STD MS	0.043	0.048	0.001	0.163	0.045	0.050	0.002	0.147
isobutyl acetate	STD MS	0.024	0.032	0.003	0.100	0.033	0.038	0.003	0.127
acetoin	STD MS	0.550	0.570	0.018	1.752	0.588	0.469	0.048	1.358
ethyl lactate	STD MS	0.023	0.038	0.000	0.120	0.037	0.043	0.000	0.120
ethanol, 2,2′-oxybis-, diacetate	RI MS	0.226	0.194	0.009	0.644	0.243	0.223	0.018	0.714
acetic acid	STD MS	96.072	3.292	88.053	98.571	94.207	4.136	87.096	97.901
1,2-propanediol, diacetate	RI MS	0.013	0.007	0.000	0.021	0.033	0.018	0.016	0.075
propanoic acid	STD MS	0.128	0.093	0.056	0.308	0.137	0.068	0.047	0.244
butanoic acid, 3-hydroxy-, ethyl ester	RI MS	0.340	0.276	0.024	0.944	0.439	0.296	0.114	0.848
1-methoxy-2-propyl acetate	RI MS	0.140	0.127	0.009	0.406	0.229	0.138	0.098	0.452
butanoic acid	STD MS	0.008	0.023	0.000	0.075	0.006	0.019	0.000	0.062
1,3-propanediol, diacetate	RI MS	0.179	0.245	0.010	0.735	0.252	0.434	0.028	1.424
isovaleric acid	STD MS	0.005	0.006	0.000	0.017	0.006	0.007	0.000	0.017
diethyl succinate	RI MS	0.019	0.026	0.000	0.083	0.027	0.029	0.000	0.073
benzyl acetate	STD MS	0.008	0.008	0.000	0.023	0.009	0.011	0.000	0.037
phenethyl acetate	STD MS	0.123	0.109	0.002	0.296	0.157	0.135	0.015	0.364
phenethyl alcohol	STD MS	0.009	0.011	0.000	0.036	0.012	0.016	0.000	0.057
butylated hydroxytoluene	STD MS	0.025	0.032	0.000	0.080	0.060	0.046	0.011	0.142
glycerol 1,2-diacetate*	MS	0.026	0.017	0.000	0.048	0.047	0.034	0.013	0.113
5-acetoxymethyl-2-furaldehyde	RI MS	0.001	0.001	0.000	0.003	0.323	0.257	0.035	0.742
1,2,3-propanetriol, 1-acetate*	MS	0.074	0.051	0.001	0.161	0.134	0.080	0.062	0.273
triacetin*	MS	0.016	0.010	0.000	0.027	0.036	0.022	0.017	0.074
1,1-ethanediol, diacetate*	MS	0.022	0.032	0.000	0.095	0.032	0.031	0.000	0.104
levulinic acid	RI MS	0.000	0.000	0.000	0.000	0.095	0.061	0.000	0.220
ethyl hydrogen succinate	RI MS	0.122	0.149	0.000	0.458	0.186	0.203	0.011	0.649
5-hydroxymethylfurfural	STD MS	0.000	0.000	0.000	0.000	0.087	0.061	0.028	0.217
N-acetyltyramine*	MS	0.015	0.015	0.000	0.048	0.022	0.019	0.000	0.053
2,3-butanedioldiacetate	RI MS	0.150	0.133	0.011	0.477	0.194	0.161	0.063	0.490
2,3-butanediol	STD MS	0.079	0.119	0.003	0.404	0.134	0.195	0.017	0.707
isoamyl acetate	STD MS	0.187	0.247	0.004	0.740	0.200	0.232	0.029	0.649

[a]STD: standard; MS: mass spectra; RI: retention index; * tentative identification.

■ RESULTS AND DISCUSSION

Impurities in the Extraction Solution from Wine Vinegar and ABM. First of all, we checked for the presence of impurities in the solution extracted by diethyl ether along with acetic acid from wine vinegar and ABM and whether they affect the isotopic data of acetic acid. We analyzed 11 samples of ABM and 10 of wine vinegar and used GC-MS/MS for quantification (Table 2).

The percentage of impurities was found to be on average 6% in the extraction solution of ABM and 4% in that of wine vinegar. The main impurities (higher than 1%) are the extraction solvent, ethyl acetate, acetoin (= 3-hydroxybutanone) and butanoic acid. Comparison of the ABM and vinegar solutions showed 5-acetoxymethyl-2-furaldehyde (= 5-formylfurfuryl acetate), levulinic acid, and 5-(hydroxymethyl)furfural (= 5-hydroxymethyl-2-furaldehyde), mostly originating from the heating of the grape must and caramel, to be significantly higher in ABM ($p < 0.001$, Tukey, Kruskall−Wallis and U-Mann−Whitney tests).

In the case of D/H analysis, the presence of impurities was overcome by carrying out a composite NMR experiment on acetic acid, as described in the Materials and Methods section. As for $\delta^{13}C$, we compared the values obtained for the extracted purified solution using EA-IRMS with those for pure acetic acid analyzed by GC/c-IRMS. We did not find any significant differences ($p < 0.001$) in the 18 vinegar and 21 ABM samples, the mean difference being 0.06 ‰ and the maximum difference 0.3 ‰.

We can conclude that, even if impurities are extracted along with acetic acid from wine vinegar and ABM, the isotopic data of acetic acid are not affected when the methods here described are adopted.

Validation of the Isotopic Methods for ABM. There exist official validated isotopic methods for acetic acid and water in wine vinegar and for ethanol in grape must. To ascertain whether the same methods can also be used for analyzing the ingredients of AMB, we assessed the repeatability and reproducibility of SNIF-NMR and IRMS analyses of acetic acid, ethanol, and water in ABM and compared these values with those obtained in vinegar and must and with those reported in the corresponding official methods.

To assess repeatability, we analyzed the same vinegar and ABM samples 10 times. The repeatability values (sr < 0.4 ppm) for $(D/H)_{CH3}$ are comparable for vinegar and ABM and are lower than the repeatability mean value of the EN 16466-1 method (sr = 0.6 ppm). The sr for $\delta^{13}C$ and $\delta^{18}O$ for both vinegar and ABM (≤0.2 ‰) are consistent with those for

Table 3. Standard Deviations of Repeatability and Reproducibility (sr, sR) and of Repeatability and Reproducibility Limits (r, R): Comparison of the Values Found in This Study with Those Using OIV and EN Methods

	$(D/H)_{CH3}$ (ppm)				$\delta^{13}C$/‰				$\delta^{18}O$/‰			
	sr	r (2.8 × sr)	sR	R (2.8 × sR)	sr	r (2.8 × sr)	sR	R (2.8 × sR)	sr	r (2.8 × sr)	sR	R (2.8 × sR)
wine-must (ethanol, OIV)	0.3	0.8	0.4	1.1	0.1	0.3	0.2	0.6	0.1	0.3	0.2	0.6
vinegar (acetic acid, OIV, EN)	0.6	1.7	0.6	1.7	0.2	0.6	0.3	0.8	0.1	0.3	0.2	0.6
vinegar (acetic acid, this study)	0.4	1.1	0.5	1.4	0.1	0.3		<0.4[a]	0.1	0.3		<0.4[a]
ABM (acetic acid, this study)	0.2	0.6	0.4	1.1	0.2	0.6		<0.4[a]	0.1	0.3		<0.5[a]
ABM (ethanol, this study)	0.5	1.4		<0.8[a]	0.2	0.6		<0.3[a]				

[a]Maximum difference between laboratories found in this study.

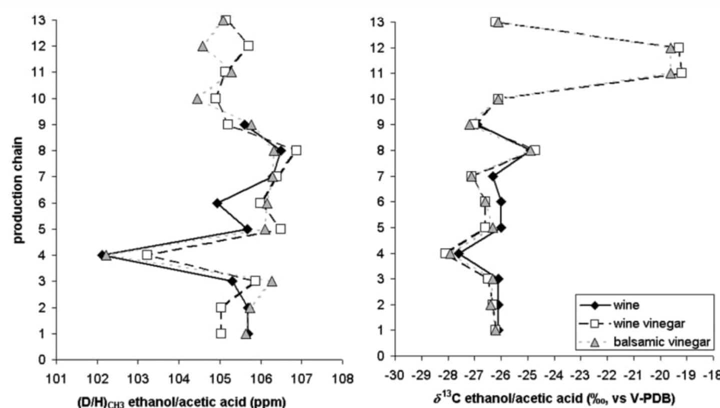

Figure 1. Variations in ethanol/acetic acid $(D/H)_I$ and $\delta^{13}C$ values between wine, wine vinegar, and ABM.

vinegar reported in the OIV and EN methods (≤0.2 ‰). The sr for $(D/H)_{CH3}$ (0.5 ppm) and $\delta^{13}C$ (0.2 ‰) of alcohol from must contained in the ABM (residue after acetic acid extraction) are also comparable to those of the OIV-MA-AS311-05 (average sr = 0.3 ppm) and OIV-MA-AS312-06 (average sr = 0.2 ‰) methods, although they were slightly higher for $(D/H)_{CH3}$.

To determine method reproducibility, 10 samples of vinegar (cane sugar acetic acid was added to some of the samples) and 5 of ABM were analyzed in 3 different laboratories, which used as solvent, diethyl ether or tert-butyl methyl ether and as lock substance, C_6H_6 or TFA. Table 3 reports a summary of the sr and sR values of the OIV and EN methods compared with those found in this study. Also shown is the repeatability limit (r), that is, the value less than or equal to which the absolute difference between two results obtained under repeatability conditions may be expected to be with a probability of 95% (calculated as 2.8 × sr)[5], and the reproducibility limit (R), that is, the value less than or equal to which the absolute difference between two results obtained under reproducibility conditions may be expected to be with a probability of 95% (2.8 × sR)[5]. The reproducibility values for $(D/H)_{CH3}$ for vinegar and ABM are comparable to each other and to the values of the EN 16466-1 method. The sR values of authentic and adulterated samples are not different. The same was found with $\delta^{13}C$ of acetic acid and $\delta^{18}O$ of water and with $(D/H)_{CH3}$ and $\delta^{13}C$ of ethanol from ABM must fermentation, which were measured only in two laboratories, the differences being always lower than the reproducibility limits for all the isotopic parameters (Table 3).

We conclude that the isotopic methods for analyzing $(D/H)_{CH3}$, $\delta^{13}C$, and $\delta^{18}O$ in grape must and vinegar can also be applied to the ingredients of ABM. The same validation data apply, except for the repeatability values of ethanol $(D/H)_{CH3}$, which are here slightly higher.

Variations between $(D/H)_{CH3}$ and $\delta^{13}C$ Values in Wine and Must and Those in the Ingredients of ABM. Figure 1 reports the $(D/H)_{CH3}$ and $\delta^{13}C$ values of ethanol obtained from wine (solid black diamonds connected by black lines), acetic acid extracted from vinegar (open squares connected by dashed lines), and balsamic vinegar (solid gray triangles connected by dotted lines) for the 13 chains, reported along the *y* axis. The mean difference for $(D/H)_{CH3}$ between wine and both vinegar and ABM was +0.3 ppm with a range of −0.7 to 1.1 ppm and therefore below the repeatability limits of the official methods reported in Table 3. The difference was not significant according to a paired *t*-test ($p < 0.001$), in line with previous findings.[6] The wine samples had different $(D/H)_I$ values, ranging from 102.1 to 106.3 ppm, and covered a large part of the typical variability of a grape product.[2]

The $\delta^{13}C$ of ethanol obtained from wine also appears not to differ from that of acetic acid after extraction from both vinegar and ABM (Figure 1), in line with previous findings.[6] The mean differences between wine and vinegar and wine and ABM are 0.4 ‰ and 0.3 ‰, respectively, with a range of −0.8 to 0.2 ‰, averagely lower than the official repeatability limits shown in Table 3. Moreover, the differences were not significant according to a paired *t*-test ($p < 0.001$). The $\delta^{13}C$ values of chains 11 and 12 for both products are very high (on average

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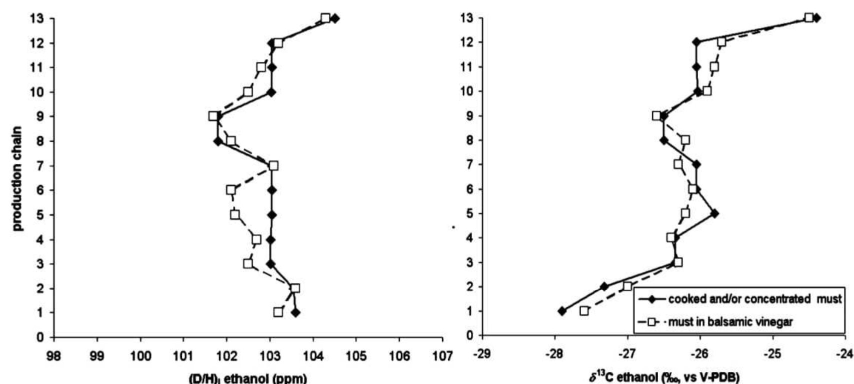

Figure 2. Variations in ethanol $(D/H)_I$ and $\delta^{13}C$ between the mixtures of concentrated and cooked must (calculated values) and the corresponding mixture in the ABM.

Table 4. Effects of Filtration, Concentration, and Addition of Caramel on the Isotopic Values of Acetic Acid and Ethanol of ABM

			acetic acid fraction		must fraction	
	density	acidity (g/100 mL)	$(D/H)_{CH3}$ (ppm)	$\delta^{13}C$/‰ vs V-PDB	$(D/H)_I$ (ppm)	$\delta^{13}C$/‰ vs V-PDB
concentrated grape must					102.9	−25.9
cooked grape must					103.2	−26.2
expected value of mix					103.0	−26.0
white wine vinegar			104.9	−26.1		
ABM (not filtered)			104.5	−26.1	102.6	−25.9
ABM (tangentially filtered)			104.3	−26.0	102.8	−26.0
ABM (with 1.79% caramel)			104.1	−26.0	102.7	−25.9
white wine vinegar		11.11	105.2	−26.2		
ABM (not concentrated)	1.169 46	6.31	105.1	−26.1		
ABM (45°Brix)	1.206 38	6.31	105.3	−26.1		
ABM (57°Brix)	1.278 19	5.71	105.6	−26.1		

−19.5 ‰) due to the addition of cane sugar acetic acid to the vinegar.

ABM is made with a mix of concentrated and cooked grape must. In this work, we produced ABM with different percentages of these two ingredients (Table 1). We analyzed the raw must and calculated the values of ethanol $(D/H)_I$ and $\delta^{13}C$ for each mix containing different quantities of concentrated and cooked must (see Table 1). Figure 2 shows a comparison between these values and the values for the ethanol obtained from fermentation of the residual solution of the corresponding ABM after acetic acid extraction. The mean differences were 0.3 ppm for $(D/H)_I$ and 0.1 ‰ for $\delta^{13}C$, ranging from −0.3 to 1 ppm and from −0.4 to +0.4 ‰, respectively. In both cases, the mean values are below the official limits of repeatability (Table 3).

Effects of technological processing stages, such as addition of caramel, tangential filtration, and concentration, were found to be nonsignificant for isotopic ratios of both acetic acid and must ethanol (Table 4): indeed, the maximum difference between values from the same experiment was always below the corresponding repeatability limit (Table 3). As for caramel, we have found by experiment that it does not ferment in the conditions adopted here, which is why its presence does not affect the isotopic values of ethanol. Anyway its low presence (maximum addition allowed 2%) should not influence the data. The absence of any effect of concentration is not consistent with what is found in the literature,[8] where it has been shown that concentration under vacuum evaporation, as in this case, can change significantly the $\delta^{18}O$ of must water, the $(D/H)_{II}$ of must ethanol and also, but to a lesser extent, $(D/H)_I$.

We conclude that there are no changes in isotopic values $(D/H)_I$ and $\delta^{13}C$ from wine to vinegar and to ABM and from the original must to ABM must, independently of concentration, filtration, and addition of caramel. This supports the suggestion that the methods recognized for vinegar and must using a composite NMR experiment for D/H can also be adopted for analysis of the ingredients in ABM. Moreover, it provides experimental evidence that the reference data of wine databanks can also be used to evaluate the authenticity of vinegar and ABM.

Detection of Adulteration of Vinegar and ABM. Previous studies have shown that C and H stable isotope ratios have a marked capacity to identify synthetic vinegars and distinguish C3 and C4 derived products, allowing the adulteration of vinegars with raw fermentation materials cheaper than those declared on the label to be detected.[9−11] Here, we investigated the ability of isotopic analysis to detect the fraudulent addition of increasing percentages of cane sugar ethanol to wine from which vinegar is derived and of synthetic vinegar to ABM. The general reference of wine, as reported by

Dordevic et al. (98.8 to 106 ppm for $(D/H)_{CH3}$ and −29.3 ‰ to −24.3 ‰),[2] can be adopted for products such as vinegar and ABM, where origin and year of production are generally not known.

Table 5 shows that the addition of more than 20% of cane sugar ethanol to the source wine can be detected because the isotopic parameters fall outside of the limits, taking into account the measurement uncertainty. In addition, the presence of 20% of synthetic acetic acid (and potentially less, since the synthetic sources cover a very wide range)[7] can be detected. In all cases, the actual detection limit depends on the isotopic values of the starting materials (wine vinegar and added substitutes).[2,12] Moreover, detection of adulteration is more efficient if the exact origin of the grapes is known, because the limits are more narrow.

Table 5. Isotopic Values of Nonauthentic Vinegar and ABM

	% grape	% cane	$(D/H)_{CH3}$	$\delta^{13}C$/‰
vinegar 1	90%	10%	106.7	−25.3
vinegar 2	86%	14%	107.3	−24.1
vinegar 3	80%	20%	107.0	−24.2
vinegar 4	67%	33%	108.5	−22.6
vinegar 5	60%	40%	109.3	−21.5
vinegar 6	58%	42%	109.1	−21.2
ABM from vinegar 1	90%	10%	106.9	−25.1
ABM from vinegar 6	58%	42%	108.3	−21.3
	% grape	% synthetic	$(D/H)_{CH3}$	$\delta^{13}C$/‰
ABM 1	60%	40%	112.4	−27.8
ABM 2	80%	20%	110.7	−27.3

■ ASSOCIATED CONTENT

Supporting Information

Single data of the studies for determining repeatability and reproducibility. This material is available free of charge via the Internet at http://pubs.acs.org.

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CHAPTER 5 OLIVE OIL

5.1 INTRODUCTION

Extra-virgin olive oil (EVOO) represents a key product in the EU market, with Europe being the main exporter and consumer worldwide, as around 98% of the world's olive trees are concentrated in the Mediterranean basin (Greece, Spain, Portugal, Italy and France). It enjoys global recognition, thanks to its nutritional value and beneficial effects on health, but has suffered a dramatic loss of consumer confidence due to increasing numbers of fraud cases. Indeed, when a product acquires a reputation extending beyond national borders it can find itself in competition with products which pass themselves off as the genuine article and take the same name.

To protect both ethical producers and consumers, European law requires that the origin of certain premium products such as EVOO must be declared on the label. This is why the EU created the PDO and PGI systems to protect and promote food produced to certain specifications (EU Reg. 1151/2012); EVOO has the most restrictive regulations (EU Reg. 1335/2013).

In the last two decades, different works have been published on the isotopic composition of olive oil (OO). The first studies showed that the $^{13}C/^{12}C$ ratio measured in bulk OO was able to detect adulteration with cheaper oils [Angerosa *et al.*, 1997; Spangenberg *et al.*, 1998]. Subsequently, researchers have found that SIRA can also be applied to characterise the geographical origin of OO, showing that the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios change according to latitude, altitude, distance from the sea and environmental conditions [Breas *et al.*, 1998; Angerosa *et al.*, 1999; Aramendia *et al.*, 2007]. Moreover, a number of other studies have focused on different combinations of $\delta^{2}H$, $\delta^{13}C$ and $\delta^{18}O$ measurements in bulk oil to characterise EVOO [Bontempo *et al.*, 2009; Camin *et al.*, 2010a; Camin *et al.*, 2010b; Iacumin *et al.*, 2009; Portarena *et al.*, 2014; Chiocchini *et al.*, 2016]. Finally, the isotope composition was measured not only in bulk samples but also in sub-components in order to obtain additional information for the authentication of OO. Combined with multivariate statistics, the carbon isotopic composition of individual fatty acids (FAs) and bulk OO provided superior discrimination between samples of different geographical origin [Faberi *et al.*, 2014].

SECTION 5.3

The use of IRMS, ^{1}H NMR and chemical analysis to characterise Italian and imported Tunisian olive oils

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Statement of the author: My personal contribution to this work mainly concerned the stable isotope ratio analysis (δ^2H, δ^{13}C and δ^{18}O) of the olive oil samples collected in this study .

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The use of IRMS, ^{1}H NMR and chemical analysis to characterise Italian and imported Tunisian olive oils

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ABSTRACT

Isotope Ratio Mass Spectrometry (IRMS), ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), conventional chemical analysis and chemometric elaboration were used to assess quality and to define and confirm the geographical origin of 177 Italian PDO (Protected Denomination of Origin) olive oils and 86 samples imported from Tunisia.

Italian olive oils were richer in squalene and unsaturated fatty acids, whereas Tunisian olive oils showed higher δ^{18}O, δ^{2}H, linoleic acid, saturated fatty acids β-sitosterol, *sn*-1 and 3 diglyceride values. Furthermore, all the Tunisian samples imported were of poor quality, with a K_{232} and/or acidity values above the limits established for extra virgin olive oils.

By combining isotopic composition with ^{1}H NMR data using a multivariate statistical approach, a statistical model able to discriminate olive oil from Italy and those imported from Tunisia was obtained, with an optimal differentiation ability arriving at around 98%.

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1. Introduction

Extra virgin olive oil is a fundamental component of the Mediterranean diet and different international regulations have been introduced to define the authenticity and quality of oil, namely EC Reg. 182/2009, followed by Implementing Regulation (EU) 29/2012 and subsequently Commission Implementing Regulation (EU) 1335/2013. Extra virgin olive oil commands a high retail value (2–4 times that of other oils) because of its sensorial qualities and purported health benefits. Indeed, this product is very rich in mono-unsaturated fatty acids (Salas, Harwood, & Martínez-Force, 2013) and polyphenols, resulting in a health claim regarding "olive oil polyphenols" approved by the European Commission (EC Regulation 432/2012). These factors and the increasing demand for olive oil have encouraged many Mediterranean countries to invest in olive oil production. Consequently, throughout Europe, there is an enormous range of olive oils of different geographical origin and quality. The importance of the geographical origin for foodstuffs has been recognised by the European Union for some time: starting from 1992 when the PDO and PGI systems were created to promote and protect foodstuffs of particular quality (Regulation 2081/92/EEC, followed by Regulation 510/2006/EEC, subsequently amended by Regulation 1151/2012/EU), and, in 2009, when labelling of origin became compulsory for extra virgin and virgin olive oil (EC Regulation 182/2009, followed by EU Reg. 29/2012 and 1335/2013). Specifically, Article 4 of the EU Reg. 29/2012 states that 'Extra virgin olive oil and virgin olive oil shall bear a designation of origin on the labelling' and confirms that in contrast with other categories of edible olive oil, the characteristics of a virgin olive oil are strictly related to the geographical origin of the olives, in addition to the specific techniques used during production. However, official olive oil quality control methods are based on the maximum or minimum limits for certain chemical components, e.g. fatty acids, sterols, alcohols or stigmastadiene (Regulation 2568/91/EEC and amendments, in particular EU Reg. 299/2013), and are unable to verify the real geographical origin of olive oil. There is, therefore, the potential for mislabelling olive oil, without the possibility of detection. This situation can influence consumers' perceptions of the benefits of consuming olive oil, and the current image of uncontrolled provenance in the olive oil market poses a considerable risk to the opportunity for economic growth in many Mediterranean countries. This is a pressing topic,

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particularly for Italy, which has 43 PDO and PGI extra virgin olive oils and is the second largest producer in the world (around 500,000 tonnes expected in 2012/13, EC Final report – October 2012), after Spain, and is also the largest consumer of extra virgin olive oils globally (International Olive Oil Council, http://www.internationaloliveoil.org). The recent scandals involving Italian olive oils, such as the one revealed in the 'New York Times', with the striking headline "Extra Virgin suicide: the adulteration of Italian olive oil" (Jan. 26th 2014), underline the need for analytical methods and statistical tools capable of effectively verifying claims of origin and/or quality. This need is felt particularly by the European Union, which during recent years has launched many calls to find methods capable of checking the traceability and authentication of food products, in particular olive oil (e.g. FP6 TRACE project, FP7 Food Integrity project, Horizon 2020 call of March 2014 on olive oil) within its funding programmes.

The many methods suggested as solutions for this issue/matter over time have included stable Isotope Ratio Mass Spectrometry (IRMS) and Nuclear Magnetic Resonance (NMR) spectroscopy.

The first studies on the stable isotope ratios of olive oils focused on adulteration of olive oil with cheaper oils, using $^{13}C/^{12}C$ measured in bulk olive oil or some sub-components (Angerosa, Camera, Cumitini, Gleixner, & Reniero, 1997; Spangenberg, Macko, & Hunziker, 1998; Spangenberg & Ogrinc, 2001). Subsequently, stable isotope ratio analysis ($^{13}C/^{12}C$, either with or without analysis of $^{18}O/^{16}O$ and $^{2}H/^{1}H$), also proved to be a good tool for characterising geographical origin (Angerosa et al., 1999; Aramendia et al., 2007; Baum, Lu, Muccio, Jackson, & Harrington, 2010; Bréas, Guillou, Reniero, Sada, & Angerosa, 1998; Camin, Larcher, Perini et al., 2010; Royer, Gerard, Naulet, Lees, & Martin, 1999), as isotope ratios change according to latitude, suggesting distance from the sea and environmental conditions during the growing of plants (water stress, atmospheric moisture and temperature) as co-factors of variability. As regards the characterisation of Italian extra virgin olive oils specifically, the results of works carried out on $\delta^{13}C$ and $\delta^{18}O$ (Chiavaro et al., 2011; Iacumin, Bernini, & Boschetti, 2009) also combined with $\delta^{2}H$ (Bontempo et al., 2009; Camin, Larcher, Nicolini et al., 2010) proved that it is possible to distinguish samples collected in different Italian macro areas with different climatic and geographical characteristics.

NMR spectroscopy is a powerful technique, able to make a contribution to food analysis (Mannina, Sobolev, & Viel, 2012). The advantage of ^{1}H NMR spectroscopy is its ability to provide a complete view of foodstuff metabolites, providing qualitative and quantitative information on major and minor compounds. In particular, the NMR approach together with a suitable statistical analysis (Mannina & Sobolev, 2011) has provided results in terms of the geographical origin (D'Imperio et al., 2007; Mannina, Fontanazza, Patumi, Ansanelli, & Segre, 2001), variety (Mannina, Patumi, Proietti, & Segre, 2001; Mannina et al., 2003) and adulteration of olive oil (Garcia-Gonzalez, Mannina, D'Imperio, Segre, & Aparicio, 2004; Mannina et al., 2009).

In recent years a further approach has combined the two techniques (NMR and IRMS) and has been successfully applied to the characterisation of Andalusian (Aramendia et al., 2010) and Mediterranean olive oils (Alonso-Salces et al., 2010).

In this study, we considered 177 Italian extra virgin olive oils and 86 olive oils produced in Tunisia and imported to Italy. Tunisia is the country from which the largest amount of European olive oil is imported. A multi-methodological approach, based on the chemical analysis provided for by European law (EC Reg. 182/2009 and amendments), on the stable isotope ratio of H, C and O and the ^{1}H NMR profile, was adopted. The aim of the work was to define the quality of olive oils imported to Italy from Tunisia, and to create a multivariate statistical model based on isotopic ratios and the ^{1}H NMR profile, capable of distinguishing Italian olive oils from those imported from Tunisia and potentially exported and mislabelled as Italian olive oils.

The novelty of the study lies in the fact that for the first time we assessed the quality of olive oil imported at customs from Tunisia to Italy and combined two powerful techniques (IRMS and ^{1}H NMR) with a robust multivariate statistical approach to discriminate Italian from Tunisian olive oils. The final aim of the work was to develop tools to protect Italian olive oil from mislabelling.

2. Materials and methods

2.1. Sampling

Tunisian olive oils ($N = 86$) were officially collected by the Italian State Forestry Department at customs points between April and November 2012. 54 samples were declared to be extra virgin olive oils and 32 lampante olive oils. Certified Italian extra virgin olive oils ($N = 177$) were officially collected by the Ministry of Agricultural, Food and Forestry Policy during the 2011–2012 harvest year. The Italian samples were made up of about three samples for each Italian PDO currently recognised according to EC Reg. 510/2006, divided between the regions as follows: 12 Abruzzo, 18 Calabria, 12 Campania, 11 Lazio, 9 Liguria, 3 Lombardy, 3 Marche, 3 Molise, 17 Apulia, 6 Sardinia, 18 Sicily, 12 Tuscany, 9 Trentino, 15 Umbria and 12 Veneto.

2.2. Chemical analysis

The acidity values, UV spectrophotometric indices (K_{232}, K_{270}, ΔK) and fatty acid composition of Tunisian samples were determined following the analytical methods described, respectively in Annex II, Annex IX and Annex X of Regulation EEC 2568/1991, as amended by EC 1989/2003 and EU 299/2013 Regulations.

Fatty acid composition analysis was carried out using a Thermo Trace GC/FID chromatograph equipped with a fused silica capillary column RTX-2330 (10% cyano-propyl-phenyl, 90% bis cyanopropyl poly siloxane, $L = 60$ m, i.d. = 0.25 mm, f.t. = 0.25 μm) Restek (Bellefonte, Pennsylvania, USA). Helium was used as a carrier gas with a constant flow rate of 0.7 mL min^{-1}. The amount of sample injected was 1 μL with a split ratio of 1:99; injector temperature was 250 °C. The oven temperature programme was 10 min at 165 °C, raised by 1 °C/min to 175 °C, then raised by 5 °C/min to 220 °C and finally held at 220 °C for 10 min; the total time was 39 min. The identification of individual FAMEs was performed by comparison of retention times with those of a standard FAME mixture (Supelco, Bellefonte, PA).

Quantification was carried out using the internal normalisation method; results were expressed as a percentage of total fatty acids, assuming complete elution of the components.

2.3. Stable isotope ratio analysis

Analysis of $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{2}H/^{1}H$ in bulk Italian and Tunisian olive oils was performed using an isotope ratio mass spectrometer (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) coupled with an elemental analyser (Flash EATM1112, Thermo Scientific) for $^{13}C/^{12}C$ measurement and with a pyrolyser (Finnigan TMTC/EA, high temperature conversion elemental analyser, Thermo Scientific) for $^{2}H/^{1}H$ and $^{18}O/^{16}O$ measurement. The analytical procedure is reported in Camin, Larcher, Perini et al. (2010) and Camin, Larcher, Nicolini et al. (2010).

The isotopic values were expressed in δ‰, as described below. They were calculated against a working in-house standard (commercial olive oil) for $\delta^{13}C$ and $\delta^{18}O$. The commercial olive oil was calibrated against the international reference materials: fuel oil

NBS-22 (IAEA-International Atomic Energy Agency, Vienna, Austria) and IAEA-CH-6 Sucrose for $^{13}C/^{12}C$, and benzoic acid-601 for $^{18}O/^{16}O$. For δ^2H, as recently suggested (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014), beside the olive oil standard (calibrated against NBS-22), we used a second standard with different δ^2H values (magnesium stearate of the FIRMS FT scheme, δ^2H value: −228‰).

The isotopic values of the aforementioned international reference materials and, therefore, also of the samples were expressed in δ‰ vs. VPDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$ and VSMOW (normalised in relation to the Vienna Standard Mean Ocean Water – Standard Light Antarctic Precipitation VSMOW-SLAP standard scale) for $\delta^{18}O$ and δ^2H, according to Coplen (2011) and Brand et al. (2014).

2.4. NMR analysis

Olive oil samples (20 μL) were dissolved in DMSO (20 μL) and $CDCl_3$ (700 μL) directly inside the 5 mm NMR tube. NMR experiments were recorded on a Bruker AVANCE 600 spectrometer operating at the proton frequency of 600.13 MHz (B_0 = 14.1 T) and equipped with a Bruker multinuclear *Z* gradient 5 mm probe head. The ^1H NMR spectra were acquired at 300 K using the following experimental conditions: number of scans 1024; π/2 pulse 8 μs; time domain (TD) 64 K data points; relaxation delay plus acquisition time 3.5 s; spectral width 18.5 ppm. ^1H NMR spectra were obtained by Fourier Transformation (FT) of FID (Free Induction Decay), applying exponential multiplication with a line-broadening factor of 0.3 Hz and a zero filling (size = 128 K) procedure. The resulting ^1H NMR spectra were phased manually. Chemical shifts were reported as compared to residual $CHCl_3$ signal, set at 7.26 ppm. The baseline was corrected using the Cubic Spline Baseline Correction routine in the Bruker Topspin software. The intensity of the 17 selected signals was measured using the semi-automatic peak picking routine of Bruker TOPSPIN software and normalised as compared to resonance at 2.251 ppm, due to α-methylenic protons of all acyl chains, set to 1000.

2.5. Statistical analysis

The data were analysed in the R statistical environment (R Core Team, 2014). The data for the two analytical methods (IRMS and NMR) were analysed by applying Random Forest discriminant analysis, which is a form of non-parametric multivariate analysis combining the most significant variables and creating models maximising the difference between groups. This kind of data analysis allows rigorous validation of the models using computer – intensive methods such as bootstrapping and simulation, making it possible to test the model's ability to correctly reclassify the samples in their own group. The higher the percentage of correct classifications, the more the model is valid, robust and, therefore, usable.

3. Results and discussion

3.1. Chemical characterisation of Tunisian olive oils imported to Italy

In Table 1, the mean values of acidity (expressed as the percentage of oleic acid), absorbance at 232 nm and 270 nm wavelengths (K_{232}, K_{270}) and Delta-*K*, as well as the fatty acid composition of Tunisian olive oils, are reported and grouped by declared type.

On the basis of free acidity, the olive oils are classified as extra virgin (⩽0.8 g/100 g), virgin (⩽2 g/100 g) and lampante (⩾2 g/100 g) olive oil (EU Reg. 299/2013, Annex I). According to free acidity, it was confirmed that the 54 Tunisian samples declared to be extra virgin olive oils could be effectively classified

Table 1
Chemical composition of 86 Tunisian olive oils collected in 2012, grouped by type (extra virgin olive oils and lampante oils).

		Acidity (% oleic ac.)	Palmitic %	Palmitoleic %	Heptadecanoic %	Stearic %	Oleic %	Linoleic %	Linolenic %	Eicosenoic %	Lignoceric %	K_{232}	K_{270}	Delta-*K*
EVOO	*N*	54	54	54	54	54	54	54	54	54	54	54	54	54
	Mean	0.30	14.4	1.7	0.0	2.4	66.2	13.7	0.57	0.22	0.07	4.13	0.246	0.011
	Std Dev	0.13	1.2	0.3	0.0	0.2	2.5	1.7	0.04	0.03	0.03	1.12	0.189	0.025
	Min	0.05	11.5	0.9	0.0	2.2	61.4	8.9	0.50	0.18	0.00	2.58	0.100	−0.001
	Max	0.63	16.7	2.2	0.1	3.0	73.0	16.2	0.65	0.30	0.10	8.74	0.947	0.079
Lampante	*N*	32	32	32	32	32	32	32	32	32	32	32	32	32
	Mean	2.68	16.8	2.2	0.0	2.6	60.0	16.8	0.68	0.21	0.07	5.98	0.282	0.009
	Std Dev	0.44	1.5	0.4	0.0	0.2	4.9	3.2	0.04	0.01	0.02	1.73	0.035	0.003
	Min	2.14	13.3	1.3	0.0	2.4	56.7	9.5	0.60	0.18	0.05	3.30	0.200	−0.001
	Max	3.43	18.3	2.5	0.1	3.0	71.1	18.9	0.71	0.24	0.10	8.30	0.355	0.013

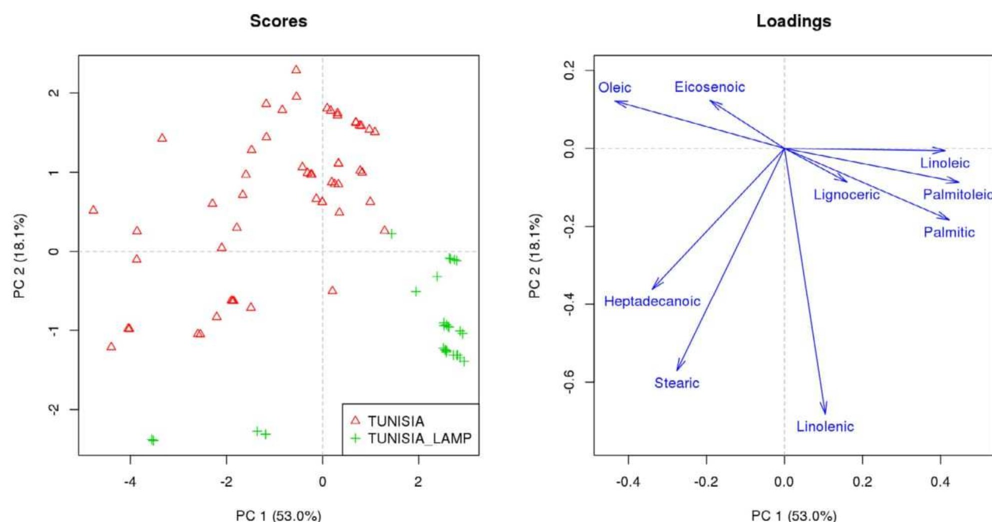

Fig. 1. PCA performed on fatty acid data.

as such, and the other 32 samples were confirmed to be lampante olive oils.

Olive oil, similarly to all vegetable oils, consists of a main saponifiable fraction (triglycerides, around 99%, Salas et al., 2013) and a lesser unsaponifiable fraction (minor components). Fatty acid composition and fatty chain distribution on the glycerol moiety may show some variability. The characteristic range of variability in fatty acid composition in olive oils is established by EC Reg. 2568/91 and EU Reg. 299/2013, as well as by the International Olive Council. In general, a prevalence of monounsaturated oleic acid, a modest amount of palmitic and stearic (saturated) acids and a fair amount of linoleic and α-linolenic acids (polyunsaturated) are observed in olive oil. The Tunisian oils analysed had a fatty acid composition compatible with that of an olive oil. To better visualise the sample fatty acid composition, PCA (Principal Component Analysis) was carried out (Fig. 1a and b). For PCA, data on myristic acid, heptadecenoic, arachic and behenic acids were not taken into account, because of the absence of variability between the samples. The first two components explained 71.1% of total dataset variability (Fig. 1a). Large positive loadings on PC 1 were seen for linoleic, palmitoleic and palmitic acid; the largest negative loading on PC 1 was seen for oleic acid (Fig. 1b). The most important variables in PC 2 were linolenic and stearic acids (both with negative loadings) (Fig. 1b). In general, lampante oils have a fatty acid composition that differentiates them from extra virgin oil. Another possible explanation of the variability is the different chemical composition of the main olive cultivar used for production. In Tunisia, olive tree cultivation takes place in all regions, covering a wide range of climatic conditions and there are two main cultivars present (Chemlali and Chétoui) (Issaoui et al., 2010). The Chemlali variety represents about 80% of national olive oil production and is grown mainly in central and southern Tunisia, where water resources are limited. The second variety, Chetoui, is widespread in the north of the country, both in mountain areas and on the plains. According to the literature, these two varieties have differences in chemical composition. In particular, with regard to the fatty acid composition, the olive oils of the Chetoui variety have a higher oleic acid content as compared to the olive oils of the Chemlali variety, which is characterised by a relatively low level of oleic acid (53–60%) and high levels of palmitic and linoleic acids (Dabbou et al., 2009; Zarrouk et al., 2009). Furthermore, olive oils made with the Chetoui variety show higher values of chlorophylls and polyphenols (Issaoui et al., 2007), and a lower content of *trans* 2-hexenal as compared to oils made with the Chemlali variety.

Spectrophotometer analysis reveals whether a product has undergone refining processes or oxidation and ageing phenomena. In particular, compounds are formed during the refining process (discoloration), such as conjugated dienes and trienes that are not primarily present in extra virgin olive oils and that absorb at specific wavelengths (232 nm and 270 nm). An increase in K_{232} shows primary oxidation with the formation of peroxides, while an increase in K_{270} indicates secondary oxidation with the formation of aldehydes and ketones. The limits for extra virgin olive oil are 2.5 for K_{232}, 0.22 for K_{270} and 0.01 for Delta-K (EU Reg. 299/2013, Annex I), while no limits have been established for lampante oils. Considering only extra virgin olive oils, all the Tunisian samples had K_{232} values above the 2.5 limit (up to 8.7), whereas ten had K_{270} values above the 0.22 limit (up to 0.95). Finally, 9 out of 54 oils had Delta-K values above the limit of 0.01 (up to 0.08). This suggests that these products are in fact probably lampante oils, the refining process, and particularly the bleaching step leading to a decrease in free fatty acids and thus acidity, but also to a simultaneous increase in the K_{270} parameter (Antonopoulos, Valet, Spiratos, & Siragakis, 2006).

3.2. Isotopic composition of Italian and Tunisian olive oils

In Table 2, the mean, standard deviation, minimum and maximum values of δ^2H, δ^{18}O and δ^{13}C of olive oils are summarised for Italian and Tunisian extra virgin olive oils and for Tunisian lampante oils. The Italian olive oils were categorized as from the North (Trentino, Veneto, Lombardia, Emilia Romagna), Centre (Liguria, Tuscany, Umbria, Abruzzo, Lazio), South 1 (Campania, Apulia) and South 2 (Calabria, Sicily, Sardinia) macro-areas, considering climatic conditions and the proximity to the sea rather than simply

Table 2
Stable isotope ratios ($^{13}C/^{12}C$, $^{2}H/^{1}H$, $^{18}O/^{16}O$) of Italian olive oils (subdivided into the North, Centre, South 1 and South 2 macro-areas) and Tunisian olive oils, the latter grouped by declared type (extra virgin olive oils and lampante oils) collected in the 2011–2012 harvest years. The superscript letters indicate the results of Unequal *N* Tukey HSD test for Italian and Tunisian olive oils ($p < 0.001$).

		Mean	Std Dev	Min	Max
North Italy	$\delta^2H/‰$	−157	5	−169	−146
	$\delta^{13}C/‰$	−30.0^{a}	0.9	−31.7	−28.3
	$\delta^{18}O/‰$	22.9^{a}	1.3	20.8	26.1
Central Italy	$\delta^2H/‰$	−148^{b}	5	−159	−134
	$\delta^{13}C/‰$	−29.2^{b}	0.7	−31.2	−27.9
	$\delta^{18}O/‰$	24.5^{b}	0.9	22.4	26.3
South Italy 1	$\delta^2H/‰$	−148^{b}	5	−158	−139
	$\delta^{13}C/‰$	−29.6^{ab}	0.7	−30.9	−28.1
	$\delta^{18}O/‰$	24.6^{b}	0.8	23.2	26.9
South Italy 2	$\delta^2H/‰$	−145^{cb}	4	−152	−136
	$\delta^{13}C/‰$	−29.2^{b}	0.8	−30.8	−27.7
	$\delta^{18}O/‰$	26.2^{c}	1.1	24.1	28.3
Tunisia declared EVOO	$\delta^2H/‰$	−143^{cd}	2	−148	−138
	$\delta^{13}C/‰$	−29.9^{a}	0.5	−30.5	−28.9
	$\delta^{18}O/‰$	25.9^{c}	0.6	24.6	27.2
Tunisia declared lampante olive oils	$\delta^2H/‰$	−140^{d}	2	−145	−136
	$\delta^{13}C/‰$	−29.0^{b}	0.1	−29.2	−28.9
	$\delta^{18}O/‰$	26.5^{c}	0.6	25.0	27.9

Table 3
Pearson coefficient of the correlations between the isotopic values and chemical parameters of Tunisian and Italian olive oils resulting highly statistically significant ($p < 0.001$).

	δ^2H	$\delta^{13}C$	$\delta^{18}O$	K_{270}	Acidity (% oleic ac.)	Stearic %	Oleic %	Linoleic %	Linolenic %
δ^2H	1.00	n.s.	0.69	n.s.	0.58	n.s.	−0.59	0.62	n.s.
$\delta^{13}C$	n.s.	1.00	n.s.	0.59	0.67	0.59	n.s.	n.s.	0.72
$\delta^{18}O$	0.69	n.s.	1.00	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

latitude, according to a previous publication (Camin, Larcher, Perini et al., 2010). As regards Italian olive oils, substantial agreement between the $\delta^{13}C$ values and literature data was found, whereas the $\delta^{18}O$ and δ^2H values found in this study covered a wider range of values, generally extending to higher levels as compared to the literature (Angerosa et al., 1999; Bontempo et al., 2009; Camin et al., 2010; Iacumin et al., 2009). However, it should be born in mind that the samples referred to in the literature and those for this work were collected in different years, and the influence of year has been demonstrated to be statistically significant (Camin, Larcher, Perini et al., 2010). As regards Tunisian olive oils, until now, no studies have previously reported on stable isotope ratios. Basically, by dividing Tunisian samples into the two declared extra virgin and lampante groups, the latter samples showed higher values for all three stable isotope ratios, in particular δ^2H and $\delta^{18}O$ (Table 2). Isotopic data for the six groups (Italian extra virgin oils grouped in macro-areas, Tunisian extra virgin and Tunisian lampante olive oils) were subjected to Unequal *N* Tukey HSD (Honestly Significantly Different) and Kruskal Wallis tests to identify statistical differences between the groups (Table 2). The results were the same for both tests and showed that $\delta^{13}C$ is not very effective in distinguishing between different geographical origins and that there is a large overlap between samples from Tunisia and Italy. On the other hand, both $\delta^{18}O$ and δ^2H values made it possible to differentiate ($p < 0.001$) Italian extra virgin olive oils from the North, Centre and South 1 areas from Tunisian olive oils. None of the stable isotope ratios was able to fully differentiate Tunisian samples from oils collected in the Italian South 2 area. The similarity of the $\delta^{18}O$ and δ^2H values in the latter Italian and Tunisian olive oils can be attributed to similar climatic conditions during the pre-harvest period.

The ability of $\delta^{18}O$ and δ^2H to distinguish olive oils produced in Tunisia and Apulia (South 1 area) is particularly interesting because this latter area is the main production region for the export of Italian extra virgin olive oil (De Gennaro, Roselli, & Medicamento, 2008).

The correlations between stable isotope ratios (for all Italian and Tunisian samples) and between stable isotope ratios and chemical parameters (only for Tunisian oils) were checked. In Table 3, the *r* values are highlighted for relationships that were shown to be highly statistically significant ($p < 0.001$) and with a *r* value greater than 0.5. The data on myristic acid, heptadecenoic, arachic and behenic acids were not taken into account, because of the absence of variability between samples. δ^2H and $\delta^{18}O$ were shown to be correlated. Except for correlation with δ^2H, $\delta^{18}O$ did not have other significant correlations and would, therefore, seem to be completely independent of the type of oil (lampante, virgin or extra virgin). On the other hand, both $\delta^{13}C$ and δ^2H were significantly correlated with acidity and the content of some acids. In particular, δ^2H correlated negatively with oleic acid and positively with linoleic acid, perhaps in relation to the different varieties of olives used to produce the oil. Indeed, the Chemlali cultivar, characterised by low levels of oleic acid and a high content of linoleic and palmitic acid, is mainly cultivated in areas where water resources are limited and is, thus, associated with higher δ^2H values, due to a higher level of evapotranspiration in leaves due to water stress (Schmidt, Werner, & Eisenreich, 2003; Schmidt, Werner, & Rossmann, 2001). Similarly, the correlation of $\delta^{13}C$ with acidity and with the content of stearic and linolenic acids, could be explained on the basis of the fact that the $\delta^{13}C$ values in plant are influenced by the availability of water, which control stomatal aperture and the internal CO_2 concentration in the leaf (O'Leary, 1995). The relationship between these acids and $\delta^{18}O$, which would be expected, was not significant, perhaps because while the hydrogen of plant lipids originates solely from the water, oxygen also derives from atmospheric CO_2 and O_2.

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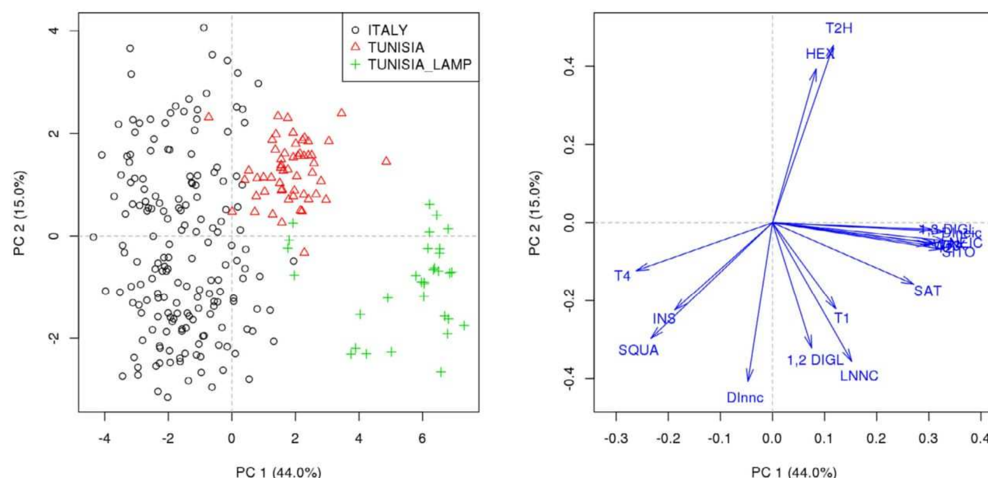

Fig. 2. PCA performed on the intensity of selected NMR variables.

3.3. ^{1}H NMR metabolite profiling of Italian and Tunisian olive oils

In order to evaluate the contribution of NMR to the geographical characterisation of olive oils, Italian and Tunisian samples were analysed using ^{1}H NMR analysis, following the protocol previously reported (D'Imperio et al., 2007; Mannina et al., 2012). The normalised intensities of 17 selected resonances sensitive to geographical origin (D'Imperio et al., 2007) were subjected to suitable statistical analysis. They are HEX = hexanal, T2H = Trans-2-hexenal, T4, T3, T2, T1 = terpene 4, terpene 3, terpene 2, terpene 1, 1,3 DIGL = sn 1,3 diglycerides, 1,2 DIGL = sn 1,2 diglycerides, Dlnnc = diallylic protons of linolenic fatty chains, Dlneic = diallylic protons of linoleic fatty chains, SQUA = squalene, INS = methylenic protons of insatured fatty chains, SAT = methylenic protons of palmitic and stearic fatty chains, WAX = wax, LNNC = methyl of linolenic fatty chains, LNEIC = methyl of linoleic fatty chains, SITO = β sitosterol.

The PCA scoreplot (Fig. 2a) showed good separation between Italian and Tunisian olive oils in the first PC, explaining 44% of total variance. From the loading plot (Fig. 2b), the most important variables for this separation were T4, SQUA and INS, with large negative loadings on PC 1, and a group of highly correlated variables containing T3, T2, 1,3 DIGL, Dlneic, WX, LNEIC and SITO, with large positive loadings on PC 1. Tunisian olive oils were separated into two groups, specifically the group on the left side including all extra virgin and only four Tunisian lampante olive oils, whereas the right side of the map shows only Tunisian lampante samples. The mean values of NMR variable intensities are shown in the supplementary materials.

Italian samples had high values for squalene and a terpene, indicated as "terpene 4", whereas Tunisian samples had a high value for β-sitosterol, saturated fatty acids, linoleic acid, *sn*-1,3 diglycerides, aldehyde compounds and two terpene compounds indicated as "terpene 2" and "terpene 3", as shown by the corresponding loadings. The low number of saturated fatty chains in Italian olive oils is an important "quality marker", making these olive oils most suitable for specific dietary regimens. Saturated fatty acids have the disadvantage of increasing the synthesis of cholesterol and promoting cardiovascular problems.

These results are in agreement with the literature, which reports that olive oils from Tunisia have a higher content of linoleic acid and a lower content of unsaturated fatty acids as compared to

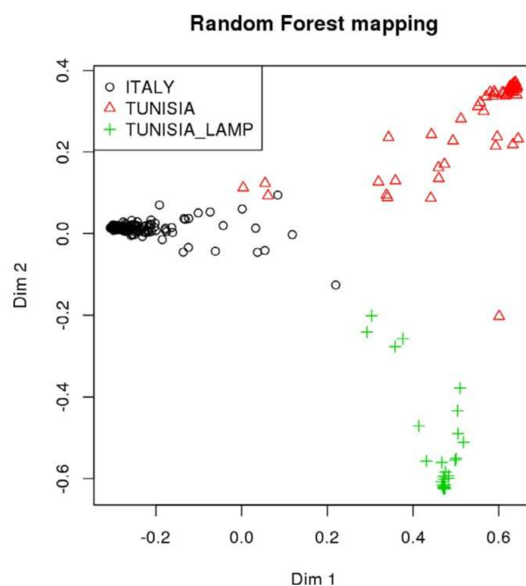

Fig. 3. Graphic representation of multivariate random forest analysis for stable isotope and ^{1}H NMR data for Italian and Tunisian extra virgin olive oils and Tunisian lampante oils.

Italian olive oils in general and Sicilian olive oils in particular (Baccouri et al., 2007). As previously reported (Section 3.1), the separation of the Tunisian olive oils into two groups could be justified by the different chemical composition of the olive oils due to the cultivar (Chemlali and Chetoui).

3.4. Multivariate analysis of ^{1}H NMR and isotopic data

We combined the 3 stable isotope ratios and 17 ^{1}H NMR resonances by applying Random Forest discriminant analysis. This is a form of non-parametric multivariate analysis that creates and

validates models by maximising the difference between groups (Breiman, 2001), as shown in previous works (Camin et al., 2012; Carvalho et al., 2013; Svetnik et al., 2003). Considering the three groups (authentic Italian extra virgin olive oils, Tunisian declared extra virgin olive oils, Tunisian lampante oils), a clear-cut separation between Italian and Tunisian samples was obtained (Fig. 3), with correct classification of 98.5% within the 3 groups and 100% distinction between Italian oils and Tunisian lampante oils in cross-validation tests.

4. Conclusion

Tunisian olive oils imported to Italy would seem overall to be of poor quality and even when declared to be extra virgin olive oils their characteristics often do not meet the standards established by law for this category. In particular, all of them showed K_{232} absorbance values above the limit allowed for extra virgin olive oil (Section 2.5).

Tunisian olive oils had δ^{18}O and δ^{2}H values significantly higher than Italian samples from the North, Centre and South 1 groups, but as these isotopic ratios are related to climatic conditions, there was an overlap with the extra virgin olive oils of South 2 areas (Sicily, Sardinia and Calabria), which have a similar climate to Tunisia during the olive ripening period.

^{1}H NMR analysis allowed us to distinguish Italian samples from Tunisian ones. Tunisian olive oils were themselves separated into two groups. This last differentiation may be attributed to the predominant type of cultivar (Chemlali or Chétoui).

By combining isotopic composition with ^{1}H NMR data using a multivariate statistical approach, a statistical model able to discriminate olive oil from Italy and oil imported from Tunisia was obtained, with an optimal differentiation ability arriving at 98.5%. The parameters included in the model are not linked to the quality of olive oil and, therefore, the model can also be used when the quality of olive oils is not comparable. In the near future, the model needs to be tested with extra virgin olive oils directly sampled at Tunisian cultivation sites and with olive oils of different origin (e.g. Spain).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.08.132.

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SECTION 5.4

Discrimination between European and non-European olive oils using stable isotope ratio analysis

Statement of the author: My personal involvement in this research started with the experimental design to measure the carbon and hydrogen isotopic value of fatty acids extracted from olive oil using GC-C\Py-IRMS after transesterification of tryglicerides. As regards method development, I personally performed all the experiments and data analysis presented in the manuscript. Moreover, I measured $\delta^{2}H$, $\delta^{13}C$ and $\delta^{18}O$ values of olive oil in bulk samples. As first author I was responsible for writing the manuscript and managing the comments and improvements to the text by the other co-authors.

INTRODUCTION

European law requires that the origin of some premium products such as EVOO be declared on the label. Official OO quality control methods are based on quantitative analysis of specific chemical compounds, *e.g.* fatty acids, sterols, alcohols or stigmastadiene (EEC Reg. 2568/91 and amendments), but it is impossible to verify the real geographical origin of EVOO using these parameters. This situation highlights the increasing demand for analytical methods and statistical tools capable of effectively verifying claims of origin.

Currently, SIRA offers one of the most promising approaches for establishing the authenticity of premium products. This is because SIRA relies on the fact that the content of stable isotopes of bio-elements (H, C, N, O, S) determined by IRMS reflects local agricultural practices and geo-climatic characteristics [Laursen *et al.*, 2016].

This study investigated the ability of IRMS to distinguish European from non-European EVOO. The study was conducted on approximately 100 EVOO samples collected worldwide in the main producing countries. $^{2}H/^{1}H$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios were analysed in bulk oil, and furthermore both $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios were determined on the four main FAs (linoleic, oleic, palmitic and stearic acids), for $^{2}H/^{1}H$ for the first time.

MATERIALS AND METHODS

Reagents and reference materials

Fatty acid methyl ester (FAME) standards at ≥99% purity (methyl linoleate, methyl oleate, methyl palmitate and methyl stearate), and heneicosane (≥99.5% purity) were purchased from Sigma-Aldrich (Milan, Italy). All other solvents and reagents (hexane, methanol and sodium hydroxide) used were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy).

Sampling

In this study a total of 101 authentic EVOOs made with different cultivars were examined. The olive oil samples were collected worldwide in the major olive oil-producing regions of Argentina (1), Australia (9), France (6), Greece (8), Italy (28), Morocco (1), Peru (1), Portugal (12), Spain

(13), Tunisia (12), Turkey (4), Uruguay (2) and the USA (4). The oil samples were collected in 30 ml dark glass bottles and kept at 4°C until analysis.

Isotopic analysis of bulk OO

Analytical method

Aliquots of 0.2 - 0.3 mg of olive oil were weighed into tin capsules to determine $^{13}C/^{12}C$ and silver capsules for quantification of $^{18}O/^{16}O$ and $^{2}H/^{1}H$. The analysis of bulk samples was performed in duplicate using an IRMS (visION, IRMS, Isoprime Ltd, UK) coupled with an elemental analyser (Vario Isotope Cube, Elementar Analysensysteme GmbH, Germany) for $^{13}C/^{12}C$ measurement.
For $^{2}H/^{1}H$ and $^{18}O/^{16}O$ measurement, an IRMS (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) was used, coupled with a pyrolyser (Finnigan ^{TM}TC/EA, high temperature conversion elemental analyzer, Thermo Scientific) equipped with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced with the IRMS through a dilutor (Conflo III, Thermo Scientific), dosing the sample and reference gases.

Data analysis

The isotopic values were calculated against working in-house standards (extra virgin olive oils), which were themselves calibrated against international reference materials: fuel oil NBS-22 (IAEA-International Atomic Energy Agency, Vienna, Austria) and IAEA-CH-6 Sucrose for $^{13}C/^{12}C$, and benzoic acid-601 for $^{18}O/^{16}O$. For $\delta^{2}H$, besides the olive oil standards (calibrated against NBS-22 and IAEA-CH-7 Polyethylene by building a linear relationship), a second standard with a different $\delta^{2}H$ value (magnesium stearate of the FIRMS FT scheme, $\delta^{2}H$ value: -228‰) was used. The isotopic values were expressed in δ‰ vs. V-PDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$ and V-SMOW (normalised in relation to the Vienna Standard Mean Ocean Water – Standard Light Antarctic Precipitation V-SMOW-SLAP standard scale) for $\delta^{18}O$ and $\delta^{2}H$.
The results showed that bulk-isotope analysis was able to discriminate between different countries on the basis of specific geo-climatic conditions (Figure 1), but not specifically between European and non-European olive oils, as is evident in Figure 2. Australian samples in particular had lower $\delta^{13}C$ values, except for one sample, whereas Uruguayan, North American and Peruvian samples had lower $\delta^{2}H$ and $\delta^{18}O$ values.

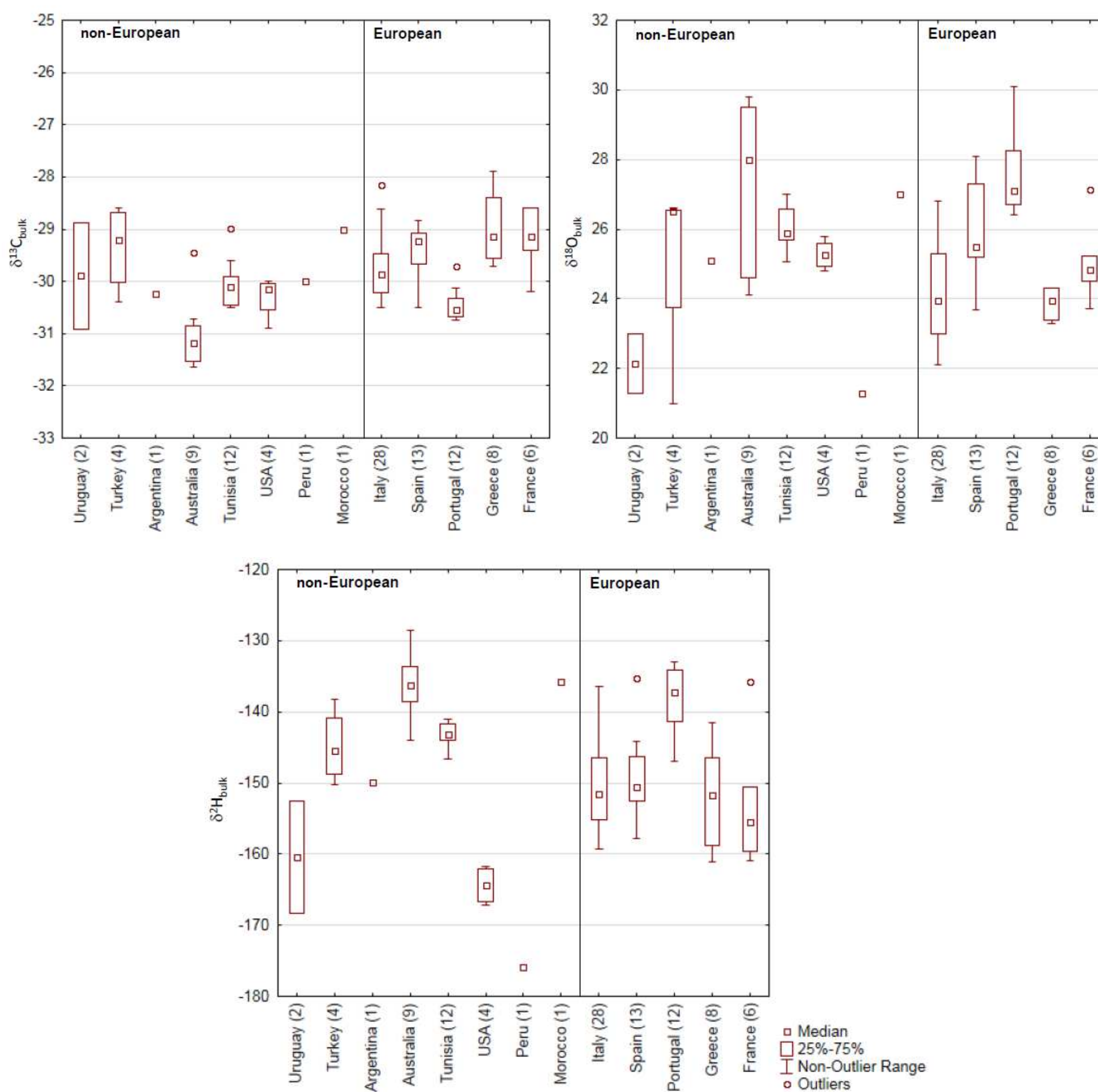

Figure1. Box-plots for extra virgin olive oil $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ values measured in bulk samples

Principal component analysis (PCA) was applied to test for differences between European and non-European olive oils, using the $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ values measured as variables (Figure 2). The first component was mainly negatively loaded by $\delta^{18}O$ and $\delta^{2}H$ values, whereas the second component was positively loaded by $\delta^{13}C$. The score plot showed that the two groups cannot be resolved on the basis of bulk isotope analysis.

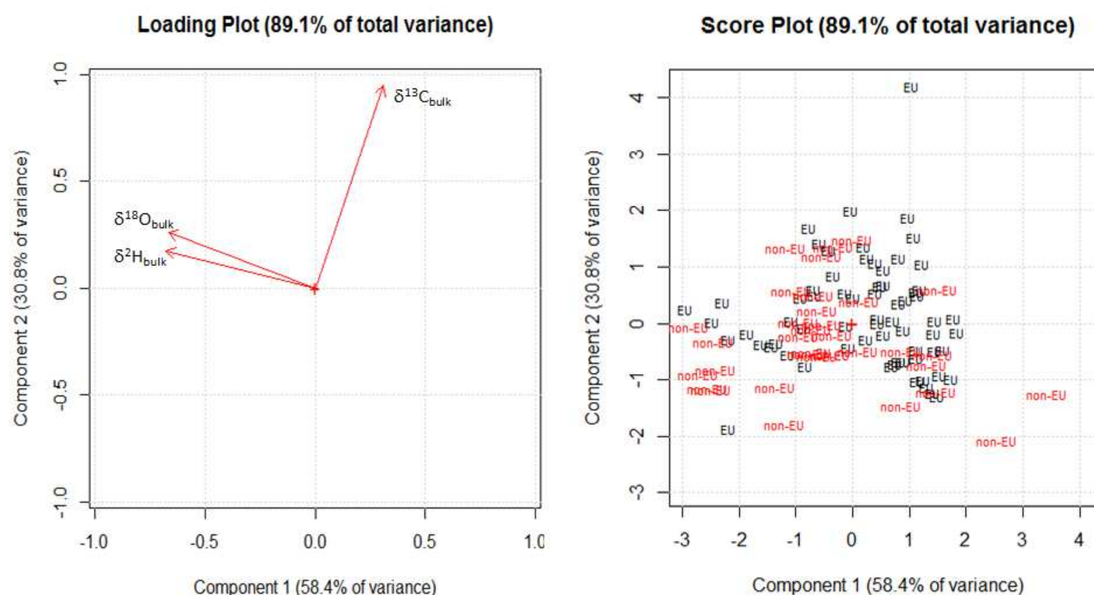

Figure 2. PCA of the $\delta^{13}C_{bulk}$, $\delta^{18}O_{bulk}$ and $\delta^{2}H_{bulk}$ values of extra virgin olive oils

Isotopic analysis of FAs

Sample preparation

For the preparation of the FAMEs via transesterification of triglycerides, 0.1g of OO sample was weighed into a 10 ml vial with 4 mL of hexane, then 1 mL of 2 M methanolic sodium hydroxide solution was added and the vial shaken for 1 min at room temperature (annex XA of EC Reg. 702/2007). The mixture was allowed to stratify until the upper layer became clear and 1 mL of the hexane solution was filtered and then injected into the GC-C-IRMS.

Analytical method

Individual FA isotopic analysis was carried out in triplicate out using Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific), interfaced with an IRMS (DELTA V, Thermo Scientific), through an open split interface and with a single-quadrupole GC-MS (ISQ Thermo Scientific) to identify the compounds. For $\delta^{13}C$ analysis, 1.0 µL of each sample was injected in split mode (1:10) with an auto-sampler (Triplus, Thermo Scientific). A BPX-70 capillary column (60 m × 0.32 mm i.d. × 0.25 µm film thickness; SGE) with He as carrier gas (at a flow of 1 mL/min) was used. The injector temperature was set at 250 °C, and the oven temperature of the GC was initially set at 50 °C, where it was held for 4 min before increasing by 30 °C/min to 170 °C, 2 °C/min to 200 °C and finally 1 °C/min to 210 °C.

Carbon isotopic analysis of individual FAs using GC-C-IRMS

For determination of $\delta^{13}C$, the eluted compounds were combusted into CO_2 and H_2O in a combustion furnace reactor, operated at 1030 °C and composed of a nonporous alumina tube (320 mm length) containing three wires (Ni/Cu/Pt, 0.125 mm diameter, 240 mm identical length) braided and centred end-to-end within the tube. Water vapour was removed with a water-removing trap, consisting of a Nafion membrane.

To monitor instrumental performance, an internal standard was added to each sample and its $\delta^{13}C$ value checked. Heneicosane was chosen as internal standard because it is not naturally present in olive oil. The carbon isotopic value of pure heneicosane (-28.8 ‰) was determined with EA-IRMS, and the differences between GC-C-IRMS and EA-IRMS values were at most ±0.2‰.

The carbon isotopic values of four FAs were determined (Figure 3): palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2).

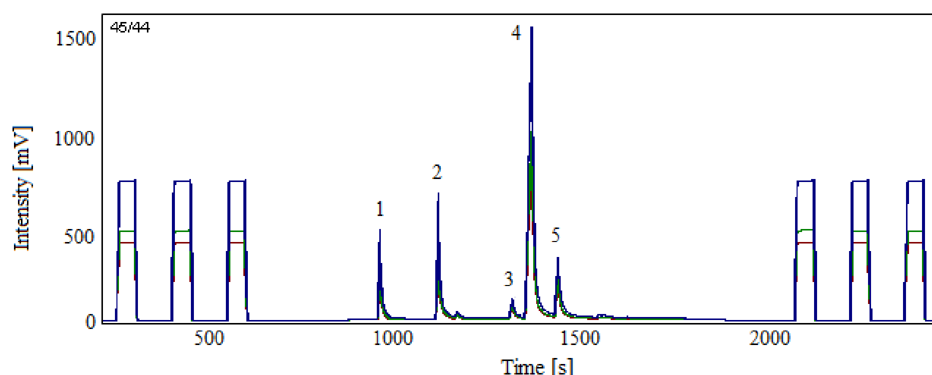

Figure 3. GC-C-IRMS chromatogram of methyl ester derivatives of FAs in an OO sample. Peaks: 1, heneicosane (internal standard); 2, methyl palmitate; 3, methyl stearate; 4, methyl oleate; 5, methyl linoleate. The three first and last peaks of each panel are reference gas signals.

To calculate the $\delta^{13}C$ values of individual FAs, a mixture of FAME reference standards was analysed before and after every three samples to account for $\delta^{13}C$ drift within the run. The instrumental data for each sample were corrected on the basis of the difference existing between the $\delta^{13}C$ value of the pure compound in GC/C-IRMS and that in EA-IRMS.

Accuracy and precision of GC-C-IRMS.

To test the accuracy of the FAME isotopic values determined, the $\delta^{13}C$ values of the mixture of standard FAMEs measured using GC-C-IRMS were compared with the isotopic values of pure

single FAMEs obtained with EA-IRMS. The $\delta^{13}C$ values determined using EA-IRMS were the means of two measurements, whereas the $\delta^{13}C$ values determined with GC-C-IRMS were the average of three runs. $\delta^{13}C$ values obtained from EA-IRMS were linearly correlated with those from GC-C-IRMS, as shown in the Figure 4, and the difference between the values measured using EA-IRMS and GC-C-IRMS was not more than ±0.2‰.

To evaluate precision, a reference FAME mixture was analysed 10 times with GC-C-IRMS. The precision (1σ) of GC-C-IRMS determination was on average ±0.2‰. To evaluate the uncertainty of measurements for all the processes, a OO sample was transesterified 10 times, and each of the samples was analysed using GC-C-IRMS. The standard deviation obtained (1σ) was on average ±0.3‰.

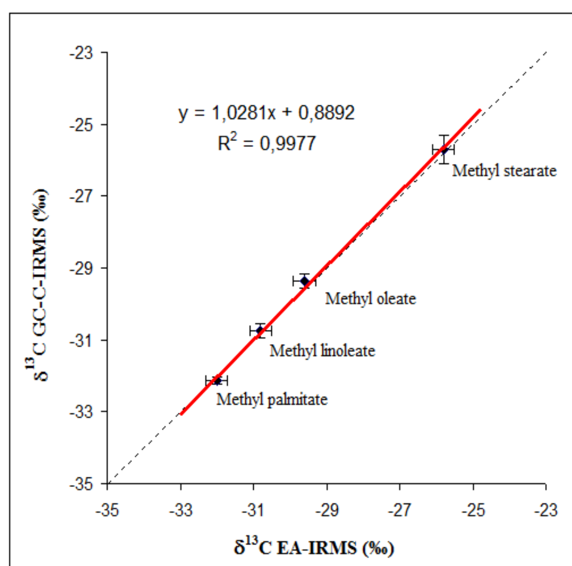

Figure 4. Isotopic measurements from GC-C-IRMS (n=3) plotted against measurements from EA-IRMS (n=2) for carbon. Error bars represent the standard deviation (±1σ) of repeated measurements.

Data analysis and corrections

The $\delta^{13}C$ value measured for the FAME is the product of the carbon native to the molecule and the contribution of the reagent (methanol) used for transesterification. An empirical correction was therefore applied to determine the effective carbon isotope value:

$$(C_n + 1)\delta^{13}C_{FAME} = C_n\delta^{13}C_{FA} + \delta^{13}C_{Me} \qquad (1)$$

where $\delta^{13}C_{FAME}$, $\delta^{13}C_{FA}$ and $\delta^{13}C_{Me}$ are the carbon isotopic values of the FAME, FA and methyl group of methanol (Me) respectively. C_n is the number of C atoms in the FA and the $\delta^{13}C$ of Me was calculated using EA-IRMS.

In Figure 5 the $\delta^{13}C$ values determined for the four main fatty acids are shown, listed according to their biosynthetic order and grouped according to their origin. As is evident in Figure 1, the European and non-European samples showed a different trend, according to the specific fatty acids considered. In particular, it should be noted that the $\delta^{13}C$ values of palmitic and stearic acids are similar for the two groups, whereas the $\delta^{13}C$ values of oleic and linoleic acids are different.

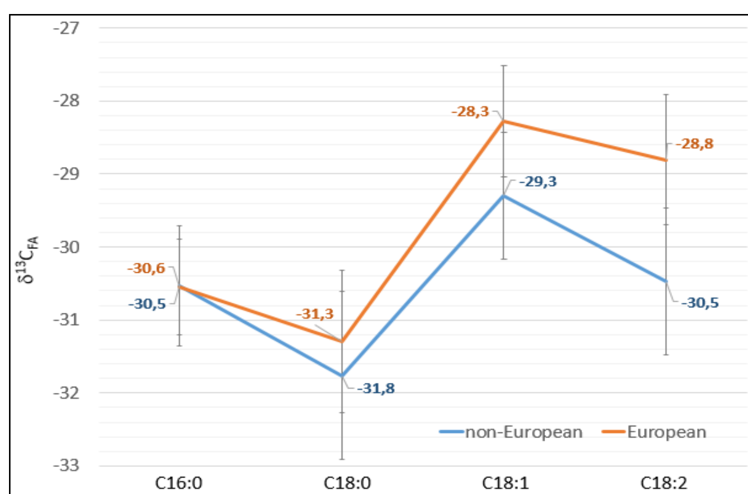

Figure 5. Trends for $\delta^{13}C$ values determined for the four main fatty acids, listed according to their biosynthesis and grouped according to European and non-European origin

Analysis of variance and Tukey's test for an unequal number of samples were carried out on the data to verify differences between European and non-European samples. The $\delta^{13}C$ of methyl oleate and linoleate were highly statistically different in the two groups of samples ($p<0.001$). Specifically, European samples generally showed higher values than non-European ones.

PCA was applied to display the samples in an unsupervised pattern recognition map (score plot), using the $\delta^{13}C$ values of fatty acids as variables (Figure 6).

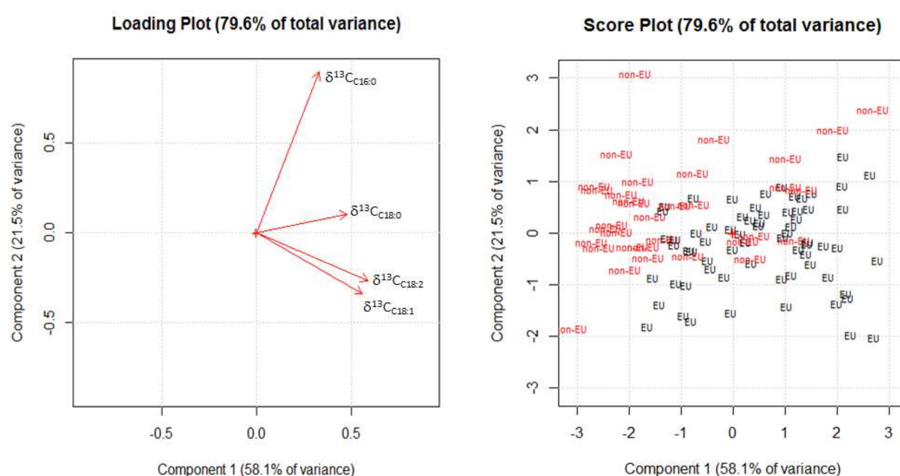

Figure 6. PCA of δ^{13}C values of FAs in extra-virgin olive oils

The PCA showed that the δ^{13}C values of fatty acids make it possible to discriminate between European and non-European olive oils, with the exception of some samples. Specifically, the first factor explained 58.1% of the total variability of the system and the second 21.5%. The first component was mainly positively loaded by the δ^{13}C determined in stearic, oleic and linoleic acids, whereas the second component was positively loaded by the δ^{13}C of palmitic acid.

Hydrogen isotopic analysis of individual FAs with GC-Py-IRMS

For measurement of δ^2H, each eluted compound was passed through a high temperature reactor, operating at 1400°C, where it was subjected to high temperature pyrolysis with development of H_2 gas. Before measuring the $^2H/^1H$ ratio, the $[H3]^+$ factor was verified to be lower than 8. To monitor instrumental performance, heneicosane was added to each sample as the internal standard and its δ^2H value checked. The hydrogen isotopic value of pure heneicosane (-190‰) was determined with TC/EA-IRMS, and the differences between GC-Py-IRMS and TC/EA-IRMS values were at most ± 2.0‰.

The hydrogen isotopic values of four FAs were determined (Figure 7): palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2).

The δ^2H values of FAs were calculated against two international reference materials by building a linear relationship: Icosanoic Acid Methyl Esters USGS70 (δ^2H value: –183.9‰) and USGS71(δ^2H value: –4.9‰).

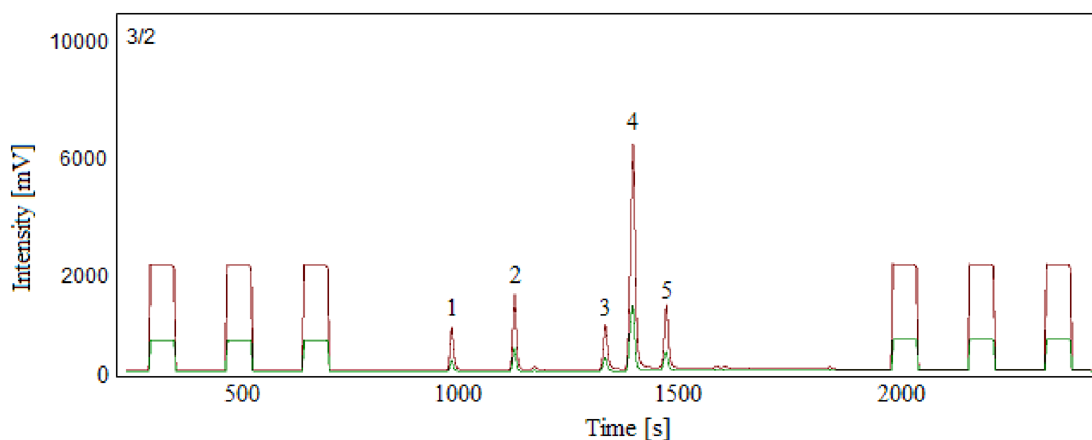

Figure 7. GC-Py-IRMS chromatogram of methyl ester derivatives of FAs in an OO sample. Peaks: 1, heneicosane (internal standard); 2, methyl palmitate; 3, methyl stearate; 4, methyl oleate; 5, methyl linoleate. The three first and last peaks of each panel are reference gas signals.

Accuracy and precision of GC-Py-IRMS

To test the accuracy of the FAME isotopic values determined, the δ^2H values of the mixture of standard FAMEs measured using GC-Py-IRMS were compared with the isotopic values of pure single FAMEs obtained with TC/EA-IRMS. The δ^2H values determined using TC/EA-IRMS were the means of two measurements, whereas the δ^2H values determined with GC-Py-IRMS were the average of three runs. δ^2H values obtained from TC/EA-IRMS were linearly correlated with those from GC-Py-IRMS, as shown in Figure 8, and the difference between the values measured using TC/EA-IRMS and GC-Py-IRMS was not more than ±1.9‰.

To evaluate precision, a reference FAME mixture was analysed 10 times using GC-Py-IRMS. The precision (1σ) of GC-Py-IRMS determination was on average ±1.0‰. To evaluate the uncertainty of measurements for all the processes, a OO sample was transesterified 10 times, and each of the samples was analysed with GC-Py-IRMS. The standard deviation obtained (1σ) was on average ±2.3‰.

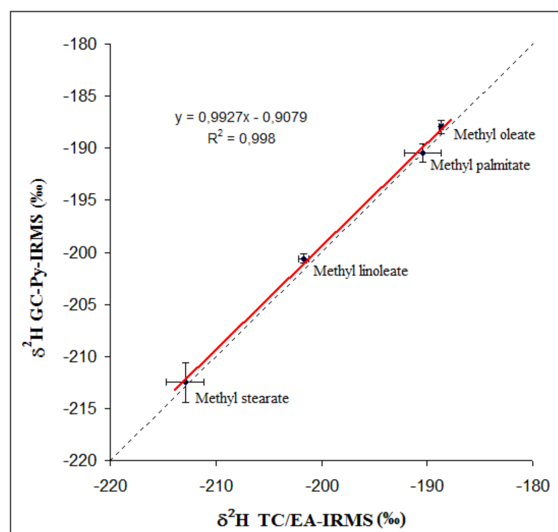

Figure 8. Isotopic measurements from GC-Py-IRMS (n=3) plotted against measurements from EA/TC-IRMS (n=2) for hydrogen. Error bars represent the standard deviation (±1σ) of repeated measurements.

Data analysis and corrections

The δ^2H values measured for FAMEs are the product of the hydrogen native to the molecule and the contribution of the reagent (methanol) used for transesterification. An empirical correction was therefore applied to determine the effective hydrogen isotope value:

$$(H_n + 3)\delta^2H_{FAME} = H_n\delta^2H_{FA} + 3\delta^2H_{Me} \qquad (2)$$

where δ^2H_{FAME}, δ^2H_{FA} and δ^2H_{Me} are the hydrogen isotopic values of the FAME, FA and methyl group of methanol (Me) respectively. H_n is the number of H atoms in the FA and the δ^2H value of Me was measured using SNIF-NMR.

Figure 9 shows the δ^2H values determined for the four main fatty acids, listed according to their biosynthetic order and grouped according to their origin. As is evident in Figure 1, the European and non-European samples showed a similar trend according to the specific fatty acids considered. In particular, non-European samples had higher δ^2H mean values for FAs than European ones.

Analysis of variance and Tukey's test for an unequal number of samples were carried out on the data to verify differences between European and non-European samples. The δ^2H of methyl oleate was statistically different in the two groups of samples ($p<0.001$).

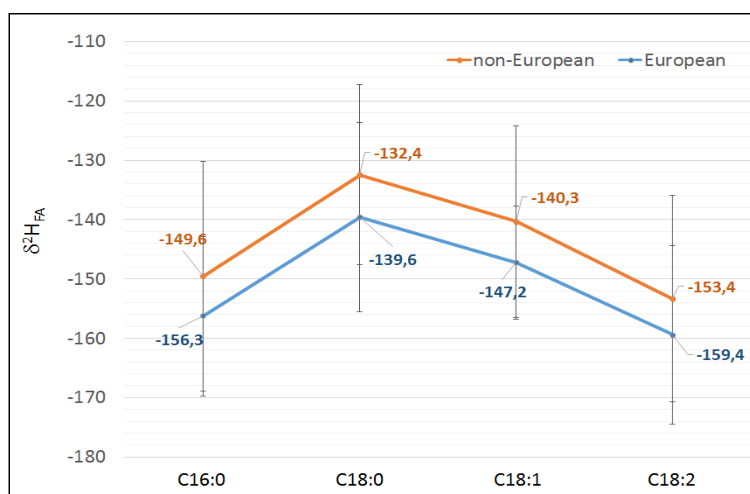

Figure 9. Trends for δ^2H values determined for the four main fatty acids, listed according to their biosynthesis and grouped according to European and non-European origin

PCA was applied using the δ^{13}C and δ^2H values of the four fatty acids as variables (Figure 10). The first two factors of PCA explained 64.7% of variance in the 8 original variables. The first component (36.2% of variance) was mainly positively loaded by the δ^2H determined in four FAs whereas the second component (28.5% of variance) was positively loaded by the δ^{13}C of four FAs. The score plot showed that the δ^2H values of the FAs decreased dispersion in the two groups, however discrimination between European and non-European olive oils did not improve significantly.

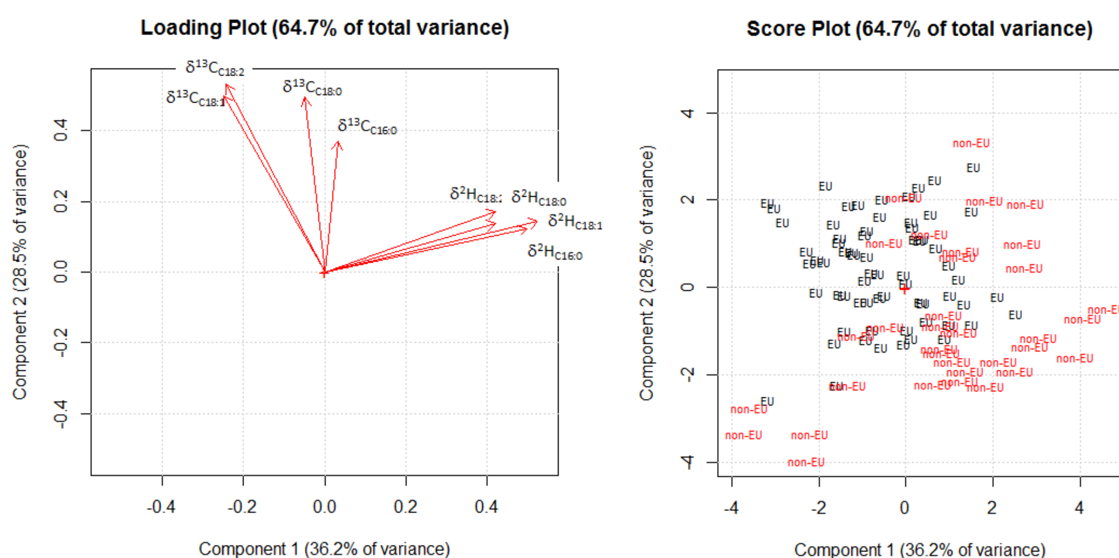

Figure 10. PCA of δ^{13}C and δ^2H values of FAs in extra-virgin olive oils

ANALYTICAL METHOD VALIDATION

To assess the reliability of the method, 10 extra-virgin olive oil samples of undisclosed geographical origin were analysed. The results of random forest analysis for origin assignment using the $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ of bulk samples and the $\delta^{13}C$ and $\delta^{2}H$ of fatty acids are shown in Table 1. The model applied had a mcc of 0.6 in 7-fold cross validation and the number of blind samples correctly assigned was 9 out of 10.

Table 1. Results of random forest analysis

Sample	Predicted origin	Real origin	
7KW	Extra UE	Tunisia	OK
BC2	UE	Argentina	NO
1QV	Extra UE	Turkey	OK
SV1	Extra UE	Australia	OK
DEF	UE	Portugal	OK
LPT	UE	Italy	OK
RP5	UE	Italy	OK
X8T	Extra UE	Uruguay	OK
A3Z	UE	Spain	OK
ZYR	UE	Spain	OK

CONCLUSION

This work demonstrates that classification of European and non-European EVOO is possible and applicable using IRMS. The results show that geographical discrimination of OO relies on the fact that isotopic composition is influenced by the geo-climatic characteristics of the area of origin. Moreover, the study highlighted the power of CSIA in comparison to bulk analysis. The isotopic fingerprint of specific sub-components adds further detailed information on local conditions (e.g. micro-climate, soil and water availability) and can therefore improve geographical discrimination.

A multivariate statistical approach based on the isotopic composition of the four FAs allowed to discriminate between European and non-European OO. In addition, by combining all the isotopic parameters measured, it was possible to obtain correct assignment of blind samples of undisclosed geographical origin.

This evidence makes it possible to conclude that in conjunction with other analytical techniques, isotope analysis may provide a useful tool for tracing the geographical origin of EVOO.

ACKNOWLEDGMENTS

This work is part of the EU FOODINTEGRITY project.

APPENDIX

Table 2. Summary of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ values determined on bulk olive oils and fatty acids

					$\delta^{13}C_{FA}$				$\delta^{2}H_{FA}$			
		$\delta^{13}C_{bulk}$	$\delta^{18}O_{bulk}$	$\delta^{2}H_{bulk}$	C16:0	C18:0	C18:1	C18:2	C16:0	C18:0	C18:1	C18:2
Extra UE	Argentina	-30.2	25.1	-150	-30.6	-29.8	-28.4	-28.7	-145	-146	-146	-166
Extra UE	Australia	-31.5	28.0	-134	-29.0	-32.1	-30.5	-31.8	-122	-119	-116	-125
Extra UE	Australia	-31.2	26.9	-129	-30.6	-32.5	-29.8	-31.0	-132	-124	-124	-140
Extra UE	Australia	-30.7	29.8	-134	-30.8	-30.6	-29.1	-28.9	-119	-119	-129	-140
Extra UE	Australia	-31.6	24.5	-136	-30.5	-32.8	-29.9	-31.0	-139	-116	-129	-141
Extra UE	Australia	-31.4	29.4	-137	-30.6	-32.7	-30.5	-31.3	-119	-109	-129	-140
Extra UE	Australia	-31.1	24.1	-130	-29.5	-31.4	-29.8	-29.7	-132	-120	-124	-146
Extra UE	Australia	-31.6	29.5	-142	-30.1	-32.4	-30.4	-31.3	-135	-124	-137	-160
Extra UE	Australia	-30.9	29.5	-139	-30.8	-31.5	-29.9	-31.4	-119	-110	-129	-125
Extra UE	Australia	-29.4	24.6	-144	-30.3	-30.2	-29.4	-31.4	-166	-159	-145	-160
Extra UE	Morocco	-29.0	27.0	-136	-30.5	-32.6	-28.0	-28.4	-137	-129	-135	-144
Extra UE	Peru	-30.0	21.3	-176	-31.2	-32.5	-29.2	-30.4	-192	-167	-180	-177
Extra UE	Tunisia	-29.0	27.0	-144	-29.7	-30.9	-28.2	-29.1	-146	-130	-138	-158
Extra UE	Tunisia	-30.5	26.6	-147	-30.4	-32.2	-29.1	-30.5	-157	-129	-140	-154
Extra UE	Tunisia	-30.5	25.8	-144	-30.9	-33.2	-29.2	-31.3	-154	-135	-134	-148
Extra UE	Tunisia	-30.3	26.6	-143	-30.8	-33.4	-29.5	-31.0	-167	-136	-135	-159
Extra UE	Tunisia	-30.4	25.1	-144	-31.0	-32.9	-29.6	-31.4	-153	-131	-140	-152
Extra UE	Tunisia	-30.5	25.7	-142	-31.3	-32.7	-29.5	-32.0	-146	-123	-137	-153
Extra UE	Tunisia	-30.1	26.7	-142	-30.6	-32.6	-28.9	-31.2	-165	-130	-136	-155
Extra UE	Tunisia	-30.1	25.2	-143	-31.1	-32.3	-29.0	-31.1	-163	-135	-137	-148
Extra UE	Tunisia	-30.0	25.9	-144	-30.9	-32.2	-28.8	-30.9	-150	-129	-137	-151
Extra UE	Tunisia	-30.1	25.7	-141	-31.2	-32.2	-29.2	-31.3	-147	-135	-139	-157
Extra UE	Tunisia	-29.6	26.2	-141	-30.7	-30.2	-28.4	-29.8	-141	-124	-136	-150
Extra UE	Tunisia	-29.8	25.9	-142	-31.2	-31.1	-28.9	-30.5	-152	-130	-139	-153
Extra UE	Turkey	-30.4	21.0	-150	-30.6	-32.9	-30.5	-31.0	-137	-135	-134	-136
Extra UE	Turkey	-28.6	26.5	-147	-29.7	-30.6	-28.0	-29.0	-141	-127	-127	-133
Extra UE	Turkey	-28.8	26.5	-144	-28.3	-30.1	-27.6	-28.5	-157	-123	-118	-138
Extra UE	Turkey	-29.6	26.6	-138	-29.4	-30.6	-28.2	-29.7	-121	-125	-126	-133
Extra UE	Uruguay	-30.9	21.3	-168	-30.4	-31.6	-29.2	-30.2	-159	-138	-170	-166
Extra UE	Uruguay	-28.9	23.0	-153	-29.0	-29.3	-28.2	-29.4	-157	-119	-151	-159
Extra UE	USA	-30.1	25.8	-162	-30.9	-29.9	-31.2	-31.0	-173	-144	-167	-179
Extra UE	USA	-30.2	25.4	-167	-31.6	-31.7	-30.5	-31.1	-177	-152	-172	-186
Extra UE	USA	-30.0	25.1	-166	-31.4	-33.1	-29.8	-29.8	-188	-172	-169	-201
Extra UE	USA	-30.9	24.8	-162	-32.5	-33.1	-29.8	-30.9	-177	-160	-166	-183
UE	France	-29.1	24.3	-155	-29.8	-32.0	-27.6	-28.3	-166	-145	-155	-180
UE	France	-30.2	23.0	-159	-31.1	-34.0	-28.8	-29.4	-191	-173	-160	-179
UE	France	-29.2	23.6	-150	-31.2	-32.9	-28.4	-29.5	-150	-132	-147	-163
UE	France	-28.6	26.9	-139	-29.5	-32.4	-27.4	-28.0	-144	-124	-136	-155
UE	France	-29.4	23.7	-158	-29.5	-32.1	-27.8	-28.3	-166	-144	-158	-185
UE	France	-28.6	24.1	-153	-30.4	-33.1	-28.2	-28.5	-158	-128	-152	-176
UE	Greece	-29.6	23.5	-158	-30.1	-31.4	-28.5	-30.0	-146	-131	-157	-177

UE	Greece	-29.5	23.5	-163	-30.5	-31.4	-28.2	-29.7	-144	-137	-144	-169
UE	Greece	-29.3	23.7	-162	-30.2	-31.7	-28.0	-29.2	-167	-146	-161	-179
UE	Greece	-28.2	23.6	-147	-29.5	-31.3	-27.1	-28.0	-158	-146	-148	-156
UE	Greece	-27.9	23.5	-144	-29.1	-30.5	-27.8	-27.2	-169	-147	-164	-190
UE	Greece	-28.6	24.1	-147	-28.9	-31.2	-28.2	-27.3	-149	-181	-154	-137
UE	Greece	-29.7	24.3	-148	-31.1	-31.9	-28.8	-28.5	-142	-116	-139	-151
UE	Greece	-29.0	24.3	-155	-29.7	-31.2	-28.6	-28.5	-153	-142	-156	-164
UE	Italy	-26.0	25.8	-146	-31.4	-30.2	-27.8	-28.6	-141	-123	-144	-162
UE	Italy	-29.8	23.9	-148	-30.4	-31.1	-27.6	-28.2	-188	-162	-151	-149
UE	Italy	-29.5	26.1	-140	-30.9	-30.0	-27.4	-27.4	-143	-123	-134	-146
UE	Italy	-30.4	23.1	-155	-30.6	-31.8	-27.3	-28.3	-150	-132	-157	-172
UE	Italy	-29.6	24.0	-147	-30.5	-31.2	-26.9	-28.0	-179	-150	-147	-146
UE	Italy	-30.2	23.4	-151	-30.0	-31.7	-27.6	-28.6	-181	-138	-153	-149
UE	Italy	-29.7	23.8	-152	-30.4	-30.5	-28.0	-28.2	-160	-132	-147	-171
UE	Italy	-30.0	22.9	-153	-30.5	-29.7	-28.0	-28.2	-150	-123	-150	-172
UE	Italy	-30.3	22.8	-155	-31.3	-29.9	-26.9	-26.6	-182	-124	-155	-148
UE	Italy	-29.8	22.7	-155	-30.6	-30.7	-27.9	-27.8	-157	-151	-160	-152
UE	Italy	-30.2	24.7	-146	-30.4	-31.1	-27.9	-28.1	-127	-132	-131	-142
UE	Italy	-28.2	26.8	-146	-30.4	-29.2	-26.8	-28.1	-179	-162	-157	-172
UE	Italy	-28.6	25.7	-146	-30.4	-29.8	-27.8	-27.9	-165	-123	-155	-141
UE	Italy	-30.4	22.8	-156	-30.6	-31.3	-28.4	-29.2	-165	-143	-155	-172
UE	Italy	-29.5	25.5	-147	-30.5	-30.4	-28.3	-28.8	-143	-129	-142	-161
UE	Italy	-30.2	23.1	-149	-30.3	-31.2	-28.6	-28.8	-151	-143	-149	-168
UE	Italy	-29.6	25.1	-155	-30.8	-32.6	-28.7	-29.7	-156	-140	-154	-177
UE	Italy	-30.3	24.1	-154	-30.8	-32.5	-29.3	-29.6	-154	-145	-153	-168
UE	Italy	-29.9	23.4	-154	-30.6	-32.3	-28.9	-29.6	-155	-178	-142	-155
UE	Italy	-29.2	24.2	-145	-30.0	-31.8	-27.9	-29.2	-144	-133	-157	-177
UE	Italy	-30.0	24.6	-148	-31.8	-32.1	-28.7	-29.4	-149	-162	-149	-114
UE	Italy	-30.1	25.1	-137	-31.4	-32.2	-28.7	-30.0	-144	-135	-138	-150
UE	Italy	-30.2	22.4	-159	-32.1	-32.4	-28.6	-30.4	-166	-142	-160	-181
UE	Italy	-28.8	26.0	-149	-30.3	-30.7	-27.5	-28.7	-163	-149	-149	-169
UE	Italy	-30.5	22.1	-156	-30.9	-32.2	-29.3	-30.1	-155	-166	-148	-137
UE	Italy	-30.5	23.0	-159	-30.1	-32.6	-29.0	-29.8	-174	-152	-158	-170
UE	Italy	-29.8	23.0	-158	-30.5	-32.9	-28.4	-29.4	-162	-146	-158	-172
UE	Italy	-28.9	25.7	-152	-31.4	-32.2	-27.6	-29.2	-153	-165	-147	-138
UE	Portugal	-30.5	28.8	-134	-31.0	-29.9	-27.9	-26.7	-135	-119	-126	-138
UE	Portugal	-30.2	28.5	-133	-30.1	-30.2	-28.9	-29.3	-160	-139	-128	-150
UE	Portugal	-32.1	27.2	-137	-31.9	-31.8	-29.3	-29.8	-171	-119	-132	-150
UE	Portugal	-30.7	26.7	-147	-30.9	-32.3	-29.0	-30.1	-145	-135	-150	-167
UE	Portugal	-30.6	26.4	-137	-31.0	-30.6	-28.1	-28.4	-153	-130	-136	-151
UE	Portugal	-30.7	27.2	-145	-30.5	-31.6	-28.9	-29.0	-171	-139	-150	-167
UE	Portugal	-30.5	30.1	-133	-30.4	-31.0	-29.2	-28.8	-145	-139	-132	-150
UE	Portugal	-30.1	27.0	-142	-30.5	-32.0	-29.2	-29.0	-143	-135	-141	-168
UE	Portugal	-30.6	27.0	-137	-31.2	-30.7	-28.4	-30.7	-149	-144	-131	-145

UE	Portugal	-30.7	28.0	-135	-31.9	-31.8	-28.7	-29.3	-137	-110	-133	-142
UE	Portugal	-29.7	26.6	-141	-31.0	-32.2	-28.7	-29.0	-152	-131	-143	-154
UE	Portugal	-30.5	26.7	-139	-30.9	-30.5	-30.3	-30.5	-152	-128	-134	-152
UE	Spain	-29.2	25.2	-151	-30.6	-29.6	-28.6	-28.9	-177	-167	-154	-150
UE	Spain	-28.9	26.8	-144	-30.2	-30.1	-28.5	-28.9	-171	-154	-150	-152
UE	Spain	-29.7	28.1	-151	-30.9	-31.2	-28.8	-28.3	-142	-122	-147	-178
UE	Spain	-28.9	27.3	-158	-30.0	-30.7	-28.3	-28.4	-150	-145	-139	-147
UE	Spain	-29.1	25.4	-152	-29.8	-30.2	-28.4	-28.4	-161	-125	-151	-175
UE	Spain	-29.1	24.9	-157	-31.3	-30.2	-26.3	-28.0	-173	-142	-164	-169
UE	Spain	-29.3	24.5	-153	-30.1	-31.3	-28.7	-28.9	-161	-144	-148	-171
UE	Spain	-28.8	27.8	-147	-29.7	-31.0	-27.6	-29.1	-151	-148	-143	-136
UE	Spain	-30.5	27.4	-135	-31.1	-31.4	-29.5	-29.4	-143	-120	-144	-146
UE	Spain	-30.2	23.7	-154	-31.0	-30.4	-27.4	-27.3	-151	-128	-137	-149
UE	Spain	-29.2	26.6	-146	-29.5	-30.1	-27.8	-28.3	-145	-121	-142	-160
UE	Spain	-29.4	25.5	-145	-30.3	-31.4	-28.9	-28.6	-154	-163	-150	-178
UE	Spain	-29.9	25.3	-146	-30.8	-31.2	-30.0	-30.4	-142	-109	-131	-147
Blind	7KW	-28.8	26.1	-143	-30.8	-31.8	-29.5	-30.4	-143	-121	-135	-160
Blind	BC2	-30.5	22.7	-176	-29.5	-32.3	-27.9	-28.4	-171	-153	-136	-158
Blind	1QV	-28.8	25.3	-143	-28.2	-29.1	-28.0	-28.4	-157	-130	-141	-142
Blind	SV1	-31.5	29.6	-141	-30.8	-31.0	-31.0	-30.9	-136	-131	-135	-168
Blind	DEF	-28.9	26.6	-154	-30.7	-31.9	-28.6	-28.9	-151	-134	-147	-161
Blind	LPT	-28.3	26.3	-146	-30.6	-30.1	-27.7	-27.8	-172	-141	-158	-175
Blind	RP5	-29.5	23.7	-157	-28.7	-32.2	-27.1	-27.2	-163	-144	-153	-163
Blind	X8T	-29.5	22.6	-157	-28.9	-29.9	-27.9	-29.3	-153	-127	-135	-164
Blind	A3Z	-29.9	29.6	-142	-29.4	-31.7	-28.1	-28.9	-161	-144	-148	-171
Blind	ZYR	-28.8	26.4	-152	-29.3	-29.8	-27.7	-28.4	-153	-129	-143	-139

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CHAPTER 6 CONCLUSIONS

In this thesis, the use of stable isotope ratios as markers for food authenticity and traceability was extensively studied and verified. IRMS methods based on bulk analysis and compound-specific analysis were developed to characterise premium products and derivatives, such as cereal crops, pasta, wine, balsamic vinegar, oenological tannins and extra-virgin olive oil.

Specifically, the isotopic composition of wheat and pasta was analysed with the aim of evaluating if and how the geographical origin and farming system affect stable isotope ratios. Firstly, the variability of the H, C, N, O and S stable isotope ratios of bulk samples along the pasta production chain (durum wheat, flour and pasta) produced using both conventional and organic farming systems in four Italian regions was investigated. The statistical model allowed particularly good differentiation of products on the basis of geographical origin (Emilia-Romagna, Tuscany, Molise and Basilicata). Subsequently, the method developed to measure the C and N isotopic composition of amino acids significantly improved discrimination between organic and conventional wheat compared to bulk analysis. With a view to the future, it may be expected that these results will lead to the development of an analytical control procedure checking on the geographical provenance of organic and conventional pasta and its raw materials.

The variation in the $^{15}N/^{14}N$ isotope ratio for the purposes of traceability was also checked for Italian wines along the oenological chain, for the first time to date. Despite the isotopic fractionation observed from soil to wine, the $\delta^{15}N$ values of vine-branches, leaves, grapes and wine reflected the variability of $\delta^{15}N$ characteristic of the provenance area. Moreover, the study showed that proline conserves the nitrogen isotopic fingerprint of the growing soil, independently of the fermentation process, type of yeast used, addition of nitrogen adjuvants and wine ageing process. Consequently, the $\delta^{15}N$ of proline can be suggested as an additional isotopic marker for the geographical characterisation of wine.

Furthermore, $\delta^{13}C$ was shown to be a tool that can be used to distinguish and characterise commercial tannins according to their botanical origin. This approach provided effective differentiation of tannins of different origin and could be used as a complementary method for the approaches based on sugar or polyphenolic composition suggested by OIV.

The same analytical approach was applied to the ingredients of “aceto balsamico di Modena IGP” (ABM) to evaluate its authenticity. No isotopic variation from wine to vinegar and ABM, and from the original must to ABM must was observed, providing experimental evidence that the analytical

procedure routinely used to verify the compliance of wine with European Regulations can also be used to evaluate the authenticity of ABM.

Finally, the isotopic composition of European and non-European extra-virgin olive oil was investigated. A dataset with the H, C and O isotopic values measured in bulk samples and the fatty acids of authentic extra-virgin olive oils collected in 13 different countries worldwide was created, allowing the distinction of two macro areas (European *versus* non-European). This study showed that the variables contributing most to discrimination of the two groups were the isotopic values of the H and C of fatty acids. Indeed, a multivariate statistical approach based on the isotopic composition of fatty acids offered good discrimination between European and non-European olive oil. Consequently, isotope analysis of fatty acids in particular could be used to verify the authenticity of extra-virgin olive oil samples, becoming an additional tool for ensuring compliance with European law. An improvement in differentiating European and non-European olive oils was also found by combining isotopic and NMR profiling methods.

The stable isotope ratio approach applied throughout this thesis proved to be highly reliable and in general it was confirmed that the isotopic composition of premium products reflects the geographical, climatic or geological composition of the site of provenance and the farming system used. Moreover, this thesis highlights that development of new compound-specific isotope analysis methods is crucial to generate more specific information, allowing more reliable food authentication and improved protection of consumers and honest producers.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Schools and training courses

September 2016 - Visiting PhD Student, Jožef Stefan Institute (Ljubljana, Slovenia).

October 2016 - Summer School: "Writing on Food. How to publish in high impact factor journals: the keys to success, with daily writing exercises", University of Bologna (Bologna, Italy).

March 2016 - School of Statistics, Fondazione Edmund Mach (S. Michele all'Adige, Italy).

January 2015 - Chemometrics School, University of Genova (Genova,Italy).

April 2014 - Stable Isotope Course: an introduction to uses in ecology and plant physiology, Technische Universität München (Freising, Germany) – University of South Bohemia (Budweis, Czech Republic).

Conferences

October 2016 - 1st Food Chemistry Conference (Amsterdam, The Netherlands).

May 2016 - 1st Isotope Ratio MS DAY, Fondazione Edmund Mach (S. Michele all'Adige, Italy).

October 2015 - 4th MS Food Day (Foggia, Italy).

September 2015 - XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology (Perugia, Italy).

July 2015 - IX In Vino Analytica Scientia (IVAS), Fondazione Edmund Mach, Laimburg Research Centre for Agriculture and Forestry (Mezzocorona, Italy).

July 2015 - Workshop: Metabolomics applications to the grapevine and wine knowledge – the CRI-FEM approach, Fondazione Edmund Mach (S. Michele all'Adige, Italy).

September 2014 - XIX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology (Bari, Italy).

July 2014 - X Congresso Italiano di Chimica degli Alimenti (Firenze, Italy).

OVERVIEW OF SCIENTIFIC PUBLICATIONS AND CONFERENCE PROCEEDINGS

Scientific publications

M. Egli, S. Hafner, C. Derungs, J. Ascher-Jenull, G. Sartori, G. Raab, F. Camin, L. Bontempo, **M. Paolini**, L. Ziller, T. Bardelli, M. Petrillo, S. Abiven, "Decomposition and stabilisation of Norway spruce needle-derived material in Alpine soils using a ^{13}C-labelling approach in the field" *Biogeochemistry.* Accepted.

M. Paolini, L. Ziller, D. Bertoldi, L. Bontempo, R. Larcher, G. Nicolini, F. Camin, "δ^{15}N from soil to wine in bulk samples and proline" *J. Mass Spec.* **2016**, 51, 668-674.

L. Bontempo, F. Camin, **M. Paolini**, C. Micheloni C., K.H. Laursen, "Multi-isotopic signatures of organic and conventional Italian pasta along the production chain" *J. Mass Spec.* **2016**, 51, 675-683.

C. Durante, L. Bertacchini, L. Bontempo, F. Camin, D. Manzini, P. Lambertini, A. Marchetti, **M. Paolini**, "From soil to grape and wine: Variation of light and heavy elements isotope ratios" *Food Chem.* **2016**, 210, 648-659.

F. Camin, A. Pavone, L. Bontempo, R. Wehrens, **M. Paolini**, A. Faberi, R. M. Marianella, D. Capitani, S. Vista, L. Mannina, “The use of IRMS, ^{1}H-NMR and chemical analysis to characterise Italian and imported Tunisian olive oils” *Food Chem.* **2015**, 196, 98-105.

M. Paolini, L. Ziller, K.H. Laursen, S. Husted, F. Camin, “Compound-Specific δ^{15}N and δ^{13}C Analyses of Amino Acids for Potential Discrimination between Organically and Conventionally Grown Wheat” *J. Agr. Food Chem.* **2015**, 63, 5841-5850.

M. Perini, **M. Paolini**, M. Simoni, L. Bontempo, U. Vrhovsek, M. Sacco, F. Thomas, E. Jamin, A. Hermann, F. Camin, "Stable isotope ratio analysis for verifying the authenticity of balsamic and wine vinegar" *J. Agr. Food Chem.* **2014**, 62, 8197-8203.

D. Bertoldi, A. Santato, **M. Paolini**, A. Barbero, F. Camin, G. Nicolini, R. Larcher, "Botanical traceability of commercial tannins using the mineral profile and stable isotopes" *J. Mass Spec.* **2014**, 49, 792-801.

R. Ferrarini, G.M. Ciman, L. Cugini, F. Lonardi, L. Bonetti, **M. Paolini**, F. Camin, "Effetti sui rapporti degli isotopi stabili dei vini" *Il corriere vinicolo.* **2014**, 87, 13-15.

Conference proceedings

M. Paolini, L. Ziller, L. Bontempo, F. Camin, “Discrimination between European and extraEuropean olive oils using stable isotope ratio analysis” Proc. 1st Food Chemistry Conference, Amsterdam (The Netherlands), 30 October-1 November 2016.

M. Paolini, F. Camin, "Using GC/IRMS analysis to improve food traceability" Proc. 1st Isotope Ratio MS DAY, S. Michele all'Adige (Italy), 09-11 May 2016.

M. Paolini, D. Bertoldi, L. Ziller, R. Larcher, G. Nicolini, F. Camin, "$\delta^{15}N$ of proline: the direct link between soil and wine" Proc. 4th MS Food Day, Foggia (Italy), 7 - 9 October 2015.

F. Camin, L. Bontempo, **M. Paolini**, C. Micheloni, K. H. Laursen, S. Husted, "Stable isotope analysis for authenticity testing of italian organic pasta: variations in $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ along the production chain" Proc. 4th MS Food Day, Foggia (Italy), 7 - 9 October 2015.

M. Paolini, "Innovative methods for isotopic analysis in organic fractions of nitrogen to enable the traceability of food" Proc. XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Perugia (Italy), 23 - 25 September 2015.

M. Paolini, D. Bertoldi, L. Ziller, C. Durante, R. Larcher, G. Nicolini, A. Marchetti, F. Camin, "Nitrogen isotope ratio from soil to wine: an initial approach in viticulture and oenology" Proc. IX IVAS 2015, Mezzocorona (Italy), 14 - 17 July 2015.

F. Camin, G. Ciman, F. Lonardi, C. Gostoli, M. Simoni, **M. Paolini**, R. Ferrarini, "Modification of isotopic ratios by the membrane contactor during wine dealcoholisation" Proc. IX IVAS 2015, Mezzocorona (Italy), 14 - 17 July 2015.

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M. Paolini, L. Ziller, C. Durante, A. Marchetti, F. Camin, "Is $\delta^{15}N$ a potential traceability tool in viticulture and oenology?" Proc. X National Congress of Food Chemistry, Firenze, 6 - 10 July 2014.