UNIVERSITÀ DEGLI STUDI DI UDINE

CORSO DI DOTTORATO DI RICERCA IN SCIENZE DEGLI ALIMENTI
CICLO XXIII

TESI DI DOTTORATO DI RICERCA

IRMS (Isotope Ratio Mass Spectrometry) and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) for the traceability and characterisation of premium products

Dottorando: Dott. Luana Bontempo

Tutor: Dott. Lara Manzocco

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GLOSSARY

Non-technical

AOAC	Association of Official Analytical Chemists
BSE	Bovine Spongiform Encephalopathy
CAP	Common Agricultural Policy
EC	European Commission
EEC	European Economic Community
EU	European Union
FAO	Food and Agriculture Organisation
IAEA	International Atomic Energy Association
IPM	Integrated Pest Management
ISMEA	Indicatori del Sistema Agroalimentare Italiano
PDO	Protected Designations of Origin
PGI	Protected Geographical Indication
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.
AMF	Arbuscular Mycorrhizal Fungi
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
CDT	Canyon Diablo Troilite - FeS from a meteorite natural sulphur deposit used as an international standard for which the $^{34}S/^{32}S$ ratio is precisely known and is defined as 0 ‰ on the $\delta^{34}S$‰ scale, now exhausted and replaced by V-CDT defined by IAEA AgS S-1 silver sulfide
CEC	Cation Exchange Capacity
D/H	The ratio of the isotope of hydrogen with atomic mass 2 (deuterium) to the isotope of hydrogen with atomic mass 1
δD‰	"Delta deuterium *per* mil". The D/H ratio expressed relative to the international standard Vienna-Standard Mean Ocean Water.
EA	Elemental Analyser
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IRMS	Isotope Ratio Mass Spectrometry
GC	Gas Chromatography
GMWL	Global Meteoric Water Line
KIE	Kinetic Isotope Effect
LMWL	Local Meteoric Water Lines
$^{15}N/^{14}N$	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 Adenosine Diphoshate
$^{18}O/^{16}O$	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.

OOA	Oxaloacetate
ox	Oxidase
PDB	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, now exhausted and replaced by V-PDB
PEP	Phosphoenolpyruvate
PGA	Phosphoglycerate
PGAL	3-Phospho-glyceraldehyde
re	Reductase
RuDP	Ribulose-1,5-diphosphate
$^{34}S/^{32}S$	The ratio of the isotope of sulphur with atomic mass 34 to the isotope of sulphur with atomic mass 32.
$\delta^{34}S$‰	"Delta Sulphur-34 *per* mil". The $^{34}S/^{32}S$ ratio expressed relative to the international standard Vienna-Canyon Diablo Troilite
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

There is an increasing demand for reliable analytical methods to verify the authenticity of the food we eat. First of all there is a growing enthusiasm for high quality food with a clear regional identity among consumers and scares such as BSE, chicken influenza and malpractice by some international food producers have added to public sensitivity regarding the validity of food origin labelling. In addition to heightened consumer awareness in the last few years, European Union policy on food has also been orientated towards safeguarding consumers. To achieve this scope the EU has on the one hand reinforced national control activities and on the other hand investigated new markers able to support food characterisation and geographical traceability, ensuring its authenticity.

Along with elemental composition, the stable isotope ratios of bioelements such as hydrogen, carbon, oxygen, nitrogen and sulphur have been used for thirty years, both separately or jointly, in order to check the authenticity of different premium products. With regard to stable isotopes, this ability is based on isotopic fractionation occurring during physical and chemical processes, as well as along metabolic pathways. For this reason, the different isotopic ratios vary according to photosynthetic and nitrogen cycles, pedological characteristics of soils, agricultural practices and geographical origin. In contrast to stable isotope ratios, which mostly depend on climatic, hydrological or geographical conditions, elements are profoundly affected by soil geology and pedological characteristics, over and beyond ‘contamination’ during processing.

Isotope ratio mass spectrometry and inductively coupled plasma mass spectrometry methods were developed to characterise and determine the authenticity of Italian and European extra virgin olive oils, olive oil squalene/squalane, Italian alpine PDO cheeses, Italian tomatoes and tomato derived products (juice, passata, paste).

In particular, the $\delta^{13}C$ and $\delta^{18}O$ of Italian extra virgin olive oils made it possible to distinguish samples from North and South Italy, whereas different δD values, determined for the first time along with $\delta^{18}O_{bulk}$, were found in oils from the Italian Adriatic and Tyrrhenian coasts, improving the differentiation of samples from Central Italy. The isotopic composition of rainfall, along with the average temperature and humidity on the two coasts, can explain these differences in the oils. The combination of elemental composition and isotopic ratios analysis extended to olive oils collected at eight European sites, allowed good discrimination in relation to geographical provenance. $\delta^{13}C$, $\delta^{18}O$ and δD were shown to be significantly correlated to geographical and climatic factors (*e.g.* latitude, distance from the coast, temperature), whereas elemental composition was related to the geological characteristics of the soil where the products were grown. Furthermore, $\delta^{13}C$ was shown to be a suitable marker for identification of squalene/squalane origin. By defining $\delta^{13}C$ threshold values of -27.4‰ for olive oil squalene/squalane, the addition of shark products can be detected starting from a minimum of 10%.

The same analytical approaches, when applied to seven PDO Italian cheeses, made it possible to characterise them, and a statistical model built on the basis of the most significant analytical parameters obtained 94% discrimination of cheese type. Furthermore $\delta^{13}C$, determined in casein or glycerol, allowed estimation of maize uptake in animal diet, making it possible to verify whether the levels established in the PDO production protocols were observed. $\delta^{18}O$ and $\delta^{15}N$ were instead shown to be very useful in differentiating mountain products.

Stable isotope ratios and elemental composition determined in tomatoes and tomato products allowed good discrimination of samples from different Italian regions. Over 95% of the samples were correctly reclassified into the production site in crossvalidation, in spite of the food processing they had undergone. The δD and $\delta^{18}O_{bulk}$ values of tomatoes and derivatives were reported for the first time in the literature.

To conclude the models developed for the specific commodities considered can be proposed as suitable tools for the detection of mislabelling and consumer protection, demonstrating that such analytical parameters can effectively contribute towards distinguishing the authenticity of commercial samples, becoming a tool for checking compliance with the law.

Keywords: stable isotope ratios, IRMS, elemental composition, ICP-MS, food, origin, traceability, authenticity.

CHAPTER 1 INTRODUCTION

1.1 FOOD AUTHENTICITY

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

1.1.1 General considerations

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

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

(i) economic adulteration of high value foods;
(ii) misdescription and/or mislabelling of geographical, botanical or species origin;
(iii) non-compliance with the established legislative standards
(iv) implementation of processing practices which are not admitted (*e.g.* irradiation, freezing).

Nowadays, objective assessment of food authenticity has become of paramount importance, as consumers come into daily contact with a wide variety of foods. Globalisation indeed means that more and more foods are traded around the world. Traceability has thus become a cornerstone of the EU’s food safety policy, representing a risk-management tool which enables those in the food industry or the authorities to withdraw or recall products which have been identified as unsafe. The increasing complexity and length of the food chain, as well as recent food scares, have also added to public sensitivity regarding the origin of food and have underlined the need for tools ensuring that foods are of a high quality and safe to be eaten when they reach the consumer.

1.1.2 EU legislation

In 1979 European controls on food labelling were introduced with Directive 112/79/EEC of the European Parliament. Additional controls and amendments have since been added, creating an array of labelling requirements.

Over two decades, most legislation regarding food safety has been standardised in the European Community. Because of the high administrative costs of standardisation the principle of mutual recognition was used for issues which did not directly endanger consumers' health. European law, and in particular the "Cassis de Dijon" ruling (case 120/78, Court of Justice of the European Communities, February 1979), now ensures that while standards might differ across countries, a country cannot oppose importation from another EU country for technical reasons (except in cases involving sanitary issues or toxic waste issues).

In 2000, the original 1979 directive and its amendments were consolidated into Directive 13/2000/EC of the European Parliament, which focuses on preventing fraudulent or deceptive practices and food adulteration. Directive 13/2000/EC requires detailed food labelling including the exact nature and characteristics of a product, enabling informed consumer choice. It also requires the list of ingredients to include the specific names of all raw materials in descending order by weight.

The reliance on mutual recognition in order to avoid trade barriers between countries, together with the need to differentiate quality products from the bulk production generated by the Common Agricultural Policy (CAP) arrangements, has resulted in more importance being given to national labelling. For example, national labels of food quality in France and Italy have appeared more and more attractive to consumers, and therefore to producers, who use them to segment the market. Pressure from countries that had developed a labelling system, largely based on geographical indications, namely France and Italy, led to the definition of a EU-wide system of labelling, in what is called the '1992 regulation of food quality'. Indeed in 1992 and subsequently in 2006 (2081/92/EEC and 510/2006/EC), the EU Regulations on Protected Designations of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG) provided a set of common rules across countries to register and protect geographical names used to identify food products and traditional production processes. These two sets of regulations are the basis of quality policy system of the EU because they ensure that only those products genuinely originating in a region can be sold as such, eliminating unfair competition and misleading products that may be of inferior quality or made with different components.

Finally, the new European Union Traceability Regulation (178/2002/EC) which came into force in January 2005, defines ‘traceability’ as “the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution”. While most value chains only allow tracing one step forward and one step back trace, a more detailed traceability system back to the seed is required, for example to ensure that products have not been genetically modified.

1.1.3 Current Perspective

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

Multi-element and isotopic analysis have been applied, separately or together, to a range of foodstuffs for about thirty years, with varying degrees of certainty in order to develop methods that could verify their authenticity. Application of a combination of isotopic and elemental compositions in order to verify the authenticity of premium regional products is based on evidence that these parameters are affected by various complementary factors. In particular stable isotope ratios mostly depend on botanical, climatic or geographical conditions, whereas mineral elements are deeply affected by geology and the pedological characteristics of soil, excluding ‘contamination’ during processing.

1.2 STABLE ISOTOPES AND ELEMENTS

1.2.1 Definition of stable isotopes

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

$$\delta‰ = \frac{R_{sample} - R_{s\tan dard}}{R_{s\tan dard}} \cdot 1000 \qquad (1)$$

where R is the ratio between heavier and lighter isotopes.

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

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

Natural variation in isotopic composition is due to the different chemical-physical characteristics of the different isotopes of a certain element, owing to different weight and nuclear spin. In a chemical/biochemical reaction or in a phase transition, these differences can both interfere with the speed of reaction (kinetic effect) and affect the energetic state of the system (thermodynamic effect).

Due to the smaller weight the lighter isotopes have greater mobility and smaller bound strength and consequently lower activation energy (kinetic effect).

The thermodynamic effect is due to the different free energy of isotopically different molecular species: heavier molecules have lower free energy, so they have greater inertia in reaction and tend to concentrate in the condensed phase.

Isotopic fractionation can also be due to situations with an altered reaction equilibrium, such as an instantaneous change in temperature, removal of a reactant or of a reaction product. This kind of fractionation (of non equilibrium, such as enzymatic reactions) determines the enrichment of a particular isotopic species, but without pre-established rules.

Subsequently, factors affecting the variability of the isotopic ratios investigated in this thesis (D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) were broadened, in order to clarify their applicative capability.

1.2.2 Natural variation in stable isotope abundance

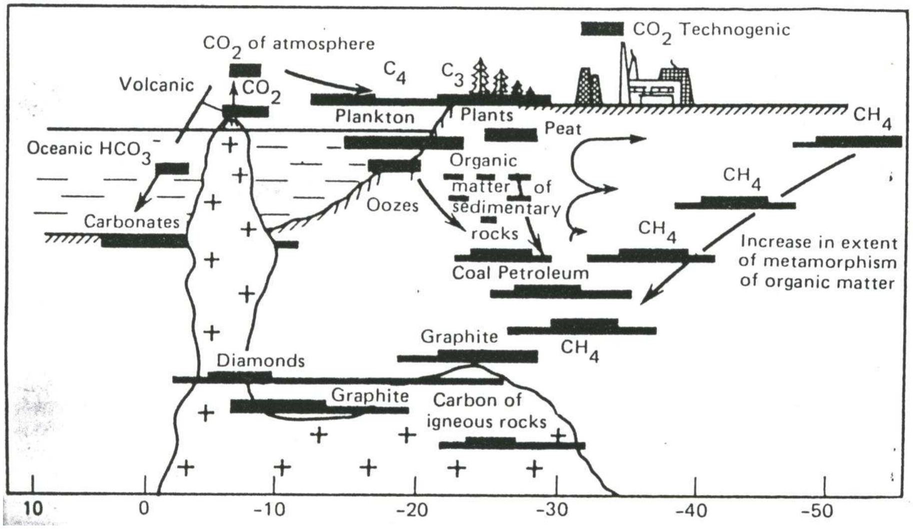
CARBON

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

- intake of carbon from the environment by organisms with ^{13}C depletion,
- exchange between atmosphere and hydrosphere leading to an enrichment of ^{13}C (Galimov, 1985),
- group of reactions in animal and vegetal bio-systems causing further fractionation.

Intake of CO_2 through photosynthesis is the main reaction involved in biological fractionation. 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, photosynthetic products show different ranges of $\delta^{13}C$ according to the kind of photosynthetic cycle (C_3, C_4 or CAM) because of the different isotopic discrimination capability of carboxylase enzymes involved in CO_2 fixation, in addition to different CO_2 concentrations in plants.

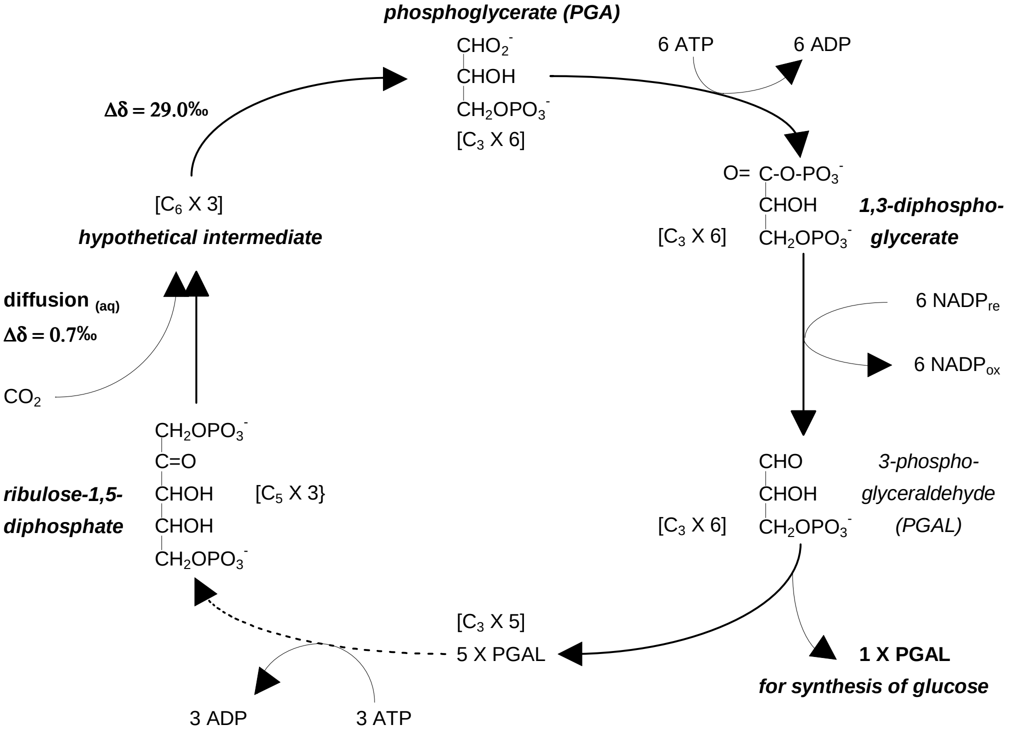
Figure 1. Scale of qualitative isotopic composition of carbon in some natural substances. (Galimov, 1985)



Calvin cycle

The main assimilation pathway of CO_2 described by Calvin, called C_3 because CO_2 is fixed in intermediate products with three atoms of carbon, is characteristic of plants from cold-temperate areas (*e.g.* tomatoes, potatoes, beetroot, wheat, rice, oats, barley, rye, soya bean, grapes, oranges and apples). Atmospheric CO_2 gets into leaf' parenchyma cells through the stomata and dissolves in the cytoplasm (Hatch and Slack, 1966; Smith and Epstein, 1971). There it binds to ribulose-1,5-diphosphate (RuDP), producing two molecules of phosphoglycerate (PGA), which are subsequently reduced to phosphoglyceraldehyde (PGAL), the first sugar of photosynthesis (Figure 2) (Taiz and Zeiger, 1998). Five out of six new PGAL molecules are used to synthesise more RuDP via a series of complex reactions driven by ATP (not shown in Figure 2). The sixth molecule of PGAL may be used to synthesise glucose (usually regarded as the end product of photosynthesis) via combinations and rearrangements. The glucose may be subsequently used to synthesise complex carbohydrates such as sucrose, structural materials such as cellulose, or energy storage compounds such as starch and lipids (Ting, 1982). Although the initial reaction of CO_2 with RuDP produces the overriding ^{13}C isotope effect associated with these species, there are many other factors which contribute to the final $\delta^{13}C$‰ value of plant material, such as 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 which utilise the C_3 pathway (Krueger and Reesman, 1982).

Figure 2. Synthesis of carbohydrate by the Calvin (C_3) cycle. Each molecule of CO_2 combines with one molecule of ribulose-1,5-diphosphate (RuDP, a five carbon sugar) to form a hypothetical unstable six carbon activated complex. This immediately splits into two molecules of phosphoglycerate (PGA, a C3 molecule). PGA is phosphorylated by ATP and then reduced by NADPre to form phosphoglyceraldehyde (PGAL, a C3 sugar). Five out of six new PGAL molecules are used to synthesise more RuDP via a series of complex reactions driven by ATP (not shown here). The sixth molecule of PGAL may be used to synthesise glucose (adapted from Ting, 1982; O'Leary, 1988; Taiz and Zeiger, 1998).

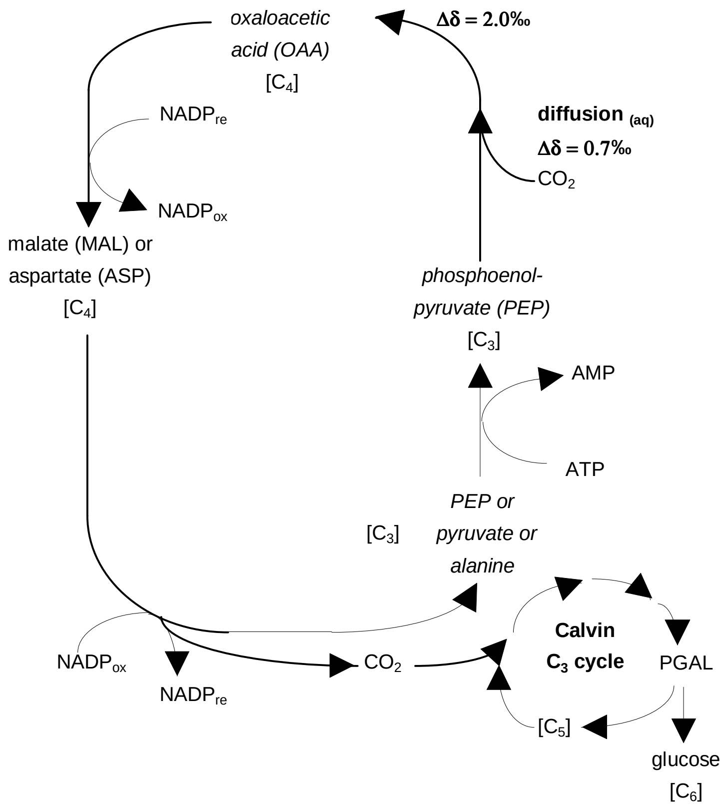


Hatch-Slack cycle

The carboxylase enzyme responsible for carbon fixation in the Calvin cycle is also capable of catalysing the oxidation of RuDP with atmospheric oxygen and this process is known as photorespiration. In other words CO_2 and O_2 are both competing substrates for the carboxylase enzyme (Taiz and Zeiger, 1998). Consequently, the C_3 mechanism will not operate with atmospheric concentrations of less than approximately 50 ppm. Below this level of concentration the plant photorespires more CO_2 than it assimilates and will eventually die (Krueger and Reesman, 1982). However, there is another group of plants that can carry out photosynthesis with conditions of high temperature, intense light, low moisture, low CO_2 and high O_2 concentrations. The mechanism of carbon fixation used by these plants was elucidated by Hatch and Slack in the late 1960s, the C_4 pathway (Hatch and Slack, 1970). Hatch-Slack plants are able to utilise CO_2 concentrations as low as 0.1 ppm. This is achieved by two sequential carboxylation reactions shown in Figure 3. When

CO_2 initially enters the leaf stomata it combines with a 3-carbon compound, phosphoenolpyruvate (PEP), to form oxaloacetate (OOA), a 4-carbon acid, which is the origin of the term *C_4* synonymous with the Hatch-Slack cycle. This first stage proceeds with a much smaller fractionation as compared to the C_3 cycle, $\Delta\delta \sim 2‰$ (O'Leary, 1981). OAA is then rapidly reduced by $NADP_{re}$ to form malate (MAL, a C_4 acid) or aminated to form aspartate (ASP, a C_4 acid). These acids are transported deeper into the C_4 plant leaves. The acids are then oxidised by $NADP_{ox}$ to form a C_3 compound and CO_2. The C_3 compound is converted to PEP, pyruvate or alanine and then to PEP by the action of ATP. The CO_2 feeds into the Calvin cycle where it is used to synthesise glucose as in Figure 2.

Figure 3. The Hatch-Slack pathway of C_4 photosynthesis. Each molecule of CO_2 combines with one molecule of phosphoenolpyruvate (PEP, a 3-carbon compound) to form a 4 carbon compound, oxaloacetic acid (OAA). OAA is then reduced by $NADP_{re}$ to form malate (MAL, a C_4 acid) or aminated to form aspartate (ASP, a C_4 acid). The acid is then oxidised by $NADP_{ox}$ to form a C_3 compound and CO_2. The C_3 compound is converted to PEP, pyruvate or alanine (not shown here) and then to PEP by the action of ATP. The CO_2 is fed into the Calvin cycle where it is used to synthesise glucose (adapted from Ting, 1982; O'Leary, 1988; Taiz and Zeiger, 1998).



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). Examples of C_4 plants are sugar cane, corn, sorghum, millet and some types of pasture grasses.

Crassulacean Acid Metabolism

There is a third group of plants which utilises an adaptive modification of the basic photosynthetic metabolism. This carbon metabolism is characterised by the storage of high concentrations of organic acids during the night and is known as *Crassulacean Acid Metabolism (CAM).* This term derives from a large succulent plant family, the *Crassulaceae*, many of which use the CAM metabolism (Ting, 1982). CAM plants tend to be cultivated in hot and dry climates and as a general rule, have their stomata closed during the day and open at night. Consequently, the plant transpires and fixes CO_2 at night and this causes a reduction in starch and other storage glucans that energise the metabolism. However, if the daytime temperature is relatively low, the stomata may open and the plant will adopt direct C_3 metabolism of CO_2. When daytime temperatures are high the stomata remain closed to prevent water loss through transpiration (Krueger and Reesman, 1982). At night, when temperatures drop, the stomata open and utilise C_4 metabolism of CO_2. The organic acids are then converted back to CO_2 for C_3 synthesis the following day. 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). Pineapple, vanilla and cacti are the most well known plants that use this metabolism.

Secondary carbon metabolism

Further fractionation between plant components from the same primary source (even at specific positions within plant compounds) is a result of kinetic isotope effects (KIEs) in metabolic processes involving side reactions or branching (Schmidt and Kexel, 1999). As a result secondary metabolites such as protein and lipid show systematic deviations from their carbohydrate precursors. As a general rule secondary metabolites tend to be relatively depleted in ^{13}C. This is most notable in lipid fractions that may differ from whole leaf carbon by as much as 10‰. This depletion may be caused by an isotope effect associated with the decarboxylation of pyruvic acid and the formation of the C_2 substrate for acetyl coenzyme A (DeNiro and Epstein, 1977). The repetition of this process during fatty acid chain construction results in alternating ^{13}C depletions (Monson and Hayes, 1982).

NITROGEN

The natural source of nitrogen is air molecular nitrogen which contains about 0.4% of ^{15}N. Through physical processes and the activity of microorganisms it is transformed into inorganic forms (nitrates, ammonia) and organic forms (amino acids, proteins) that are present and available in soil. The natural cycle of nitrogen in the environment is relatively complex as compared to the other bio-elements: carbon, hydrogen, oxygen and sulphur. It is cycled between the atmosphere, plants and microbes and occurs in a variety of reduced and oxidised forms (Table 2). According to the extent of each of these processes, which are mainly affected by the depth of soil, type of vegetation and climate, the δ^{15}N values of soils can vary considerably, generally standing between -10 and +15‰. In particular water stress and distance from the sea lead to an enrichment of ^{15}N in the soil (Heaton et al., 1987).

Table 2. Processes in nitrogen cycle that due to an 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.

In crop soils, the main factor affecting δ^{15}N is fertilisation practices. Synthetic fertilisers, produced from atmospheric nitrogen using the Haber process, show δ^{15}N values between -4 and +4‰ whereas organic fertilisers are enriched in ^{15}N, ranging between +0.6 and +36.7‰ (manure between +10 and +25‰; Bateman et al., 2007). Thus intensive use of organic fertilisers, can lead to a considerable enrichment in the ^{15}N of nitrogen compounds in soil (Kreitler, 1975; Bateman et al., 2007). $\delta^{15}N$ values in plants are generally correlated to those of nitrates and ammonia 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). Uptake does not cause any substantial fractionation, whereas enzymatic reactions such as nitrate reduction or transamination greatly affect isotopic composition (Yoneyama et al., 1995). Leguminous and nitrogen-fixing plants are a particular case as they can fix nitrogen directly from the air, showing $\delta^{15}N$ values around 0‰ (Yoneyama et al., 1995).

OXYGEN

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

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

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

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

These calculations do not agree with the mean isotopic composition of world precipitation ($\delta^{18}O$ = -4‰), proving that from an isotopical point of view evaporation and condensation are non-equilibrium processes (mainly determined by low humidity level, temperature, wind and degree of salinity).

Precipitation occurs when humidity is 100%, so isotopic fractionation is mainly due to temperature. Oceanic vapour from subtropical areas moves towards the poles, becoming cooler and condensing in the form of precipitation, becoming depleted in heavier isotopes that concentrate in initial rainfall (Figure 4). Consequently, latitude is a factor discriminating oxygen isotopic fractionation. This situation is clear in Figure 5, where it can be seen that ^{18}O depletion in precipitation increases with latitude. Thus, equatorial water is richer in heavier isotopes than water at the poles with an intermediate situation of *continuum* according to latitude. However, in the same figure considerable deviations can be noted (*e.g.* on the east coast of South America or in the Atlantic Ocean between Mexico and Scandinavia), due to warm oceanic currents. In addition to the 'latitude' effect there is a 'continental' effect due to the distance from the sea, related to the vapour masses moving over continents (with the increasing distance from the primary source of vapour the $\delta^{18}O$ of precipitation decreases due to previous precipitation) causing precipitation along the coasts which is isotopically richer than that in continental areas (mean depletion of -2.8‰/1000 km from the coast). Moreover, different altitudes inland lead to ^{18}O depletion of around -0.15‰ to -0.5‰ *per* 100 meters of height, because at higher altitude there is lighter vapour. Finally, deviation is also due to seasonal trends; during the summer there is ^{18}O enrichment, especially inland.

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

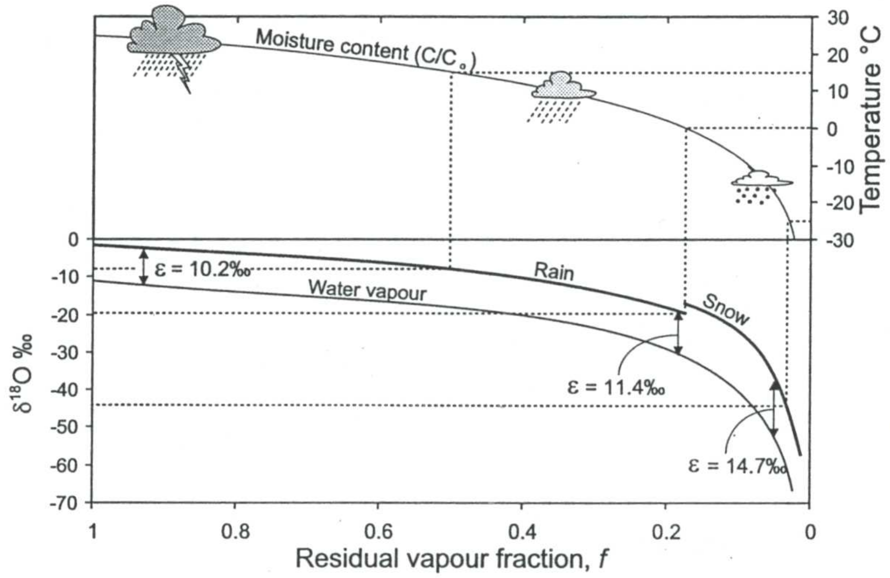
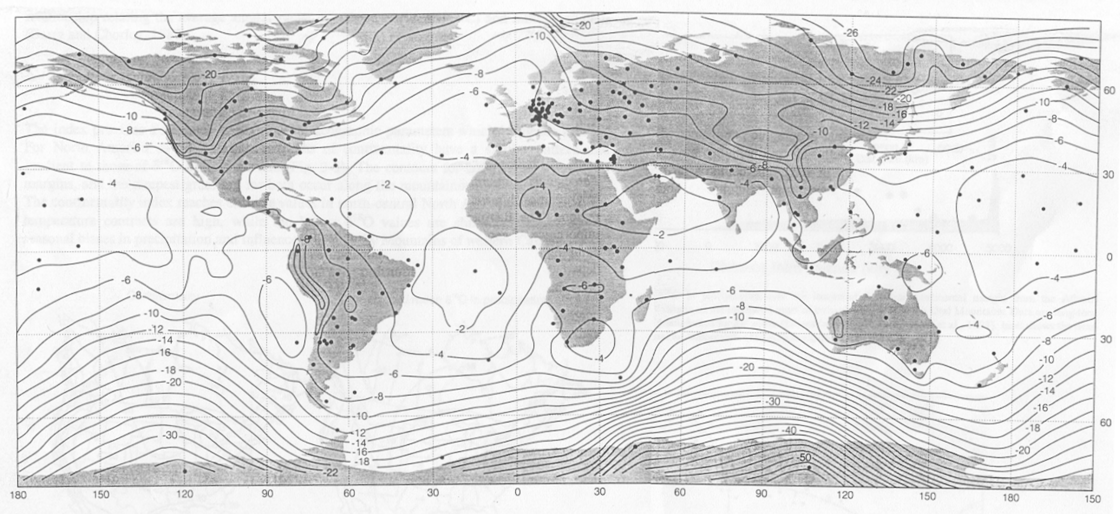


Figure 5. Distribution of mean $\delta^{18}O$ values for precipitation in 1992 and 1993 (Rozanski, 1993)



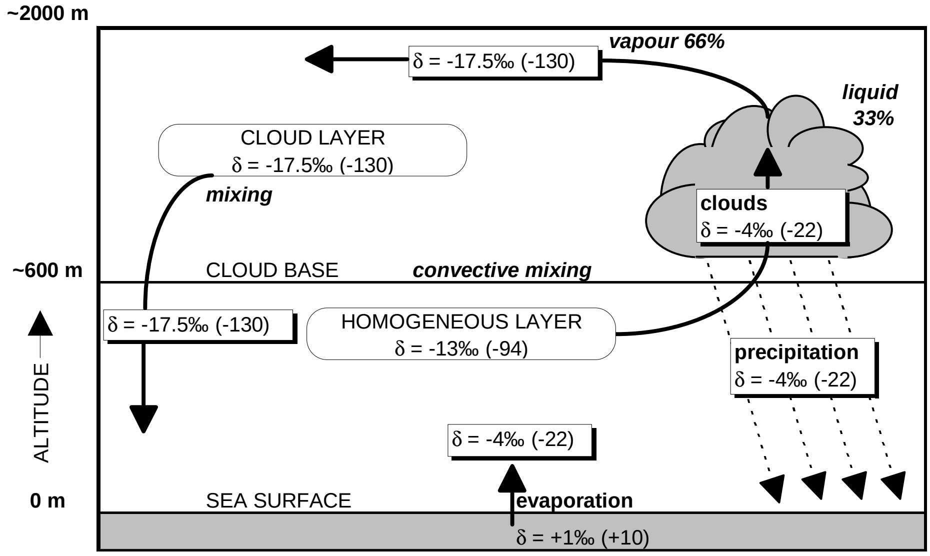
To summarise, the factors affecting $\delta^{18}O$ content in meteoric water are latitude, distance from the sea, altitude and seasonal trends. Ground water has an isotopic composition related to the mean annual isotopic composition of precipitation water and its $\delta^{18}O$ depends only on geographical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

In plants, the isotopic composition of vegetal water is related to the water absorbed from the soil, so it is affected by the factors mentioned above. Furthermore, vegetal water in the leaf suffers an isotopic fractionation during evapotraspiration processes which are affected by temperature and relative humidity and which lead to an enrichment in heavier isotopes. Vegetal compounds derive their oxygen atoms from vegetal water but also from air CO_2 and O_2 with $\delta^{18}O$ values that are essentially constant and stand at around +40.3/+42.5‰ and +23.5/+23.8‰ respectively. Oxygen integration in organic compounds through metabolic processes causes considerable isotopic fractionation. For example, the $\delta^{18}O$ of cellulose is correlated with the $\delta^{18}O$ of leaf water, with an enrichment of around 27‰ caused by the isotopic fractionations occurring during exchanges between the carbonylic group and water (Schmidt et al., 2001; Barbour, 2007).

HYDROGEN

The 100% difference in the mass of hydrogen isotopes gives rise to markedly different physico-chemical properties, in particular rates of reaction. This means that physical processes such as evaporation, condensation and biological processes, involving enzyme catalysed reaction, can produce a much greater natural variation in the abundance of deuterium (White, 1988) as displayed in Figure 6.

Figure 6. 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 δD‰ (adapted from Yurtsever and Gat, 1981)



The primary source of all organic hydrogen is the hydrosphere, which is depleted in deuterium as compared to natural waters. The variation in D in the hydrosphere follows an analogous pattern to the ^{18}O variation discussed above. The relationship between oxygen and hydrogen isotope patterns in the hydrosphere was first characterised by Craig in 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 Global Meteoric Water Line (GMWL) (Dansgaard, 1964) defined by the following equation:

$$\delta D‰ = 8 * \delta^{18}O + 10 \tag{2}$$

So, as for oxygen, meteoric water that has passed through the meteorological cycle of evaporation, condensation and precipitation ultimately makes up the groundwater and shows systematic geographical isotope variation (Yurtsever and Gat, 1981). Decreasing temperatures cause a progressive heavy-isotope depletion of precipitation when water vapour from oceans in equatorial regions moves to higher latitudes and altitudes (Craig, 1961). Evaporation of water from the oceans is a fractionating process that decreases the concentration of the heavy isotopomers of water ($HD^{16}O$, $HH^{18}O$) in the clouds as compared to the sea. As the clouds move inland and gain altitude, further evaporation, condensation and precipitation occurs decreasing the deuterium concentration. Consequently, ground water reflects this isotopic gradient from the coast to inland areas (Dansgaard, 1964).

For land plants, a further pre-assimilation affects the isotopic composition of the water substrate. The hydrogen present in plant material originates from the water taken up by the roots (Ziegler et al., 1976). The water is transported through the plants xylem system and the isotopic composition of the xylem water is the same as that of water taken in by the roots, so water is taken into the leaves without a change in isotopic composition. Evapotranspiration of water through the leaf stomata enriches the remaining water with heavier isotopomers. This is the result of differences in the vapour pressure of the HHO and HDO water isotopomers, which results in the enrichment of deuterium (and ^{18}O) in leaf tissue water and the depletion of deuterium (and ^{18}O) in water vapour. Typical leaf water and fruit water enrichments are around 20 to 40‰. There are no apparent differences in the degree of enrichment of deuterium in the leaf water of plants utilising the Calvin (C_3), Hatch-Slack (C_4) or Crassulacean acid metabolism (CAM) photosynthetic pathways (Bricout, 1982). Therefore, it is expected that crop growing regions with relatively low humidity, where the rate of evaporation from the leaf is higher, will lead to plant materials with relatively enriched δD‰ values (Martin et al., 1986). Another factor affecting the extent of D enrichment in plant products growing in similar temperate climates, is the timing of maturation. For example, the sugar present in the fruits of the Cabernet-Sauvignon grapevine variety is significantly enriched as compared to the Carignan variety, in vines grown in the same region (Martin et al., 1986). This observation is related to the fact that both the photosynthetic and maturation cycles of Cabernet-Sauvignon take place during hotter periods than in the case of the Carignan species. This ultimately affects the rate of evapotranspiration such that the varietal origin of agricultural products can be differentiated for plants grown in similar geographical locations.

During CO_2 and H_2O fixation in plant photosynthesis into primary assimilates, such as carbohydrate, deuterium depletion results in δD‰ values of –90 to -180‰. During assimilation the type of photosynthetic route used by the plant (C_3, C_4 or CAM) affects the degree of fractionation observed in metabolic hydrogen (Sternberg et al., 1984). The shoots of different plants grown under exactly the same conditions exhibited mean metabolic δD‰ values of -132‰, -91‰ and -74‰ for C_3, C_4 and CAM species respectively (Ziegler et al., 1976). Furthermore, relationships between the variation of δD‰ values of cellulose according to δD‰ of leaf water and temperature (T) have been defined (Yapp and Epstein, 1982a; Yapp and Epstein, 1982b):

$\delta D‰_{\text{cellulose}} = 0.9\ \delta D‰_{\text{leaf water}} - 22‰$

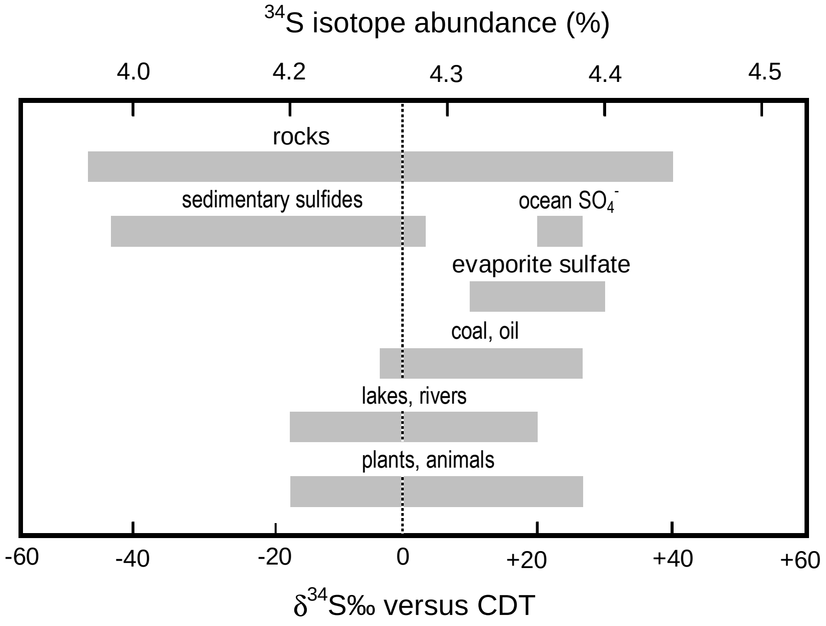
$\delta D‰_{\text{cellulose}} = 5.8\ T - 134‰$

Secondary metabolites exhibit systematic shifts between different groups of plant components, due to biochemically induced fractionation. The δD‰ 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 δD‰ of triglycerdes 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 a –110 to –140‰ depletion relative to carbohydrate. It is important to note that these last observations relate specifically to δD‰ measurement of carbon-bound or non-exchangeable hydrogen atoms in plant materials. This does not apply to the labile hydrogen attached to oxygen in hydroxyl moieties, which can readily exchange with water (Dunbar and Schmidt, 1984).

SULPHUR

The exact isotopic abundance of sulphur in a chemical substance depends on its source and hence on the formation mechanism. ^{34}S variation in the terrestrial environment is shown in Figure 7. $^{34}S/^{32}S$ ratio is thought to be fixed in our solar system, and has been since its formation, as demonstrated in many meteorites, which have values consistently around 22.22‰. The bulk sulphur isotopic ratio of the Earth is very similar to this value (Thode et al., 1961). Sulphate reduction through biological activity strongly differentiates between the two isotopes, according to the kind of reducing bacteria and electron donors and the enzymatic reaction rate (Detmers et al., 2001). Sea water sulphate enters into many of the large scale oxidation reduction processes which take place in the sea and leads to a basically uniform $\delta^{34}S$ value of around +21‰ for seawater (Rees et al., 1978). Sea spray, deriving from a well-mixed reservoir of oceanic sulphate, is one of the most important sources of atmospheric sulphur. On the other hand, combustion of sulphur containing fossil fuels and certain industrial processes involving sulphur compounds represent the main anthropogenic sources of primary SO_2 present in the atmosphere.

Figure 7. ^{34}S isotope variation ranges of sulphur pools (adapted from Winkler, 1984)



Oxidation of H_2S deriving mainly from natural processes (volcanic eruptions and forest fires) may produce secondary SO_2 (Saunders and Wood, 1973). 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). Biogenic sulphur is another important atmospheric sulphur source and when released from soils and wetlands is characteristically depleted in ^{34}S (Wadleigh and Blake, 1999; Mast et al., 2001).

Bacteria metabolise the oxygen in sulphate to oxidise organic material in a similar way with denitrification, thus producing isotopically light hydrogen sulphide (Schidlowski, 1982). The majority of this light H_2S is then incorporated into sedimentary sulphide. This reduction is associated with a number of KIEs that produce fractionation of -30 to -40‰. The $\delta^{34}S$‰ values of sulphides are therefore relatively depleted as compared to their marine sulphate source material at ~20‰. Although reactions of assimilatory sulphate reduction by plants and kinetic S-isotope effects on them must be identical to those of bacterial dissimilatory sulphate reduction, the S-isotope fractionations in plants are far smaller (Tanz and Schmidt, 2010). Bulk plant sulphur is depleted by only 1-2‰ in relation to its primary sources, soil and sea spray sulphate or SO_2 from the atmosphere. From plant material the $\delta^{34}S$ signal is transferred to animals and not essentially altered in food chains. Hence, at first glance plant assimilatory sulphate reduction, the provision of “organic sulphur” from sulphate, and sulphur metabolism in animals seem to proceed without significant sulphur isotope fractionation. The $\delta^{34}S$ values of animal samples basically reflect diet, but show some ^{34}S enrichment in relation to this, depending on the animals' state of nutrition, trophic level, and individual tissue (Tanz and Schmidt, 2010).

1.2.3 Natural variation in elemental composition

The composition of plants reflects the bio-available and mobilised nutrients present in the underlying soils in which they were grown. Elemental availability depends on several factors, such as soil pH, humidity, porosity, clay and humic complex (Kim and Thornton, 1993). Consequently, the range of soils present and bioavailability mean that elemental composition may provide unique markers in food, characterising geographical origin. With regard to manufactured foodstuffs, 'contamination' and changes during processing also play a role in the final elemental composition (Nicolini et al., 2004).

The primary factors affecting the mobility and availability of each mineral element in soil are:

- the characteristics of the specific element (radius, charge, oxidation state, free or combined form) and its interaction with other elements;
- soil characteristics (pH, Eh, cation exchange capacity - CEC, quantity of organic matter);
- presence of microorganisms and plants;
- climatic conditions that can influence pedogenetic processes.

Ion mobility is strictly related to ionic potential, which is the ratio of electric charge to the radius of the ion. This proportion measures the charge density at the surface of the ion and thus its ability to bind water molecules in a solution (Kabata-Pendias, 2001; Violante, 2002). On the basis of ionic potential it is possible to identify three classes of ions: those with a potential lower than 30 and higher than 95, which are soluble as free ions (*e.g.* K^+, Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}) and oxyanions respectively, and those with a potential of between 30 and 95, which tend to precipitate as hydroxides (*e.g.* Fe^{3+}, Al^{3+}) (Kabata-Pendias, 2001; Violante, 2002). Ionic mobility can also vary as a result of processes such as ionic exchange, immobilisation in secondary mineral stones and intake by plants.

One of the soil characteristics that most affects the cycle of elements in soil is CEC. This complex is made up of charged material, which may or may not be dependent on pH (*e.g.* clays, humic matter, colloidal substances), and oxides/hydroxides whose structural elements can be replaced by other elements with a similar encumbrance. The extent of CEC suggests the absorption capacity (defined as the temporary retention of ions on a solid surface) of a soil.

pH is another very important factor in determining the chemical and physical characteristics of soil because in addition to microbial activity, it also affects the charge of clay, complexation of organic substances, precipitation, dissolution, redox reactions and the dispersion and mobility of colloids (Adriano, 2001). In particular, an acidic environment causes a decrease in bacterial population and their activities and a slowdown in the mineralisation of organic matter. In general the ability of soil to retain mineral elements increases as pH increases, because several elements (such as Fe, Mn, Cu, Al, Zn) are less mobile and soluble in basic environments. Other elements, such as Mo, form oxyanions and are more available in alkaline conditions.

Together with pH, redox potential affects the mobility of polyvalent ions because some elements, such as Fe or Mn, are more soluble in reduced form, whereas other elements, such as Cd or Cu, are more soluble when are oxidised (Violante, 2002; Adriano, 2001).

In vivo, intake of elements by plants is affected not only by soil characteristics but also by the physiology of the different species, by reactions taking place in the rhizosphere (desorption, adsorption, precipitation, dissolution) and by interactions between elements and microorganisms (Kabata-Pendias, 2001; Basta et al., 2005).

The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms (Gregory, 2006). The rhizosphere contains many bacteria that feed on sloughed-off plant cells and the proteins and sugars released by roots (Lynch and Whipps, 1990). This release of exudates can affect the availability of elements because root exudation increases in stressful situations and in the event of food shortage (Rengel, 1997; Neumann and Römheld, 2002; Gregory, 2006). Exudates contain substances with chelation or complexant characteristics that can create stable complexes with elements of the insoluble mineral phase, making them re-absorbable by roots (Jones, 1998; Gregory, 2006; Jimenez et al., 2007).

Elements enter plants mainly through the roots but also *via* leaves. This last pathway can affect plant mineral composition in the event of sources of ‘contamination’ in the crop-growing environment (*e.g.* foundries, mines, polluted areas) or in the case of leaf treatments.

The entry of elements *via* the roots may happen according to or against the gradient and by diffusion or using carriers and proteic channels. However, at all events the absorption of elements is related to their chemical and physical characteristics and not to their biological role. Therefore, an element such as Rb without a biological role in the plant may be absorbed as Rb^+ instead of K^+ because of their similar physical-chemical characteristics. Intake is also affected by the existence of interaction between ions in solution and by a different affinity towards carriers: for example K^+, Rb^+ and Cs^+ or SO_4^{2-} and SeO_4^{2-} are in competition with each other, Ca^{2+} decreases absorption of Mg^{2+} and promotes intake of K^+ (Marschner, 1986; Kabata-Pendias, 2001).

In the roots elements are complexed to ligands with a low molecular weight or are incorporated in metabolites in order to allow transport to aerial parts. During transport ions may be subjected to interaction with other ions or with the negatively charged xylematic wall or may be re-absorbed by xylematic parenchyma. These processes are affected by ion charge and concentration, the presence of other ions or complexing agents, diameter and the charge density of the xylematic vessels. Different elements can be accumulated in different organs of the plant depending on element concentration and plant species.

Finally climate, particularly temperature, humidity and photoperiod, also affects absorption capability, because it is decisive in the development of the roots and determines the degree of evapotraspiration.

The elemental composition of plants shows extensive variability due to all the factors described above, thus different compositions are reported for different vegetal species grown on the same soil (Wyttenbach, 1998) but also for plants of the same species grown in different conditions (Miyasaka and Grunes, 1997).

Elemental composition in food and feed depends not only on the natural content but also on the conditions in which they are produced and processed (Anastasio et al., 2006; Castineira Gomez et al., 2004; Mihucz et al., 2006; Tatár et al., 2007). Metals and alloys are used as food contact materials, mainly in processing equipment, containers and household utensils, but also in foils for wrapping foodstuffs. They play a role as a safety barrier between the food and the exterior. They are often covered by a surface coating, which reduces migration in foodstuffs. When they are not covered, these food contact materials can give rise to the migration of metal ions into foodstuffs and may therefore endanger human health if the total metal content exceeds the recommended health exposure limits, if any, or bring about an unacceptable changes in the composition of foodstuffs or deterioration in their organoleptic characteristics (Council of Europe, http://www.coe.int). In the presence of moisture and oxygen, metal may experience corrosion, the rate and extent of this depending on the composition of the aqueous medium (mainly the presence of acids or salts), the solubility of the formed compounds in the medium and their rate of removal.

The release of elements from packaging to food, in particular heavy metals, may take place as the result of a series of diffusion processes subject to both thermodynamic and kinetic control. In particular, Cd, Pb, Ni, Sb, Sn, As, Ba, Cr, Hg and Se may be released from plastic articles or paper for packaging from inorganic additives as pigments, antioxidants or stabilisers (Conti, 2008).

1.2.4 Stable isotopes and elemental composition in food authentication

Stable isotope and multi-element analysis, measured using Isotope Ratio Mass Spectrometry (IRMS) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), have taken on increasing importance in determining the authenticity of fruits, vegetable and food of animal origin for producers and food controls. The relevant literature is summarised in Table 3, which shows the various commodity groups investigated, the parameters measured and the corresponding references.

Table 3. Summary of the relevant literature relating to the use of multi-element and multi-isotopic analysis of food commodities to determine the geographical origin (adapted from Kelly et al., 2005 and Gonzalvez et al., 2009)

Commodity	**Parameters measured**	**Reference**
Meat		
Beef	$^{18}O/^{16}O$	Hegerding et al., 2002
Beef	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Boner and Forstel, 2004
Beef	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$	Renou et al., 2004a
Beef	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Bahar et al., 2005
Beef	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Schmidt et al., 2005
Beef	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Bahar et al., 2008
Beef	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, ME	Heaton et al., 2008
Beef	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$	Nakashita et al., 2008
Beef	D/H, $^{18}O/^{16}O$	Horacek et al., 2010
Beef	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Guo et al., 2010
Lamb	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Piasentier et al., 2003
Lamb	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Sacco et al., 2005
Lamb	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Camin et al., 2007
Lamb	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Moreno-Rojas et al., 2008
Lamb	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Perini et al., 2009
Poultry and beef	$^{18}O/^{16}O$, ME	Franke et al., 2008
Dairy products		
Butter	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Rossmann et al., 2000
Butter	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Balling and Rossmann, 2004
Cheese	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Manca et al., 2001
Cheese	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, ME	Pillonel et al., 2003
Cheese	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Camin et al., 2004
Cheese	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Pillonel et al., 2004
Cheese	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Manca et al., 2006
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$	Kornexl et al., 1997
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Rossmann et al., 1998
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Brescia et al., 2003
Milk	$^{18}O/^{16}O$	Renou et al., 2004b
Milk	$^{18}O/^{16}O$	Ritz et al., 2005
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$	Crittenden et al., 2007
Milk	$^{18}O/^{16}O$	Engel et al., 2007
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Molkentin and Giesemann, 2007
Milk	ME	Benincasa et al., 2008
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, D/H	Camin et al., 2008
Milk	$^{13}C/^{12}C$	Molkentin, 2009
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Sacco et al., 2009
Milk	D/H, $^{18}O/^{16}O$	Chesson et al., 2010
Milk	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Molkentin and Giesemann, 2010
Milk and Cheese	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Brescia et al., 2005
Wine		
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Breas et al., 1994
Wine	ME	Baxter et al., 1997
Wine	ME	Jakubowski et al., 1999
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Rossmann et al., 1999
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Kosǐr et al., 2001
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Ogrinc et al., 2001
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Cristoph et al., 2003
Wine	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Cristoph et al., 2004
Wine	D/H, $^{18}O/^{16}O$, ME	Gremaud et al., 2004
Wine	ME	Coetzee et al., 2005
Wine	ME	Angus et al., 2006
Wine	ME	Galgano et al., 2008

Table 3. (cont.)

Commodity	**Parameters measured**	**Reference**
Fruits and Vegetables		
Pistachios	ME	Anderson et al., 2005
Potatoes	ME	Di Giacomo et al., 2007
Strawberries, blueberries, pears	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Perez et al., 2006
Welsh onions	ME	Ariyama et al., 2004
Welsh onions, onions, black soybeans	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$	Horita et al., 2008
Beverages		
Coffee	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$	Weckerle et al., 2002
Orange juice	$^{13}C/^{12}C$	Simpkins et al., 2000a
Orange juice	ME	Simpkins et al., 2000b
Orange juice	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Rummel et al., 2010
Tea	ME	Marcos et al., 1998
Tea	ME	Moreda-Pineiro et al., 2001
Tea	ME	Moreda-Pineiro et al., 2003
Tea	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Pilgrim et al., 2010
Cereal crops		
Cereals	$^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$, ME	Goitom-Asfaha et al., in print
Rice	ME	Yasui and Shindoh, 2000
Rice	$^{13}C/^{12}C$, $^{18}O/^{16}O$, ME	Kelly et al., 2002
Rice	ME	Oda et al., 2002
Soybean	ME	Kosaka et al., 2006
Wheat	$^{13}C/^{12}C$, $^{15}N/^{14}N$	Brescia et al., 2002a
Wheat	$^{13}C/^{12}C$, $^{15}N/^{14}N$, ME	Brescia et al., 2002b
Other commodities		
Hazelnuts	ME	Oddone et al., 2009
Honey	Review	Anklam, 1998
Honey	D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$	Schellenberg et al., 2010
Vegetable oils	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Breas et al., 1998
Vegetable oils	$^{13}C/^{12}C$, $^{18}O/^{16}O$	Angerosa et al., 1999
Vegetable oils	ME	Jimenez et al., 2003
Vegetable oils	ME	Jimenez et al., 2004
Vegetable oils	ME	Benincasa et al., 2007

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CHAPTER 2 AIMS AND OUTLINE OF THE THESIS

2.1 AIMS OF THE THESIS

The general aim of this thesis was to verify the applicability of the analysis of multielement stable isotope ratios using IRMS (Isotopic Ratio Mass Spectrometry) and elemental composition using ICP-MS (Inductively Coupled Plasma Mass Spectrometry) in food authenticity. In particular, analytical approaches based on these techniques were developed for the traceability of different premium products, such as PDO Italian and European extra virgin olive oils, olive oil squalene and squalane, Italian alpine cheeses, and Italian tomato and its derivates (juices, passata, pastes). The origin of these products must be declared by law, but at the moment no analytical methods exist that are capable of verifying the truthfulness of the assertions on labels.

In detail the research focused on:

- characterisation of Italian extra virgin olive oils: creation and validation of a dataset with isotopic values and elemental profiles;

- geographical traceability of extra virgin olive oils from eight European sites: creation and validation of a dataset with isotopic values and elemental profiles and subsequently creation and validation of a statistical – mathematical model usable to verify the authenticity of commercial samples;

- development of an analytical approach to distinguish squalene and squalane from olive oil from those produced using shark liver oil;

- characterisation of seven Italian cheeses from alpine regions: creation and validation of a dataset with isotopic values and elemental profiles and subsequently creation and validation of a statistical – mathematical model usable to verify the authenticity of commercial samples;

- traceability of Italian tomato products along the production chain: creation and validation of a dataset with isotopic values and mineral profiles and subsequently creation and validation of a statistical – mathematical model for the whole production line (tomatoes, juice, passata, paste).

2.2 OUTLINE OF THE THESIS

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

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

In Chapter 3 analysis of the stable isotope ratios of bioelements and of elemental composition is described in general for its use for the premium products considered in this thesis.

Each of the subsequent three chapters is devoted to an individual commodity and to related publications, presenting the results obtained during the PhD thesis.

Specifically, Chapter 4 focuses on the possibility of improving traceability of the geographical origin of extra virgin olive oils using stable isotope ratios and elemental composition. These two analytical techniques were applied to authentic Italian samples in Section 4.1 and to oils collected at eight European sites in Section 4.3. Section 4.2 focuses on the determination of stable isotope ratios in Italian olive oils produced on the Adriatic and Tyrrhenian coasts. In Section 4.4 $\delta^{13}C$ and δD analysis was investigated as a possible tool for checking on the authenticity of commercial olive oil squalene and squalane samples, which may be adulterated with cheaper but illegal products from shark liver.

The isotope ratios of alpine PDO cheeses, combined with elemental composition, were investigated in Chapter 5 as markers for the geographical origin of products and the feeding regime of animals, also with a view to use as a tool for verifying the correct application of PDO protocols.

In Chapter 6 the combination of isotopic and elemental composition was investigated as a tool for differentiating tomatoes and derivatives (juice, passata, paste) from different Italian regions, regardless of the technological processes adopted.

Chapter 7 gives a general discussion about the traceability models obtained investigating stable isotope ratios and elemental composition of olive oils, cheeses, tomatoes and derivatives and about their future application possibilities. The last part of the chapter, on the one hand gives some indications on where future application of stable isotope and elemental analyses might be expected or are already in progress and, on the other hand describes the limitations of this analytical approach emerged throughout this thesis.

CHAPTER 3 IRMS AND ICP-MS METHODS

3.1 PREPARATION OF THE SAMPLES

Isotopic ratios and elemental analysis was performed on:

- bulk sample for determination of $\delta^{13}C$, $\delta^{18}O$, δD, $\delta^{15}N$ and $\delta^{34}S$ in olive oil and tomato products;
- extracted glycerol for determination of $\delta^{13}C$ and $\delta^{18}O$ in olive oil and cheese;
- extracted casein for measurement of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in cheese;
- extraction acidified water solution for measuring the concentration of elements in olive oil;
- pure squalene/squalane for measurement of $\delta^{13}C$ and δD;
- pure squalane in extraction solutions of cosmetic products after GC separation for measurement of $\delta^{13}C$ and δD;
- mineralised samples for determination of elemental composition in tomato products and cheese.

The detailed preparation procedures are fully described in the relative chapters.

3.2 ISOTOPE RATIO MASS SPECTROMETRY METHOD

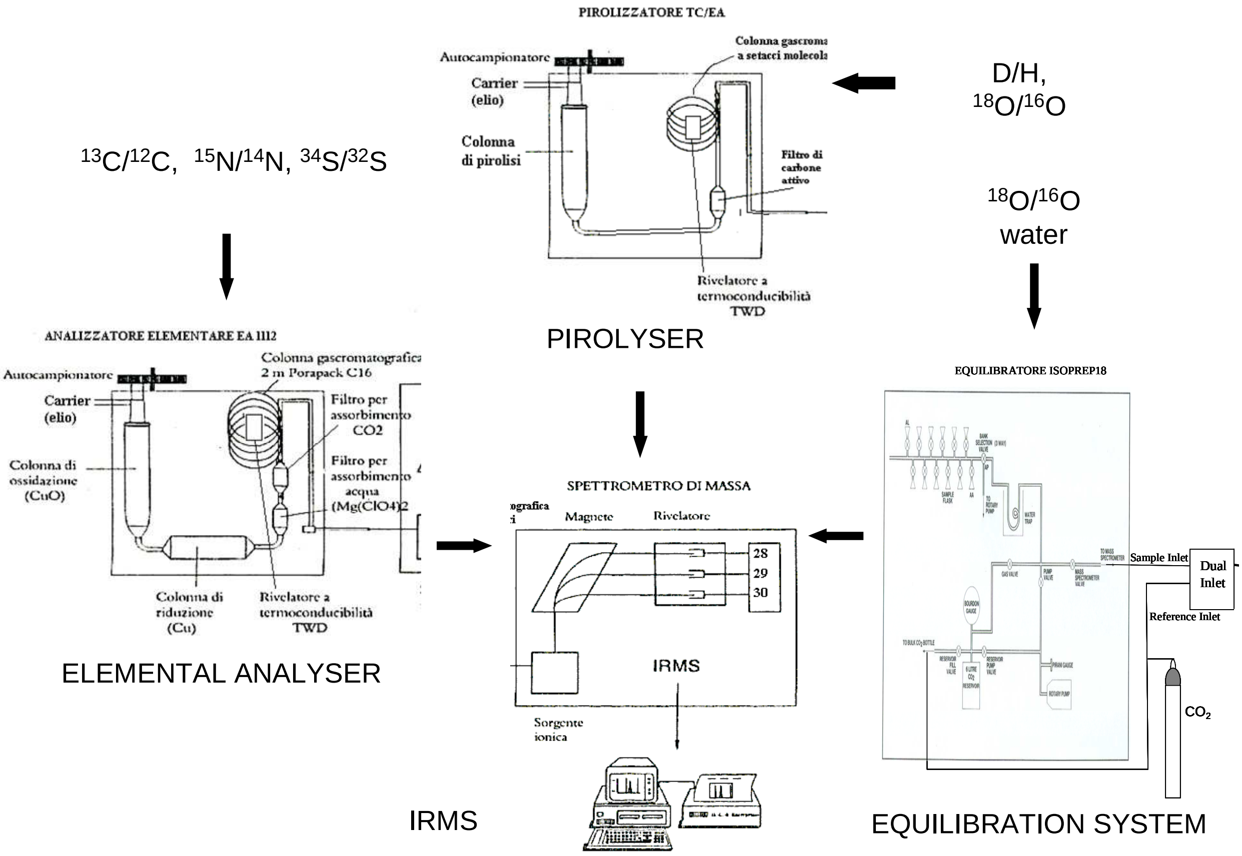
For analysis of stable isotope ratios, the sample was weighed in tin or silver capsules. The quantities of sample weights ranged from around 0.3 mg to 3 mg depending on the commodity and the isotope ratio analysed. More details on the methods are provided in the specific chapters.

Analysis was carried out using an IRMS (Isotope Ratio Mass Spectrometer, Delta Plus XP ThermoFinnigan, Bremen, Germany) equipped with an elemental analyser (Flash EA 1112, ThermoFinnigan) for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ determination and a pyrolyser (Finnigan^{TM} TC/EA, ThermoFinnigan) for $\delta^{18}O$ and δD measurement (Figure 8).

In detail, for determination of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ the sample was dropped into an oxidation column inside the elemental analyser, where it combusted and oxidised due to the high temperature, high level of oxygen and presence of CuO, with development of CO_2, N_2 and SO_2.

For measurement of $\delta^{18}O$ and δD the sample was dropped into a high-temperature conversion/elemental analyser where it was subjected to high temperature pyrolisis with development of hydrogen and carbon monoxide gases. 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 special care in maintaining dry conditions during analysis were ensured, in order to obtain reproducible results. Then the weighed samples were put in the carousel of the autosampler and stored in a desiccator above P_2O_5 for at least 24 h. The carousel was then inserted into the autosampler, equipped with a suitable cover. During measurement, dryness was guaranteed by flushing nitrogen continuously over the samples.

Figure 8: IRMS diagram



The developed gases (CO_2, N_2, CO, H_2, SO_2) were then separated in a GC column at 60°C and transferred from the elemental analyser or pyroliser after the separation into the ion source of an IRMS by the gas carrier helium (130 mL/min). In each analytical batch, every ten samples, a working in-house standard was analysed and used to calculate the isotope ratios values of the samples (see below).

Analysis of $\delta^{18}O$ in vegetal water was performed directly on the fresh sample (homogenised tomato, juice, passata or paste) using an IRMS (VG Isogas, Middlewich, UK) with a dual-inlet system, interfaced with a CO_2 preparation system (Isoprep 18, VG Isotech, Middlewich, UK), according to the ENV 12141 method (Ministerial Decree 23rd September 2005). Isoprep18 allows the equilibration of oxygen exchange between sample water and an ultrapure CO_2 with a known oxygen isotope ratio. In the dual-inlet system the CO_2 gas obtained from the sample was alternated rapidly with a standard CO_2 (of known $^{18}O/^{16}O$ value) by means of a system of valves, so that a number of comparative measurements could be made for both gases. In each analytical batch of 24 samples, 4 working in-house standards were analysed and used to calculate the isotope ratio values of the samples (see below).

In IRMS the gases are ionised in the ionic source and ions pointed through a semicircular flight tube. In the flight tube they are subjected to a constant magnetic field and, depending on the acceleration potential applied, only ions with specific masses can hit the electron multiplier detector (Table 4).

Table 4. Masses measured using IRMS to determine stable isotope ratios

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

The isotope ratios were expressed in δ‰ versus V-PDB (Vienna – Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW (Vienna – Standard Mean Ocean Water) for $\delta^{18}O$ and δD, Air for $\delta^{15}N$ and V-CDT (Vienna – Canyon Diablo Troilite) for $\delta^{34}S$ according to the equation (1). Sample analysis was carried out in duplicate thus calculating the mean values. The isotopic values were calculated against working in-house standards which were themselves calibrated against international reference materials. The specific working in-house standards and international reference materials used for the different commodities analysed are mentioned in the specific chapters, along with the detailed methods. The precision of measurements, expressed as a standard deviation when measuring the same sample ten times, is specified for each commodity in the relevant chapters.

3.3 INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY METHOD

Before analysis the samples were mineralised in closed vessels using a microwave oven digestor (Mars5 or MarsXpress, CEM Matthews, NC-North Carolina, USA) in the case of cheese and tomato products or extracted in an acidified water solution in the case of olive oil. All the materials were washed with 5% HNO_3 and rinsed with Milli-Q water (Millipore Corporation, Billerica, MA- Massachusetts, USA) before use. Details of the preparation methods are provided in the relative chapters.

Analysis of Li, Be, B, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Nb, Mo, Ru, Ag, Cd, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb, Lu, Ta, Re, Ir, Pt, Tl, Pb and U was carried out with an ICP-MS (Inductively Coupled Plasma Mass Spectrometer, Agilent 4500 or 7500ce Agilent Technologies, Tokyo, Japan) equipped with an ASX-520 autosampler (Cetac Technologies Inc., Omaha, NE) (Figure 9). The sample was aspirated and nebulised through a Babington type or microconcentric nebuliser (Agilent Technologies) in a quartz spray chamber and introduced into a Fassel type torch, where it was ionised using an Ar plasma whit a temperature range of between 7500 and 10000 °K. The ion beam was focused using electro-magnetic lenses towards a quadrupole where the ions were separated according to their mass to charge ratio. In the 7500ce model an Octopole Reaction System (ORS®, Agilent Technologies) was positioned before the quadrupole in order to remove polyatomic interferences, using He and

H_2 as collision and reaction gases, respectively. Finally the ion beam arrived at an electron multiplier detector. The principal instrumental parameters (Table 5) were optimised before each analytical batch, monitoring the sensitivity and resolution of the elements Li, Y and Tl, distributed along the range of interest (masses 7, 89 and 205).

Figure 9. ICP-MS diagram

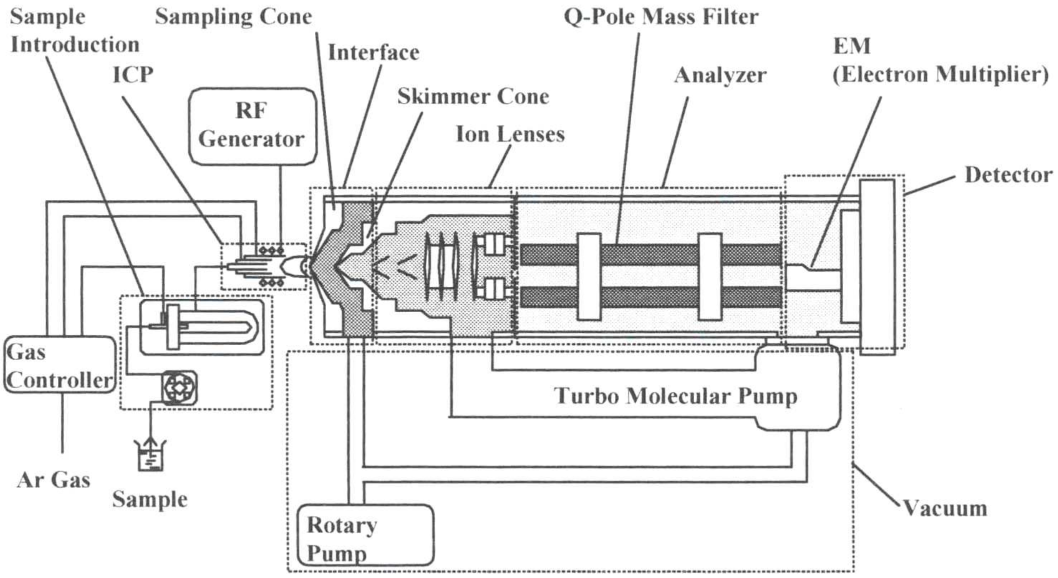


Table 5. ICP-MS instrumental parameters optimised before analysis

Parameter	Ranges	Parameter	Ranges
RF Power	1500W	Octopole cell entrance voltage (when collision/reaction cell is off)	-30 / -34V
Spray chamber temperature	2℃	Octopole cell entrance voltage (when collision/reaction cell is on)	-30 / -40V
Carrier gas flow	0.8 / 0.9 L/min	Octopole cell exit voltage (when collision/reaction cell is off)	-30 / -34V
Make up gas flow	0.2 / 0.3 L/min	Octopole cell exit voltage (when collision/reaction cell is on)	-44 / -54V
Extraction lens 1 voltage	0.0 / 1.4V	Octopole QC Focus (when collision/reaction cell is off)	3V
Extraction lens 2 voltage	-160 / -120V	Octopole QC Focus (when collision/reaction cell is on)	-11V
Omega bias voltage	-20 / -30V	Octopole bias (when collision/reaction cell is off)	-7V
Omega lens voltage	-0.6 / -0.2V	Octopole bias (when collision/reaction cell is on)	18V
QP bias (when collision/reaction cell is off)	-3.5V	Cell gas flow (H_2)	3.0 / 4.0 mL/min
QP bias (when collision/reaction cell is on)	-14.5V	Cell gas flow (He)	4.0 / 4.5 mL/min

Before analysis, the ICP-MS was calibrated using external standard solutions (Table 6). To minimise the matrix effect, mainly due to the nebulisation system, an on-line internal standard solution of Sc, Rh and Tb (3 mg/L) was continuously introduced during analysis.

Table 6. Concentration levels of the standard solutions used for the ICP-MS calibration

Element	Concentrations of the standard solutions	Element	Concentrations of the standard solutions
Li	0.05, 0.5, 5, 50 ppb	Ag	0.05, 0.5, 5, 50 ppb
Be	0.05, 0.5, 5, 50 ppb	Cd	0.05, 0.5, 5, 50 ppb
B	0.6, 6, 60, 600 ppb	Sn	0.005, 0.05, 0.5, 5 ppb
Na	0.05, 0.1, 0.5, 5, 50 ppm	Sb	0.005, 0.05, 0.5, 5 ppb
Mg	0.1, 0.5, 5, 50 ppm	Te	0.005, 0.05, 0.5, 5 ppb
Al	0.5, 5, 50, 500, 1000, 5000 ppb	Cs	0.05, 0.5, 5, 50 ppb
P	0.5, 5, 50, 500 ppm	Ba	0.5, 1, 5, 50, 500 ppb
K	0.5, 1, 5, 50 ppm	La	0.002, 0.02, 0.2, 2, 5, 20 ppb
Ca	0.5, 1, 5, 50 ppm	Ce	0.002, 0.02, 0.2, 2, 5, 20 ppb
V	0.05, 0.5, 5, 50 ppb	Pr	0.002, 0.02, 0.2, 2, 5, 20 ppb
Cr	0.05, 0.5, 5, 50 ppb	Nd	0.002, 0.02, 0.2, 2, 5, 20 ppb
Mn	0.05, 0.5, 5, 50 ppb	Sm	0.002, 0.02, 0.2, 2, 5, 20 ppb
Fe	0.5, 1, 5, 50, 500 ppb	Eu	0.002, 0.02, 0.2, 2, 5, 20 ppb
Co	0.05, 0.5, 5, 50 ppb	Gd	0.002, 0.02, 0.2, 2, 5, 20 ppb
Ni	0.05, 0.5, 5, 50 ppb	Dy	0.002, 0.02, 0.2, 2, 5, 20 ppb
Cu	0.05, 0.5, 5, 50 ppb	Ho	0.002, 0.02, 0.2, 2, 5, 20 ppb
Zn	0.05, 0.5, 5, 50 ppb	Er	0.002, 0.02, 0.2, 2, 5, 20 ppb
Ga	0.05, 0.5, 5, 50 ppb	Tm	0.002, 0.02, 0.2, 2, 5, 20 ppb
Ge	0.05, 0.5, 5, 50 ppb	Yb	0.002, 0.02, 0.2, 2, 5, 20 ppb
As	0.05, 0.5, 5, 50 ppb	Lu	0.002, 0.02, 0.2, 2, 5, 20 ppb
Se	0.05, 0.5, 5, 50 ppb	Ta	0.1, 1, 10, 100 ppb
Rb	0.5, 1, 5, 50, 500 ppb	Re	0.1, 1, 10, 100 ppb
Sr	0.5, 1, 5, 50, 500 ppb	Ir	0.005, 0.05, 0.5, 5 ppb
Y	0.002, 0.02, 0.2, 2, 5, 20 ppb	Pt	0.05, 0.5, 5, 50 ppb
Nb	0.1, 1, 10, 100 ppb	Tl	0.05, 0.5, 5, 50 ppb
Mo	0.05, 0.5, 5, 50 ppb	Pb	0.05, 0.5, 5, 50 ppb
Ru	0.5, 1, 5, 50 ppb	U	0.05, 0.5, 5, 50 ppb

Sample preparation and analysis were carried out in duplicate. The limit of detection (LOD) for each element was calculated as three times the standard deviation of the signal of the blank samples, extracted/mineralised and analysed ten times. The specific LODs obtained in analysing the different commodities are detailed in the relevant chapters.

Accuracy was checked by analysing a standard reference material in each analytical batch. Recovery of the elements is detailed for each commodity in the specific chapters. The precision of the method, evaluated by preparing and analysing a sample ten times, and reproducibility, evaluated by preparing and analysing a specific sample in each analytical batch, both calculated as RSD%, are detailed in the specific chapters.

CHAPTER 4 OLIVE OILS AND RELATED PRODUCTS

4.1 INTRODUCTION

European and Italian law require that the origin of some premium products, such as virgin and extra virgin olive oils, squalene/squalano, PDO cheeses, tomatoes and derivatives, must be declared on the label. This is particularly important with the growth and promotion of “added value” regional foods such as those produced under “Designated Origin” labels. But at the moment no analytical methods exist that are capable of verifying the truthfulness of the assertions on labels so, to achieve this aim the EU is urging to investigate new markers able to support food characterisation and geographical traceability assuring its genuiness.

World olive oil production currently stands at around 2.9 million tonnes (2009 data provided by FAO, http://faostat.fao.org/) and the olive oil industry employs about 2.7 million people, of whom approximately two million are ‘family producers’ living in the less economically favoured regions of southern Europe (http://www.oliveoiltimes.com). Indeed European producers on the Mediterranean basin – Greece, Spain, Portugal, Italy and France – supply the bulk of overall production. Thus olive oil is a fundamental part of the farmers' income and represents as much as 15% of the gross agricultural domestic product in countries such as Greece (Luchetti, 2002). Extra virgin olive oil commands a high retail value (2 to 4 times that of other oils) because of its organoleptic qualities and the purported health benefits of consuming oils high in mono-saturated fatty acids (Gurr, 1999). These factors and the increasing demand for olive oil have encouraged many Mediterranean countries to invest in olive oil production. Consequently throughout Europe there is an enormous range of quality olive oils. However, when a product acquires a reputation extending beyond national borders it can find itself in competition with products which pass themselves off as the genuine article and take the same name. This unfair competition not only discourages producers but also misleads consumers. This is why the European Union created the PDO and PGI systems in 1992 to promote and protect foodstuffs of particular quality (Regulation 2081/92/EEC subsequently replaced with Regulation 510/2006/EEC). Furthermore in February 2009 labelling of origin for virgin and extra virgin olive oils became compulsory by European law (Regulation 182/2009/EC). Nevertheless, within the framework of limits prescribed by European regulations there is potential for mislabelling olive oil without risk of detection. Indeed, official olive oil quality control methods are based on maximum or minimum limits of certain chemical components, *e.g.* fatty acids, sterols, alcohols or stigmastadiene (Regulation 2568/91/EEC and amendments), and it is impossible to verify the real geographical origin of olive oil using these. This situation can influence consumers’ perception of the benefits of consuming olive oil and the current image of uncontrolled provenance in the olive oil market poses a considerable risk to the opportunity for economic growth in many Mediterranean countries. In particular, Italy has the highest number of PDO and PGI registered foodstuffs in Europe (N=206) and extra virgin olive oil is the second most important commodity, with forty recognised brands (Wesseler, 2010). So this commodity is very important for the Italian economy, being Italy the second largest producer in the world (588,000 tons in 2009, data provided by FAO, http://faostat.fao.org/) after Spain and the largest consumer (International Olive Oil Council, http://www.internationaloliveoil.org). This situation highlights the increasing demand for analytical methods and statistical tools capable of effectively verifying claims of origin.

Mediterranean countries, which are the main producers of olive oil, are also the most important production areas of olive oil squalene. Squalene ($C_{30}H_{50}$) is mainly used as adjuvant in vaccines and, once hydrogenated to squalane ($C_{30}H_{56}$), as an emollient and hydrating agent in cosmetic products. Squalene is mainly produced from the liver oil of deep sea sharks, despite the fact that deepwater sharks have an extremely low reproductive rate and many are endangered species. Currently thousands of endangered deep sea sharks are killed each year purely to supply squalene. Environmental and ethical concerns regarding shark fishing have led to the extraction of squalene from vegetable sources, such as olive oil distillates obtained through the physical/chemical refining (de-acidification and/or deodorization) of lamp olive oil. However, squalene produced from olive oil offers a lower yield and requires longer processing times, therefore involving higher costs. This explains why in recent years shark squalene has been marketed at around one-third of the price of olive oil squalene.

In 2006 the European Union imposed deep-sea shark fishing limits in the North-East Atlantic, and since 2008 some important cosmetic firms have declared that they have stopped using shark squalene. Furthermore, the so-called ‘Animal Testing Ban’ legislation (Directive 15/2003/EC) has forbidden the carrying out of any tests on animals for cosmetic purposes within the EU. As a consequence many countries have also prohibited the use of raw materials of animal origin, including shark squalene/squalane, in cosmetic formulations.

Up to now it has not been possible to easily differentiate the two different origins of the product and this has encouraged the illegal addition of shark to olive oil squalene/squalane or often the selling of shark products as olive oil derivates. It is therefore important to develop and validate innovative analytical methods able to identify their origin and the presence of shark squalene/squalane in olive oil based products.

4.2 STABLE ISOTOPE RATIOS AND ELEMENTAL COMPOSITION OF OLIVE OILS

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

As compared to other commodities, few studies have been done on the elemental composition of olive oils. This situation is due to the high organic load and viscosity and very small mineral content of olive oils, which makes the development of a simple and effective preparation method and subsequent analysis of elemental composition difficult. Several approaches have been used, such as emulsion in water with the aid of surfactants or solvents (Anthemidis et al., 2005; De Souza et al., 2005; Jimenez et al., 2003; Castillo et al., 1999), liquid-liquid extraction (Dugo et al., 2004), wet ashing (Lo Coco et al., 2003) and total microwave digestion (Zeiner et al., 2005, Benincasa et al., 2007; Cindric et al., 2007).

Interest in the elemental composition of olive oils initially focused on the presence of toxic elements (*e.g.* Pb, Cd) and of copper and nickel as catalysts for oxidative reactions affecting the flavour and stability of oils (De Souza et al., 2005). In the last few years, studies have also been carried out to verify whether the mineral profile could be a useful marker of geographical origin, cultivar, harvesting period and adulteration with cheaper vegetable oils (Dugo et al., 2004; Benincasa et al., 2007; Cindric et al., 2007).

To date, no papers are available in the literature regarding the isotopic ratio of hydrogen (D/H) in olive oil and no studies have been developed on the use of the combination of the two analytical approaches, stable isotope ratios and elemental composition. Furthermore, no studies have investigated whether analysis of the stable isotope ratios can distinguish olive oil from shark squalene/squalane and thus detect the presence of shark squalene/squalane in olive oil based products.

SECTION 4.3

Characterisation of authentic Italian extra virgin olive oils by stable isotope ratios of C, O and H and mineral composition

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Aim

In order to verify the potential use of stable isotope ratios in determining the geographical origin of olive oils, around five hundred of authentic PDO and PGI extra virgin olive oils produced throughout Italy and officially sampled by the Italian Ministry of Agricultural, Food and Forestry Policy, were analysed to establish a yearly databank of isotopic reference values, as has been done since 1987 for wine (Regulation 555/2008/EC). In particular $^{13}C/^{12}C$ in bulk oil and extracted glycerol and $^{18}O/^{16}O$ in glycerol were determined in all the samples. Moreover, a preparation and analysis method to determine elemental composition was developed. Elemental composition, $^{18}O/^{16}O$ and, for the first time, the D/H in bulk oil of around one hundred 2005 extra virgin olive oils were investigated as further markers of provenance.

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Characterisation of authentic Italian extra-virgin olive oils by stable isotope ratios of C, O and H and mineral composition

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A B S T R A C T

The paper shows the isotopic ratios (^{13}C/^{12}C, D/H, ^{18}O/^{16}O) in oil and extracted glycerol and the mineral composition of authentic PDO and PGI Italian extra-virgin olive oils, officially collected from 2000 to 2005 (N 539) to establish a national databank. ^{13}C/^{12}C and ^{18}O/^{16}O increased from Trentino to Sicily, each year distinguishing Northern Italy from Sicily and Calabria. Significant differences were found among the years and in some cases also between PDOs from the same region. ^{13}C/^{12}C and ^{18}O/^{16}O in bulk oil were significantly correlated with those in glycerol. D/H, measured in 2005 for the first time in oil, showed promising geographical discrimination capability. The content of 26 elements – Li, Rb, Cs, La, Ce and Yb rarely reported in the literature – was measured in well settled 2005 oils after ultrasound acid extraction.

1. Introduction

Olive oil is one of the most important commodities produced in Italy, which is the second largest producer in the world (630,000 tons in 2006–2007) and the largest consumer (International Olive Oil Council, http://www.internationaloliveoil.org). European law (EEC Reg. No. 2568/91) provides producers with the opportunity of indicating the geographical origin of extra-virgin olive oil using the protected denomination of origin (PDO) or the protected geographic indication (PGI), but it does not indicate specific analytical methods to check the authenticity of these indications.

In the last few years attention has been focused on authentication of the geographical origin of olive oil using in particular the profiles of volatile compounds (Vichi, Pizzale, Conte, Buxaderas, & Lopez-Tamames, 2007), sterols (Alves, Cunha, Amaral, Pereira, & Oliveira, 2005) or free fatty acids (D'Imperio, Dugo, Alfa, Mannina, & Segre, 2007), nuclear magnetic resonance fingerprinting (Rezzi et al., 2005) and also stable isotope ratios and mineral content. Moreover, because of its financial importance and role in the Mediterranean diet, olive oil has been investigated for other purposes, such as the identification of defects (e.g. rancid taste, presence of vegetable water or muddy sediment), pollutants (e.g. pesticides or metals) or fraud (e.g. mixing with hazelnut oil) using various analytical approaches, e.g. several chromatographic techniques (Aparicio & Aparicio-Ruiz, 2000), headspace gas chromatography-mass spectrometry (Lopez-Feria, Cardenas, Garcia-Mesa, Fernandez-Hernandez, & Valcarcel, 2007), Fourier transform infrared spectroscopy (Tay, Singh, Krishnan, & Gore, 2002), X-ray scattering (Bortoleto, Pataca, & Bueno, 2005), potentiometric stripping analysis (Lo Coco, Ceccon, Circolo, & Novelli, 2003) or inductively coupled plasma optical emission spectrometry (ICP-OES) (De Souza, Mathias, Da Silveira, & Aucelio, 2005).

With regard to stable isotope ratio analysis, the ^{13}C/^{12}C measured using elemental analyser – isotopic ratio mass spectrometry (EA-IRMS) or gas chromatography/combustion/isotopic ratio mass spectrometry (GC/C-IRMS) in bulk olive oil or in some sub-components (individual fatty acids or aliphatic alcohol and sterols) has been shown to be useful for detecting the adulteration of olive oil with cheaper pomace olive oil or with other vegetable oils (Angerosa, Camera, Cumitini, Gleixner, & Reniero, 1997; Spangenberg, Macko, & Hunziker, 1998). Moreover ^{13}C/^{12}C, especially in combination with the ^{18}O/^{16}O of bulk oil, proved to be a good tool for characterising geographical origin. Royer and co-workers (Royer, Gerard, Naulet, Lees, & Martin, 1999) studied the ^{13}C/^{12}C of the palmitic, oleic and linoleic fatty acids of olive oils, observing differences between French and Italian olive oils as compared to Greek ones and achieved regional classification of the Greek olive oils. Some authors (Bréas, Guillou, Reniero, Sada, & Angerosa, 1998;

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Angerosa et al., 1999) found that both the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ of olive oils from Italy, Greece, Spain, Tunisia, Morocco and Turkey change according to the latitude, suggesting as co-factors of variability the distance from the sea and environmental conditions during the growing of plants (water stress, atmospheric moisture and temperature). Finally, Aramendia et al. (2007) observed that the $^{18}O/^{16}O$ values of bulk olive oils were influenced by the variety of the olives and by their geographical origin, but not by the altitude, ripening degree and harvesting date of olives. To our knowledge, no papers are available in the literature regarding the isotopic ratio of deuterium/hydrogen (D/H) in olive oil.

With regard to the mineral content of olive oil, interest initially focused on the presence of toxic lead or cadmium elements and of copper and nickel as catalysts for oxidative reactions affecting the flavour and stability of oils (De Souza et al., 2005). In the last few years, studies have also been carried out to verify whether the mineral profile could be a useful marker of geographical origin, cultivar, harvesting period and adulteration with cheaper vegetable oils (Benincasa, Lewis, Perri, Sindona, & Tagarelli, 2007; Cindric, Zeiner, & Steffan, 2007; Dugo, La Pera, Giuffrida, Salvo, & Lo Turco, 2004). The high organic load and viscosity and very small mineral content of olive oils were a handicap to the development of a simple and effective preparation method and subsequent analysis. Several approaches were used, such as emulsion in water with the aid of some surfactant or solvent (Anthemidis, Arvanitidis, & Stratis, 2005; Castillo, Jimenez, & Ebdon, 1999; De Souza et al., 2005; Jimenez, Velarte, & Castillo, 2003), liquid–liquid extraction (Dugo et al., 2004), wet ashing (Lo Coco et al., 2003) and total microwave digestion (Benincasa et al., 2007; Cindric et al., 2007; Zeiner, Steffan, & Cindric, 2005). Of the analytical techniques, the following were more frequently applied: electrothermal and graphite furnace atomic absorption spectrophotometry (Cindric et al., 2007; Dugo et al., 2004), derivative potentiometric stripping (Dugo et al., 2004; Lo Coco et al., 2003) and, increasingly in the last few years, inductively coupled plasma–optical emission spectrometry (Anthemidis et al., 2005; Zeiner et al., 2005; Cindric et al., 2007) and plasma–mass spectrometry (ICP–MS) (Benincasa et al., 2007; Castillo et al., 1999; Jimenez et al., 2003).

The present work focuses on the $^{13}C/^{12}C$ in bulk oil and extracted glycerol and $^{18}O/^{16}O$ in glycerol of 539 authentic PDO and PGI extra-virgin olive oils produced from 2000 to 2005 throughout Italy, sampled by the Ministry of Agricultural, Food and Forestry Policy and analysed to establish an yearly databank of isotopic reference values. This was done to evaluate the geographic authenticity of commercial samples, as has been done since 1987 for wine (EEC Reg. No. 2729/2000). Moreover, the mineral composition, the $^{18}O/^{16}O$ and, for the first time, the D/H in bulk oil of around one hundred 2005 extra-virgin olive oils are shown and discussed for their variability.

2. Materials and methods

2.1. Sampling

Authentic and well settled extra-virgin olive oils ($N = 539$) were officially collected by the Ministry of Agricultural, Food and Forestry Policy from 2000 to 2005 in the production regions of the only one PGI and the 34 out of 37 PDOs recognised at the present in Italy, according to the EC Reg. No. 510/2006. Traditionally, each PDO defines multi-varietal oils (e.g. PDO Chianti can include up to 76 varieties). The sampling tried to cover all the harvest time, the variability of the multi-varietal blends and the production area. $^{13}C/^{12}C$ in bulk olive oil and the extracted glycerol, as well as $^{18}O/^{16}O$ in the glycerol, were measured in 2000 ($N = 82$), 2001 (102), 2002 (66), 2003 (95), 2004 (58) and 2005 (136) samples. In 2005, measurement of the $^{18}O/^{16}O$ and D/H of bulk olive oil also took place. Finally, the mineral content of a selection of 99 samples of 2005 was measured.

2.2. Chemicals

2.2.1. Isotopes

All the solutions were prepared with Milli-Q water (18M Ωcm resistivity; Millipore, Bedford, MA). Sodium hydroxide 2N (RP grade; Carlo Erba Reagents, Milan, Italy), hydrochloric acid at 37% (RP; Carlo Erba Reagents), diethyl ether (Normapur; VWR International, Leuven, Belgium), ethanol at 96% (Sigma–Aldrich GmbH, Steinheim, Germany), tin and silver capsules (Säntis analytical AG, Teufen, Switzerland), P_2O_5 at 97% (Sigma–Aldrich GmbH) and nitrogen gas at 99.999% (Linde Gas, Milan, Italy) were used.

The isotopic values (expressed in $\delta‰$, as described below) were calculated against working in-house standards (commercial olive oil and glycerol), calibrated against international reference materials: fuel oil NBS-22 (IAEA-International Atomic Energy Agency, Vienna, Austria) and sugar IAEA-CH-6 (IAEA) for $^{13}C/^{12}C$ measurement; IAEA-CH-6 (IAEA) for $^{18}O/^{16}O$ and NBS-22 for D/H. Whereas in the past the data regarding $^{18}O/^{16}O$ in glycerol were usually calibrated against glycerol used in the European project SMT4-CT98-2236 (Camin et al., 2004), in this work they were calibrated against the IAEA-CH6 value ($\delta^{18}O = +36.4‰$ vs. V-SMOW) assigned since 2005 (Boschetti & Iacumin, 2005) and accepted in the European TRACE project (contract No. FP6-2003-FOOD-2-A 006942).

The isotopic values of the aforementioned international reference materials and therefore also of the samples were expressed in $\delta‰$ vs. V-PDB (Vienna - Pee Dee Belemnitella) for $\delta^{13}C$ and V-SMOW (Vienna – standard mean ocean water) for $\delta^{18}O$ and δD, according to the following formula: $[(Rs-Rstd)/Rstd] \times 1000$, where Rs is the isotope ratio measured for the sample and Rstd is the isotope ratio of the international standard.

2.2.2. Elements

Nitric acid at 69.5% (Superpure; Merck, Darmastadt, Germany), hydrochloric acid at 37% (ACS; Riedel-deHaën, Seelze, Germany), ICP Multielement Standard Solution VI (Merck), Multielement Calibration Standard 1 (Agilent Technologies, Santa Clara, CA, USA), and Cesium 1000 ug/ml (Ultra Scientific, Bologna, Italy) were used. Standard solutions were diluted and stabilized with the addition of a 1% HNO_3 and 0.2% HCl solution. SRM 2387 'Peanut butter' (National Institute of Standard and Technologies, Gaithersburg, MD, USA) was used as standard reference material to check the accuracy of the method. Sc 0.1 mg/L, Rh 0.1 mg/L and Tb 0.1 mg/L were used as internal standards. All the glassware was rinsed with nitric acid (5% v/v) and twice with milli-Q water before use.

2.3. Apparatus

2.3.1. Isotopes

The analysis was performed using an isotopic ratio mass spectrometer (IRMS) (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) coupled with an Elemental Analyser (Flash EA^{TM}1112, Thermo Scientific,) for $^{13}C/^{12}C$ measurement and with a Pyrolyser (Finnigan^{TM}TC/EA, high temperature conversion elemental analyzer, Thermo Scientific,) for D/H and $^{18}O/^{16}O$ measurement. To separate the gases, the Elemental Analyser was supplied with a Porapack QS (3 m; 6 × 4 mm, OD/ID) GC column and the Pyrolyser with a Molecular Sieve 5A (0.6 m) GC column. The devices were 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.

2.3.2. Elements

The analysis was performed using an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with an autosampler ASX-520 (Cetac Technologies Inc., Omaha, NE, USA). After extraction, the samples were introduced into a Scott spray chamber using a MicroMist nebulizer and then into a Fassel type torch. An octopole reaction system (ORS) using He and H_2 as collision and reaction gases, respectively, was used to remove polyatomic interferences.

2.4. Sample preparation and analysis

2.4.1. Isotopes

Glycerol was obtained through hydrolysis of 20 ml of oil in NaOH, acidification of the solution, extraction of fatty acids and purification by under vacuum distillation, according to the method described for fat from cheese in Camin et al. (2004).

Aliquots of 0.3 mg of sample were weighed in tin capsules for determination of $^{13}C/^{12}C$ and silver capsules for quantification of $^{18}O/^{16}O$ and $^{2}H/^{1}H$.

For $^{13}C/^{12}C$, the precision of measurement, expressed as standard deviation when measuring an oil sample 10 times, was 0.1‰.

For $^{18}O/^{16}O$ and D/H analysis, the samples were stored in a desiccator above P_2O_5 for at least 24 h, then weighed into silver capsules and put into the auto-sampler equipped with a suitable cover. During measurement, dryness was guaranteed by flushing nitrogen continuously over the samples. The pyrolyser temperature was 1450 °C. The D/H and $^{18}O/^{16}O$ ratios of bulk olive oils were measured simultaneously in one run. The IRMS measured first D/H and then, following the magnet jump, $^{18}O/^{16}O$, taking about 10 min for each sample. Before measuring D/H, the H3 factor, which allows correction of the contribution of $[H3]^+$ to the m/z 3 signal (Sessions, Burgoyne, & Hayes, 2001), was verified to be lower than 9. The precision of measurement, expressed as standard deviation when measuring an oil sample 10 times, was 0.3‰ for $^{18}O/^{16}O$ and 2‰ for D/H.

2.4.2. Elements

About 15 g of sample were weighed into a 50 ml conical vial of polypropylene (PP) and 15 ml of 1% HNO_3/0.2% HCl water solution was added. The mixture was thoroughly shaken for 30 s using a vortex mixer and immediately placed in an ultrasonic bath (170 W × 5 min) to extract the trace elements from the oil to the acid solution. The mixture was centrifuged (4000 rpm × 5 min) to separate the two phases. The upper oil phase was accurately removed by aspiration and the lower aqueous phase transferred into a clean PP vial and subjected to ICP-MS analysis of Li, B, Na, Mg, K, Ca, Mn, Co, Cu, Ga, Se, Rb, Sr, Mo, Cd, Cs, Ba, La, Ce, Nd, Sm, Eu, Yb, Tl, Pb, and U. Isotopes and ORS gases are shown in Table 1. Extraction and analysis was carried out in duplicate. The accuracy of the extraction method was evaluated in a natural oil sample spiked with a defined aliquot of the reference material (0.6 g of 'semi-solid' peanut butter mixed into 15 g of oil until thoroughly combined). The oil and fortified mixture were both extracted and analysed 10 times. Recoveries were calculated on the difference of the mean content of the spiked and the un-spiked samples. The detection limit (DL) of each element was calculated as three times the standard deviation of the signal of the blank sample, extracted and analysed ten times, whereas the blank sample was prepared using Milli-Q water to substitute the oil sample in the extraction step. Precision (RSD%) was evaluated by preparing and analyzing an oil sample 10 times. DL and RSD% are shown in Table 1.

Table 1
Instrumental conditions and mineral content distribution of well settled extra-virgin Italian olive oils (ORS: collision cell octopole reaction system)

Element	Isotope	ORS mode	Unit	DL	Number of Samples > DL	RSD (%)	25° Percentage	Median	75° Percentage	90° Percentage	Maximum	Range (Minimum–Maximum)	Literature
Li	7	–	µg/kg	0.005	56	18	–	0.007	0.013	0.023	0.208	–	–
Na	23	He	mg/kg	0.04	42	27	–	–	0.100	0.211	1.105	28.8–38.0	d,n
Mg	26	He	mg/kg	0.014	62	20	–	0.019	0.055	0.109	0.495	0.056–3.8	d,f,g
K	39	He	mg/kg	0.06	68	20	–	0.163	0.645	1.702	9.94	< 0.001–0.19	d,n
Ca	40	H_2	mg/kg	0.03	12	16	–	–	–	0.380	0.950	< 0.05–26.9	a, b, d,n
Mn	55	–	µg/kg	0.01	60	21	–	0.211	0.630	1.43	10.0	< 1–200	a, b, c, d, f, m, n
Co	59	–	µg/kg	0.004	20	13	–	–	–	0.012	0.033	0.023–5450	a, b, c, d, f, m, n
Cu	63	He	µg/kg	0.13	88	21	0,237	0.360	0.689	1.45	26.3	< 1–4510	a, c, d, f, g, h, l, m, n
Rb	85	–	µg/kg	0.03	83	21	0,041	0.110	0.375	1.03	13.4	–	–
Sr	88	–	µg/kg	0.04	15	17	–	–	–	0.483	3.85	1.52–48.9	b
Cs	133	–	µg/kg	0.003	67	20	–	0.004	0.005	0.012	0.819	–	–
Ba	137	–	µg/kg	0.29	22	21	–	–	–	0.543	2.49	< 0.15–700	a, c, g
La	139	–	µg/kg	0.0017	46	24	–	–	0.006	0.040	2.94	–	–
Ce	140	–	µg/kg	0.0027	50	24	–	0.003	0.008	0.046	4.72	–	–
Sm	147	–	µg/kg	0.0009	27	24	–	–	0.001	0.004	0.111	0.004–0.226	i
Eu	151	He	µg/kg	0.0002	39	20	–	–	0.001	0.002	0.023	< 0.009–0.021	b
Yb	171	–	µg/kg	0.0004	61	20	–	0.001	0.001	0.002	0.041	–	–
Pb	208	–	µg/kg	0.02	80	21	0,195	0.372	0.725	1.50	8.46	< 0.42–79.9	a, c, d, f, g, h, l, m, n
U	238	–	µg/kg	0.001	67	18	–	0.001	0.007	0.015	0.119	<0.25	c

a: Anthemidis et al. (2005); b: Benincasa et al. (2007); c: Castillo et al. (1999); d: Cindric et al. (2007); e: Dugo et al. (2004); f: Jimenez et al. (2003); g: La Pera et al. (2002); h: Lo Coco et al. (2000); i: Lo Coco et al. (2003); l: Martin-Polvillo et al. (1994); m: Solinas et al. (1987); n: Zeiner et al. (2005).

Table 2

Median, minimum and maximum values of δ^{13}C (bulk olive oil and glycerol) and of δ^{18}O values of glycerol (‰ vs. V-PDB and V-SMOW, respectively) in the Italian olive oil databank (2000–2004) and results of the non-parametric statistical test (Kruskall–Wallis' test) among crops

Region	N_{toc}	Parameter	2000				2001				2002				2003				2004			
			N	Median	Minimum	Maximum	*N*	Median	Minimum	Maximum	*N*	Median	Minimum	Maximum	*N*	Median	Minimum	Maximum	*N*	Median	Minimum	Maximum
Trentino	37	δ^{13}C bulk	5	−30.7	−31.8	−29.7	23	−30.7	−32.4	−30.1	3	−29.7	−30.4	−29.7	3	−29.5	−29.5	−29.4	3	−30.7	−30.8	−30.4
		δ^{13}C glycerol		−33.6	−34.7	−32.7		−33.5	−34.6	−32.7		−32.8	−33.3	−31.8		−32.2	−32.4	−31.9		−33.4	−33.5	−33.2
		δ^{18}O glycerol		29.2	27.8	29.2		29.1	28.0	30.5		29.3	28.5	30.2		30.9	30.8	32.4		29.3	29.2	29.3
Veneto	20	δ^{13}C bulk	3	−30.3	−30.9	−30.3	3	−30.3	−30.7	−30.3	3	−31.1	−31.2	−30.7	5	−28.8	−29.0	−27.5	6	−30.1	−31.4	−29.0
		δ^{13}C glycerol		−32.4	−33.9	−32.3		−32.5	−32.6	−32.1		−32.6	−33.3	−32.0		−31.5	−31.8	−29.2		−32.0	−33.5	−31.0
		δ^{18}O glycerol		31.3	29.1	31.7		29.8	29.5	30.8		28.9	28.0	30.3		32.7	30.2	33.1		30.6	29.7	33.1
Lombardia	16	δ^{13}C bulk	4	−30.3	−30.6	−29.9	3	−30.4	−30.6	−30.3					5	−29.1	−29.3	−28.6	4	−31.0	−31.4	−29.4
		δ^{13}C glycerol		−32.5	−33.6	−32.1		−32.2	−32.3	−31.9						−30.7	−31.3	−29.8		−32.8	−33.1	−31.7
		δ^{18}O glycerol		30.2	28.1	31.3		29.8	29.7	30.1						31.5	31.3	32.1		30.3	29.6	33.2
Emilia-	13	δ^{13}C bulk	2	−29.2			3	−29.6	−29.8	−29.4	2	−30.0			6	−29.1	−29.3	−28.7				
Romagna		δ^{13}C glycerol		−31.6				−31.6	−31.6	−31.3		−32.3				−31.2	−31.7	−30.7				
		δ^{18}O glycerol		29.7				29.8	29.1	30.1		29.7			31.1	30.9	32.4					
Liguria	18	δ^{13}C bulk	3	−30.3	−30.6	−30.1	3	−29.7	−30.0	−29.1	3	−30.3	−30.5	−29.6	6	−28.8	−29.1	−28.2	3	−30.0	−30.2	−29.6
		δ^{13}C glycerol		−33.3	−33.4	−33.1		−32.2	−32.7	−31.7		−32.7	−33.3	−32.6		−31.1	−31.7	−30.9		−33.1	−33.1	−32.6
		δ^{18}O glycerol		31.3	31.1	31.3		31.8	31.6	33.2		29.8	29.5	29.9		32.3	31.5	32.6		31.4	30.4	32.0
Tuscany	23	δ^{13}C bulk	5	−29.3	−30.0	−28.3	6	−29.3	−30.3	−29.0	9	−30.4	−30.8	−29.9	3	−28.7	−29.5	−27.9				
		δ^{13}C glycerol		−31.0	−32.3	−30.2		−31.3	−32.1	−30.9		−32.5	−33.3	−31.4		−31.4	−31.7	−30.5				
		δ^{18}O glycerol		32.9	30.9	34.2		31.0	30.2	31.8		31.2	28.4	33.1		31.9	31.6	32.8				
Umbria	30	δ^{13}C bulk	4	−29.3	−29.5	−29.2	15	−29.4	−30.0	−28.1					11	−28.9	−29.5	−28.3				
		δ^{13}C glycerol		−30.1	−31.4	−29.7		−30.9	−32.0	−29.6						−30.6	−31.7	−29.8				
		δ^{18}O glycerol		31.8	30.5	33.7		32.1	30.0	33.4						32.6	31.4	33.4				
Abruzzo	30	δ^{13}C bulk	10	−28.7	−29.7	−28.1					6	−29.4	−30.1	−29.0	8	−28.3	−28.5	−28.1	6	−29.1	−30.0	−28.4
		δ^{13}C glycerol		−30.4	−31.3	−28.8						−30.8	−31.5	−29.9		−29.8	−30.1	−29.7		−31.0	−31.8	−30.3
		δ^{18}O glycerol		32.9	31.1	34.1						31.6	30.6	32.2		33.4	33.2	33.5		32.6	31.2	33.3
Lazio	50	δ^{13}C bulk	10	−29.2	−30.3	−28.1	12	−28.8	−29.5	−28.0	12	−30.1	−31.1	−29.6	12	−28.6	−29.3	−27.8	4	−29.3	−29.9	−28.9
		δ^{13}C glycerol		−30.7	−32.1	−28.5		−30.3	−31.2	−28.9		−31.1	−32.8	−29.6		−29.8	−31.3	−28.4		−31.0	−31.7	−30.3
		δ^{18}O glycerol		33.3	31.8	33.5		32.8	30.6	33.5		31.6	29.9	33.3		33.0	32.4	33.4		32.5	32.2	32.9
Campania	35	δ^{13}C bulk	9	−29.4	−29.7	−27.5	9	−28.2	−29.3	−27.4	9	−30.0	−30.8	−28.9	6	−28.5	−29.5	−27.9	2	−28.8	−29.0	−28.6
		δ^{13}C glycerol		−31.5	−32.5	−30.0		−30.4	−31.1	−29.8		−31.7	−33.5	−29.7		−30.5	−31.5	−29.7		−30.8	−31.1	−30.5
		δ^{18}O glycerol		33.3	31.9	34.4		33.0	31.2	34.3		31.2	27.6	31.9		33.1	31.2	33.9		32.0	31.7	32.3
Apulia	55	δ^{13}C bulk	18	−29.2	−30.4	−28.2	22	−28.9	−30.0	−26.6	5	−30.6	−30.8	−30.0					10	−29.6	−30.6	−28.5
		δ^{13}C glycerol		−31.4	−32.3	−29.9		−30.8	−31.8	−29.3		−32.1	−34.4	−32.0	00					−31.8	−33.0	−30.7
		δ^{18}O glycerol		31.7	30.3	35.7		31.9	31.1	34.2		31.0	29.8	31.7						31.9	30.5	32.6
Calabria	24	δ^{13}C bulk									8	−29.8	−31.3	−28.3	9	−29.4	−30.1	−28.8	7	−29.2	−30.2	−29.0
		δ^{13}C glycerol										−31.0	−32.0	−29.9		−30.5	−31.1	−30.2		−31.0	−31.3	−30.4
		δ^{18}O glycerol										32.9	30.7	34.1		33.5	31.3	34.8		34.8	32.5	35.6
Sicily	52	δ^{13}C bulk	9	−28.4	−28.7	−28.0	3	−28.3	−28.5	−27.0	6	−28.5	−29.4	−27.7	21	−28.8	−30.0	−27.7	13	−29.1	−29.8	−28.0
		δ^{13}C glycerol		−30.4	−31.4	−29.3		−29.3	−29.4	−28.4		−29.5	−31.8	−28.8		−30.2	−31.5	−29.0		−30.6	−31.8	−29.1
		δ^{18}O glycerol		35.5	32.5	36.2		33.9	33.4	35.1		33.7	33.1	34.8		33.0	30.6	35.0		34.2	32.4	34.6
Total	403	δ^{13}C bulk	82	-29.3^{ab}	−31.8	−27.5	102	-29.2^{ab}	−32.4	−26.6	66	-30.0^{c}	−31.3	−27.7	95	-28.9^{a}	−30.1	−27.5	58	-29.4^{bc}	−31.4	−28.0
		δ^{13}C glycerol		-31.2^{b}	−34.7	−28.5		-31.2^{b}	−34.6	−28.4		-31.7^{b}	−34.4	−28.8		-30.6^{a}	−32.4	−28.4		-31.3^{b}	−33.5	−29.1
		δ^{18}O glycerol		30.0^{ab}	25.2	33.6		29.0^{b}	25.4	32.5		28.7^{b}	25.0	32.2		30.1^{a}	27.6	32.4		29.7^{ab}	26.7	33.1

Different letters correspond to significantly different median values ($p < 0.001$) *N* = number of samples.

2.5. Statistical analysis

The data were statistically evaluated according to the procedures of the software Statistica 7.1 (StatSoft Italia srl, Padua, Italy). Non parametric tests (Kruskall–Wallis and multiple bilateral comparison) were applied because of the low and unequal numbers of samples per group and the not always normal distribution (Soliani, 2003).

3. Results and discussion

3.1. Stable isotope ratios

In Table 2 the median, minimum and maximum values of bulk and glycerol $\delta^{13}C$ and of glycerol $\delta^{18}O$ were summarised for 2000–2004 production and the different Italian regions listed according to the latitude. $\delta^{18}O$ was measured in the glycerol instead of in the bulk oil because the former was assumed to be more closely related to the isotopic characteristics of ground water (Schmidt, Werner, & Rossmann, 2001).

The $\delta^{13}C$ values measured in glycerol were always lower than in bulk olive oil, with a mean difference ± standard deviation of 1.87 ± 0.67, confirming previous results (Zhang, Buddrus, Trierweiler, & Martin, 1998). Moreover, $\delta^{13}C_{glycerol}$ and $\delta^{13}C_{bulk}$ of the 403 samples were significantly correlated ($\delta^{13}C_{glycerol} = 1.1114 \times \delta^{13}C_{bulk} + 1.4057$; $p < 0.001$). If we consider 2000, 2001, 2002 and 2004 individually, the intercepts ranged from −0.3075 to +6.3585 and the slopes from 1.0435 to 1.2841. The correlation for 2003, also significant, showed particular and extreme values, both as regards intercept (−7.0134) and slope (0.8162).

Significant differences ($p < 0.001$) were found among the years of harvest (Table 2). In particular 2003, well-known as one of the hottest years in the last few decades in Italy, was different ($p < 0.001$) for at least one isotopic parameter from all the other years, showing enrichment in the heavier isotopomeres.

All the isotopic parameters showed a trend for the values to increase from Trentino to Sicily in all years (Table 2), with this being less evident in 2003. This trend, already observed in wine (Rossmann et al., 1996; Rossmann et al., 1999), is probably positively related to vicinity to the sea and dryness of the climate and negatively to latitude, as suggested by some authors (Angerosa et al., 1999; Bréas et al., 1998). Indeed, the $\delta^{13}C$ values of plant compounds are influenced by the availability of water, relative humidity and temperature, which control stomatal aperture and the internal CO_2 concentration in the leaf (O'Leary, 1995). The $\delta^{18}O$ of carbohydrates and their immediate descendants, such as glycerol, is correlated to the $\delta^{18}O$ of leaf water (Schmidt et al., 2001), which reflects the isotopic composition of groundwater and average precipitation in the region – mainly related to latitude, distance from the sea and altitude (Clark & Fritz, 1997) – and the extent of evapotranspiration, mainly influenced by humidity and temperature (Rossmann et al., 1999).

For statistical evaluation, we grouped the regions into four clusters on the basis of their latitude and the similarity of their isotopic ratios: North (Trentino, Veneto, Lombardia, Emilia Romagna), Centre (Liguria, Tuscany, Umbria, Abruzzo, Lazio), South-1 (Campania, Apulia) and South-2 (Calabria, Sicily). Applying the non parametric test of Kruskall–Wallis (Fig. 1), olive oil from Northern Italy showed isotopic values for at least one parameter significantly lower ($p < 0.01$) than for the South-2, Centre and South-1 in 5, 3 and 2 years respectively, out of the 5 years. Central Italy was never separated from South-1, whereas it was different from South-2 in two out of 5 years. The two southern macro areas could be distinguished from one another in three out of 5 years.

Comparing the few PDOs with at least five samples within each region and each year, we observed some significant differences ($p < 0.01$). In Lazio, the two 'Canino' and 'Sabina' PDOs were significantly different in 2000, 2002 and 2003 for $\delta^{13}C$ and in 2001 for $\delta^{18}O$. This could be ascribed to the inland location of the production area of 'Sabina', nearer Rieti than the 'Canino' area, closer to the sea near Tuscany, as well as to the possible effect of the prevailing olive cultivar (Aramendia et al., 2007). In Abruzzo and Apulia the comparison was only possible for one year (2000 and 2001, respectively). Significant differences in the $\delta^{13}C$ values were observed between 'Aprutino Pescarese' and 'Colline Teatine' in Abruzzo and between 'Dauno' and 'Collina di Brindisi' in Apulia.

For the 2005 samples, along with the aforementioned parameters, the $\delta^{18}O$ and δD in bulk oil were also measured (Table 3). δD was investigated as a possible additional parameter for the

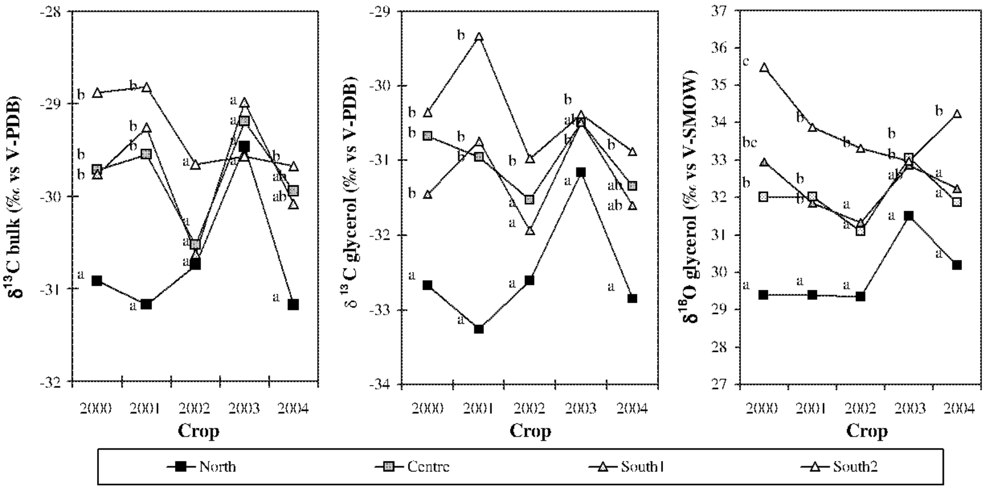

Fig. 1. Median values of the $\delta^{13}C$ (bulk olive oil and glycerol) and the $\delta^{18}O$ (glycerol) for North, Centre, South-1 and South-2 Italian olive oils (2000–2004) and results of the non-parametric statistical test (Kruskall–Wallis' test) among North, Centre, South-1 and South-2 Italian olive oils; different letters correspond to significantly different median values ($p < 0.01$).

Table 3

Median values of $\delta^{13}C$ (bulk olive oil and glycerol), $\delta^{18}O$ (bulk and glycerol) and 8D (bulk oil) for North, Centre, South-1 and South-2 Italian olive oils produced in 2005 and results of the non-parametric statistical test (KruskallWallis' test)

	N	$\delta^{13}C$ bulk (‰ vs. V-PDB)			$\delta^{13}C$ glycerol (‰ vs. V-PDB)			$\delta^{18}O$ glycerol (‰ vs. V-SMOW)			$\delta^{18}O$ bulk (‰ vs. V-SMOW)			$\delta^{2}H$ bulk (‰ vs. V-SMOW)		
		Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
North	45	-30.9^{a}	−31.9	−29.8	-33.9^{a}	−35.0	−32.0	28.0^{a}	26.9	31.0	20.2^{a}	19.1	22.4	-159^{a}	−165	−145
Centre	45	-30.1^{b}	−30.9	−29.0	-32.3^{b}	−33.5	−30.6	30.5^{b}	28.9	32.3	22.3^{b}	20.3	23.7	-153^{b}	−162	−142
South-1	19	-29.6^{bc}	−30.9	−29.2	-32.0^{b}	−33.3	−31.0	30.1^{b}	29.6	31.6	22.2^{b}	21.8	23.1	-149^{bc}	−156	−140
South-2	27	-29.2^{c}	−30.9	−27.9	-31.0^{c}	−32.5	−28.9	32.8^{c}	30.6	35.7	24.5^{c}	22.8	26.8	-148^{c}	−155	−137

Different letters correspond to significantly different median values $p < 0.01$.

characterisation of geographical origin, being influenced in plant products by the isotopic composition of the primary hydrogen source (source water through the leaf water) and by the geographical and climatic factors mentioned above for $\delta^{18}O$, together with their biosynthetic pathways (Schmidt, Werner, & Eisenreich, 2003).

As compared to previous years (Fig. 1), the median values of the isotopic parameters were lower, as a consequence of the rainy and cold climate characterising 2005.

The $\delta^{18}O$ values measured in bulk were significantly correlated with those in glycerol ($p < 0.001$):$\delta^{18}O_{bulk}$=0.837*$\delta^{18}O_{glycerol}$ −3.2213 ($R^2 = 0.8772$), the first always being lower, with a mean difference ± standard deviation of 8.15‰ ± 0.66.

The $\delta^{18}O$ and δD values in bulk olive oil were also correlated ($p < 0{,}001$), as happens in water (Clark & Fritz, 1997); the correlation equation is $\delta^2H_{bulk} = (-208{,}1\ + 2{,}5091) \times \delta^{18}O_{bulk}$, but the R^2 value (0.4582) is low.

The $\delta^{18}O$ values of bulk olive oil showed the same capability as the $\delta^{18}O$ of glycerol in terms of distinguishing the four regional groups. The δD values showed similar capability to that of $\delta^{13}C$ and $\delta^{18}O$ to differentiate the four groups.

3.2. Mineral composition

The recoveries of the extraction method evaluated with the NIST sample were generally satisfactory for all the certified elements, being 82% for Zn, 84% for Mn, 90% for Ca, 92% for Mg, 95% for K, and 101 for Na. The precision of the analytical method ranged from 13% to 27% for the different elements (Table 1). Such values can be deemed satisfactory, considering the very low content of elements in olive oil.

Tables 1 and 4 show the concentration of the elements quantifiable over the DL in at least 10 samples. The content of Mo, Cd and Tl were below the respective DLs (0.18, 0.02 and 0.005 μg/kg respectively) in all the samples. Ga was found in quantifiable amounts (DL = 0.004 μg/kg) only in one Terra di Bari PDO oil (0.023 μg/kg). B was found in measurable amounts (DL = 1 μg/kg) only in five samples, with a maximum of 12.2 μg/kg for a Monte Etna PDO oil, in agreement with the results shown by Eschnauer for wines from grapes grown in volcanic areas (Eschnauer, 1982). Se was only detectable (DL = 0.014 μg/kg) in seven samples, with a maximum of 0.021 μg/kg, far below the content reported by Dugo et al. (2004), for 50 Sicilian oils analysed using cathodic stripping potentiometry and by Benincasa et al. (2007) for 36 oils from 4 Central–Southern Italian regions digested by microwave and analysed using ICP–MS. Nd was only quantifiable (DL = 0.023 μg/kg) in nine samples, with a maximum value of 0.932 μg/kg in a Terra di Bari PDO oil.

Tables 1 and 4 show the distribution of the mineral element content in the total sampling and for each PDO. As regards the content of Li, Rb, Cs, La, Ce and Yb shown in Table 1, we could not find other data for extra-virgin olive oils in the literature, while Mg, Ca, Mn, Sr, Sm, Eu and U were found in the concentration ranges reported in the literature. Na and K were measured in notably lower and higher amounts respectively, as compared to olive oils from Croatia analysed by Zeiner et al. (2005), and Cindric et al. (2007). The Co and Cu content were also in the ranges reported in literature, with the exclusion of the maximum values found for some of the aforementioned Croatian oils. The Ba content agreed with that found by Jimenez et al. (2003), and Anthemidis et al. (2005), but was far below the maximum content (700 μg/kg) measured by Castillo et al. (1999), using a semi-quantitative ICP–MS approach with direct emulsion nebulisation of the oil sample. The Pb content was low and always below the legal limits (0.1 mg/kg, EEC Reg. No. 466/2001), probably as a consequence of the increasingly widespread use of adequate equipment throughout the olive oil processing chain.

Table 4
Mineral content of well settled 2005 extra-virgin Italian olive oils displayed for region and for PDO or PGI

			South Italy										Centre Italy					North Italy	
		Region	Calabria			Apulia	Sicily						Lazio		Tuscany		Umbria	Veneto	Trentino - Veneto
		PDO/ PGI	Alto Crotonese	Bruzio	Lametia	Terra diBari	Monte Etna	Monti Iblei	Val Demone	Valdi Mazara	Valli del Belice	Valli Trapanesi	Canino	Sabina	Lucca	Toscano PGI	Umbria	Veneto	Garda
Element	Unit	No. Obs.	3	2	3	10	3	3	3	3	3	3	6	6	3	3	12	7	26
Li	µg/kg	median		0.003	0.010	0.007	0.031	0.013		0.011	0.007	0.008	0.004		0.006	0.006		0.016	0.003
		max	0.010	0.006	0.013	0.039	0.208	0.046	0.008	0.013	0.007	0.010	0.018	0.012	0.014	0.007	0.029	0.064	0.091
Na	mg/kg	median			0.049		0.280	0.189	0.066				0.069			0.060		0.154	
		max	0.124		0.100	0.170	1.11	0.492	0.161	0.129		0.052	0.326	0.124		0.133	0.340	0.609	0.312
Mg	mg/kg	median				0.042	0.082	0.087	0.015	0.047	0.016		0.031			0.034		0.050	0.046
		max	0.016		0.017	0.225	0.495	0.139	0.017	0.081	0.017	0.016	0.110	0.056		0.047	0.053	0.104	0.264
K	mg/kg	median		0.038	0.180	0.415	0.726	1.171	0.116	0.418	0.079		0.033			0.292	0.109	0.424	0.516
		max	0.32	0.08	0.22	3.79	9.94	1.70	0.17	0.90	0.10	0.12	0.56	1.13	0.12	0.43	0.64	2.06	3.13
Ca	mg/kg	median																	
		max				0.395	0.447	0.603											0.950
Mn	µg/kg	median			0.408	0.598	0.491	0.351		0.134			0.312	0.058		0.921		0.392	0.507
		max			0.566	10.0	3.42	1.21		0.522			3.61	0.655	0.173	1.17	0.623	1.49	2.87
Co	µg/kg	median				0.003	0.006												
		max				0.033	0.024	0.030		0.009				0.005	0.014		0.030	0.012	0.031
Cu	µg/kg	median	0.160	0.334	0.343	0.404	2.107	0.446	0.360	0.434	0.311	0.171	0.251	0.127	0.416	0.324	0.258	0.583	0.493
	µg/kg	max	0.237	0.363	0.952	2.37	15.8	1.47	0.689	0.472	0.885	0.430	0.663	0.824	0.778	0.355	0.810	1.72	26.3
Rb	µg/kg	median		0.040	0.108	0.394	0.199	0.628	0.040	0.178	0.060	0.036	0.237	0.173		0.214	0.058	0.336	0.183
		max	0.263	0.049	0.175	4.19	13.4	1.59	0.081	0.802	0.065	0.074	2.98	2.69	0.055	0.214	0.584	1.013	1.110
Sr	µg/kg	median																	
	µg/kg	max					1.23	3.85					1.12						1.40
Cs	µg/kg	median	0.003	0.008	0.004	0.004	0.004	0.012	0.004	0.004	0.005	0.004	0.006	0.004	0.003	0.003	0.004	0.004	
		max	0.035	0.012	0.005	0.012	0.038	0.014	0.004	0.013	0.005	0.005	0.819	0.011	0.012	0.004	0.012	0.007	0.012
Ba	µg/kg	median																	
	µg/kg	max				2.05	2.49	0.550					1.13			0.309	0.435	1.34	0.695
La	µg/kg	median			0.064	0.032	0.002						0.016			0.009	0.001	0.002	
		max			0.158	2.94	0.008	0.023		0.004			0.225	0.012		0.035	0.040	0.011	0.005
Ce	µg/kg	median		0.002	0.043	0.058							0.020			0.013		0.004	0.003
	µg/kg	max		0.004	0.111	4.72	0.018	0.045		0.008			0.161	0.024		0.056	0.056	0.013	0.009
Sm	µg/kg	median			0.005	0.003							0.001			0.001			
		max			0.010	0.111		0.002					0.015	0.001		0.004	0.003		0.002
Eu	µg/kg	median		0.0010	0.0017	0.0021	0.0004									0.0004			
	µg/kg	max	0.0020	0.0020	0.0017	0.0226	0.0008	0.0004		0.0020	0.0020	0.0021	0.0032	0.0021		0.0028	0.0025	0.0023	0.0006
Yb	µg/kg	median	0.0012	0.0007	0.0019	0.0022		0.0007		0.0006	0.0011	0.0011	0.0007	0.0012		0.0012	0.0003	0.0007	
		max	0.0017	0.0014	0.0032	0.0412	0.0015	0.0009		0.0017	0.0017	0.0015	0.0032	0.0022		0.0033	0.0030	0.0020	0.0011
Pb	µg/kg	median	0.180	0.262	0.595	0.380	211	0.439	0.815	0.292	0.300		0.329	0.234	0.790	0.345	0.516	0.691	0.260
	µg/kg	max	1.47	0.299	1.50	2.69	3.86	0.972	1.24	0.733	0.409	0.468	1.84	0.464	2.60	0.573	2.76	0.904	8.46
U	µg/kg	median			0.0021	0.0021	0.0104	0.0008	0.0009		0.0030		0.0130	0.0050		0.0067	0.0012	0.0314	0.0023
		max	0.0014		0.0176	0.0401	0.0802	0.0017	0.0016		0.0145	0.0067	0.1190	0.0939		0.0211	0.0044	0.0131	0.0390

Comparison between the mineral content of the individual PDOs was not an aim of this first work carried out on Italian extra-virgin olive oils in agreement with the Italian Ministry of Agricultural, Food and Forestry Policy. At all events – taking into account only the 3 PDOs with at least 10 samples each, namely Garda, Umbria and Terra di Bari – a trend toward higher Mg, Ca, Mn and Sr content would seem to characterise the Garda oils, especially if compared to the Umbrian oils, possibly related to the soil of the region, mainly originating from dolomitic limestone rock.

4. Conclusions

This paper, shows the results of the largest investigation ever carried out on multi-element stable isotope ratio and mineral composition using IRMS and ICP-MS in authentic PDO and PGI Italian extra-virgin olive oils. The study was done in collaboration with the Ministry of Agricultural, Food and Forestry Policy to establish a national databank for olive oils.

The stable isotope ratios of carbon, oxygen and hydrogen in olive oil were shown to increase from Trentino to Sicily, making it possible to distinguish Northern Italy from Sicily and Calabria each year and confirming the trend observed for other commodities such as wine. Significant differences were found among the years of harvest and in some cases also between PDOs from the same region. The $\delta^{13}C$ and $\delta^{18}O$ values in bulk oil were significantly correlated with those in glycerol. The $\delta^{18}O$ in glycerol showed the same capability to differentiate the geographic origin as $\delta^{18}O$ in bulk, whereas in some years the $\delta^{13}C$ of glycerol showed a better capability to discriminate as compared to bulk. Because the discriminating capability achievable using $\delta^{13}C$ in glycerol is the same as that of $\delta^{18}O$ in bulk, it would seem preferable to directly measure the isotopic ratios in bulk, making savings in terms of time and costs. The δD values, measured in 2005 for the first time in oil, showed promising geographical discrimination capability.

The content of each mineral element in well settled olive oil, measured after ultrasound acid extraction, was basically low and similar to that seen in the literature. The capability to discriminate on the basis of minerals in PDOs could only be checked for few oils produced in specific areas with different geology and requires further research in order to be confirmed.

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Results

$\delta^{13}C_{glycerol}$ and $\delta^{13}C_{bulk}$ were significantly correlated like $\delta^{18}O_{glycerol}$ and $\delta^{18}O_{bulk}$. Thus, as their ability to differentiate geographical origin is more or less the same, it would seem preferable to measure the isotopic ratios in bulk directly, making savings in terms of time and costs. All the isotopic parameters, including the δD of bulk oil, showed a trend for the values to increase from Trentino to Sicily in all years. Consequently, the isotopic parameters made it possible to distinguish samples from macro areas such as North and South Italy, whereas differentiation between oils from Central and South Italy was more difficult.

After development of a suitable method for the preparation and analysis of elemental composition, a dataset with elemental profiles was created and validated. The elemental composition of samples of some PDOs (*e.g.* from Trentino and Umbria) showed different content for some elements, probably in relation to the different geological characteristics of the soil in which the plants are grown.

These results suggest that further investigations should be carried out on the use of the parameters investigated for differentiating samples from areas with different climatic and geological characteristics, highlightening the factors affecting them and relations between them.

SECTION 4.4

Coast and year effect on H, O and C stable isotope ratios of Tyrrhenian and Adriatic Italian olive oils

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Aim

As Italy has a unique morphology and climate, the rainfall along the Tyrrhenian and Adriatic coasts originates in different areas. As a consequence of this situation it was noticed that the δ^{18}O values of rain samples from the two coasts were different (Longinelli and Selmo, 2003; Longinelli et al., 2006). δ^{18}O and δD in fresh water correlate on a worldwide scale according to Global Meteoric Water Line – GMWL (relationship (2); Craig, 1961), which represents the average of Local Meteoric Water Lines (LMWL) differing from the GMWL as a consequence of local climatic and geographical factors (Lachniet and Patterson, 2006; Gammons et al., 2006; Njitchoua et al., 1999; Peng et al., 2004). As the δD and δ^{18}O of vegetable compounds are broadly related to their values in ground water, the δD and δ^{18}O of olive oils from the Tyrrhenian and Adriatic coasts should reflect the differences found in their rainfall values. This ‘coast’ effect could be useful in differentiating of Italian olive oil samples from Central Italy. To verify this, the δD, δ^{18}O and δ^{13}C values of around two hundred samples of authentic Italian PDO and PGI extra virgin olive oils from the Adriatic and Tyrrhenian coasts collected in three different years were determined. The data measured were related with climatic data in order to explain their variability. Particular attention was paid to the innovative D/H parameter of bulk olive oil, which has not been investigated, with the exception of the previously reported study, limited to a single year, and to its relationship with ^{18}O/^{16}O.

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Coast and year effect on H, O and C stable isotope ratios of Tyrrhenian and Adriatic italian olive oils

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The paper discusses the $^2H/^1H$, $^{18}O/^{16}O$ and $^{13}C/^{12}C$ ratios of 196 authentic Italian extra-virgin olive oils produced in 3 years on the Tyrrhenian and Adriatic coasts. The $^2H/^1H$ and $^{18}O/^{16}O$ ratios were linearly and positively correlated. The year of production influenced mainly $^{18}O/^{16}O$ in relation to the amount of rainfall and the atmospheric humidity in the period of oil accumulation in the olives. The $^2H/^1H$ ratio significantly distinguished the olive oils produced on the Adriatic coast from those on the Tyrrhenian coast in each year. This coast effect is a consequence of the different sources and isotopic compositions of the rainfall and the different climatic conditions on the two coasts. The paper contributes towards understanding the influence of climatic factors on isotopic variability and towards improving the traceability of the geographical origin of olive oils, using $^2H/^1H$ as a more innovative parameter. Copyright © 2009 John Wiley & Sons, Ltd.

Interest in $^2H/^1H$ and $^{18}O/^{16}O$ (measured using isotope ratio mass spectrometry and usually expressed as δ^2H and $\delta^{18}O$) in fresh water can be traced back to the studies of Craig in the 1960s.[1] He discovered that $\delta^{18}O$ and δ^2H in fresh water correlate on a worldwide scale according to a relationship (approximately $\delta^2H = 8^*\delta^{18}O + 10$), which has since then been known as the Global Meteoric Water Line (GMWL). This relationship represents an average of Local Meteoric Water Lines (LMWL) that differ, both in slope and intercept, from the GMWL as a consequence of local climatic and geographic factors.[2–5] An important parameter defined by these relationships is the intercept, called d_{excess} in meteoric waters,[6] defined for a slope of 8 as $d_{excess} = \delta^2H - 8^*\delta^{18}O$, which on a global basis averages about 10‰, although it varies regionally according to humidity, wind speed and sea surface temperature during primary evaporation.[7]

Since then many studies have been carried out in the field of environmental isotope hydrogeology, with different aims, such as determination of the origin and dynamics of groundwater or its salinity,[8–12] surveying the variations in the composition of the water in geothermal reservoirs induced by earthquakes,[13] the study of water recharge of rivers and lakes,[3,14] and paleoclimatic investigations.[15]

Italy has a particular morphology and climate. The mountainous barrier represented by the Alps in the north divides Italy from the European continent, protecting the peninsula from cold winds originating in the first (north-east) and fourth (north-west) quadrants. Moreover, the Apennines, running from north to south along the narrow peninsula wedged in the Mediterranean basin, separate the eastern coast on the Adriatic Sea from the western coast on the Tyrrhenian Sea. The rainfall along the Tyrrhenian coast derives from Mediterranean water vapour carried by westerly winds from the Atlantic Ocean. These air masses and the inflow of vapour from the Balearic and Tyrrhenian basin are at least partially dammed by the Apennine ridge. The Italian Adriatic coast is characterised by the presence of dry and cold north-easterly winds blowing from European continental areas and by south-easterly winds carrying water vapour from the warmer eastern Mediterranean area.[16] It has been reported that vapour from the western Mediterranean area is characterised by $\delta^{18}O$ values ranging between −15 and −11‰ and δ^2H values between −98 and −84‰, whereas the atmospheric moisture which moves from the European continent has $\delta^{18}O$ values between −19 and −15‰ and corresponding δ^2H values between −123 and −82‰.[17] To our knowledge there are no data are available regarding the isotopic composition of the vapour carried by the south-easterly winds.

Longinelli *et al.*[16,18] provided a first 'overall' isotopic map of Italian rainfall water and noticed differences between the $\delta^{18}O$ values of rain samples from the Tyrrhenian and Adriatic coasts. As far as we know, few studies have considered this geographical effect of these differences on isotopes in food commodities, with the exception of some general comments in two papers,[19,20] where the authors alluded to differences in the $(^2H/^1H)_I$, $(^2H/^1H)_{II}$ of ethanol (measured using site-specific natural isotope fractionation-nuclear magnetic resonance, SNIF-NMR) and the $\delta^{18}O$ values of water in relation to Italian wines from the Adriatic and Tyrrhenian coasts.

Olive oil is a commodity produced throughout Italy and is of primary economic importance. It has been reported that the $\delta^{18}O$ value, together with $\delta^{13}C$ and more recently δ^2H, is able to differentiate between olive oils of different

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geographical origin, according to the latitude, distance from the sea and the environmental conditions during the growing of the plants (humidity, temperature and amount of rainfall).[21–24]

The aim of this paper is to verify whether the aforementioned 'coast effect' is also relevant to the stable isotope ratios of hydrogen, oxygen and carbon of extra-virgin olive oils and how this effect is influenced by the climatic conditions (amount of rain, humidity, temperature) of the production year. Particular attention is paid to the innovative parameter $^2H/^1H$ of bulk olive oil that, with the exception of a previous study limited to a single year,[23] has not been investigated, and to its relationship with $^{18}O/^{16}O$.

EXPERIMENTAL

Sampling plan

Authentic samples of PDO (Protected Denomination of Origin) and PGI (Protected Geographic Indication) extra-virgin olive oils (N = 196) were collected in 2005, 2006 and 2007 (Table 1) under the supervision of the Italian Ministry of Agricultural, Food and Forestry Policy. The sampling was organised in order to cover all traditional harvest periods and production areas, taking into account the multi-varietal blends provided for in the individual PDOs and PGIs listed in European Council Regulation (EC) No. 510/2006. The oils were collected along the Adriatic and Tyrrhenian coasts in a narrow belt of 30 km, to reduce the so-called 'continental effect'.[22–24] Samples of Sabina and Tuscia PDOs (N = 25) – from areas located, respectively, at up to 60 and 45 km from the sea – were included to achieve continuity in the sampling along the Tyrrhenian coast (Fig. 1).

Climatic data

Temperature (°C), rainfall (mm) and humidity (%) data were taken from the official journals of the Italian Air Force (2006, 2007, 2008).[25–29] We selected the 12 weather stations (Adriatic coast: Trieste, Cervia, Rimini, Termoli, Brindisi; Tyrrhenian coast: Sarzana, Capo Mele, Pisa, Grosseto, Civitavecchia, Grazzanise, Capo Palinuro) situated closest to the sampling sites. For each station we considered the total amount of rainfall and the average of minimum and maximum values for both temperature and humidity; then we averaged the data for each coast. As oil accumulates in the mesocarp of olives in roughly 20 weeks,[30] we used climatic data from August to December, the harvesting month of most of the samples.

Table 1. Italian administrative regions of the 196 extra-virgin olive oils

Production year	Adriatic coast	Tyrrhenian coast
2005	Friuli (2)	Liguria (3)
	Abruzzi (7)	Tuscany (5)
	Marche (3)	Latium (12)
	Molise (5)	Campania (9)
	Apulia (10)	Calabria (3)
2006	Emilia-Romagna (2)	Liguria (3)
	Abruzzi (8)	Tuscany (6)
	Marche (6)	Latium (15)
	Molise (4)	Campania (8)
	Apulia (15)	Calabria (3)
2007	Friuli (1)	Liguria (3)
	Emilia-Romagna (2)	Tuscany (6)
	Abruzzi (8)	Latium (18)
	Marche (2)	Campania (10)
	Apulia (14)	Calabria (3)

Stable isotope ratio analysis

The $^2H/^1H$ and $^{18}O/^{16}O$ ratios of bulk olive oil were measured in one run using an isotope ratio mass spectrometer (Delta Plus XP, ThermoFinnigan, Bremen, Germany) following pyrolysis of the sample in a high-temperature conversion/elemental analyser (TC/EA, Thermo Finnigan). To obtain reproducible results for $^2H/^1H$ and $^{18}O/^{16}O$, during the analysis, a high temperature (1450°C) and a H_3 factor (to correct for the contribution of the $[H_3]^+$ to the *m/z* 3 signal[31]) of less than 8 ppm/nA were employed, and special care was taken to maintain dry conditions. The samples were stored in a desiccator above P_2O_5 for at least 24 h before analysis then placed into an autosampler equipped with a suitable cover. During the measurements, dry conditions were guaranteed by flushing nitrogen continuously over the samples. Measurement of $^{13}C/^{12}C$ in bulk olive oil was carried out using the istope ratio mass spectrometer coupled with an elemental analyser (Flash EA^{TM}1112, ThermoFinnigan). More details of the methods are provided by Camin *et al.*[23]

The isotope ratio values were normalised against international reference materials, NBS-22 fuel oil (IAEA-International Atomic Energy Agency, Vienna, Austria) for $^2H/^1H$ and $^{13}C/^{12}C$ measurement (assigned values for δ^2H and $\delta^{13}C$ were −120‰ and −29.8‰, respectively) and IAEA-CH-6 (IAEA) sugar for $^{18}O/^{16}O$ and $^{13}C/^{12}C$ determination (assigned values for $\delta^{18}O$ and $\delta^{13}C$ were +36.4‰ and −10.4‰). They were expressed in δ‰ versus the international materials 'Vienna Pee Dee Belemnite' (V-PDB) for $\delta^{13}C$ and 'Vienna Standard Mean Ocean Water' (V-SMOW) for $\delta^{18}O$ and δ^2H, according to the following formula: $[(R_s - R_{std})/R_{std}] \times 1000$, where R_s is the isotope ratio measured for the sample and R_{std} is the isotope ratio of the international standard. The repeatability, as one standard deviation of ten consecutive determinations of the same sample, was ± 1‰ for δ^2H, ± 0.3‰ for $\delta^{18}O$ and ±0.1‰ for $\delta^{13}C$.

The data were processed using analysis of variance (ANOVA), Tukey's test for an unequal number of samples and Pearson's correlation test using the statistical software package Statistica 8.1 (Statsoft Italia srl, Padua, Italy).[32]

RESULTS AND DISCUSSION

Year and coast effect on $^2H/^1H$ and $^{18}O/^{16}O$

Table 2 shows the distribution of $\delta^{18}O$ and δ^2H values displayed by coast and production year. The values of $\delta^{18}O$ and δ^2H over three years were significantly ($p < 0.001$) correlated ($\delta^2H = 2.663 * \delta^{18}O - 210.04$; $R = 0.5697$), with a linear relationship similar to that recently found in around 100 olive oils produced in 2005 in a wider area including all the Italian PDOs.[23]

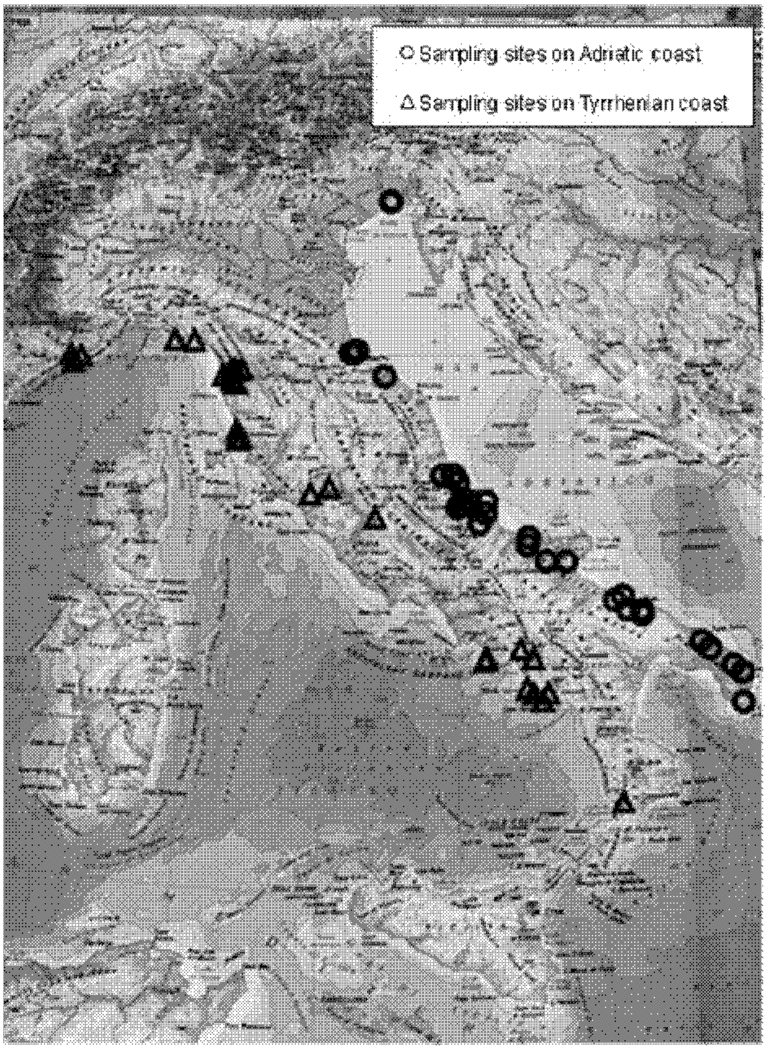

Figure 1. Sites of sampling of Adriatic and Tyrrhenian olive oils.

Table 2. Year and coast effect on the values of δ^2H (‰, vs. V-SMOW), δ^{18}O (‰, vs. V-SMOW) and δ^{13}C (‰, vs. V-PDB) of the olive oils (N = number of samples; significance level is indicated with different letter types: capital for $p < 0.001$, small for $p < 0.01$; CE = 'coast effect', YE = 'year effect')

		2005					2006					2007					2005–2007			
Area	Parameter	N	Mean	Std. Dev.	YE	CE	N	Mean	Std. Dev.	YE	CE	N	Mean	Std. Dev.	YE	CE	N	Mean	Std. Dev.	CE
Adriatic and	δ^2H	59	−151	5	A	—	70	−149	5	A	—	67	−144	5	B	—	196	−148	6	—
Tyrrhenian coasts	δ^{18}O	59	22.2	0.6	A	—	70	23.2	0.9	B	—	67	24.5	1.0	C	—	196	23.4	1.2	—
	δ^{13}C	59	−30.0	0.5	A	—	70	−30.0	0.7	A	—	67	−28.8	0.7	B	—	196	−29.6	0.9	—
Adriatic coast	δ^2H	27	−154	4	A	A	35	−153	4	a	A	27	−149	4	b	A	89	−152	4	A
Tyrrhenian coast	δ^2H	32	−148	3	A	B	35	−146	3	A	B	40	−141	4	B	B	107	−144	4	B
Adriatic coast	δ^{18}O	27	22.1	0.6	A	n.s.	35	23.0	0.7	B	n.s.	27	24.1	0.8	C	a	89	23.0	1.1	a
Tyrrhenian coast	δ^{18}O	32	22.4	0.6	A	n.s.	35	23.4	1.0	B	n.s.	40	24.8	1.0	C	b	107	23.6	1.3	b
Adriatic coast	δ^{13}C	27	−29.9	0.5	A	n.s.	35	−30.0	0.5	A	n.s.	27	−29.1	0.7	B	a	89	−29.7	0.7	n.s.
Tyrrhenian coast	δ^{13}C	32	−30.0	0.5	A	n.s.	35	−30.1	0.9	A	n.s.	40	−28.6	0.7	B	b	107	−29.5	1.0	n.s.

DOI: 10.1002/rcm

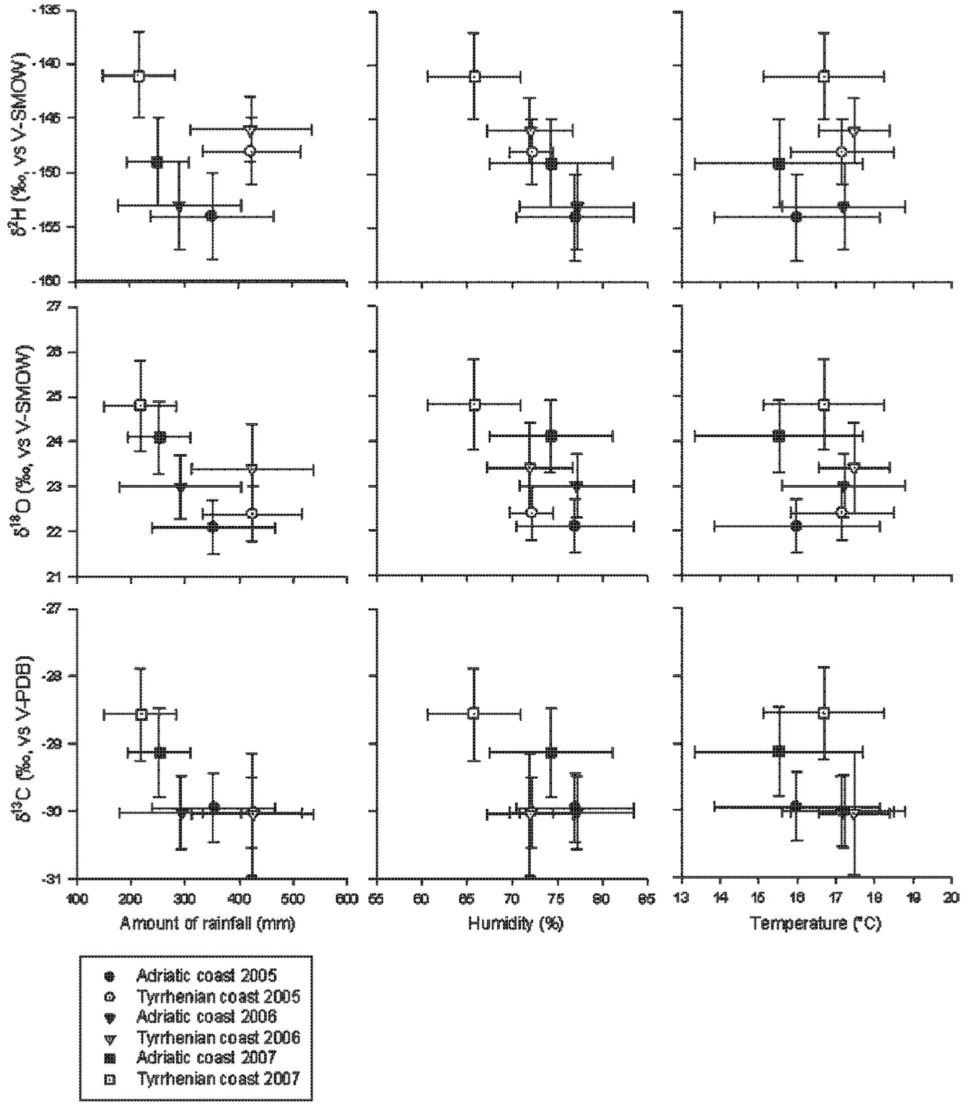

Figure 2. Relationship between isotopic values (δ^2H, $\delta^{18}O$ and $\delta^{13}C$) of olive oils and meteorological conditions (amount of rainfall, humidity and temperature) in 2005, 2006 and 2007 of Tyrrhenian and Adriatic coasts. Amount of rainfall, humidity and temperature = total amount of rainfall and the average of minimum and maximum values for both humidity and temperature measured in at least five weather stations in the period August–December.[25–29] The symbols represent the mean of each group and the whiskers correspond to one standard deviation of the groups.

The correlation was also significant ($p < 0.001$) when considering the samples from the Adriatic ($\delta^2H = 1.989 * \delta^{18}O - 197.75$; $R = 0.4678$) and Tyrrhenian coasts ($\delta^2H = 2.137 * \delta^{18}O - 194.93$; $R = 0.6342$) separately. The slope and intercept for the two coasts were significantly different ($p < 0.05$ and < 0.01, respectively). On the other hand the d_{excess} values calculated for rain collected near our sampling sites by other authors[16] were not significantly different.

Considering the coast and production year separately, the significance of the correlation was confirmed only in 2006 on both coasts ($p < 0.05$), probably because of the more limited variability in the range of values.

When processed by ANOVA, both for $\delta^{18}O$ and δ^2H, the two 'year' and 'coast' sources of variance were highly significant ($p < 0.001$) while their interaction was not significant. The significance of year and coast effects was also confirmed using non-parametric statistical approaches (U of Mann-Whitney or Kruskal-Wallis and multiple bilateral comparison tests).[33]

Year effect

Highly significant differences were found between years on both the Tyrrhenian and the Adriatic coast. The 2007 samples for both $\delta^{18}O$ and δ^2H showed significantly higher values sampling areas. Significant correlations between the δ^2H values of the rainfall water and the oil were evident for the data of both coasts and for those of the Adriatic coast alone. The correlation was practically absent in the case of the data for the Tyrrhenian coast, due to the smaller range of variability of the data. The data for the olive oils from both coasts showed a mean $\Delta\delta^2$H of around −109‰ relative to the rainfall water data, as a consequence of the deuterium depletion occurring in plants, when the hydrogen of water is incorporated into photosynthetic products and during the biosynthetic pathway of lipids.[36,38,39]

Year and coast effect on ^{13}C/^{12}C

Table 2 shows the distribution of the δ^{13}C values of olive oil displayed by coast and production year. δ^{13}C discriminates 2007 clearly from 2005 and 2006 probably as a result of the lower humidity and rainfall (Fig. 2), as discussed above for δ^2H, confirming what has been shown for products and derivatives of plant origin.[40–42] With regard to the coast effect, although in previous studies δ^{13}C was shown to be capable of differentiating the geographical origin of oils,[22,23,43] in this work it only distinguished between the two coasts in 2007 ($p < 0.01$). The bigger difference in humidity in 2007 on the two coasts (8.5% between the mean values) could justify the different δ^{13}C values of the Adriatic and Tyrrhenian oils.

In order to check the existence of statistical correlations with the climatic factors, the δ^{13}C values of the olive oils were related to the yearly climatic data of the closest weather station. Considering only highly significant correlations ($p < 0.001$), the δ^{13}C values of the Tyrrhenian oils correlated inversely to precipitation (approximately −0.5‰ δ^{13}C/100 mm). The correlations with the other climatic factors were not statistically significant, confirming what was found by Iacumin *et al.*[24]

CONCLUSIONS

Italian olive oils from the Adriatic and Tyrrhenian coasts can be distinguished mainly on the basis of their δ^2H values. The isotopic composition of rainfall, along with the average temperature and humidity on the two coasts, can explain these differences in the oils.

Differences in rainfall and humidity seem to be the principal reason for the annual variability in terms of the isotopic values of olive oils.

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Results

The δD and $\delta^{18}O$ of bulk olive oils were shown to be correlated, as in fresh water, but the slope and intercept of the relationships found for the Adriatic and Tyrrhenian coasts were significantly different. Highly significant differences were found between years on both the Tyrrhenian and Adriatic coasts. Differences in rainfall and humidity seemed to be the principal reason for this annual variability, whereas temperature did not appear to be influential. Significantly different δD values were found between oils from the Adriatic and Tyrrhenian coasts, whereas $\delta^{18}O$ was shown to be less significant. The isotopic composition of rainfall, along with average temperature and humidity on the two coasts, can explain these differences in the oils.

Development of further studies is desirable in order to explore whether the ability of stable isotope ratios and elemental composition to differentiate olive oil provenance, as highlighted in these first two exploratory studies, could also be ‘exported’ to areas outside Italy.

SECTION 4.5

Isotopic and elemental data for tracing the origin of European olive oils

Camin F., Larcher R., Nicolini G., Bontempo L., Bertoldi D., Perini M., Schlicht C., Schellenberg A., Thomas F., Heinrich K., Voerkelius S., Horacek M., Ueckermann H., Froeschl H., Wimmer B., Heiss G., Baxter M., Rossmann A., Hoogewerff J.

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Aim

As stable isotope ratios and elemental composition have been shown to be a promising tool for differentiating Italian olive oils in the studies previously presented, it was decided to apply the same parameters to the differentiation of authentic olive oils collected at eight different European sites with different climatic and geological characteristics. The study aimed to examine more closely the relationship of isotopic and elemental parameters with geographical, geological and climatic factors and consequently to test their usefulness in terms of the geographical differentiation of European olive oils. This ability was also tested in relation to the composition of corresponding surface waters. For this purpose, more than two hundred European extra virgin olive oils were collected at olive mills following the milling of multivarietal olives at eight European sites (Trentino, Tuscany, Sicily, Algarve, Carpentras, Barcelona, Chalkidiki and Lakonia). At the same eight sites more than three hundred of surface waters were also sampled. Meteorological data on the sites during the period of maturation of the olives was obtained from the closest weather station. H, C, and O stable isotope ratios and elemental composition were determined in all the olive oil samples, H and O isotope ratios and elemental concentration were measured in the fresh surface waters.

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Isotopic and Elemental Data for Tracing the Origin of European Olive Oils

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H, C, and O stable isotope ratios and the elemental profile of 267 olive oils and 314 surface waters collected from 8 European sites are presented and discussed. The aim of the study was to investigate if olive oils produced in areas with different climatic and geological characteristics could be discriminated on the basis of isotopic and elemental data. The stable isotope ratios of H, C, and O of olive oils and the ratios of H and O of the relevant surface waters correlated to the climatic (mainly temperature) and geographical (mainly latitude and distance from the coast) characteristics of the provenance sites. It was possible to characterize the geological origin of the olive oils by using the content of 14 elements (Mg, K, Ca, V, Mn, Zn, Rb, Sr, Cs, La, Ce, Sm, Eu, U). By combining the 3 isotopic ratios with the 14 elements and applying a multivariate discriminant analysis, a good discrimination between olive oils from 8 European sites was achieved, with 95% of the samples correctly classified into the production site.

KEYWORDS: Olive oil; European origin; traceability; IRMS; ICP-MS

INTRODUCTION

On February 4, 2009, the European Union (EU) Member States agreed to compulsory origin labeling for virgin and extra virgin olive oils (EC Regulation 182/2009) to avoid consumers being misled about their true characteristics and origin. This highlights the increasing demand for analytical methods and statistical tools capable of effectively verifying claims of origin.

The stable isotope ratios of bioelements, as well as the elemental profile, measured by isotope ratio mass spectrometry (IRMS) and inductively coupled plasma–mass spectrometry (ICP-MS) have been shown to be useful markers for tracing the geographical origin of several foods, including olive oils (*1–5*).

The H, C, and O isotopic composition of olive oils and of plant material in general is related to the climatic conditions (relative humidity, temperature, amount of precipitation) and geographical characteristics (distance from the sea or other evaporation source, altitude, latitude) of the area where the plants grow (*6–9*) and to the plant variety (*3*). The $^{13}C/^{12}C$ ratios of plant compounds are affected by the botanical origin of the plant (discrimination between C3 and C4 plants) (*10*) and by several environmental and physiological factors that influence the stomatal conductance and the intercellular and ambient CO_2 concentration, such as relative humidity, temperature, amount of precipitation, water stress, plant age, and maturation (*8, 11–14*). The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of plant material reflect (a) the ratios of water uptake by the plant (linked to latitude, elevation, distance from the evaporation source, temperature, and amount of precipitation) (*15, 16*), (b) the evaporative and diffusional effects during transpiration (affected by relative humidity, temperature, isotope composition of water vapor) (*9, 17*), and (c) the biosynthetic pathways including the isotopic exchange between organic molecules and plant water (*17–19*).

The elemental content of plants is mainly related to the geological and pedoclimatic characteristics of the site of growth (*20*). Vegetable-derived manufactured products such as olive oil will have a trace element content related not only to that of soil but also to farming, olive collection, manufacturing, refining, and storage processes (*21–23*).

Within the framework of the European TRACE Project, 267 olive oils collected from eight European sites were analyzed for H, C, and O stable isotope ratios and their elemental profiles. H and O isotope ratios and elemental composition were also measured in 314 fresh surface waters from the same sites. The aim of the study was to investigate if olive oils produced in areas with different climatic and geological characteristics could be discriminated on the basis of isotopic and elemental data.

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This capability was also tested in relation to the composition of the corresponding surface waters.

To our knowledge, such a multielement and multi-isotopic study on the traceability of authentic European olive oils and of the related source surface waters has never been reported, thus far.

MATERIALS AND METHODS

Samples. Two hundred and sixty-seven European extra virgin olive oils were collected at the mill from the milling of multivarietal olives from 8 European sites during the 2005 and 2006 harvests. At the 8 sites, also 314 surface waters were sampled during all four seasons of 2005 (**Table 1**). The mean values of 5 month temperature (°C), relative humidity (%), and 5 month total amount of rainfall (mm) were taken from the closest weather station: Web sites http://www.meteotrentino.it, Arco (TRE); http://www.ilmeteo.it/, Sesto Fiorentino (TOS) and Assoro/Enna (SIC); http://www7.ncdc.noaa.gov/, Faro/Almanacil (ALG) and Carpentras (CAR); the Servei Meteorològic de Catalunya, Barcelona (BAR); the Hellenic National Meteorological Service through Prof. Michael Komaitis (Agricultural University of Athens, Greece), Mikra (CHA), and Kalamata/Gythi (LAK). When not available (CHA, LAK, ALG, CAR), relative humidity was downloaded from the site http://www.wunderground.com. As oil accumulates in the mesocarp of olives in roughly 20 weeks (*24*), we used climatic data from August to December. Latitude, longitude, distance from the sea, and altitude were recorded either in the field by GPS or deduced from "Google Earth". The geological classification was deduced from the U.S. Geological Survey map of Europe, the European soil map, and the IGME5000 geological map of Europe (Pawlewicz, M. J.; Steinshouer, D. W.; Gautier, D.L. (2003) Map Showing Geology, Oil and Gas Fields, and Geologic Provinces of Europe including Turkey; Open File Report 97-470I: Central Region Energy Resources Team, U.S. Department of the Interior, U.S. Geological Survey [http://pubs.usgs.gov/of/1997/ofr-97-470/OF97-470I/index.htm]; European Soil Database (v 2.0) European Soil Bureau Network and the European Commission, EUR 19945 EN, March 2004 [http://eusoils.jrc.it/]).

Stable Isotope Ratio Analysis. The analysis of stable isotope $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{2}H/^{1}H$ (D/H) ratios of bulk olive oils was performed in different European laboratories, using a range of isotope ratio mass spectrometers (Delta plus XL, Delta Plus XP, Delta V, Delta S, Thermo-Finnigan, Bremen, Gremany; Isoprime, AP2003, GV Instruments Ltd., Manchester, U.K.; Optima Micromass) connected to a pyrolyzer (TC/EA, ThermoFinnigan; EuroPyrOH, Eurovector 3000) for $^{18}O/^{16}O$ and D/H or an elemental analyzer (Flash EA 1112, 1110, 1108 ThermoFinnigan; Costech ECS4010; NA2100 Proteins, Carlo Erba, Milan, Italy; Vario EL III, Elementar Analysensysteme GmbH, Hanau/Germany) for $^{13}C/^{12}C$. The analytical conditions are reported in Camin et al. (*5*).

The $^{18}O/^{16}O$ and D/H ratios of water were measured using isotope ratio mass spectrometers (SIRA II, VG Fisons, Middlewich, U.K.; Thermo Delta V) connected with a water/CO_2 equilibration system (ISOPREP, VG Fisons) and/or a pyrolyzer (TC/EA ThermoFinnigan).

The isotope ratios were expressed in δ‰ versus V-PDB (Vienna − Pee Dee Belemnite) for $\delta^{13}C$ and V-SMOW (Vienna − Standard Mean Ocean Water) for $\delta^{18}O$ and δD, according to the following formula: $[(R_x - R_{std})/R_{std}] \times 1000$, where R_x is the isotope ratio measured for the sample and R_{std} is the isotope ratio of the international standard. The values were calculated against in-house oil standards, which were themselves calibrated against international reference materials: fuel oil NBS-22 (IAEA, http://curem.iaea.org/catalogue/SI/index.html) and sugar IAEA-CH-6 (IAEA) for $^{13}C/^{12}C$, benzoic acid IAEA-601 (IAEA) and IAEA-CH-6 (IAEA, with $\delta^{18}O = +36.4$‰ vs V-SMOW) (*25*) for $^{18}O/^{16}O$ and NBS-22 for D/H.

The standard deviation of repeatability (S_r) for oil was 0.1‰ for $\delta^{13}C$, 0.4‰ for $\delta^{18}O$, and 1‰ for δD, whereas that for water was 0.1‰ for $\delta^{18}O$ and 1‰ for δD. To ascertain the interlaboratory reproducibility, one extra virgin olive oil interlaboratory comparison material (EVOO ICM) and one water ICM sample were used in each laboratory as quality control material. The standard deviation of reproducibility was good for $\delta^{13}C$ ($S_R = 0.1$‰), for δD of water and oil ($S_R = 2$‰), and for $\delta^{18}O$ of water ($S_R = 0.2$‰), but not for $\delta^{18}O$ of oil ($S_R = 2$‰). The olive oil $\delta^{18}O$ values were therefore normalized by assigning by agreement a value of +27.0‰ to EVOO ICM. Calculating S_R of normalized $\delta^{18}O$ values of around 50 olive oils measured in at least two laboratories, we obtained a fair value (0.6‰).

Table 1. Number of Samples and Data of Geographical, Geological, and Climatic Characteristics (*T*, Temperature; RH, Relative Humidity) of the Provenience Area

	no. of oils								2005			2006		
site	2005	2006	no. of water	latitude (deg)	longitude (deg)	distance from the sea (km)	altitude (m asl)	geology	mean *T* (°C)	mean RH (%)	precipitation (mm)	mean *T* (°C)	mean RH (%)	precipitation (mm)
Trentino, Italy (TRE)	28	28	40	45.9	10.9	129	211	limestone	12.2	75	424	13.6	73	394
Carpentras, France (CAR)	20	20	40	44.3	5.2	97	384	limestone	13.4	69	265	15.3	67	279
Tuscany, Italy (TOS)	18	21	40	44.0	11.4	83	343	shale/mudstone/clay/loess	14.8	73	617	16.7	68	302
Barcelona, Spain (BAR)	8	2	36	41.7	2.0	35	362	shale/mudstone/clay/loess	13.2	73	405	14.8	75	349
Chalkidiki, Greece (CHA)	20	20	40	40.1	23.9	1	23	acid magmatic	16.2	60	140	16.3	60	206
Sicily, Italy (SIC)	20	20	40	37.8	14.6	32	672	shale/mudstone/clay/loess	12.8	69	218	16.0	68	332
Algarve, Portugal (ALG)	7	7	38	37.2	−7.1	5	27	limestone	18.8	67	270	19.9	72	341
Lakonia, Greece (LAK)	11	17	40	36.6	22.4	1	105	limestone	17.9	59	629	18.0	60	269

Table 2. Limits of Detection

element/ isotope	LOD oil (μg/kg)	LOD water (mg/L)	element/ isotope	LOD oil (μg/kg)	LOD water (mg/L)	element/ isotope	LOD oil (μg/kg)	LOD water (mg/L)
Li/7	0.008	0.0006	Ni/60	1	0.00020	Ba/137	0.12	0.00012
B/11	0.17	0.015	Cu/63	0.10	0.00005	La/139	0.002	0.00005
Na/23	20	0.51	Zn/66	6	0.00005	Ce/140	0.0050	0.00005
Mg/26	4	0.11	Ga/71	0.001	0.00005	Nd/146	0.004	0.00005
Al/27	3	0.0015	Se/78	0.014	0.0082	Sm/147	0.0010	0.00005
K/39	20	0.005	Rb/85	0.001	0.00006	Eu/151	0.0002	0.00005
Ca/40	25	0.02	Sr/88	0.3	0.0025	Yb/171	0.0004	0.00005
V/51	0.007	0.00036	Mo/98	0.050	0.0002	Lu/175	0.02	0.00005
Mn/56	0.2	0.0002	Cd/111	0.005	0.00005	Tl/205	0.0040	0.00005
Co/59	0.002	0.00006	Cs/133	0.001	0.00005	Pb/206 + 207 + 208	0.1	0.00005
						U/238	0.001	0.00006

Table 3. Mean and Standard Deviation (SD) of δ^{13}C, δ^{18}O, and δD of European Olive Oils and Surface Waters[a]

year			TRE	CAR	TOS	BAR	CHA	SIC	ALG	LAK
Oils										
2005	δ^{13}C ‰ vs V-PDB	mean	−31.0 d	−29.4 b	−30.2 c	−29.3 bc	−29.3 b	−29.1 ab	−28.6 ab	−28.4 a
		SD	0.4	0.2	0.3	0.2	0.6	0.5	0.3	0.6
	δ^{18}O ‰ vs V-SMOW	mean	20.2 e	24.8 b	21.9 d	23.4 c	23.4 c	24.8 b	27.1 a	25.1 b
		SD	0.4	0.5	0.5	0.6	0.6	0.6	0.8	0.7
	δD ‰ vs V-SMOW	mean	−159 e	−150 bc	−154 dce	−151 bcd	−157 de	−146 ab	−139 a	−142 a
		SD	3	2	2	4	5	5	2	5
2006	δ^{13}C ‰ vs V-PDB	mean	−30.8 e	−29.1 bc	−30.1 d	−29.3	−29.4 c	−28.6 ab	−28.5 ab	−28.3 a
		SD	0.5	0.5	0.3		0.2	0.2	0.3	0.4
	δ^{18}O ‰ vs V-SMOW	mean	21.6 d	25.5 b	22.3 d	24.5	23.5 c	26.6 a	27.1 a	24.1 c
		SD	0.4	1.4	0.8		1.5	0.3	0.5	0.3
	δD ‰ vs V-SMOW	mean	−156 c	−149 b	−148 b	−144	−160 c	−141 a	−145 ab	−144 ab
		SD	3	8	3		3	2	2	3
2005 + 2006	δ^{13}C ‰ vs V-PDB	mean	−30.9 f	−29.3 d	−30.1 e	−29.4 cd	−29.4 d	−28.9 bc	−28.6 ab	−28.4 a
		SD	0.5	0.4	0.3	0.2	0.4	0.4	0.3	0.5
	δ^{18}O ‰ vs V-SMOW	mean	20.9 f	25.1 bc	22.1 e	23.6 cde	23.5 d	25.7 b	27.1 a	24.5 c
		SD	0.8	1.1	0.7	0.8	0.5	1.0	0.6	0.7
	δD ‰ vs V-SMOW	mean	−157 cd	−150 b	−151 b	−150 abc	−158 d	−144 a	−142 a	−143 a
		SD	3	6	4	5	5	4	4	4
Waters										
	δ^{18}O ‰ vs V-SMOW	mean	−9.9 d	−7.2 c	−6.6 bc	−6.6 bc	−7.3 c	−6.3 b	−3.4 a	−6.3 b
		SD	0.4	0.4	1.6	0.6	0.8	0.8	0.9	0.9
	δD ‰ vs V-SMOW	mean	−65 e	−48 d	−44 cd	−41 c	−43 cd	−40 c	−19 a	−31 b
		SD	2	3	6	5	7	5	5	7

[a] The two oils from 2006 Barcelona and the "evaporated" surface waters were not considered in the statistical test (see the text). The significance of HSD for unequal *N* Tukey is reported: groups of one row with different letters are statistically different ($p < 0.001$).

Elemental Analysis. The analyses were performed using an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with an ASX-520 autosampler (Cetac Technologies Inc., Omaha, NE). An octopole reaction system (ORS) was used to remove polyatomic interferences, using He and H_2 as collision and reaction gas, respectively.

The samples were prepared and analyzed according to the method of Camin et al. (5), with minor modifications. The extracting water solution was prepared with 6.7% H_2O_2 (30% Superpure, Merck, Darmstadt, Germany), 1% HNO_3 (Superpure, Merck), and 0.2% HCl (ACS; Riedel-deHaën, Seelze, Germany). All of the materials were washed with 5% HNO_3 and rinsed with Milli-Q water before use. Sample preparation and analysis were carried out in triplicate. The limit of detection (LOD) of each element was calculated as 3 times the standard deviation of the signal of the blank samples, extracted and analyzed 10 times (**Table 2**).

Because an international vegetable oil is not available, accuracy was checked by using two spiked samples in each analytical run. The first spiked sample was prepared by adding one defined aliquot (around 0.5−1 g) of the standard reference material NIST 2387 (semisolid peanut butter) to 15 g of a natural olive oil and mixed thoroughly. The second sample was obtained by spiking 15 g of olive oil with 40 mg of SPEX s-23 100z (organometallic multistandard certified mineral oil). The natural oil and the fortified mixtures were extracted and analyzed three times. Recovery was calculated on the difference of the mean content of the spiked and the unspiked samples. Using the NIST standard, the recoveries for the six elements were 82% for Zn, 84% for Mn, 90% for Ca, 92% for Mg, 95% for K, and 101% for Na, whereas those obtained using the SPEX standard for the 15 elements were lower (B, 88%; Na, 80%; Mg, 67%; Al, 53%; K, 92%; Ca, 84%; V, 63%; Mn, 71%; Ni, 65%; Cu, 61%; Zn, 68%; Mo, 66%; Cd, 71%; Ba, 65%; Pb, 67%), perhaps as a consequence of the difficulty of obtaining a well-homogenized spiked sample.

The precision (RSD%) of the analytical method, evaluated by preparing and analyzing an oil sample 10 times, ranged from 13 to 27% for the different elements. These values can be deemed satisfactory, considering the very low content of elements in olive oil.

Statistical Analysis. The data were statistically evaluated using Statistica v 8 (StatSoft Italia srl, Padua, Italy).

Table 4. Pearson Coefficient, Significance and Regression Coefficient (Intercept and Slope) of the Correlation between Isotopic Values and Geographical and Climatic Factors[a]

		$\delta^{13}C$	$\delta^{18}O$	δD	latitude (DD)	longitude (DD)	distance from the coast (km)	altitude (m asl)	temp (°C)	relative humidity	rain (mm)
						Olive Oil					
$\delta^{13}C$	*r*		0.82	0.62	−0.80	**0.13**	−0.75	**0.07**	0.59	−0.61	−0.29
	r^2		0.67	0.38	0.64	**0.02**	0.56	**5×10^{-3}**	0.35	0.38	0.09
	signif		<0.001	<0.001	<0.001	**0.0377**	<0.001	**0.2687**	<0.001	<0.001	<0.001
	intercept		−38.30	−17.67	−20.46	**−29.75**	−28.71	**−29.63**	−33.70	−22.20	−28.85
	slope		0.370	0.079	−0.218	**0.015**	−0.014	**3×10^{-4}**	0.273	−0.108	−0.002
$\delta^{18}O$	*r*	0.82		0.70	−0.67	**−0.16**	−0.58	**0.21**	0.51	−0.41	−0.32
	r^2	0.67		0.49	0.45	**0.03**	0.34	**0.04**	0.26	0.17	0.10
	signif	<0.001		<0.001	<0.001	**0.0076**	<0.001	**0.001**	<0.001	<0.001	<0.001
	intercept	76.69		53.38	40.50	**24.13**	25.05	**23.09**	15.84	34.41	25.31
	slope	1.796		0.197	−0.405	**−0.042**	−0.024	**0.002**	0.512	−0.159	−0.005
δD	*r*	0.62	0.70		−0.56	**−0.19**	−0.37	0.27	0.43	**−0.17**	**0.003**
	r^2	0.38	0.49		0.31	**0.03**	0.13	0.08	0.18	**0.03**	**1×10^{-5}**
	signif	<0.001	<0.001		<0.001	**0.0023**	<0.001	<0.001	<0.001	**0.0047**	**0.9548**
	intercept	−8.80	−209.38		−100.93	**−148.63**	−147.53	−153.20	−173.96	**−134.61**	**−150.84**
	slope	4.803	2.483		−1.196	**−0.171**	−0.054	0.009	1.530	**−0.238**	**2×10^{-4}**
						Water					
$\delta^{18}O$	*r*			0.93	−0.62	−0.26	−0.55	−0.47	0.54	−0.30	**−0.01**
	r^2			0.87	0.39	0.07	0.390	0.22	0.29	0.09	**0.0002**
	signif			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	**0.8297**
	intercept			−1.60	6.19	−6.35	−5.94	−5.96	−12.93	−1.15	**−6.86**
	slope			0.125	−0.319	−0.047	−0.022	−0.003	0.409	−0.085	**−0.0001**
δD	*r*		0.93		−0.76	**−0.07**	−0.72	−0.54	0.70	−0.51	**0.05**
	r^2		0.87		0.58	**0.01**	0.52	0.29	0.49	0.26	**0.002**
	signif		<0.001		<0.001	**0.22**	<0.001	<0.001	<0.001	<0.001	**0.4292**
	intercept		5.62		77.78	**−41.40**	−33.10	−34.37	−101.25	32.07	**−43.91**
	slope		6.982		−2.938	**−0.102**	−0.216	−0.023	3.984	−1.102	**0.004**

[a] Numbers given in boldface are evidence of the nonsignificant correlation ($p \geq 0.001$). DD, decimal degrees.

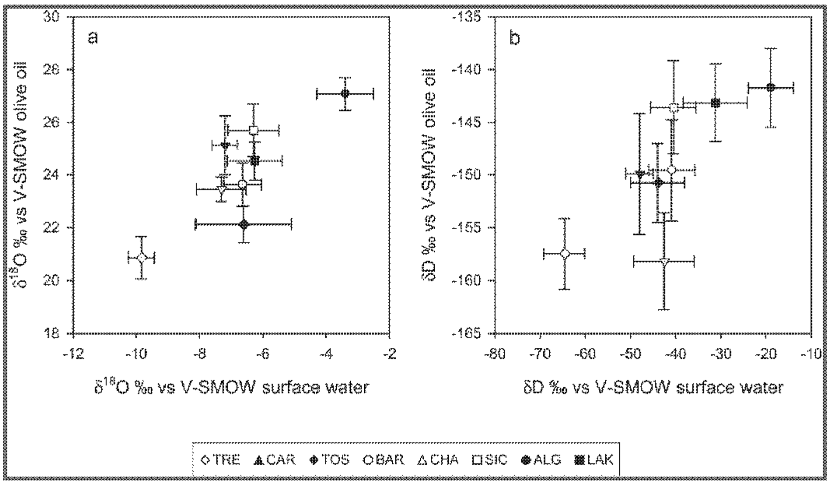

Figure 1. Plot of the mean values and the respective standard deviations of $\delta^{18}O$ (**a**) and δD (**b**) values of olive oil against those of water.

RESULTS AND DISCUSSION

Stable Isotope Ratios. In **Table 3** the mean and standard deviation of $\delta^{13}C$, $\delta^{18}O$, and δD values of olive oils, grouped according to origin and production year and ordered in inverse proportion to latitude, are shown. By applying the Kolmogorov–Smirnov test (26), the isotopic data was normally distributed into the groups.

The isotopic parameters were significantly ($p < 0.001$, Person's correlation test) correlated with each other (**Table 4**). The linear

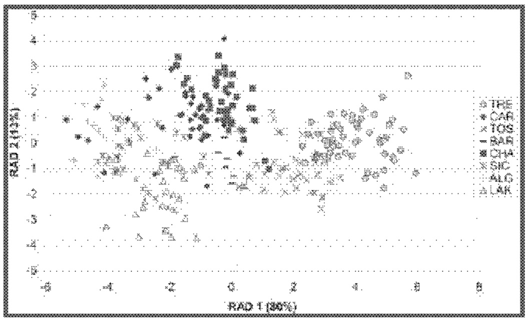

Figure 2. Canonical discriminant analysis of δ^2H, δ^{13}C, and δ^{18}O of the olive oils from the eight European sites: scatterplot of the first two canonical variables.

Table 5. Reclassification Discriminant Analysis of δ^2H, δ^{13}C, and δ^{18}O of the Olive Oils from the Eight European Sites: Results of Classification Matrix

	% correctly classified	TRE	CAR	TOS	BAR	CHA	SIC	ALG	LAK
TRE	91	51	0	5	0	0	0	0	0
CAR	70	0	28	1	0	3	4	4	0
TOS	87	5	0	34	0	0	0	0	0
BAR	40	0	1	1	4	3	1	0	0
CHA	90	0	1	3	0	36	0	0	0
SIC	63	0	8	0	2	0	25	3	2
ALG	57	0	0	0	0	0	6	8	0
LAK	79	0	0	0	0	0	6	0	22

equation of δD versus δ^{18}O values ($\delta D = 2.48 \times \delta^{18}O - 209.38$) is similar to the one found for Italian PDO olive oils ([$\delta D = 2.5091 \times \delta^{18}O - 208.1$, Camin et al. (*5*); $\delta D = 2.663 \times \delta^{18}O - 210.04$, Bontempo et al. (*6*)]. It therefore can be inferred that olive oil δD and δ^{18}O values correlate on a worldwide scale, likewise to water [Global Meteoric Water Line (*27*)].

δ^{13}C and δ^{18}O showed significant correlation (Pearson's correlation test, $p \leq 0.001$) to latitude, distance from the coast, temperature, relative humidity, and amount of precipitation of the last 5 months before harvest, even if in some cases with very low r^2, whereas δD correlates with latitude, distance from the coast, altitude and temperature (**Table 4**). The deviation of the δD values from the geographical and climatic gradient for Chalkidiki for both years (**Table 3**) could be justified on the basis of the olive size, because Chalkidiki olives were quite big (3 cm long and 1.5 cm round), for example, in comparison to olives from Lakonia, Carpentras, and Trentino (1−1.5 long and ∼0.5 cm round) and therefore could be subjected to different kinetic fractionation during evapotranspiration (*28*). In contrast to previous studies (*6*, *7*), specific weather stations were considered for each site, and we found more significant correlations with temperature and relative humidity. The slope and intercept of the linear equations of δ^{18}O versus latitude, temperature, and humidity are comparable to those of the literature (*6*, *7*).

ANOVA and Honestly Significantly Different (HSD) for unequal *N* Tukey tests highlight statistically significant ($p < 0.001$) differences in the values of all three isotopic parameters among the olive oils produced in the eight sites (**Table 3**), both within each year or considering the two years together. The two olive oils from Barcelona were excluded from the statistical evaluation of 2006, because they were not representative from a statistical point of view. Comparing the two years' data, we found differences ($p < 0.001$) between 2005 and 2006 data for δ^{18}O in TRE, SIC, and LAK and for δD in TRE, SIC, TOS, andALG. The different climatic conditions of the last 5 months (period of oil accumulation, see **Table 1**) between the two years can justify these differences (*6*).

Considering the two years together, δ^{13}C allows to discriminate ($p < 0.001$) TRE; TOS; the group CAR, BAR, CHA; the group ALG, LAK; most of all along a latitudinal gradient, except for TOS and CAR, for which the climatic conditions play a significant role. The δ^{18}O improves the discrimination between ALG and LAK and together with δD between CAR and CHA.

Similarly to olive oils, the δ^{18}O and δD values of the surface waters (**Table 3**) collected from the same sites were significantly affected by the site (ANOVA test) and correlated (Pearson's test, $p \leq 0.001$) with temperature, relative humidity, latitude, distance from the sea, and altitude (**Table 4**), in some cases with very low r^2. Of the 314 water samples, we do not consider 20 from ALG, 11 from TOS, and 4 from each CAR, TRE, and SIC, because the *d*-excess value > 2‰ indicated high water evaporation (*29*).

As expected, δ^{18}O and δD values of surface waters correlated with each other: most of the samples were along the Global Meteoric Water Line (GMWL; $\delta D = 8 \times \delta^{18}O + 10$), whereas Lakonia and Chalkidiki along the Mediterranean water line ($\delta D = 8 \times \delta^{18}O + 20$) (*30*).

In comparison to water, olive oils are enriched in ^{18}O to around 30‰ and depleted in D to about 120‰, as a consequence of the fractionation occurring in plants during the photosynthesis and biosynthetic pathways of lipids (*18*, *19*).

The parametric HSD for unequal *N* Tukey test applied to the water samples recognizes fewer groupings for δ^{18}O with respect to the olive oils and less deviation for both elements to the climatic and geographical (latitude, distance from the sea) gradient. By plotting the mean values of olive oil against those of water (**Figure 1**), a relationship between the isotopic composition of olive oils and that of the local water is evident, even if not unequivocal, due to the effect of climatic and physiological factors on evapotraspiration processes in plants.

To assess the discrimination efficiency for olive oil origin, a multivariate analysis of the two years' data was carried out by discriminant analysis (using standard procedure), which maximized the differences between the groups by means of a linear combination of the variables (*31*). By applying canonical discriminant analysis, three different independent discriminant functions (RAD) were computed. The combination of the first two canonical variables RAD1 (80%) and RAD2 (13%) accounted for 93% of variability (scores plot shown in **Figure 2**). RAD1 is loaded negatively with δ^{18}O (standardized coefficient, −0.72) and δ^{13}C (−0.52), whereas RAD2 is mainly determined negatively by δ^2H (−1.08) and positively by δ^{18}O (0.80). Some geographical groupings are evident, as confirmed by applying the reclassification discriminant analysis, where 77.9% of the 267 samples were correctly reclassified (**Table 5**).

Elemental Composition. Of the 31 analyzed elements, Ca was present in detectable amounts in 98% of samples, Rb in 92%, K and Mg in 88%, Cu in 82%, Zn in 67%, U in 61%, La in 57%, Mn in 55%, Ba in 53%, Ce in 53%, Cs in 51%, Na in 50%, Pb in 49%, B in 44%, Sm in 39%, Co in 38%, Yb in 36%, Nd in 35%, Eu in 34%, Sr in 32%, Li and V in 31%, Ga in 21%, Al in 19%, Ni in 10%, Cd in 7%, Tl in 4%, Se in 3%, and Mo and Lu in 1% of the samples.

Table 6 shows the median contents quantified for each element in the 267 olive oil samples for each site and for the 3 geological classes, as well as the median content in the 313 surface waters for the 3 geological classes (1 water from BAR was not measured). Median values were displayed because of the non-normal

Table 6. Median Values of Elements of European Olive Oils and Results for Geological Origin of HSD for Unequal *N* Tukey Test ($p < 0.05$) Applied on Normalized and Box−Cox Transformed Data

	olive oils (μg/kg)													surface waters (mg/L)					
	shale/clay/mudstone/loess					limestone						acid magmatic		shale/clay/ mudstone/loess		limestone		acid magmatic	
element	SIC	TOS	BAR	median	signif	TRE	LAK	ALG	CAR	median	signif	CHA	signif	median	signif	median	signif	median	signif
Li	0.009	<0.008	0.016	<0.008		<0.008	<0.008	0.018	0.068	<0.008		<0.008		0.00706	a	0.00128	b	0.00526	a
B	<0.17	<0.17	0.40	<0.17		0.81 <0	<0.17	0.19	0.81	<0.17		<0.17		0.0525	a	0.0240	b	0.0174	b
Na	36	34	22	31		<20	<20	65	231	34		<20		22.10	b	11.41	c	51.49	a
Mg	12	15	14	15	b	9	28	97	58	19	a	13	ab	14.07		8.09		17.06	
Al	<3	<3	<3	<3	b	<3	<3	<3	5	<3	a	<3	ab	0.0028	b	<0.0015	b	0.0096	a
K	184	85	204	156	a	62	54	403	47	62	b	92	a	2.280		1.531		2.506	
Ca	95	117	73	100	b	011	144	361	401	133	a	128	ab	74.45	a	52.54	b	29.27	c
V	<0.007	0.016	<0.007	0.009	a	<0.007	<0.007	<0.007	<0.007	<0.007	b	<0.007	ab	<0.00036	b	<0.00036	b	0.00050	a
Mn	<0.2	<0.2	1.1	<0.2	c	<0.2	0.6	3.7	0.7	0.5	b	0.6	a	0.000	b	0.015	c	0.0158	a
Co	<0.002	<0.002	0.02	<0.002		<0.002	<0.002	0.025	0.010	<0.002		0.003		0.00016		0.00014		0.00014	
Ni	<1	<1	<1	<1		<1	<1	<1	<1	<1		<1		0.00089		0.00027		0.00043	ab
Cu	0.31	0.35	0.87	0.3		0.33	0.310	1.55	0.36	0.3		0.51		0.00114	a	0.36	b	0.00026	b
Zn	7	11	7	8	b	170	12	40	21	12	a	11	a	0.0017		0.0017		0.0009	
Ga	<0.001	<0.001	0.005	<0.001		<0.001	<0.001	0.005	0.001	<0.001		<0.001		0.00150	a	0.00061	b	0.00150	a
Se	<0.014	<0.014	<0.014	<0.014		<0.014	<0.014	<0.014	<0.014	<0.014		<0.014		<0.0082	b	<0.0082	a	<0.0082	a
Rb	0.133	0.043	0.157	0.113	a	0.024	0.031	0.254	0.045	0.039	b	0.077	a	0.00056		0.00061		0.00049	
Sr	<0.3	<0.3	<0.3	<0.3	c	<0.3	<0.3	1.2	1.9	<0.3	b	<0.3	a	0.51870	a	0.14988	b	0.30638	b
Mo	<0.050	<0.050	<0.050	<0.050		<0.050	<0.050	<0.050	<0.050	<0.050		<0.050		0.00060	a	0.00024	b	0.00047	a
Cd	<0.005	<0.005	<0.005	<0.005		<0.005	<0.005	0.007	<0.005	<0.005		<0.005		<0.00005	b	<0.00005	a	<0.00005	a
Cs	<0.001	<0.001	0.01	<0.001	b	0.006	<0.001	0.043	0.011	0.006	a	<0.001	ab	<0.00005	b	<0.00005	a	<0.00005	a
Ba	<0.12	0.13	0.18	<0.12		0.360	<0.12	0.33	0.57	0.36		0.37		0.06	a	0.01811	b	0.03912	ab
La	<0.0020	<0.0020	0.0375	<0.0020	b	<0.0020	0.0046	0.0296	0.0194	0.0052	a	0.0037	a	<0.00005	c	<0.00005	b	0.00015	a
Ce	<0.0050	0.0083	0.0522	<0.0050	b	<0.0050	0.05050	0.0505	0.05	0.0081	a	<0.0050	ab	<0.00005	c	<0.00005	b	0.00018	a
Nd	<0.004	<0.004	0.018	<0.004		<0.004	0.005	0.025	00.0	0.005		<0.004		<0.00005	c	<0.00005	b	0.00013	a
Sm	<0.0010	<0.0010	0.0072	<0.0010	b	<0.0010	<0.0010	0.0078	0.0048	0.0015	a	<0.0010	ab	<0.00005	c	<0.00005	b	<0.00005	a
Eu	<0.0002	<0.0002	0.0051	<0.0002	b	<0.0002	<0.0002	0.0052	0.0049	0.0003	a	<0.0002	ab	<0.00005	b	<0.00005	a	<0.00005	a
Yb	<0.0004	<0.0004	0.0046	<0.0004		<0.0004	<0.0004	0.0047	0.0050	0.0005		<0.0004		<0.00005	b	<0.00005	a	<0.00005	a
Lu	<0.02	<0.02	<0.02	<0.02		<0.02	<0.02	<0.02	<0.02	<0.02		<0.02		<0.00005		<0.00005		<0.00005	
Tl	<0.004	<0.004	<0.004	<0.004		<0.004	<0.004	<0.004	<0.004	<0.004		<0.004		<0.00005	b	<0.00005	a	<0.00005	a
Pb	<0.1	<0.1	0.5	<0.1		<0.1	<0.1	1.5	0.6	0.1		<0.1		<0.11.5		<0.11.5		<0.00005	
U	<0.001	<0.001	0.046	<0.001	b	0.003	0.021	0.0	00.0	0.005	b	0.018	a	0.00071	b	0.00025	c	0.00061	a

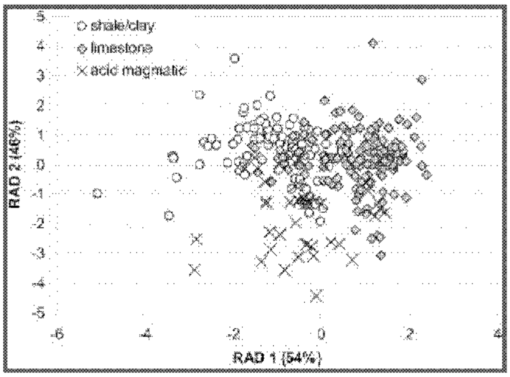

Figure 3. Canonical discriminant analysis of the elemental content of the olive oils from three geological origins: scatterplot of the first two canonical variables.

distribution of the raw data (Kolmogorov−Smirnov and Shapiro−Wilk tests).

For the statistical elaboration we considered all of the raw data, also including the values lower than the detection limit. For every sample, the content of each element was normalized by dividing it by the sum of the content of all the elements. This was done to minimize possible problems due to salt crystallization in organic matrix and settling.

After the Box−Cox transformation, a normal distribution of data was obtained for each element, with the exception of Li.

ANOVA and HSD for unequal *N* Tukey tests, performed on Box−Cox-transformed data, highlight statistically significant ($p < 0.05$) differences in the content of 16 elements (Mg, Al, K, Ca, V, Mn, Ni, Zn, Rb, Sr, Ce, Sm, Cs, La, Eu, U) among the olive oils produced in the 3 different geological zones (**Table 6**). Al and Ni were excluded from the multivariate statistical elaboration as they were quantifiable (amount > LOD) in <30% of samples.

The canonical multivariate discriminant (standard) analysis performed on olive oils using the 14 elements produced 2 canonical variables (RAD 1 and RAD 2) explaining 54 and 46% of variability, respectively (**Figure 3**). The elements with higher standardized coefficients were Cs (0.88), V (−0.82), and Rb (−0.81) for RAD 1 and Sm (−0.67), Ce (0.59), U (−0.73), Ca (−0.70), and Rb (−0.63) for RAD 2.

Overall, 76% of the samples were correctly classified into the 3 geological typologies: 83% of limestone samples, 63% of acid magmatic samples, and 72% of clay/shale samples were classified correctly; 17 oil samples collected on a limestone geology were erroneously classified as belonging to a clay/shale zone and 6 to a magmatic zone, and 9 and 6 samples collected on an acid magmatic geology were misclassified as belonging to a limestone and clay/shale zone, respectively, whereas 20 samples obtained in a clay/shale zone were assigned to a limestone zone and 5 to an acid magmatic zone.

Of the 14 discriminant elements for oils, 10 also proved to be significant (ANOVA, $p < 0.05$) for surface water differentiation, although the median values of the three geological types were not always in the same order in olive oils and waters. This could be due to possible physiological effects such as root absorption or plant translocation/accumulation or to the different solubilities of the elements in water and oils.

By applying multivariate discriminant analysis on the 14 elements, 82% of the 313 surface water samples were correctly classified into the 3 geological groups (the elemental profile of 1 water from BAR is missing). Twenty-two of 115 water samples collected on clay/shale geology were erroneously classified as belonging to a limestone zone, whereas 27 of 158 samples collected in a limestone zone and 5 of 40 collected in an acid magmatic zone were assigned to a clay/shale geology. Two samples collected on a limestone geology were classified as belonging to a magmatic zone, and 1 sample belonging to the acid magmatic geology was assigned to a limestone zone.

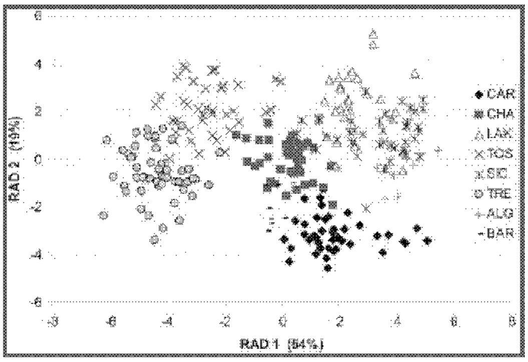

Figure 4. Canonical discriminant analysis of the isotopic and elemental composition of the olive oils from the eight European sites: scatterplot of the first two canonical variables.

Table 7. Reclassification Discriminant Analysis of the H, C, and O Stable Isotope Ratios and Elemental Composition of the Olive Oils from the Eight European Sites: Results of Classification Matrix

	% correctly classified	TRE	CAR	TOS	BAR	CHA	SIC	ALG	LAK
TRE	95	53	0	2	0	1	0	0	0
CAR	98	0	39	0	1	0	0	0	0
TOS	97	1	0	38	0	0	0	0	0
BAR	100	0	0	0	10	0	0	0	0
CHA	98	0	0	1	0	39	0	0	0
SIC	95	0	0	0	0	0	38	0	2
ALG	100	0	0	0	0	0	0	14	0
LAK	86	0	0	0	0	0	3	1	24

The selected 14 elements seem to be a good starting point to geologically characterize the origin of olive oils, as well as surface water.

Geographical Discrimination of Olive Oils Using Isotopic and Elemental Analysis. By combining the 3 isotopic ratios and the 14 elements described above, an improved separation of samples produced at the 8 sites is obtained (**Figure 4**). Using standard and forward stepwise discriminant analysis, which kept all 14 selected trace elements and the 3 isotopic ratios in the model, 7 canonical variables were identified. The first and second variables account for 73% of the variability (54 and 19%, respectively). The higher standardized coefficients were obtained for δ^{18}O (0.74), δ^{13}C (0.58), La (0.56), and Ce (−0.83) for RAD 1 and for U (0.81), Ce (0.87), Ca (0.65), V (0.60), La (−1.02), and Sr (−0.58) for RAD 2. RAD3 (12%), loaded mainly by K (0.82), Ca (0.81), U (0.52), δD (−0.75), and Sm (−0.50), improves the separation between Lakonia and Sicily.

A total of 95.5% of the olive oil samples could be correctly classified to the production site (**Table 7**).

To test the predictive discrimination power and the stability of the model, some of the analyzed samples were used as unknowns to validate the model built on the basis of the remaining cases. In detail, 3 different sets of 26 olive oils (10% of the original database; 4 SIC, 4 TOS, 4 CAR, 4 CHA, 5 TRE, 3 LAK, 1 BAR, 1 ALG randomly selected) were removed from the data, and each time the model was calculated on the remaining 241 cases and was validated with all 267 samples (including the excluded oils). In all analyses, around 95% (from 95.1 to 95.5%) of the samples were correctly classified.

The reclassification of the unknown samples indicates the stability of the model.

To use the model for verifying claim of origin of commercial olive oils, a larger number of authentic samples must be analyzed, considering also other European production sites, as has been done since 1987 for wine (EU Regulation 555/2008).

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Results

The isotopic ratios of olive oil were shown to be related to geographical and climatic factors at the site where the plants were grown. In particular, $\delta^{13}C$ and $\delta^{18}O$ were significantly correlated to latitude, distance from the coast, temperature, relative humidity and the extent of precipitation in the five months before harvest, whereas δD correlated with latitude, distance from the coast, altitude and temperature. It was possible to characterise the geological origin of olive oils by using the content of fourteen elements (Mg, K, Ca, V, Mn, Zn, Rb, Sr, Cs, La, Ce, Sm, Eu, U). By combining the three isotopic ratios with the fourteen elements and applying multivariate discriminant analysis, good discrimination between olive oils from the eight European sites was achieved, with 95% of the samples correctly classified into the production site.

SECTION 4.6

Stable isotope ratios of carbon and hydrogen to distinguish olive oil from shark squalene-squalane

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Aim

Up to now it has not been possible to easily differentiate if squalene and squalane derive from shark or olive oil, so it is therefore important to develop and validate innovative analytical methods able to identify their origin and the presence of shark squalene/squalane in olive oil based products.

In this study stable isotope ratios of carbon and hydrogen of authentic samples of olive oil and shark squalene/squalane were investigated in order to verify if they can distinguish the two different origins of these compounds and thus detect the illegal presence of shark compounds in olive oil based products.

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Stable isotope ratios of carbon and hydrogen to distinguish olive oil from shark squalene-squalane

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Squalene and its hydrogenated derivate squalane are widely used in the pharmaceutical and cosmetic fields. The two compounds are mainly produced from the liver oil of deep sea sharks and from olive oil distillates. Squalene and squalane from shark cost less than the same compounds derived from olive oil, and the use of these shark-derived compounds is unethical in cosmetic formulations. In this work we investigate whether $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios can distinguish olive oil from shark squalene/squalane and can detect the presence of shark derivates in olive oil based products. The $^{13}C/^{12}C$ ratios (expressed as $\delta^{13}C$ values) of bulk samples and of pure compounds measured using isotope ratio mass spectrometry (IRMS) were significantly lower in authentic olive oil squalene/squalane (N: 13; -28.4 ± 0.5‰; -28.3 ± 0.8‰) than in shark squalene/squalane samples (N: 15; -20.5 ± 0.7‰; -20.4 ± 0.6‰). By defining $\delta^{13}C$ threshold values of -27.4‰ and -26.6‰ for olive oil bulk and pure squalene/squalane, respectively, illegal addition of shark products can be identified starting from a minimum of 10%. $^{2}H/^{1}H$ analysis is not useful for distinguishing the two different origins.

$\delta^{13}C$ analysis is proposed as a suitable tool for detecting the authenticity of commercial olive oil squalene and squalane samples, using IRMS interfaced to an elemental analyser if the purity is higher than 80% and IRMS interfaced to a gas chromatography/combustion system for samples with lower purity, including solutions of squalane extracted from cosmetic products. Copyright © 2010 John Wiley & Sons, Ltd.

Squalene ($C_{30}H_{50}$) is an isoprenoid [(6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene, Fig. 1(a)]. It is a precursor of metabolic intermediates in the biological synthesis of sterols[1] and is largely used for synthesis of active derivatives used in the pharmaceutical field. Many authors have attributed anti-oxidant and anti-ageing properties to squalene[2–6] and it has also shown positive action against tumour metastasis and carcinoma in rats.[7–9] It has a shelf-life of more than 2 years if kept inside sealed containers away from light and oxygen.

Several tests have shown squalene and its hydrogenated derivate, squalane ($C_{30}H_{56}$, Fig. 1(b)), to have an emollient and hydrating effect: the latter compound is widely used in the cosmetics field, as it maintains its properties, including viscosity, unchanged in a range from −30 to 250°C. Squalene and squalane are highly lipophilic compounds that are well absorbed through the skin and used as emollient and hydrating agents, protecting against external agents (air, light, UV rays and environmental pollution).[10–13]

Squalene is mainly produced from olive oil distillates, obtained using a process of physical/chemical refining (de-acidification and/or deodorisation) of olive oil, and from the liver oil of deep sea sharks.

Olive oil squalene predominantly originates in Mediterranean countries, which are the main producers of olive oil. Virgin olive oil has a squalene content of 0.4–0.6%, while distillates from physical refining have a squalene content of about 8–12% and those from alkali deodorising (currently less common) around 21%.[14–16] Olive oil squalene is obtained using two extraction processes. The most common involves esterification of distilled fatty acids to obtain triglycerides, from which squalene is separated through high vacuum distillation. This process is time-consuming and very complicated, but it gives a high level of purity (>95%). The second, less common, involves the treatment of distilled fatty acids with a solution of KOH, transforming them into soap, then extracting the unsaponifiable fraction with hexane. Squalene obtained using this process has a maximum purity of 82–83% and is rich in saturated paraffin extracted from the solvent. Further processing is then required to obtain a product of acceptable purity (>92%), implying a low yield and high costs.

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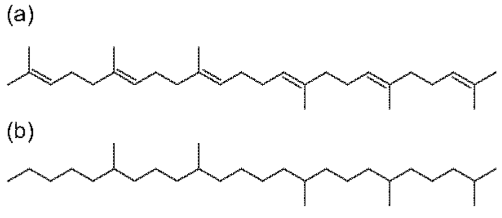

Figure 1. Chemical structures of squalene (a) and squalane (b).

Apart from olive oils, other vegetable oils have a negligible content of squalene and/or the extraction is not economically reasonable. Amaranth oil, for example, is a potential source of squalene,[17] since its seeds contain 7% oil, with a squalene content of 6–8% but, because of the high price of this oil, its limited production and high extraction costs, squalene production from amaranth oil is not economically viable.

With regard to animal squalene, about 25% of the weight of a deep sea shark (average weight of 100 kg) is made up of its liver, which contains about 50% oil, of which 50–80% is squalene. Squalene, with a purity of >98%, is obtained directly from the liver oil, after a single distillation phase in a vacuum, at temperatures of 200–230°C.

Nearly 70 hours of processing are required to obtain olive oil squalene with a purity higher than 92%, whereas only 10 hours are required to obtain shark squalene with a purity higher than 98%. Moreover, the squalene yield is significantly higher from processing shark liver oil (with 50–80% of squalene) than with olive oil (distillates from physical refining: 8–12% and from alkali deodorising: 21%). This explains why in recent years shark squalene has been marketed at around one-third of the price of olive oil squalene.

Because of the so-called 'Animal Testing Ban' legislation (EU Directive 2003/15/EC), it has been forbidden within the EU to carry out any tests on animals for cosmetic purposes. As a consequence many countries have also prohibited the use of raw materials of animal origin, including shark squalene/squalane (SQ), in cosmetic formulations. Indeed, the slaughter of animals purely for the extraction of one component to be used in cosmetics products is now ethically unacceptable. It has been estimated that more than 350 000 sharks are illegally slaughtered each year in the Atlantic (from Spain to Angola) and Pacific (east of New Zealand and China) Oceans, purely for the production of squalene (about 2.500 tons/year). After removal of the liver, the remains of the sharks are thrown back into the sea.

Up to now it has not been possible to easily differentiate the two different origins of the product and this has encouraged the illegal addition of shark to olive oil squalene/squalane or often the selling of shark SQ as olive oil SQ. It is therefore important to develop and validate innovative analytical methods able to identify the origin of SQ and the presence of shark SQ in olive oil based products.

In two previous studies,[18,19] it was shown that the presence of some minor steroid compounds can characterise the origin of squalene ('stigmastane' for olive oil and 'colestane' for shark), but recent advanced technological purification processes can cause a decrease or the total elimination of these marker compounds. A more recent study[20] has highlighted that the deuterium (^{2}H) distribution, measured using SNIF-NMR (site-specific isotopic fractionation–nuclear magnetic resonance), in olefinic, methylene and methyl sites is different in olive oil, shark and synthetic squalene.

In this work we investigate whether measurement of the stable isotope ratios of C (^{13}C/^{12}C) and H (^{2}H/^{1}H) using isotope ratio mass spectrometry (IRMS) can distinguish olive oil from shark squalene/squalane and can detect the presence of shark SQ in olive oil based products.

EXPERIMENTAL

Samples

Thirteen authentic samples of olive oil and fifteen of shark squalene/squalane, four mixtures, four commercial olive oil samples and two samples of squalane extracted from cosmetic products in n-heptane solution were considered (Table 1).

The authentic samples come from different European areas as well as from Japan and Korea, which are the most important producers and consumers of this product. The samples of squalane extracted from cosmetic products were obtained according to the following procedure: 10 g of the product was made up to 100 mL by adding water and acidified with HCl to pH 2. NaCl was added and the mixture heated to 80°C for 5 min and extracted threer times with 50 mL of petroleum ether. The three extracts were combined, washed with water, added to $Na_2(SO_4)$ and filtered. After evaporation of petroleum ether using a rotavapor, squalane was recovered in n-heptane.

The purity of the samples expressed as a percentage of squalene or squalane was quantified using gas chromatographic (GC) analysis.[18,19]

^{13}C/^{12}C analysis of bulk squalene and squalane samples using EA/IRMS

Aliquots of 0.3 mg of sample were weighed in tin capsules (Säntis Analytical AG, Teufen, Switzerland) and introduced by means of an autosampler (AS 200; Thermo Scientific, Bremen, Germany) into a Flash EA 1112 (Thermo Scientific) elemental analyser (EA), where they were burnt quantitatively to CO_2 and H_2O (H_2O removed using a $Mg(ClO_4)_2$ filter) in the presence of O_2 and CuO. The CO_2 was flushed through the ConFlo III dilutor device (ThermoFinnigan, Bremen, Germany) into the isotope ratio mass spectrometer (DELTA V; Thermo Scientific) where the signal intensities of the isotopomers at *m/z* 44 and 45 were determined. The uncertainty of measurement, calculated as 2 × standard deviation of intra-laboratory reproducibility, was 0.3‰ (see below for the definition of the unit '‰').

^{13}C/^{12}C analysis of pure squalene and squalane after GC separation

A volume of 1 μL of solution (2 μL of squalene and 1 μL of squalane samples in 1 mL of n-pentane; VWR, Milano, Italy) was injected using a 10 μL syringe (Hamilton, Bonaduz, Switzerland) with an autosampler (GC-PAL; GC Analytics AG, Zwingen, Switzerland) into a gas chromatograph

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Table 1. Description of the samples, δ^{2}H and δ^{13}C values of bulk samples and δ^{13}C of pure squalene/squalane (SQ) after GC separation. Mean and standard deviation, as well as minimum and maximum values and 95% of variability (mean ± std dev × '*t student*'), are shown for olive oil and shark SQ. USP: United States Pharmacopoeia

	Purity (%)			δ^{2}H ‰ vs. V-SMOW	δ^{13}C ‰ vs. V-PDB **EA/IRMS**	δ^{13}C ‰ vs. V-PDB **GC/C-IRMS**
Olive oil squalene						
1	82	Italy	February 2009	−170	−27.8	−26.8
2	94	Turkey	January 2009	−170	−28.7	−28.0
3	86	Spain	March 2009	−163	−28.0	−28.7
4	88	Spain	December 2005	−163	−28.3	−29.3
5	83	Spain	April 2009	−166	−28.1	−26.9
6	98	Turkey	March 2009	−163	−29.1	−27.9
7	84	Spain	June 2009	−164	−27.9	−28.0
mean				*−165*	*−28.3*	*−27.9*
std. dev.				*3*	*0.5*	*0.9*
Olive oil squalane USP grade						
8	98	Turkey	April 2009	−140	−28.8	−29.2
9	95	France	February 2009	−170	−27.9	−28.3
10	93	Netherlands	July 2008	−172	−28.6	−28.6
11	95	Spain	November 1999	−249	−28.5	−28.4
12 – from 6	95	Turkey	May 2009	−171	−28.9	−28.5
13	98	Corea	December 2009	−250	−29.1	−29.0
mean				*−192*	*−28.6*	*−28.7*
std. dev.				*46*	*0.4*	*.04*
Olive oil squalene/squalane (N. 13)						
mean				**−178**	**−28.4**	**−28.3**
std. dev.				**33**	**0.5**	**0.8**
mean – t * std. dev.				**−249**	**−29.4**	**−29.9**
mean + t * std. dev.				**−107**	**−27.4**	**−26.6**
min				**−250**	**−29.1**	**−29.3**
max				**−140**	**−27.8**	**−26.8**
Shark squalene						
14 – shark liver raw oil	80	Spain	December 2008	−158	−21.1	−20.8
15 – shark liver raw oil	85	Portugal	January 2009	−185	−21.1	−21.3
16	95	Spain	January 2009	−171	−20.5	−20.7
17	97	Spain	March 2009	−168	−20.5	−20.7
18	90	Portugal	March 2009	−157	−21.0	−20.8
19 – shark liver raw oil	85	Spain	November 2008	−158	−20.9	−20.6
20	99	Japan	January 2009	−171	−21.2	−21.4
21 – shark liver raw oil	75	Portugal	July 2009	−184	−21.0	−20.9
22	99	Portugal	December 2008	−168	−20.6	−19.9
23– decholesterolised shark liver raw oil	98	Spain	August 2009	−182	−20.9	−20.3
24– distilled squalene (from N. 30)	97	Spain	August 2009	−163	−20.3	−20.0
Mean				*−170*	*−20.8*	*−20.6*
std. dev.				*10*	*0.3*	*0.5*
Shark squalane USP grade						
25	98	Spain	January 2009	−273	−20.5	−20.4
26	99	Korea	January 2009	−175	−19.2	−19.5
27	99	Japan	January 2009	−210	−19.7	−20.1
28	99	Korea	January 2009	−174	−19.1	−19.6
mean				*−208*	*−19.6*	*−19.9*
std. dev.				*47*	*0.6*	*0.4*
Shark squalene/squalane (N. 15)						
mean				**−180**	**−20.5**	**−20.4**
std. dev.				**29**	**0.7**	**0.6**
mean – t * std. dev.				**−242**	**−21.9**	**−21.6**
mean + t * std. dev.				**−118**	**−19.1**	**−19.3**
min				**−273**	**−21.2**	**−21.4**
max				**−157**	**−19.1**	**−19.5**
Commercial mixture and squalene/squalane samples						
29 – decholesterolised shark oil (70% squalene) + olive oil deodoriser distillates 30% (4% squalene)	74	Italy	July 2009	−163	−20.3	−20.4
30–50% shark liver oil + 50% fatty acids distillate from olive oil deodorisation	67	Spain	March 2009	−154	−24.9	−19.9
31 – after esterification of 30	79	Spain	March 2009	−153	−25.0	−20.9
32 – after distillation at 230°C of 31	64	Spain	March 2009	−149	−23.3	−19.7
33 – commercial squalane	84	Italy	February 2006	−178	−26.5	−26.0

(*Continues*)

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Table 1. (Continued)

	Purity (%)			δ^2H ‰ vs. V-SMOW	$\delta^{13}C$ ‰ vs. V-PDB **EA/IRMS**	$\delta^{13}C$ ‰ vs. V-PDB **GC/C-IRMS**
34 – commercial squalane	88	France	July 2008	−267	−26.2	−25.7
35 – commercial squalene	90	Italy	July 2009	−164	−27.9	−27.5
36 – commercial squalene	95	Italy	July 2009	−147	−21.0	−20.0
Squalane from commercial cosmetic products						
37	0.5		November 2009			−20.8
38	2		November 2009			−20.6

(Agilent 6890A; Agilent Technologies, Wilmington, DE, USA) equipped with a J&W Scientific HP-5 column, 30 m length,. 0.32 mm I.D., film: crosslinked 5% diphenyl 95% dimethylpolysiloxane, 0.25 μm film thickness (Agilent Technologies), in split mode (1:25). The injector temperature was set to 290°C and the temperature program was as follows: initial column temperature, 160°C; holding time, 2 min; temperature increased at a rate of 15°C/min, to 240°C; holding time,10 min; temperature increased at a rate of 10°C/min, to 290°C; holding time, 7 min.

After chromatographic separation, the squalene/squalane was burnt quantitatively to CO_2 and H_2O in an oxidation reactor. The solvent was vented with a back-flush valve during the first 400 s of analysis, to avoid damage and inactivation of the oxidation column. The oxidation reactor consisted of a 32 cm long alumina tube in which three thin (0.125 mm diameter) braided wires were placed: one nickel oxide, one copper oxide and one platinum. The tube was kept at a temperature of 940°C and was flushed with a flow of oxygen every 40 samples to re-oxidise the catalysts. Water was eliminated by a water-removing trap, consisting of a Nafion membrane. Carbon dioxide, carried by an helium stream, was then introduced into the ion source of the DELTA V isotope ratio mass spectrometer by an open split interface. The peak of squalene or squalane was identified by previously measuring pure commercial compounds under the same operating conditions. The uncertainty of measurement was 0.5‰.

$^2H/^1H$ analysis of bulk squalene and squalane samples

Aliquots of 0.3 mg of sample were weighed in silver capsules (Säntis Analytical AG), stored in a desiccator above P_2O_5 at 97% (Sigma Aldrich GmbH, Steinheim, Germany) for at least 24 h, then placed, equipped with a suitable cover, into the autosampler. During the measurement procedure, dryness was guaranteed by flushing nitrogen at 99.999% purity continuously over the samples. When analysis began, the capsules were dropped into the column of the pyrolyser (Thermo Finnigan TC/EA, high-temperature conversion elemental analyser; Thermo Scientific) where, at a temperature of 1450°C, the hydrogen atoms of the sample were quantitatively converted into H_2. The H_2 was flushed into 0.6 m GC column containing 5 Å molecular sieve (maintained at a temperature of 60°C) and through the ConFlow III device into the Finnigan DELTA XP isotope ratio mass spectrometer (Thermo Scientific). Before measuring the D/H ratio, the H_3 factor,[21] which allows correction of the contribution of $[H_3]^+$ to the m/z 3 signal, was checked to be less than 9 ppm/nA, as suggested by the instrument manufacturer. The uncertainty of measurement was 2‰.

Expression of the results

The isotopic values were calculated against working standards (commercial squalene and squalane), calibrated against international reference materials: fuel oil NBS-22 (IAEA – International Atomic Energy Agency, Vienna, Austria) and sugar IAEA-CH-6 (IAEA) for $^{13}C/^{12}C$ and NBS-22 for $^2H/^1H$ measurement.

The isotopic ratio values of the aforementioned international reference materials and therefore also of the samples were expressed in δ‰, versus V-PDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$ and V-SMOW (Vienna Standard Mean Ocean Water) for δ^2H, according to the following formula: $[(Rs - Rstd)/Rstd] \times 1000$, where Rs is the isotope ratio measured for the sample and Rstd is the isotope ratio of the international standard.

RESULTS AND DISCUSSION

$\delta^{13}C$ of bulk squalene and squalane samples

The primary source of carbon for terrestrial plants is atmospheric CO_2 with a mean $\delta^{13}C$ value of around −8‰,[22] whereas for the marine system it is the dissolved inorganic carbon pool used by microalgae and phytoplankton, which are at the lower trophic levels of the marine food web. The dissolved inorganic carbon pool has a $\delta^{13}C$ value of around 0‰,[23] significantly higher than that of atmospheric CO_2.[24]

During the process of incorporation of source C into terrestrial and aquatic vegetal compounds, significant isotopic discrimination against the heavier isotope (^{13}C) takes place. C3 terrestrial plants, such as olive trees, have a mean $\delta^{13}C$ value of −27‰, whereas marine phytoplankton and microalgae have $\delta^{13}C$ values close to −22‰ and −15‰, respectively.[23] The $\delta^{13}C$ of fish reflects that in their diet, with an enrichment of less than 1‰ per trophic level (e.g.[25]). Inside plant or animal tissue the different compounds can have a characteristic isotope composition (e.g. lipids have the lowest ^{13}C content, e.g.[26–28]), because the different metabolic pathways involved in their synthesis are followed by the characteristic isotope fractionation effect.

The $\delta^{13}C$ values of bulk olive oils[29–31] and of olive oil fatty acids[32] have been shown to be around −32 ÷ −27‰ and −37 ÷ −29‰, respectively. The values of marine fish were found to vary from −23 to −15‰,[33–36] considering both wild and farmed fish, mainly salmon, and mainly bulk lyophilised fish. Of the different fish tissues, liver has the lowest ^{13}C content, probably due to the higher content of lipid.[36]

The biosynthetic pathway of squalene is similar in animals and plants: the synthesis of mevalonate from acetyl-CoA, the formation of isoprenoid units from mevalonate by loss of CO_2 and the condensation of six isoprenoid units to form squalene.[37,38] $\delta^{13}C$ values of -28.8 ± 0.3‰ and -30.8 ± 0.2‰ were reported for the squalene of leaves from the C3 plant gymnosperm cedar *Cryptomeria japonica*,[38] and of −21.0‰ for deep sea shark oil,[39] whereas $\delta^{13}C$ values for olive oil or shark squalene and squalane are not available to our knowledge. We have assumed that shark SQ should have significantly higher $\delta^{13}C$ values than olive oil SQ. Table 1 summarises the $\delta^{13}C$ values of olive oil and shark squalene and squalane samples used in this work.

In order to consider larger groups, squalene and squalane were considered together. At least for olive oil, the $\delta^{13}C$ bulk values were not significantly different between squalene and squalane ($p < 0.001$; Mann-Whitney U test; software Statistica version 8; StatSoft Italia srl, Padua, Italy). Moreover, considering squalane sample number 12 produced by hydrogenation of squalene sample number 6, it appears that the hydrogenation process does not lead to significant isotopic fractionation.

A mean $\delta^{13}C$ value of −28.4‰ (±0.5‰) for olive oil and of −20.5‰ (±0.7‰) for shark SQ bulk samples was found. There is therefore a clear difference between the two origins (olive oil and shark liver oil). The shift (7.9‰) is very similar to that observed between the carbon source for terrestrial and marine systems.

Considering the 95% upper confidence limit of olive oil SQ, a threshold value of −27.4‰ for olive oil squalane/squalene samples (purity > 80%) can be identified.

In order to quantify the minimum percentage addition of shark SQ detectable through measurement of the $\delta^{13}C$ value of the bulk sample, a graph was created on the basis of the mean and the 95% confidence limits ($t_{student} \times$ std. dev.) of the two groups and increasing the percentage addition of shark to olive oil squalene/squalane from 0 to 100% (Fig. 2). The mean values of the mixture were calculated as the sum of the mean values of the two groups, multiplied by the percentage of contribution to the mixture, whereas the standard deviation was the sum of the std. dev. of the two groups multiplied by the percentage of contribution, according to the *law of error propagation* in the case of the sum of two or more variables. The validity of the graph was confirmed by analysing nine prepared mixtures: squalene sample number 1 (80%, 50%, 20%) + sample number 22 (20%, 50%, 80%); squalene sample number 6 (80%, 50%, 20%) + sample number 20 (20%, 50%, 80%); squalane sample number 9 (80%, 50%, 20%) + sample number 26 (20%, 50%, 80%) (Fig. 2). The limit of −27.4‰ made it possible to detect illegal addition of shark products starting from a minimum of 10%. Addition of shark SQ at levels higher than 30% were detectable in all the cases (Fig. 2).

On the basis of this limit, three of the four commercial samples declared to be olive oil squalene or squalane (purity >80%) were found to have had shark SQ added (more than 70% in one sample and more than 20% in the other two samples).

$\delta^{13}C$ of pure squalene and squalane after GC separation

As the purity of some authentic samples was not optimal and up to 25% of other components were present, measurement of the $\delta^{13}C$ of pure SQ after GC separation was also carried out.

The $\delta^{13}C$ values of the pure SQ were shown to be significantly correlated with the $\delta^{13}C$ of the bulk samples

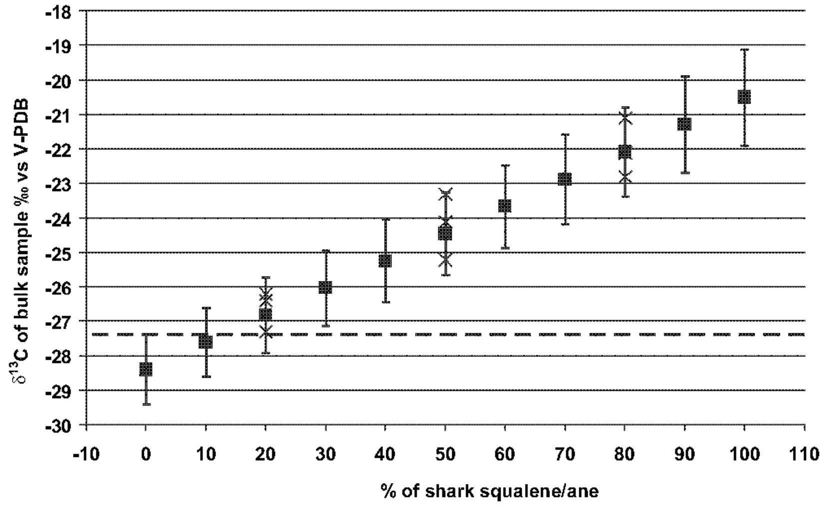

Figure 2. Graphs of $\delta^{13}C$ values of hypothetical mixtures of shark and olive oil squalene/squalane bulk samples to detect the minimal percentage of shark SQ detectable in a mixture. Percentage of olive oil squalene/ane = 100 − % of shark squalene/ane. Broken line defines the threshold limit for olive oil squalene/ane. Square: mean value. Bars: 95% confidence limit. x: measured values of prepared mixtures.

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($\delta^{13}C_{SQ} = 0.98 \times \delta^{13}C_{bulk} - 0.34$; $R^2 = 0.98$) and to be on average 0.1 ± 0.5‰ higher. For olive oil, the correlation was less good (olive oil: $\delta^{13}C_{SQ} = 0.74 \times \delta^{13}C_{bulk} - 7.26$, $R^2 = 0.20$; shark: $\delta^{13}C_{SQ} = 0.70 \times \delta^{13}C_{bulk} - 6.13$; $R^2 = 0.71$) and the difference between the $\delta^{13}C$ values of pure SQ and those of the bulk sample was higher (olive oil: 0.2 ± 0.7‰; shark: 0.0 ± 0.3‰). For shark SQ the shift for all the samples (from −0.5 to +0.7‰) was similar to the measurement uncertainty, whereas for olive oil it varied from −0.9 to +1.2‰, particularly for the less pure squalene samples. This higher shift is probably because the other components in olive oil samples (mainly C8–C18 saturated paraffin) have a different isotope composition from squalene: e.g. C16 and C18 olive oil fatty acids have $\delta^{13}C$ values between −30 and −34‰;[32] n-alkane, n-alkanoic acid and n-alkanol of C3 gymnosperm and angiosperm (such as olive) plants between −29 and −40‰.[40] The lower values of some pure SQ than of the bulk samples are not easily explainable.

Considering the values of mean, standard deviation, minimum, maximum and 95% confidence limits (Table 1), it is clear that the analysis of pure SQ using GC/C-IRMS does not improve the ability to distinguish between the two origins (olive oil and shark) and therefore for samples with a content of SQ greater than 80% it is preferable to measure the bulk samples directly.

GC/C-IRMS measurement of $\delta^{13}C$ is very useful for identifying the origin of SQ in the case of particularly impure commercial samples, as for samples 30–32 (Table 1), in which the presence of around 50% fatty acids significantly decreases the $\delta^{13}C$ value of pure squalene. Moreover, the GC/C-IRMS technique can be used to determine the origin of squalane present in cosmetic products, because the extracted solution in n-heptane can be analysed directly.

For pure olive oil squalene/squalane, considering as for the bulk samples the 95% upper confidence limit, a threshold $\delta^{13}C$ value of −26.6‰ can be employed. After having created a graph with the same rules as followed for bulk samples (Fig. 2), it is evident that this limit makes it possible to detect the presence of more than 40% shark SQ in all the possible mixtures and that in most cases an addition of more than 10% is detectable (Fig. 3).

In all the commercial mixtures and in both the squalane solutions extracted from cosmetic products (Table 1), more than 80% shark SQ was found.

δ^2H of bulk squalene and squalane samples

In higher plants such as the olive, the hydrogen derives from the water taken in by the plant and is incorporated into the vegetal compounds through NADPH.[41] The δ^2H values of water are related to the climatic conditions in the area (temperature, amount of precipitation) and consequently to its geographical characteristics (latitude, elevation, distance from the evaporation source) and can vary worldwide from around −60 to +1‰ in precipitation.[42] During photosynthesis and lipid biosynthesis significant hydrogen isotopic fractionation occurs mainly for sterols (decrease in δ^2H by about −250‰), phytol and sesquiterpenese (decrease of −330‰).[41] The δ^2H values of aliphatic hydrocarbons record the isotopic signature of the source water, because they resist exchange of inorganic hydrogen into organic hydrogen[43] and, as for water, they can vary extensively.

Values from −165 to −133‰ have been found for European olive oils,[29–31] values of −258 ± 3‰ and −266 ± 0.3‰ for the squalene of *Cryptomeria japonica* [38] leaves and between −100 and −200‰ for the n-alkane of several C3 gymnosperm and angiosperm plants from Japan and Thailand.[40]

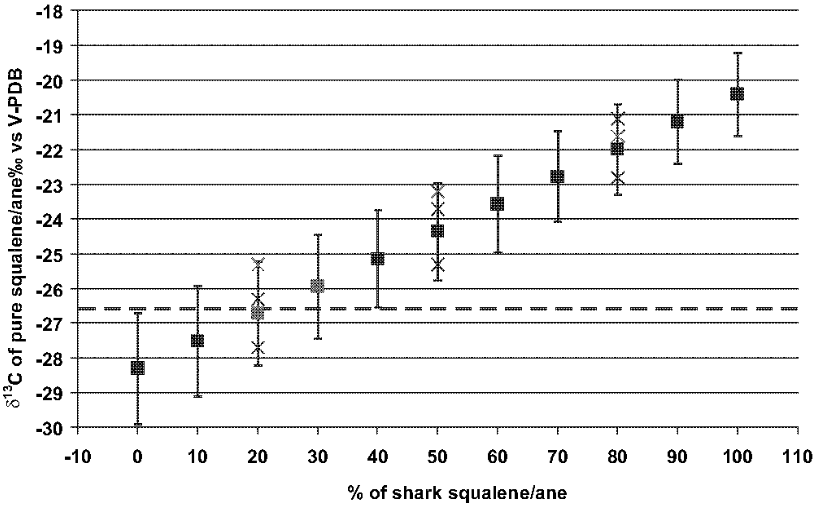

Figure 3. Graphs of $\delta^{13}C$ values of pure squalene/squalane in hypothetical mixtures of shark and olive oil samples to detect the minimal percentage of shark SQ detectable in a mixture. Percentage of olive oil squalene/ane = 100 − % of shark squalene/ane. Broken line defines the threshold limit for olive oil squalene/ane. Square: mean value. Bars: 95% confidence limit. x: measured values of prepared mixtures.

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The stable hydrogen isotope composition of fish reflects that of their environment.[44] Up to 35% of fish tissue hydrogen derives from environmental water, whereas the remaining part comes from feed, with negligible trophic δ^2H fractionation.[45] Phytoplankton was found to have significantly lower δ^2H values than terrestrial plants from the same locations, because the latter are subject to evaporation-transpiration processes.[46] Similarly to δ^{13}C, we therefore would expect a different H isotope composition in olive oil and shark squalene, although we have to take into account the fact that different provenance areas could mask the differences between plant and animal compounds, due to the relationship of δ^2H values with the environment. Indeed, δ^2H values of -161 and -171‰ (therefore similar to those of European olive oil) have been found for the lipids of marine plants and animals, respectively, coming from South California, USA.[47]

We did not find significant differences between the δ^2H values of olive oil (-165 ± 3‰) and those of shark squalene (-170 ± 10‰) (Table 1). The differences found by Deiana[20] between olive oils and shark squalene concerned the ^{2}H pattern measured by SNIF-NMR in the methyl/methylene/vinyl hydrogen atoms, but they are not evident if we consider the entire compound. The δ^2H values of squalane samples were not taken into account because during the hydrogenation process of squalene, squalane would include exogenous hydrogen, which would mask the isotopic composition of the original hydrogen included in the biosynthesis.

As a consequence of the low discrimination capability of δ^2H analysis, compound-specific δ^2H measurement by GC/pyrolysis-IRMS was not performed.

CONCLUSIONS

An analytical method was developed to distinguish olive oil from shark squalene and squalane samples and to detect the illegal addition of shark derivates to olive oil based products. The method consisted of δ^{13}C measurement using EA/IRMS (Elemental Analyser/Isotope Ratio Mass Spectrometry) for bulk squalene/squalane samples (purity >80%), and GC/C-IRMS (Gas Chromatography-Combustion/Isotope Ratio Mass Spectrometry) for samples with lower purity, including solutions of squalane extracted from cosmetic products. The method makes it possible to detect the presence of more than 10% shark squalene/squalane in a mixture.

δ^{13}C analysis can be proposed as a suitable tool for detecting the authenticity of commercial olive oil squalene and squalane samples.

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Results

$^{13}C/^{12}C$ (expressed as $\delta^{13}C$ values) were found to be significantly lower in authentic olive oil squalene/squalane (-28.4‰ ± 0.5‰) than in shark squalene/squalane samples (-20.5‰ ± 0.7‰). D/H analysis is not useful for distinguishing the two different origins.

By defining $\delta^{13}C$ threshold values of -27.4‰ for olive oil squalene/squalane, addition of shark products can be identified starting from a minimum of 10%. On the basis of this limit, squalane extracted from several commercial cosmetic products was found to have been produced from shark liver oil.

Thus $\delta^{13}C$ analysis can be proposed as an official way of detecting whether any batch of commercial squalene or squalane and of squalane within cosmetic formulations, has come from shark liver or olive oil.

The proposed method will promote the production of squalene from olive oil and will discourage the illegal fishing of deep-sea sharks, contributing towards preserving sharks from extinction.

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CHAPTER 5 CHEESES

5.1 INTRODUCTION

As for olive oil, law requires that the origin of PDO/PGI cheeses must be declared on the label but does not indicate useful analytical tools to verify the truthfulness of what is asserted on the label. At global level, cheese is an important agricultural product. According to the Food and Agricultural Organization of the United Nations, over 19 million tons of cheese were produced worldwide in 2009 (data provided by FAO, http://faostat.fao.org/). The largest cheese producer is the United States, accounting for 25% of world production, followed by Germany, France and Italy. With production standing at over 1.1 million tons of cheese, Italy has the largest number of cheeses with PDO and PGI denominations after France, (N = 39, data provided by the Italian Ministry of Agricultural, Food and Forestry Policy, http://www.politicheagricole.it). In the case of fresh milk and PDO/PGI cheeses, both Italian and European law (Ministerial Decree 14th January 2005, Regulation 510/2006/EC) require indication of the origin of milk and raw materials used for manufacture on the label. However, as for olive oil, official methods for analysing cheese (Regulation 273/2008/EC) do not provide useful tools able to verify their real geographical origin, particularly important in the case of dairy products with special regulatory status or commanding a price premium based on their provenance. In order to protect the consumer and honest producers from mislabelling, it is necessary to develop objective methods capable of discriminating PDO cheeses from cheaper imitation products and which can therefore be used to check on the authenticity of products on the market.

5.2 STABLE ISOTOPE RATIOS AND ELEMENTAL COMPOSITION OF CHEESES

As stable isotope ratios combined with elemental composition have been shown to be a powerful tool for characterising olive oil, it was decided to try adopting the same approach for the characterisation of different kinds of cheese.

Indeed, of the various methods available, analysis of the stable isotope ratios of bioelements and elemental composition seems to be most suitable for this purpose (Gonzalvez et al., 2009). Isotope ratios have been successfully used for identification of the type of diet supplied to animals (Camin et al., 2004; Camin et al., 2008; Knobbe et al, 2006; Kornexl et al., 1997) and for differentiating products from different countries. In particular, the stable isotope ratios of carbon and nitrogen in casein have made it possible to distinguish Pecorino produced in Sardinia from that produced in Sicily and Apulia (Manca et al., 2001) and buffalo mozzarella originating from two areas in southern Italy (Brescia et al., 2005). Together with the stable isotope ratio of other bioelements (S and H in casein and C and O in glycerol), this analytical approach also made it possible to separate different European cheeses from France, Italy and Spain (Camin et al., 2004) and Peretta cheese from Sardinia from competitors’ cheeses produced in northern Europe (Manca et al., 2006).

The elemental composition of dairy products was first investigated particularly in relation to detecting the species of animal providing the milk (Fresno et al., 1995; Benincasa et al., 2008) or determining specific elements, mainly toxic, introduced to the human diet, with the related legal and nutritional implications (Brätter et al., 1998; McKinstry at al., 1999). More recently, and with varying degrees of certainty, elemental concentration has also been applied to the characterisation of milk and cheese. In particular this made it possible to differentiate buffalo mozzarella from two areas of southern Italy (Brescia et al., 2005) and Emmental, Edam and caprine cheeses from several European countries (Suhaj and Korenovska, 2008). To date, only Pillonel et al. (2003) have used isotopes and elements jointly in the dairy sector, specifically to authenticate Emmental-type cheeses produced in Switzerland, Germany, France, Austria and Finland. Their very encouraging results showed that $\delta^{13}C$, δD and $\delta^{15}N$ stable isotope ratios allowed good discrimination between geographically distant regions, whereas elemental composition contributed more towards distinguishing relatively close regions.

SECTION 5.3

Elemental and isotopic characterisation of typical Italian alpine cheeses

Bontempo L., Larcher R., Camin F., Hölzl S., Rossmann A., Horn P., Nicolini G.

International Dairy Journal (in press)

Aim

In this study elemental composition, $\delta^{13}C$ of casein and glycerol, $\delta^{15}N$ and $\delta^{34}S$ in casein and $\delta^{18}O$ in glycerol were determined in cheeses with PDO status or with a PDO application underway (Asiago, Fontina, Montasio, Spressa, Puzzone, Toma and Vezzena) produced in alpine/pre-alpine Italian regions. The ultimate aim of this work was to characterise the kinds of cheese considered, firstly to support requirements for their recognition, and then to verify the possibility of creating a traceability model based on analytical data which could be used to protect these PDO cheeses from mislabelling and to verify the authenticity of commercial cheeses.

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Elemental and isotopic characterisation of typical Italian alpine cheeses

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Abstract

Seven kinds of cow milk cheese (Asiago, N=16; Fontina, N=16; Montasio, N=16; Puzzone, N=14; Spressa, N=15; Toma, N=16; Vezzena, N=16) produced in alpine and pre-alpine Italian areas are described using the isotopic ratios of C, N, O, S and Sr and the contents of 49 mineral elements. A multivariate discriminant analysis based on Ba, Ca, K, Mg, Rb, $\delta^{13}C_{casein}$, $\delta^{15}N$ and $\delta^{18}O$ correctly classified 94 % of the 109 samples with a maximum of 100% in the case of Fontina and Puzzone. $\delta^{13}C_{casein}$ and $\delta^{13}C_{glycerol}$ allowed to estimate the maize uptake in the animal's diet and PDO protocols observance.

1. Introduction

The stable isotope ratios of light and heavy elements, in combination with the content of several mineral elements, have been used in the last few years as a powerful tool to characterise several food commodities: *e.g.* olive oils (Camin et al, 2010a; Camin et al., 2010b), orange juices (Rummel, Hoelzl, Horn, Rossmann & Schlicht, 2010) and tea (Pilgrim, Watling & Grice, 2010). For milk and dairy products, elements and stable isotopes ratios have frequently been used but very rarely in combination.

Coni and co-workers (Coni, Bocca & Caroli, 1999; Coni, Bocca, Ianni & Caroli, 1995) highlighted that curdling and salting, as well as releases from manufacturing equipment, are the main critical points in the uneven distribution of elements in the different sub-products (e.g. whey, curd). Fresno and co-workers (Fresno, Prieto, Urdiales & Sarmiento, 1995) reported significant differences in the content of P, K, Mg, Zn, Fe and Mn in goat's and cow's milk, whereas Benincasa et al. (Benincasa, Lewis, Sindona & Tagarelli, 2008) were able to discriminate milk supplied by cows and water buffaloes using Ca, P, Ga, Zn, Mn, B and S content. In a few studies elemental composition has also been used to distinguish the geographical origin of dairy products. Brescia and co-workers

(Brescia, Monfreda, Buccolieri & Carrino, 2005) identified Li and K as useful markers for distinguishing milk and mozzarella cheeses from Apulia and Campania, whereas Sacco et al. (2009) distinguished Southern Italian milk from Central European samples using the combination of some elements and basic compositional parameters.

Isotopic ratios have been used for the identification of the type of diet by Camin et al. (Camin, Perini, Colombari, Bontempo & Versini, 2008; Camin, Wietzerbin, Cortes, Haberhauer, Lees & Versini, 2004), Knobbe et al. (2006) and Kornexl et al. (Kornexl, Werner, Rossmann, & Schmidt, 1997), confirming that the $\delta^{13}C$ of bulk milk, as well as of casein and glycerol extracted from milk or cheese, is related to the amount of C3 and C4 plants in animal diet. On the contrary, the reliability of milk and casein $\delta^{15}N$ for reconstructing dietary composition is lower, being affected not only by dietary composition (the presence of N-fixing plants) but also by pedology, aridity, distance from the sea and fertilisation practices adopted in the area where the feed was grown. Crittenden et al. (2007) were able to distinguish cow's milk from different regions in Australasia using the $\delta^{18}O$ isotope ratios of milk water and the $\delta^{13}C$ of skim milk and casein, confirming the isotope fractionation patterns predicted on the basis of latitude and climate. Camin et al. (2004), considering cheeses from Spain, Italy and France, found that the $\delta^{15}N$ and $\delta^{34}S$ of casein are mostly influenced by the geographic and climatic conditions in the area (aridity, closeness to the sea, altitude). Fortunato and co-workers (2004) demonstrated that the $^{87}Sr/^{86}Sr$ ratio in cheeses from different sources (alpine and pre-alpine areas, Bretagne, Finland, Canada and Australia) agreed with local geological properties. Very recently, the stable isotope ratios of light elements have also been recognised as authentication markers for Grana Padano cheese (EC 2009/C 199/11).

To date, only Pillonel et al. (2003) have used isotopes and elements jointly in the dairy sector, specifically to authenticate Emmental-type cheeses produced in Switzerland, Germany, France, Austria and Finland. Their very encouraging results showed that $\delta^{13}C$, δD, $\delta^{15}N$ and $^{87}Sr/^{86}Sr$ stable isotope ratios allowed good discrimination between geographically distant regions, whereas elemental composition contributed more towards distinguishing relatively close regions. The same authors consider the combination of different analytical approaches a very effective tool for compositional characterisation and geographical location of milk and dairy products. This combination could be particularly helpful in the case of fresh milk and PDO cheeses, for which Italian and European law (DM 14 January 2005, EC 2081/92) requires indication of the origin of milk and raw materials used for manufacture on the label.

Our study investigates the possibility of using isotopic and elemental analysis to distinguish cheeses produced in alpine and pre-alpine Italian areas.

2. Material and methods

2.1. Sampling

We considered seven kinds of 'semicotto di media stagionatura' (curd cooked at 40-48 °C, semi-matured) cheeses, five with PDO status (Asiago, EC 1107/96 and 1200/07; Fontina, EC 1107/96; Montasio, EC 1107/96; Spressa, EC 2275/03; Toma, EC 1107/96) and two traditional products (Puzzone and Vezzena, with PDO request underway), produced in alpine/pre-alpine Italian regions (Friuli, Piemonte, Trentino, Valle d'Aosta and Veneto) exclusively using cow's milk. This type of cheese is manufactured by setting the curd at about 32-37 °C, breaking down and cooking it at about 40-48 °C and finally pressing the curd in moulds. This production procedure is traditional in alpine regions and allows an effective whey purge process at about 25 °C in 12-24 hours. After a few days, the cheeses are immersed in a brine solution or processed with dry salt for a period ranging from a few hours (14h) to 3 months. After the salting process, the cheeses are matured for a period ranging from 15 days to 18 months.

Authentic cheeses (N=109) were collected directly from the producers at technological maturity over a period of 3 years in March, June, September and December, in order to include the maximum variability of production and changes in animal diet (percentages of *e.g.* grass, maize silage, flour).

For each cheese, a radial segment of about 1 kg was sampled, with the crust removed, carefully homogenised with an electric mixer (Type 4249, Braun GmbH, Kronberg, Germany), vacuum-packed and stored at -20 °C up to the time of analysis.

The elemental composition and $\delta^{13}C$ and $\delta^{15}N$ in casein, $\delta^{13}C$ and $\delta^{18}O$ in glycerol were measured in all the samples, whereas $\delta^{34}S$ in casein (N=85) and $^{87}Sr/^{86}Sr$ in bulk (N=69) were quantified only in a reduced subset.

2.2. Elemental analysis

Around 0.65 g cheese was weighed directly into a quartz vessel (QXP-1500, CEM, Matthews, USA), with the addition of 4mL HNO_3 (Superpure 65%, Merck, Darmstadt, Germany), 2 mL H_2O_2 (Superpure 30%, Merck) and 1 mL Y internal standard solution (500 mg L^{-1}, Merck) for volume correction. The samples were mineralized in closed vessels using a microwave oven digestor (Mars5, CEM).

Analysis of Al, B, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Sr and Zn was carried out with an ICP–OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Optima 3300 Dual View, Perkin Elmer, Norwalk, USA) using a solution of Tb 2 mg L^{-1} (BDH-Aristar, BDH Laboratory Supplies, Pool, UK) as on–line internal standard. Ag, Be, Cd, Ce, Co, Cs, Dy, Er, Eu, Ga, Gd, Ge, Ho, Ir, La, Li, Lu, Nb, Nd, Pb, Pr, Pt, Rb, Re, Ru, Sb, Sm, Ta, Te, Tl, Tm, U, V and Yb were analysed using an ICP–MS (Inductively Coupled Plasma – Mass Spectrometer, HP–4500 series, Hewlett–Packard Co., Corvallis, USA) with an on–line internal standard solution of Sc 1 mg L^{-1}, Rh 0.5 mg L^{-1} and Tb 0.25 mg L^{-1} all supplied by BDH-Aristar.

The accuracy of the whole process was assured using the certified material 'Whole milk powder'(SRM 8435, NIST-National Institute of Standards & Technology, Gaithersburg, MD, USA) in each analytical batch. Mean recoveries for the certified elements were: 80% for Pb, 100% for Ba, Ca, Cu, Mg, Na, Zn, K and Sr, 110% for B and Mn, 120% for Fe and Mo.

The precision (RSD%) of the process, evaluated by preparing and analysing a cheese sample 10 times, was under 10%, with the exclusion of Ir (11%), Mn (11%), Pt (11%), Ga (13%), Ni (14%), Ce (15%), Cr (16%), Ru (16%), Fe (18%), Al (21%).

2.3. Isotopic analysis

$\delta^{13}C$ and $\delta^{15}N$ were determined in the casein extracted from cheese (Manca et al., 2001) whereas $\delta^{13}C$ and $\delta^{18}O$ were measured in the glycerol extracted (Camin et al., 2004), using an IRMS (Isotopic Ratio Mass Spectrometer; Delta plus XP ThermoFinnigan, Bremen, Germany) after total combustion or pyrolysis of the sample (about 0.6 mg casein and 0.8 mg glycerol) in an Elemental Analyser (EA Flash 1112 ThermoFinnigan) or Pyrolyser (TC/EA, ThermoFinnigan) as described in Camin et al. (2004).

$\delta^{34}S$ was determined in casein using an IRMS (Isoprime, AP2003, GV Instruments Ltd., Manchester, UK) after combustion (about 3 mg casein) in an Elemental Analyser (Vario EL III Elementar Analysensysteme GmbH, Hanau, Germany) (Manca et al., 2006).

The isotope ratios were expressed relatively to international standards (Vienna - Pee Dee Belemnite for $\delta^{13}C$; Vienna - Air for $\delta^{15}N$; Vienna – Standard Mean Ocean Water for $\delta^{18}O$; Canyon Diablo Triolite for $\delta^{34}S$) in δ‰ according to the general formula:

$\delta(‰)=[(R_{sample}-R_{standard})/R_{standard}]*1000$,

where R is the ratio between the heavier isotope and the lighter one.

The values were calculated against international reference materials (NBS-22 fuel oil for $\delta^{13}C$; IAEA-NO3 nitrate for $\delta^{15}N$; glycerol used in the European project SMT4-CT98-2236 for $\delta^{18}O$; NBS-123 ZnS for $\delta^{34}S$) provided by the International Atomic Energy Agency (Vienna, Austria). The precision of measurement, expressed as 2 times the standard deviation when measuring the same sample 10 times, was 0.2, 0.3, 0.5 and 0.3‰ respectively for $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$.

For strontium isotope analysis aliquots of about 100 mg of cheese were dried under a infrared lamp and then ashed in a muffle oven for 2 hours at 850 °C. Remaining ashes were dissolved in 0.5 mL of 9N HNO_3. Purification and accumulation of Sr was achieved using PTFE columns filled with 0.025 g of Sr-specific crown-ether resin (Sr-Spec®, EIChroM Industries, Darien, IL, USA; recipe modified from Horwitz, Chiarizia & Dietz, 1992 and Pin & Bassin, 1992). About 50 mg of Sr was loaded in a mixture of $TaCl_5$, HF, HNO_3, H_3PO_4 and H_2O ("Birk's solution") on single-band W filaments. Strontium isotope ratios were measured in static mode using a TIMS (Thermal Ionisation Mass Spectrometer, MAT 261.5, ThermoFinnigan). Measured isotope values were normalized for mass fractionation using the naturally invariant value for $^{88}Sr/^{86}Sr$ of 8.37521 and the exponential fractionation law. Accuracy and precision of the mass spectrometer runs were controlled by analysing reference material $SrCO_3$ NBS 987 and natural in house standards. During the period of analyses the mean $^{87}Sr/^{86}Sr$ of NBS 987 was 0.710231 ± 0.000027 (1 standard deviation, N=22). The accuracy of measured samples was estimated to be around ± 0.000040 or better. Total blanks for strontium were in the pico gram range and so had no significant influence on measured isotope values.

2.4. Statistical analysis

The data were statistically evaluated using Statistica v8 software (StatSoft Inc., Tusla, Oklahoma, USA) procedures (Kolmogorov-Smirnov test, Box-Cox transformation, ANOVA, Honestly Significantly Different Tukey's test for unequal N, Kruskall Wallis test, multiple bilateral comparison, Pearson's correlation, multivariate Canonical Discriminant Analysis).

3. Results and discussion

3.1. Elemental and isotopic composition

Table 1 shows the distribution statistics for elemental and isotopic composition. The element content was expressed as dry matter in order to correct the effects of different degrees of moisture and ripening. Of the 49 elements analysed, only the distribution of Al, B, Ba, Ca, Co, Cr, Cs, Cu, Fe, Ga, K, Mg, Mn, Mo, Na, Rb, Sr, V and Zn are given, these elements being detectable in at least 65% of the samples. Ag, Cd, Ce, Dy, Er, Eu, Gd, Ge, La, Li, Lu, Nb, Nd, Ni, Pb, Pr, Re, Ru, Sb, Sm, Ta, U and Yb were present in detectable amounts only in a lower % of samples, whereas none of the samples showed detectable amounts of Be, Ho, Ir, Pt, Te, Tl and Tm. All the data were normally distributed (Kolmogorov-Smirnov test) in each of the seven types of cheese, with the exception of B, Ce, Cu, Mo and V, which were normalised using Box-Cox transformation before any further statistical evaluation.

Table 2 shows the correlation coefficients, slopes and intercepts of the regression lines between parameters that were shown to be statistically significant ($P < 0.001$) in Person's correlation test.

The $\delta^{13}C$ of casein and glycerol were shown to be closely correlated, confirming the previous results of Camin et al. (2004) and Pillonel et al. (2003). Higher δ-values in casein as compared to glycerol could be explained by ^{13}C depletion occurring during the synthesis of lipids. The $\delta^{15}N$ and $\delta^{34}S$ values, affected by similar variability factors such as fertilisation practices, climatic conditions, closeness to the sea *etc.* were not significantly correlated, confirming previous results found in cow's milk from alpine regions (Rossmann, Kornexl, Versini, Pichlmayer & Lamprecht, 1998). This significant correlation ($r = 0.77$) had been observed on European scale for cow's and sheep cheeses from France, Italy and Spain by Camin et al. (2004).

Both $\delta^{13}C_{casein}$ and $\delta^{13}C_{glycerol}$ correlated significantly, positively with.$\delta^{15}N$ and negatively with $^{87}Sr/^{86}Sr$ ($P < 0.001$). No statistically significant correlations were found between isotopic and elemental parameters.

Some 'technological' elements correlated with each other, as expected (Coni et al., 1999; Fresno et al., 1995) and in particular Fe correlated positively with Al and Mn, as did Zn with Cr. Some of the elements of the I and II groups of the periodic table correlated with each other as reported by Cichoscki et al. (Cichoscki, Valduga, Valduga, Tornadijo & Fresno, 2002) and Fresno et al. (1995) for Spanish cheeses. In particular Cs and Rb correlated positively with Ba, K positively with Mg, Na negatively with Sr.

3.2. Product differentiation

Table 1 shows the statistically significant differences ($P < 0.001$) between cheeses in relation to values for 12 parameters. Such differences were confirmed by applying both parametric statistical approaches (ANOVA, HSD Tukey's test for unequal N) and non-parametric statistical approaches (Kruskall Wallis and multiple bilateral comparison tests, applied to raw data). In particular, statistically lower Ca content was found in Toma, Ca and Mg in Fontina, Ba, Ga and Rb in Montasio and K in Puzzone. According to Coni et al. (1999), Fresno et al. (1995) and Pillonel et al. (2003), such differences may depend on manufacturing processes, in particular coagulation conditions, salting, releases from manufacturing equipment and concentrates fed to the animals, as well as geographical origin.

Both $\delta^{13}C_{casein}$ and $\delta^{13}C_{glycerol}$ made it possible to discriminate Fontina from Montasio. As regards the former cheese, on the basis of the isotope values and using the relation given by Camin et al. (2008), we can estimate that the maize uptake in the animals' diet was at maximum/no more than 10%, fully complying with the 20% level established in the PDO production protocol. As regards Montasio, no limits have been established for maize in the PDO protocol and the higher values observed by us suggest a contribution of about 45-70%. For Asiago, Puzzone and Toma no limits have been established in the production protocol and the estimated contributions ranged between 20% and 40%, 16% and 20% and 40% and 60% respectively. Spressa and Vezzena production protocols provide for a maximum use of fodder, usually with a high but not legally established maize content, of 50% in the diet and the estimated contributions were 10-17% and 19-25% respectively.

Asiago and Montasio showed statistically higher $\delta^{18}O$ values than Fontina, Puzzone and Spressa, probably because the latter cheeses are produced at higher altitude and with a lower contribution of C4 plants, which have higher $\delta^{18}O$ and $\delta^{13}C$ values (Camin et al., 2008; Schmidt, Werner & Rossmann, 2001).

$\delta^{15}N$ made it possible to distinguish Toma and Vezzena, the latter with significantly lower values, confirming what has previously been observed in cheeses (Pillonel et al., 2003) and lamb meat samples (Camin et al., 2007) produced at high altitudes as compared to products from lower lying areas.

$^{87}Sr/^{86}Sr$ significantly distinguished Asiago and Montasio from Spressa, Fontina and Toma ($P < 0.001$) at lower values. Old acidic rock such as granite shows the highest $^{87}Sr/^{86}Sr$ ratios, whereas mafic and carbonate-rich rocks have the lowest (Figure 1) (Pillonel et al., 2003). Indeed, the lowest $^{87}Sr/^{86}Sr$ values were measured in Asiago and Montasio cheeses, the ratio of the former being clearly influenced by Tertiary basalt underlying pasture, while that of the latter is influenced by the river deposit sediment deriving from Mesozoic and Tertiary basalt in the Carnic Alps. In the area of Lavarone, where Vezzena is produced, Mesozoic carbonate-rich rocks with low $^{87}Sr/^{86}Sr$ values prevail, whereas values close to zero (Puzzone), are typical for cheeses from alluvial plain terraces with large catchment areas.

Standard multivariate Canonical Discriminant Analysis (CDA) was performed on the data in order to check for the possibility of discriminating between cheeses. $^{87}Sr/^{86}Sr$ and $\delta^{34}S$ were not considered because they were only measured in a subset of samples. Twelve parameters (Ba, Ca, Cu, Ga, K, Mg, Rb, Zn, $\delta^{13}C_{casein}$, $\delta^{15}N$, $\delta^{13}C_{glycerol}$, $\delta^{18}O$) were shown to be significantly different at HSD for unequal N Tukey and multiple bilateral comparison tests.

To make the model more robust against interference due to Cu and Zn releases from dairy equipment, we did not use these elements in the CDA. We also removed $\delta^{13}C_{glycerol}$ as it is significantly correlated (Pearson's correlation coefficient r = 0.966) with $\delta^{13}C_{casein}$ and we chose to retain the latter because casein is more easily extractable from the bulk cheese. Finally, Ga was removed by the CDA model as not statistically significant.

The first 3 canonical variables (Rad. 1, Rad. 2, Rad. 3) explained 91 % of total variability (Figures 2a and 2b). Rad.1 was loaded positively with $\delta^{13}C_{casein}$, Ba and $\delta^{18}O$ (standardised coefficients 1.58, 1.39 and 0.64 respectively) and negatively with Ca (-0.59). Rad. 2 was mainly loaded positively with $\delta^{13}C_{casein}$ (0.58) and Mg (0.57) and negatively with $\delta^{15}N$ (-1.18), K (-0.60) and Ba (-0.53) whereas Rad. 3 was loaded positively by Rb (1.09) and Mg (0.65) and negatively by Ba (-0.78).

Overall, 94 % of the samples were correctly classified (Table 2), with a re-classification percentage of 100 % in the case of Fontina and Puzzone.

Model predictivity and stability were proved by re-calculating 3 different models after removing 20 randomly selected cheeses (corresponding to 20% of the original database) each time and applying these models to the original data-set, obtaining correct classification for at least 90%.

In conclusion, the paper defined the compositional characteristics of authentic traditional alpine and pre-alpine Italian cheeses using multi-element stable isotope ratios and mineral elements. Such analytical parameters can effectively contribute towards distinguishing different kinds of cheese and supporting the demand for or the existence of a Protected Designation of Origin.

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Table 1

Elemental and isotopic composition of the cheeses.

	ASIAGO (N=16)		FONTINA (N=16)		MONTASIO (N=16)		PUZZONE (N=14)		SPRESSA (N=15)		TOMA (N=16)		VEZZENA (N=16)	
	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.
Al (mg kg^{-1})	2.35	1.01	2.63	0.79	2.88	1.49	2.26	1.03	2.38	1.30	2.79	1.50	1.90	0.88
B (mg kg^{-1})	0.37	0.21	0.41	0.24	0.53	0.26	1.09	1.21	0.46	0.33	0.56	0.26	0.56	0.31
Ba (mg kg^{-1})	1.16^{ab}	0.43	0.74^{ac}	0.28	0.53^{c}	0.13	1.22^{ab}	0.22	1.57^{b}	0.28	1.23^{ab}	0.67	0.97^{abc}	0.27
Ca (mg kg^{-1})	14.4^{ab}	1.56	12.9^{a}	2.01	13.3^{ab}	1.25	12.7^{ab}	1.63	15.9^{b}	1.27	12.3^{a}	1.18	14.6^{ab}	1.94
Co (mg kg^{-1})	7.43	2.99	6.45	2.15	6.66	3.32	9.57	5.46	8.09	3.59	7.39	4.18	8.31	3.50
Cr (mg kg^{-1})	0.26	0.10	0.25	0.08	0.25	0.10	0.29	0.18	0.38	0.22	0.24	0.09	0.27	0.09
Cs (mg kg^{-1})	4.68	2.43	6.67	2.49	3.15	1.82	6.69	4.08	8.98	4.61	7.24	6.08	6.95	2.88
Cu (mg kg^{-1})	5.20^{ab}	4.03	6.19^{ab}	5.33	1.38^{a}	3.40	3.35^{a}	5.79	13.8^{c}	2.90	0.45^{a}	0.36	8.90^{bc}	2.93
Fe (mg kg^{-1})	1.85	0.63	2.25	0.33	2.03	0.69	2.07	1.01	2.36	2.09	2.02	0.52	1.95	0.32
Ga (mg kg^{-1})	51.0^{ab}	28.5	38.6^{ab}	25.2	21.3^{a}	8.18	64.0^{ab}	28.1	80.9^{b}	36.8	60.9^{ab}	32.4	43.9^{ab}	26.3
K (mg kg^{-1})	1.69^{a}	0.17	1.73^{ab}	0.23	1.69^{ab}	0.22	1.49^{b}	0.41	1.95^{a}	0.22	1.78^{a}	0.21	1.80^{a}	0.21
Mg (mg kg^{-1})	0.54^{a}	0.05	0.43^{b}	0.07	0.49^{ab}	0.06	0.46^{ab}	0.11	0.56^{a}	0.06	0.45^{ab}	0.06	0.55^{a}	0.10
Mn (mg kg^{-1})	0.28	0.05	0.31	0.06	0.27	0.05	0.29	0.09	0.32	0.05	0.37	0.11	0.33	0.07
Mo (mg kg^{-1})	0.23	0.12	0.45	0.14	0.20	0.12	0.30	0.14	0.20	0.08	0.19	0.13	0.32	0.13
Na (mg kg^{-1})	12.5	3.05	11.6	3.13	11.2	3.66	9.66	2.82	13.2	2.59	13.3	2.98	11.6	2.18
Rb (mg kg^{-1})	2.57^{ab}	1.34	3.29^{ab}	1.18	1.41^{a}	0.44	2.07^{ab}	0.63	5.37^{b}	2.81	4.02^{ab}	3.32	4.74^{b}	2.04
Sr (mg kg^{-1})	4.76	1.53	4.73	1.55	4.45	1.94	6.31	2.67	5.72	1.33	4.52	0.87	3.55	0.86
V (mg kg^{-1})	11.9	8.13	12.2	7.08	12.3	7.90	11.2	6.83	12.5	7.03	18.3	8.38	8.18	4.77
Zn (mg kg^{-1})	65.0^{ab}	5.11	57.4^{a}	5.96	59.9^{a}	5.00	65.7^{ab}	8.42	74.0^{b}	5.96	56.4^{a}	5.65	74.5^{b}	7.68
$\delta^{34}S_{casein}$	5.2	0.5	4.5	0.6	5.2	0.5	4.6	0.4	5.0	0.3	5.4	0.9	4.9	0.4
$^{87}Sr/^{86}Sr$	-1.748^{a}	0.942	0.572^{b}	0.896	-2.209^{a}	1.933	-0.332^{ab}	0.257	0.718^{b}	0.143	0.458^{b}	0.925	-0.695^{ab}	0.177
$\delta^{18}O_{glycerol}$	18.0^{a}	0.9	14.9^{b}	2.6	17.9^{a}	0.8	15.5^{ab}	1.1	15.8^{ab}	0.8	17.0^{ab}	1.1	16.2^{ab}	1.4
$\delta^{13}C_{casein}$	-20.0^{ab}	1.4	-23.3^{c}	0.6	-18.1^{a}	0.6	-21.7^{bc}	0.5	-22.2^{c}	0.5	-20.1^{ab}	2.4	-22.0^{c}	0.8
$\delta^{15}N_{casein}$	4.9^{a}	0.4	4.6^{abc}	0.3	4.8^{abc}	0.4	4.3^{bc}	0.2	4.6^{ac}	0.2	5.2^{a}	0.4	4.2^{b}	0.4
$\delta^{13}C_{glycerol}$	-25.1^{a}	1.6	-29.5^{d}	0.7	-22.6^{b}	0.9	-26.8^{ac}	0.8	-27.7^{cd}	0.8	-25.1^{ab}	3.2	-27.7^{cd}	1.2

Table 2

Pearson' correlation coefficient (r), intercept (I) and slope (S) of the correlations between the analytical parameters. Only the statistically significant relationships ($P < 0.001$) are shown.

		Al	B	Ba	Ca	Co	Cr	Cs	Fe	Ga	K	Mg	Mn	Na	$\delta^{13}C_{casein}$	$\delta^{13}C_{glycerol}$	$^{87}Sr/^{86}Sr$
Cr	r				0.44												
	I				-0.06												
	S				0.02												
Fe	r	0.42															
	I	1.66															
	S	0.24															
Mg	r		-0.42		0.75						0.43						
	I		0.55		-0.04						0.22						
	S		-0.06		0.04						0.17						
Cs	r			0.56													
	I			-0.87													
	S			6.26													
Ga	r			0.77		0.50		0.64									
	I			-5.52		15.5		32.2									
	S			50.4		6.28		3.74									
Mn	r			0.38				0.40	0.36								
	I			0.28				0.30	0.25								
	S			0.04				0.004	0.04								
Rb	r			0.65				0.75		0.64			0.40	0.37			
	I			0.80				1.99		1.66			-0.89	-0.20			
	S			2.18				0.22		0.03			13.6	0.31			
Zn	r				0.68		0.38					0.64					
	I				26.4		57.2					39.6					
	S				2.79		28.2					49.6					
Sr	r													-0.36			
	I													7.74			
	S													-0.23			
$\delta^{15}N$	r														0.54	0.53	
	I														7.38	7.18	
	S														0.13	0.10	
$\delta^{13}C_{glycerol}$	r														0.98		-0.45
	I														0.94		-26.7
	S														1.30		-0.82
$\delta^{13}C_{casein}$	r																-0.45
	I																-21.4
	S																-0.63

Table 3

Results of the classification matrix of the cheeses based on Ba, Ca, K, Mg, Rb, $\delta^{13}C_{casein}$, $\delta^{15}N$ and $\delta^{18}O$, achieved applying Reclassification Discriminant Analysis

	% correctly classified	ASIAGO	FONTINA	MONTASIO	PUZZONE	SPRESSA	TOMA	VEZZENA
ASIAGO	88	14	0	0	0	1	1	0
FONTINA	100	0	16	0	0	0	0	0
MONTASIO	94	1	0	15	0	0	0	0
PUZZONE	100	0	0	0	14	0	0	0
SPRESSA	93	0	0	0	0	14	0	1
TOMA	81	1	0	1	0	1	13	0
VEZZENA	100	0	0	0	0	0	0	16
Total	94	16	16	16	14	16	14	17

Fig. 1. Synoptic diagram showing the $^{87}Sr/^{86}Sr$ variances (mean values and standard deviations) of Asiago, Fontina, Montasio, Puzzone, Spressa, Toma and Vezzena cheeses.

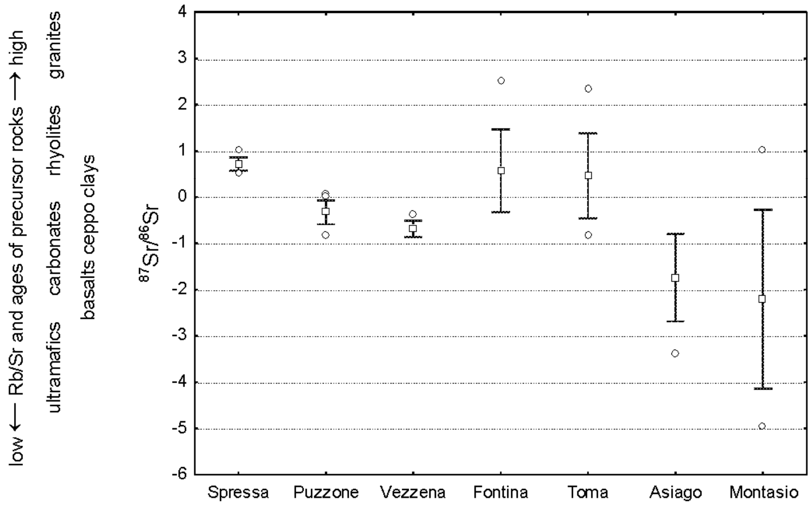


Fig. 2a. Canonical Discriminant Analysis of the isotopic and elemental composition of the cheeses. Scatterplot of the first two canonical variables.

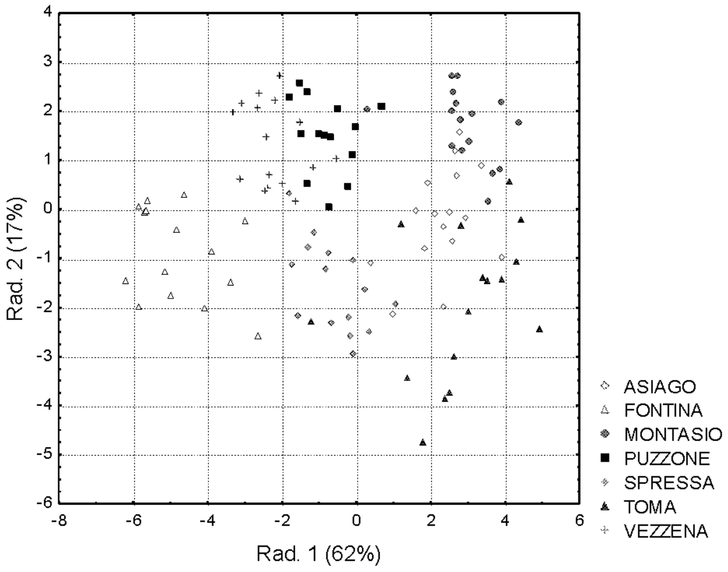
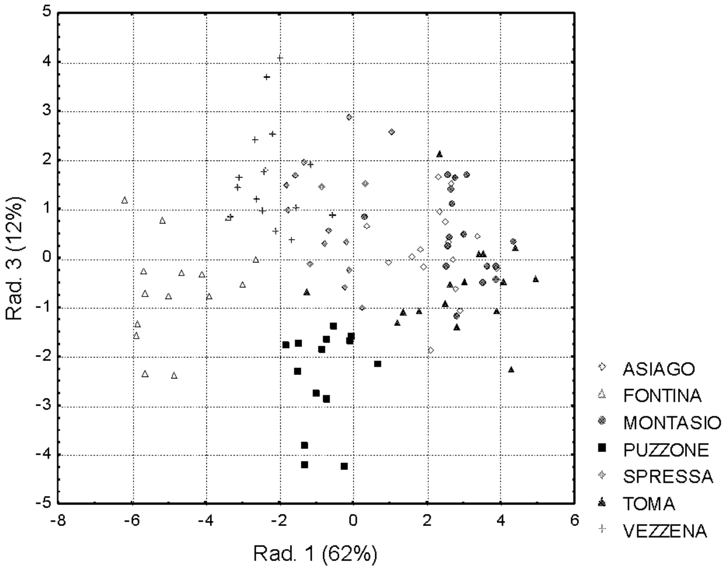


Fig. 2b. Canonical Discriminant Analysis of the isotopic and elemental composition of the cheeses. Scatterplot of the first and the third canonical variables.



Results

Both $\delta^{13}C_{casein}$ and $\delta^{13}C_{glycerol}$ made it possible to estimate maize uptake in the animals' diet, allowing researchers to verify whether the levels established in the PDO production protocol were observed. Cheeses produced at higher altitude showed $\delta^{18}O$ and $\delta^{15}N$ values statistically lower as compared to products from lower lying areas. Therefore these parameters could be very useful in the differentiation of mountain products. The statistical model (Canonical Discriminat Analysis) built on the basis of the most significant analytical parameters (Ba, Ca, K, Mg, Rb, $\delta^{13}C_{casein}$, $\delta^{15}N_{casein}$, $\delta^{18}O_{glycerol}$) achieved 94 % of product type discrimination, 100% in the case of Fontina and Puzzone. Thus this model can be proposed as a suitable tool for the detection of mislabelling and for consumer protection, demonstrating that such analytical parameters can effectively contribute towards distinguishing different kinds of cheese and supporting the demand for or the existence of a Protected Designation of Origin.

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CHAPTER 6 TOMATOES AND DERIVATIVES

6.1 INTRODUCTION

As previously stated about olive oils and PDO/PGI cheeses, law establishes that the geographical origin of fresh tomatoes used in passata production must be declared on the label but does not indicate which parameters should be used to establish whether the declared origin is truthful.

After potatoes, the tomato is the second most important vegetable crop in the world. World production currently stands at around 141 million tons of fresh fruit produced in 2009 on an area of around 5 million hectares (data provided by FAO, http://faostat.fao.org/).

In recent years the quantity of tomatoes consumed around the world has increased on average by 3% yearly (http://www.researchandmarkets.com/reports/c55316), perhaps due to the healthy properties ascribed to tomatoes and in particular to some of its components, such as lycopene.

Tomato products are the most popular sauce ingredient for Italians, who use around 550 million kilograms of them a year (http://www.coldiretti.it). More specifically, each family buys on average 31 kg of tomato products a year, preferring peeled tomatoes (12 Kg), passata (11 Kg), pulp (5 Kg) and paste (3 Kg) in descending order. This type of product is of major importance for the Italian economy, as Italy is the third largest producer of tomatoes of paste varieties in the world with around 5 million tons, after the USA (around 11 million tons) and China (around 6.5 million tons) (data provided by ISMEA, September 2009 report, http://www.ismea.it), and the biggest exporter of tomato products, to the tune of 700 million dollars, according to 2007 figures (data provided by FAO, http://faostat.fao.org). However, in the last few years the outlook for the production and trading of paste tomato varieties has changed rapidly. In 2008 around 780 thousand tons of passata were manufactured in Italy, but only about 436 thousand tons were entirely Italian product (data provided by ISMEA, September 2009 report). During the last ten years, Italian imports of paste from China have quadrupled (+272 %), becoming the preeminent import and during 2010 will reach around 100 million kilograms, representing about 15% of Italian fresh tomatoes used for processing. The opportunity of palming off products originating outside the European Community as Italian products leads to a range of problems. Firstly the consumer's purchase is not guaranteed and he has greater difficulty in obtaining the genuine product, but above all he is exposed to possible health risks because the regulations in countries outside the EU are less restrictive than European regulations. This situation also has a negative influence on farmers, who are underpaid for their products, seeing a decline of around 10% in 2010. The situation worsens if one considers that the Italian tomato industry currently employs about 20 thousand people, including around eight thousand farms cultivating tomatoes on 85 thousand hectares of land and supplying 173 factories (http://www.coldiretti.it).

Italian law has intervened in the question of 'tomatoes', mainly with the scope of promoting food safety and avoiding the detrimental effect of economic fraud on consumers and reputable traders.

The Ministerial Decree 23rd September 2005 of the Italian Ministry of Agricultural, Food and Forestry Policy first of all specifies that passata is "a product obtained directly from sound ripe fresh tomatoes, having the colour, flavouring and taste characteristics of the fruit from which it originates, made by squeezing, possible separation of peels and seeds and partial water removal so that the refractometric extract ranges between 5 and 12 Brix degrees (±3%) net of added salt". The same decree indicates the ranges for some parameters (total sugar content, pH, Howard Mould Count - HMC, mineral traces, lactic acid, presence of peels and seeds) and the ingredients permitted (salt, acidity regulators, spices, aromatic herbs). Finally, the decree establishes that it is not permitted the concentration of tomato juice above 12 Brix degrees and its following dilution to obtain reconstituted passata. $\delta^{18}O$ measurement in vegetal water (UNI ENV 12141 method) is indicated as the official method for detecting the fraudulent addition of water. Vegetal water has $\delta^{18}O$ values significantly higher than those for source water, following isotopic fractionation processes in plants.

Since 15 June 2006, the geographical origin of the fresh tomatoes used in passata production must be declared on the label (Ministerial Decree 17th February 2006). However this decree does not establish which parameters should be used to establish whether the declared origin is correct.

6.2 STABLE ISOTOPE RATIOS AND ELEMENTAL COMPOSITION OF TOMATOES

Few papers have been written on the stable isotope ratios of bioelements in tomatoes and tomato products. In the last few years consumers have tended to prefer organically produced food, which is generally considered to be healthier than conventional products. Therefore many studies have been carried out in order to verify this platitude scientifically. Nakano and Uehara (2007) found that the $\delta^{15}N$ values of tomatoes were similar to those of the fertiliser applied for organic fertilisation, while for chemical fertilisers the values were between the $\delta^{15}N$ values of the fertiliser applied and the soil. The $\delta^{15}N$ values of fruit were below +3.1‰ for chemical fertilisers and over +8.7‰ for organic fertilisers, so they suggested a threshold of around +4‰ to distinguish the two agricultural practices. Schmidt and colleagues (2005) measured the $\delta^{18}O$, $\delta^{34}S$, $\delta^{13}C$ and $\delta^{15}N$ values of tomatoes cultivated organically and conventionally. As expected the $\delta^{15}N$ values of products from organic cultivations were quite high, whereas those of fruit from conventional production were relatively low. Furthermore they found that the $\delta^{13}C$ values of tomatoes grown in the Netherlands were extremely negative. This situation was due to the feeding of CO_2 into greenhouses, recycled from methane heating plants. Bateman and colleagues (2007) reported that the different types of fertilisers commonly used in organic and conventional systems result in differences in the nitrogen isotope composition of tomatoes. They found a mean $\delta^{15}N$ value of 8.1‰ for organically grown tomatoes and a mean value of −0.1‰ for those grown conventionally. However the overlap between the $\delta^{15}N$ values of the organic and conventional datasets suggested that the use of this parameter had some limitations. So Kelly and Bateman (2010) combined trace element and nitrogen isotope data and improved the correct classification of tomato samples. These results suggested that systematic differences in the concentrations of certain elements (Mn, Ca, Cu, Zn) occurred between crops cultivated under organic and conventional regimes as a result of the presence of elevated levels of arbuscular mycorrhizal fungi (AMF) in ‘organic soils’.

Other studies focused on the behaviour of stable isotope ratios in the different parts of tomato plants or in stressful situations (Choi et al., 1997; Terwilliger et al., 1996). In particular Bong and colleagues (2008) proved that $\delta^{18}O$ and δD in fast-growing fruits such as tomatoes are subject to greater evaporation than in slow-growing fruits (plums and grapes) under the same climatic conditions, maybe because evaporation intensity depends on the season during which plants grow more intensively. Kanai and colleagues (2008) showed that $\delta^{13}C$ and $\delta^{15}N$ in tomato fruit have an opposite trend in the case of N-deficiency. In particular, ^{13}C, fed through the leaf *via* $^{13}CO_2$, initially accumulated mostly in the fruit but over the course of time receded in the fruit and increased in the leaves and other parts of the stem. Nitrogen deficiency did not influence ^{13}C content in fruits. ^{15}N, fed through the roots, dislocated preferentially to the leaves and then to the fruit, however nitrogen deficiency significantly increased partitioning in favour of the fruit.

Pfammatter et al. (2004) used $\delta^{18}O$ in vegetal water to discriminate tomatoes from five different Swiss cantons. The same parameter was useful for differentiating Swiss tomatoes from fruits from Belgium, Israel, Spain, the Netherlands, Morocco and Italy.

Trifirò and colleagues (2007), considered the $\delta^{18}O$ of vegetal water in fresh fruits, passata, paste and diluted paste, verifying the usefulness of this parameter, statutory according to Italian law (Ministerial Decree 23rd September 2005), in detecting the fraudulent addition of water during the production of passata. $\delta^{18}O$ values increased in tomato products concentrated by evaporation and passata showed $\delta^{18}O$ values significantly higher than for products obtained by dilution of concentrates.

Many papers have been written on the mineral composition of tomatoes but the topic has mainly been investigated from a nutritional point of view. In particular, toxic (Pb, Cd, As, Al) and biologically essential (*e.g.* Ca, Mg, K, P, Cr) elements were determined in tomatoes in order to verify mean intake through the human diet (Bosque et al., 1990; Bratakos et al., 2002; Ekholm et al., 2007; Bronkowska et al., 2008; Demirbas, 2010; Karavoltsos et al., 2008). As for stable isotope ratios, some studies have focused on differences in the mineral composition of organic and conventionally grown tomatoes. Rossi and colleagues (2008) compared the heavy metal content of tomatoes grown using conventional, integrated pest management (IPM) and organic farming techniques. They found that conventional tomato samples showed the lowest contamination levels for Cu and Cd. No significant differences in Cd levels were observed between tomatoes grown with IPM systems and those grown using organic techniques, whereas higher levels of Cu were found in IPM products. Organic samples showed the highest levels of Pb but this situation could be explained by higher input of the metal through manure. However, the levels of contaminants detected in this study were markedly lower than the maximum limits allowed by law. In two different studies, Moral and co-workers (2002) and Cenariu and colleagues (2008) also found that tomato plants cultivated organically generally showed the highest mineral content. Specifically, Moral et al. found that there was a higher concentration of Cd in the aerial part of tomato plants grown in organic soils than in those cultivated conventionally. Moreover the same authors investigated the displacement of Cd in different parts of tomato plant. In particular they found that the fruit accumulates lower levels of Cd than other parts of the plant. Cd in the fruit of the tomato was one order of magnitude lower than Cd in leaves. Gundersen and co-workers (2001) determined the concentration of some major and trace elements in tomato fruit grown in three different substrate systems (soil, rockwool irrigated with two different nutrient solutions). They found that concentrations of Ca, Cd, Fe, Mn, Mo, Na, Ni, Sr, and Zn were also significantly different for tomatoes grown on the different substrates. The concentrations of Ca, Cd, Fe, Mn Na, Ni, Sr, Zn Cu, K, Mg, P, Sn, and V were significantly different for different harvest times. It is worth noting that the authors found a Cd concentration more than 15 times higher and a Ca concentration 50-115% higher in soil-grown fruit than in rockwool-grown fruit.

Finally, a few authors have considered the elemental composition of tomatoes and in one case paste for the purposes of characterising them and differentiating products from different countries or cultivars (Loiudice et al., 1995; Imhof et al., 2004; Hernandez-Suarez et al., 2007; Hernandez-Suarez et al., 2008; Guil-Guerrero and Rebolloso-Fuentes, 2009). Imhof and colleagues (2004) investigated the amount of Cd, Pb, Co, Ni, Cu, Zn, Mn, Mo, Li, Na, K, Rb, Mg, Ca, Sr, Al and Ba in tomatoes. They were able to distinguish tomatoes grown on different substrates (hors-sol, sol and field grown) and from different countries (Swiss, Spain and the Netherlands). Hernandez-Suarez and colleagues (2007, 2008) found that concentrations of K, Mg, Ca, Na, Fe, Cu, Zn and Mn in tomatoes are affected by many factors, such as cultivar, cultivation method, production region and sampling period. Finally, LoFeudo and co-workers (2010) were able to discriminate between tomato samples cultivated in different Italian areas and between tomato paste samples from different countries using elemental composition (Italy, China, Greece, California).

SECTION 6.3

Traceability along the production chain of Italian tomato products on the basis of stable isotopes and mineral composition

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Rapid Communications in Mass Spectrometry (in press)

Aim

The aim of this work was to verify how $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$, δD and the mineral content of tomatoes and derivatives vary along the production chain and secondly, to evaluate their usefulness as geographical origin markers independently of the technological process applied.

Specifically, the study determined $\delta^{13}C$, $\delta^{15}N$, δD, $\delta^{34}S$ and the $\delta^{18}O$ of bulk and $\delta^{18}O$ of vegetable water, as well as the elemental composition of around three hundred authentic Italian samples of tomato, tomato juice, passata and paste officially collected by the Italian Ministry of Agricultural, Food and Forestry Policy in Emilia Romagna, Apulia and Piedmont. δD and $\delta^{18}O_{bulk}$ have never been reported in the literature to date.

Traceability along the production chain of Italian tomato products on the basis of stable isotopes and mineral composition

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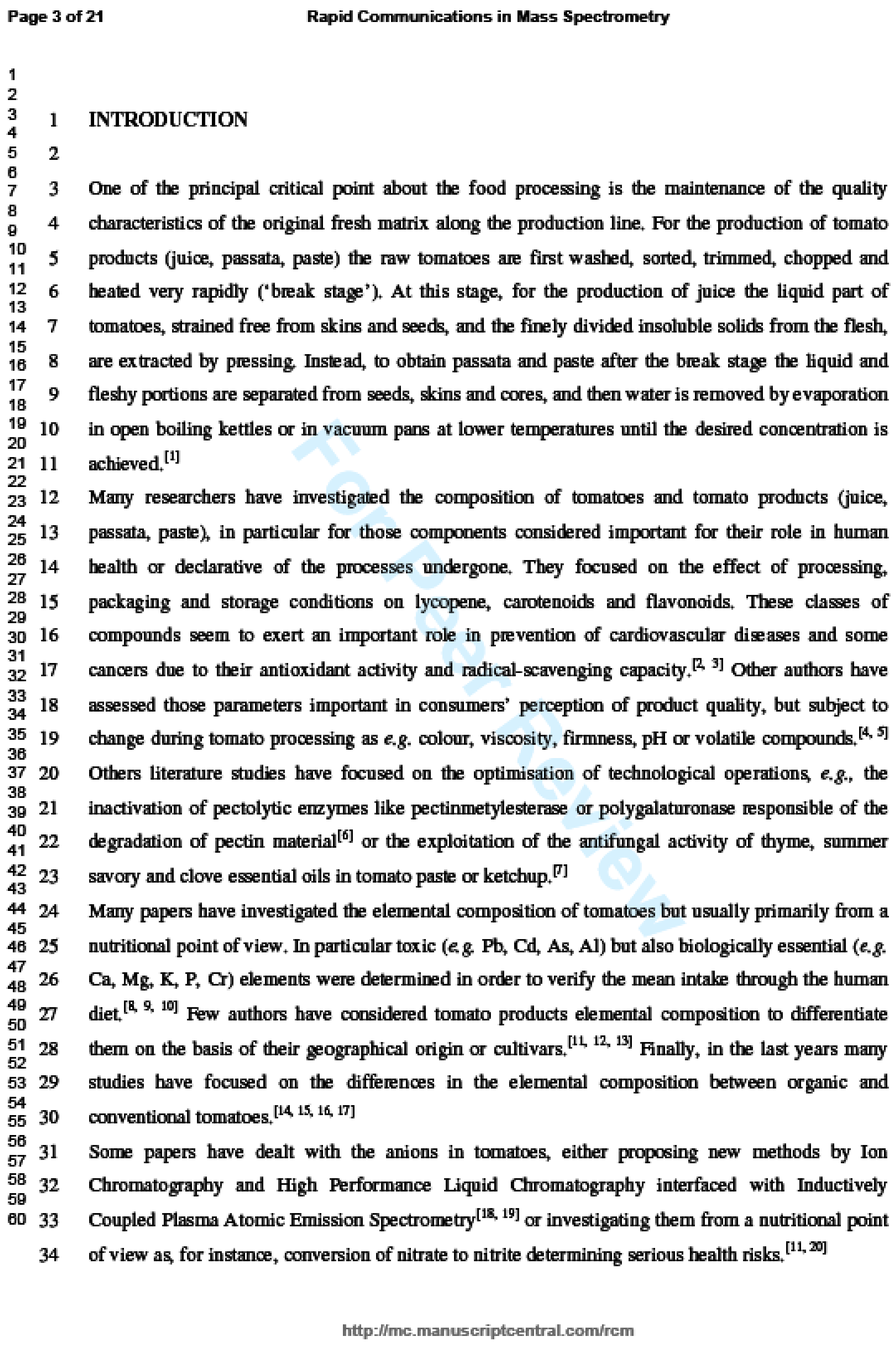
Abstract

The paper shows the variability of stable isotope ratios and mineral composition in tomato and derivatives along the production chain (juice, passata and paste) in order to evaluate the possibility of tracing the geographical origin. The ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, D/H, $^{34}S/^{32}S$ and the content of Li, Be, B, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Mo, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Tm, Yb, Ir, Tl, Pb, U and of nitrates, chlorides, sulphates and phosphates were measured by Isotope Ratio Mass Spectrometry, Inductively Coupled Plasma Mass Spectrometry and Ion Chromatography, respectively. Tomato products were from three Italian regions (Piedmont, Emilia-Romagna, Apulia). By applying a linear discriminant analysis on seventeen of these parameters (Gd, La, Tl, Eu, Cs, Ni, Cr, Co, $\delta^{34}S$, $\delta^{15}N$, Cd, K, Mg, $\delta^{13}C$, Mo, Rb and U) an excellent discrimination among products from the three regions was achieved. Irrespective of the processing technology, over 95% of the samples were correctly reclassified in crossvalidation into the production site. The use of these parameters will allow the development of analytical control procedures usable to check the geographical provenance of Italian tomatoes and derivatives.

Keywords:

geographical origin; IRMS; ICP MS; IC; passata



INTRODUCTION

One of the principal critical point about the food processing is the maintenance of the quality characteristics of the original fresh matrix along the production line. For the production of tomato products (juice, passata, paste) the raw tomatoes are first washed, sorted, trimmed, chopped and heated very rapidly ('break stage'). At this stage, for the production of juice the liquid part of tomatoes, strained free from skins and seeds, and the finely divided insoluble solids from the flesh, are extracted by pressing. Instead, to obtain passata and paste after the break stage the liquid and fleshy portions are separated from seeds, skins and cores, and then water is removed by evaporation in open boiling kettles or in vacuum pans at lower temperatures until the desired concentration is achieved.[1]

Many researchers have investigated the composition of tomatoes and tomato products (juice, passata, paste), in particular for those components considered important for their role in human health or declarative of the processes undergone. They focused on the effect of processing, packaging and storage conditions on lycopene, carotenoids and flavonoids. These classes of compounds seem to exert an important role in prevention of cardiovascular diseases and some cancers due to their antioxidant activity and radical-scavenging capacity.[2, 3] Other authors have assessed those parameters important in consumers' perception of product quality, but subject to change during tomato processing as *e.g.* colour, viscosity, firmness, pH or volatile compounds.[4, 5] Others literature studies have focused on the optimisation of technological operations, *e.g.*, the inactivation of pectolytic enzymes like pectinmetylesterase or polygalaturonase responsible of the degradation of pectin material[6] or the exploitation of the antifungal activity of thyme, summer savory and clove essential oils in tomato paste or ketchup.[7]

Many papers have investigated the elemental composition of tomatoes but usually primarily from a nutritional point of view. In particular toxic (*e.g.* Pb, Cd, As, Al) but also biologically essential (*e.g.* Ca, Mg, K, P, Cr) elements were determined in order to verify the mean intake through the human diet.[8, 9, 10] Few authors have considered tomato products elemental composition to differentiate them on the basis of their geographical origin or cultivars.[11, 12, 13] Finally, in the last years many studies have focused on the differences in the elemental composition between organic and conventional tomatoes.[14, 15, 16, 17]

Some papers have dealt with the anions in tomatoes, either proposing new methods by Ion Chromatography and High Performance Liquid Chromatography interfaced with Inductively Coupled Plasma Atomic Emission Spectrometry[18, 19] or investigating them from a nutritional point of view as, for instance, conversion of nitrate to nitrite determining serious health risks.[11, 20]

Little information is available on the stable isotope ratios of bioelements in tomato and its derivatives (D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ expressed as δD, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ respectively). Kelly and Bateman[17] and Nakano and Uehara[21] focused on the differences in $\delta^{15}N$ values between organically (higher values) and conventionally (lower values) grown fruits, whereas Schmidt et al.[22] considered also $\delta^{18}O$ of water contained in fruits (vegetal water), $\delta^{34}S$ and $\delta^{13}C$, being this last heavily affected by the recycling of CO_2 from methane' heating system in greenhouses. Other studies were focused on the behaviour of stable isotope ratios in tomato plants or in stressful situations.[23] In particular Bong and colleagues[24] measured $\delta^{18}O$ and δD of vegetal water in fast- and slow- growing fruits proving that fast-growing fruits like tomato experience much stronger evaporation than slow-growing fruits whereas Kanai and colleagues[25] showed that $\delta^{13}C$ and $\delta^{15}N$ of tomato fruits have an opposite trend in case of N-deficiency. Pfammatter et al.[26] used $\delta^{18}O$ of vegetal water to discriminate Swiss tomatoes from different cantons and from foreign fruits. Only Trifirò and colleagues[27] considered the $\delta^{18}O$ of vegetal water in fresh fruits, passata, paste and diluted paste, verifying the usefulness of this statutory parameter provided by the Italian law (Ministerial Decree 23rd September 2005) to detect fraudulent addition of water during production of passata. They showed that this value increases in tomato products during concentration by evaporation and that passata obtained by dilution of concentrates with water shows significantly lower values.

Mineral and isotopic compositions have been used separately or together since thirty years to support food characterization and geographical traceability assuring authenticity.[28, 29, 30, 31, 32, 33, 34] To date, the impact along the production line from fresh material to final food product of the different processing steps on the mineral and isotopic compositions of tomatoes and derivatives have been poorly investigated.

The aim of this paper is firstly to fill this lack of knowledge verifying how $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$, δD and the mineral content in tomatoes and derivatives vary along the production chain and, secondly, to evaluate their usefulness as geographical origin markers independently on the technological process applied.

MATERIALS AND METHODS

Sampling

320 authentic Italian samples of tomato (N= 80), tomato juice (N= 79), passata (N= 104) and paste (N= 57) were officially collected in eight firms by the Italian Ministry of Agricultural, Food and Forestry Policy in the period August-September of 2007 and 2008 harvests. The samples were from

Emilia Romagna, Apulia, Piedmont, three of the most important Italian producers of paste tomato varieties (data provided by ISTAT – Istituto Nazionale di Statistica; http://agri.istat.it). Immediately after collection the samples were frozen and stored at -20 °C until the analyses. $\delta^{13}C$, $\delta^{15}N$, δD, $\delta^{34}S$ and $\delta^{18}O$ of bulk and $\delta^{18}O$ of vegetable water, as well as the elemental composition and the content of nitrates, chlorides, phosphates and sulphates were measured in all of the samples. Water content of the samples, determined accordingly to the Italian Official methods (Ministerial Decree 3rd February 1989), was used to normalize mineral parameters re-calculating their concentration respect to dry matter content (d.m.). Before analysis raw tomatoes were well homogenized with a high speed Ultra-Turrax® mixer (T25 Basic, Ika®-Werke Gmbh & Co., Staufen, Germany). Juices, passata, pastes and the homogenized tomatoes were also freeze-dried for the analysis of $\delta^{13}C$, $\delta^{15}N$, δD, $\delta^{34}S$ and $\delta^{18}O$ of bulk.

Analysis of Chlorides, Nitrates, Sulphates and Phosphates

About 10 g of fresh sample (homogenized tomato, juice, passata or paste) was directly weighted in a polypropylene vial and homogenized with an Ultra-Turrax® mixer after adding till 50mL Milli-Q ultrapure laboratory grade water at 18,2 MΩ×cm (Millipore Corporation, Billerica, MA-Massachusetts, USA),. The resulting slurry was centrifuged at 8000 rpm for 5 minutes. For the analysis of anions the supernatant liquid was filtered 0.45 µm (Minisart, Sartorius AG, Goettingen,Germany), diluted 60 times in the case of pastes and 20 for the other kinds of sample and analysed. The analysis of chlorides, nitrates, sulphates and phosphates was performed using an Ion Chromatograph (Dx 120, Dionex Corporation, Sunnyvale, CA, USA), equipped with a 4 x 250 mm HPLC column (IonPac AS 14A, Dionex). The mobile phase consisted of a $Na_2CO_3/NaHCO_3$ (8mM:1mM) solution pumped isocratically in the system at a rate of 1 mL/min.

Stable Isotope Ratio Analysis of Light Elements

Analysis of $\delta^{18}O$ in water was performed directly on the fresh sample (homogenized tomato, juice, passata or paste) using an Isotope Ratio Mass Spectrometer (VG Isogas, Middlewich, UK) interfaced with a CO_2 equilibration system (Isoprep 18, VG Isotech, Middlewich, UK), according to the ENV 12141 method (Ministerial Decree 23th September 2005).

Around 1.7mg of freeze-dried sample was weighted in tin or silver capsule for the analysis of $\delta^{13}C$ and $\delta^{15}N$ in one run, 8 mg for $\delta^{34}S$ and 0.5 mg for $\delta^{18}O$ and δD in one run. For $^{34}S/^{32}S$, around 8 mg of the oxidating agent V_2O_5 was added in the capsule in order to optimise the combustion of the sample. The analysis was carried out using an Isotope Ratio Mass Spectrometer (Delta Plus XP ThermoFinnigan, Bremen, Germany) equipped with an elemental analyzer (Flash EA 1112, ThermoFinnigan) for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ determinations and a pyrolyser (Finnigan^{TM} TC/EA, ThermoFinnigan) for $\delta^{18}O$ and δD measurements.

The isotope ratios were expressed in δ‰ versus V-PDB (Vienna – Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW (Vienna – Standard Mean Ocean Water) for $\delta^{18}O$ and δD, Air for $\delta^{15}N$ and V-CDT (Vienna – Canyon Diablo Troilite) for $\delta^{34}S$ according to the following formula:

$$\delta‰ = [(R_s - R_{std})/R_{std}]*1000$$

where R_s is the isotope ratio measured for the sample and R_{std} is the isotope ratio of the international standard. Sample analysis was carried out in duplicate. The isotopic values were calculated against working in-house standards (casein for $\delta^{13}C$, $\delta^{15}N$, δD and $\delta^{18}O$ of bulk, and water for $\delta^{18}O$ of vegetal water), which were themselves calibrated against international reference materials: L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 for $^{13}C/^{12}C$; L-glutamic acid USGS 40 and potassium nitrate IAEA-NO3 for $^{15}N/^{14}N$; benzoic acid IAEA-601 for $^{18}O/^{16}O$. The $\delta^{34}S$ values were calculated against barium sulphates IAEA-SO-5, NBS 127 (IAEA) and a calibrated casein working standard through the creation of a linear equation. The D/H values were corrected against the same casein reference material with an assigned value of δD, according to the "comparative equilibration technique".[35] Details of the analytical procedure were reported in previous publications.[36, 37, 38]

The precision of measurement, expressed as one standard deviation when measuring the same sample 10 times, was 0.1‰ for $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ of water, 0.3‰ for $^{18}O/^{16}O$ of bulk, 0.4‰ for $^{34}S/^{32}S$ and 2‰ for D/H.

Elemental Analysis

Around 2 g of fresh sample (homogenized tomato, juice, passata or paste) was weighed directly into a PFA Teflon® vessel (Xpress, CEM, Matthews, NC-North Carolina, USA) and added with 4 mL HNO_3 (Superpure 65%, Merck, Darmstadt, Germany), 5 mL of MilliQ water and 1 mL Re internal standard solution (800 µg/L, Merck) for volume correction. The samples were mineralized in the closed vessels using a microwave oven digestor (MarsXpress, CEM).

Analysis of Li, Be, B, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Mo, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Tm, Yb, Ir, Tl, Pb and U was carried out with an Inductively Coupled Plasma Mass Spectrometer (Agilent 7500ce Agilent Technologies, Tokyo, Japan) equipped with an ASX-520 autosampler (Cetac Technologies Inc., Omaha, NE). An Octopole Reaction System (ORS®, Agilent Technologies) was used to remove polyatomic interferences, using He and H_2 as collision and reaction gas, respectively.

All of the materials were washed with 5% HNO_3 and rinsed with Milli-Q water before use. Sample preparation and analysis were carried out in duplicate. The limit of detection (LOD) of each element was calculated as 3 times the standard deviation of the signal of the blank samples, extracted and analyzed 10 times (Table 1). Because an international standard of tomato or tomato derivative is not available, accuracy was checked using two vegetal standard reference materials, NIST 1547 ('Peach leaves', NIST-National Institute of Standards & Technology, Gaithersburg, MD, USA) and BCR 100 ('Beech leaves', Institute for Reference Materials and Measurements - IRMM, Geel, Belgium) in each analytical run. The recoveries of the 34 elements (B, Na, Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Sn, Sb, Cs, Ba, La, Ce, Nd, Sm, Eu, Gd, Yb, Pb and U) were satisfactory and between 75% and 120% (Table 2).[13] The precision of the method, evaluated by preparing and analyzing a sample 10 times and expressed as RSD ranged from 3 to 10% for all the elements, except Ga (13%). The reproducibility, evaluated by preparing and analyzing a specific sample of passata in each analytical batch, and calculated as RSD of 22 analytical runs, ranged between 4% and 21%. These values can be deemed satisfactory, considering the very low content of some elements in tomato and derivatives.

Statistical Analysis

The data were evaluated using the statistical environment R (http://www.R-project.org/) and the packages MASS[39] and Statistica v 8 (StatSoft Inc., Tusla, Oklahoma, USA).

RESULTS AND DISCUSSION

In Table 3 the median, 25th and 75th percentile values of $\delta^{13}C$, $\delta^{18}O_{bulk}$, δD, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O_{water}$, of elements and anions in tomatoes, juices, passata and pastes are summarised for Piedmont, Emilia Romagna and Apulia.

In broad terms, $\delta^{18}O_{bulk}$, $\delta^{18}O_{water}$ and particularly δD show a trend for the median values to increase from North (Piedmont) to South (Apulia) within each product and also when considering all the products together. The $\delta^{18}O$ of plant products originates from CO_2, H_2O and O_2 and correlates with water,[40] so it reflects the isotopic composition of groundwater and average precipitation in the region – mainly related to latitude, distance from the sea and altitude – and the extent of evapotranspiration, mainly influenced by humidity and temperature.[29] Similarly, the hydrogen present in plant material originates from the water taken up by the roots[41] and the subsequent evapotranspiration process of water through the leaf stomata enriches the remaining water in the heavier isotopomer. Therefore, it is expected that growing regions with relatively low humidity, where the rate of evaporation from the leaf is higher, result in plant materials relatively enriched in deuterium.[41] $\delta^{18}O_{water}$ values are similar to those reported by Bong et al. in Korean tomatoes,[24] by Schmidt et al. in Dutch and German tomatoes[22] and by Trifirò et al. in Italian tomatoes, passata and pastes.[27] Instead Pfammatter and co-workers reported lower $\delta^{18}O_{water}$ values for Swiss tomatoes.[26] δD and $\delta^{18}O_{bulk}$ have not been reported in the literature so far. They are significantly correlated in tomatoes ($\delta D = 4.66 * \delta^{18}O_{bulk} - 170.55$; R = 0.52), juices ($\delta D = 6.90 * \delta^{18}O_{bulk} - 221.57$; R = 0.62) and pastes ($\delta D = 7.16 * \delta^{18}O_{bulk} - 221.64$; R = 0.65) but not in passata ($\delta D = 5.02 * \delta^{18}O_{bulk} – 173.01$; R = 0.41). The relationship, statistically significant, found considering all the kind of samples together was $\delta D = 6.85 * \delta^{18}O_{bulk} - 220.50$ (R = 0.60). The relationships found had an intercept (d_{excess}) similar to those found in Italian olive oils.[33, 34, 42] $\delta^{15}N$ values agreed with those reported by Bagnaresi et al.,[43] Kelly and Bateman,[17] Schmidt et al.[22] and Nakano and Uehara[21] in tomatoes grown both organically and conventionally. Very interesting are the $\delta^{15}N$ values of Emilia Romagna that in half of the tomatoes were higher than 7‰, suggested as the minimum threshold value for organically grown vegetables,[22] probably indicating a more extensive use of organic fertilizers than in Apulia or Piedmont. Interestingly, $\delta^{34}S$ values of Piedmont are higher than those of Emilia Romagna and Apulia, probably due to the different geology of growth soil[31] and are similar to those found by Schmidt and colleagues in German tomatoes.[22] In general $\delta^{13}C$ values of tomatoes agree with those found by Schmidt and colleagues[22] even if some samples of the present study, surely grown in soil, showed values lower than -28‰ (up to -29.1‰), level suggested by the same authors as possible threshold of greenhouse cultivated tomatoes.

Of the forty-six analysed elements, Li, B, Na, Mg, P, K, Ca, Mn, Fe, Ni, Cu, Zn, Se, Rb, Sr, Mo, Cd, Ba and Ce are present in detectable amounts in 100% of samples, Co, Y, Nd, U, Cs, La, Al, As, Gd, Yb, Sm, Ge, Cr and Sn in between 90 and 99% of the samples, Dy, Be, Ho and Eu in at least 80%, Pb, Pr, Sb and V in at least 70%, Tm in 68%, Ga in 46%, Ir in 26%, Ag and Tl in less than 20% of the samples. It is possible to note that lanthanides' distribution in tomato products is in accordance with the Oddo-Harkins rule, as reported in other commodities[44] and the even-numbered nuclides are more stable than the odd-numbered ones. It is noteworthy that the rare earth elements (RREs) concentration in Emilia Romagna samples is always far below the values found in Piedmont and Apulia products, probably reflecting the different RREs distributions in the growth soils. RREs concentrations are in agreement with the values reported by Spalla and co-workers,[45] confirming also that the light REEs (La, Ce, Pr, Nd and Sm) content is significantly higher than the heavy REEs (Gd, Dy, Ho, Tm and Yb). K, P, Ca, Mg, Zn, Mn, Rb, Sr, Co, Se and Al content are in the ranges reported in literature[8, 9, 10, 11, 14, 15, 16, 17, 46] except with those reported by Nakano and Uehara[21] that measured values of P, K, Ca and Mg higher than ours in tomatoes grown with different fertilizers. The Na content in tomatoes agrees with that found by Thybo et al.,[15] Kelly and Bateman,[13] LoIudice et al.[11] and Guil-Guerrero with Rebolloso-Fuentes,[20] but is higher than the contents measured by Incrocci et al.[46] in tomatoes grown in soilless cultures and lower than those reported by Demirbas for Turkish tomatoes,[16] Hernandez-Suarez et al. for tomatoes from Tenerife island[12] and Gundersen et al. for tomatoes grown in greenhouses.[14] Cu, Ni, Pb and Cd values are lower than those reported by Ekholm et al. in tomatoes grown in Finland[9] and higher than those measured by Gundersen et al.[14] but are in agreement with the data reported by other authors.[16] As and Cr values are far below those measured respectively by Bronkowska et al. in tomatoes grown in a region with copperworks[10] and by Bratakos et al. in Greek tomatoes.[8]

Chlorides and phosphates contents in tomatoes are similar to those reported in literature, whereas sulphates content is lower than that quoted in two previous studies.[7, 14] Nitrates concentration is far below the values found by Hertz and Baltensperger[18] and by Guil-Guerrero and Rebolloso-Fuentes.[20]

Multivariate evaluation of the data

For the statistical analysis, the raw data of elements are taken into account, also including the values lower than the detection limit. Furthermore the element and anionic compositions are expressed as dry matter in order to correct the effects of different degrees of moisture of tomato products. As isotopic and mineral data were normally distributed (Kolmogorov-Smirnov and Shapiro-Wilk tests) they were submitted to ANOVA and Honestly Significantly Different (HSD) for unequal N Tukey tests to evaluate statistically significant differences due to product type and geographical origin before applying a multivariate evaluation. Tests highlight statistically significant ($p < 0.001$) differences in the values of δD, $\delta^{18}O_{bulk}$, $\delta^{18}O_{water}$, B, Cr, Sr, Ba, phosphates, sulphates and chlorides (Table 4) for the differently processed tomato products. Highly significant differences ($p < 0.001$) in the values of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O_{bulk}$, $\delta^{34}S$, $\delta^{18}O_{water}$, Li, Mg, P, K, Cr, Fe, Co, Ni, Ga, As, Se, Mo, Cd, Sn, Sb, Ba, La, Eu, Gd, Tm, Tl, Pb and U are found for samples of different geographical origin. The differences of D/H and $^{18}O/^{16}O$ passing from tomato to paste were easily predictable on the basis of water loss upon evaporation. Cr concentration shows a strong increase from raw tomatoes to tomato products (mean values of about 300 µg/kg d.m. and 950 µg/kg d.m. respectively) suggesting a possible release from stainless steel manufacturing system. However, since this parameter was found to be highly significant also in the differentiation of the geographical origin, it was decided to keep it in the model. The same considerations apply to Ba that was found to be highly significant in the differentiation of samples based on both geographical origin and processing criteria. Phosphates content is shown to be heavily affected by the kind of product with a decrease of its values from tomatoes and juices to passata and pastes (about 6000 mg/kg d.m. and 8500 mg/kg d.m. respectively) while it does not seem to be influenced by geographical origin. Therefore, it was decided to remove it for the statistical analysis, just like the variables B, Sr and sulphates.

Before applying multivariate analysis of the data, they were submitted to a cluster analysis with the aim to reduce the number of variables. Ce, Pr, Nd and La; Gd, Dy, Yb, Y, Ho, Sm, V and Al; Co and Mn, were shown to be highly correlated each other. On this basis only La, Gd and Co were kept into the model whereas Ce, Pr, Nd, Yb, Y, Ho, Sm, V, Al and Mn were not considered. Na and chlorides were removed for the statistical analysis because salt is normally added during production of passata as is evident for chlorides in Table 4.

After this selection, 40 out of 56 variables were considered. Using robust PCA (Principal Component Analysis)[47] we identified 5 outliers, which were excluded from the training set. Three out of the five outliers were two juices and a passata collected in the same firm in Apulia, even if at different times, and showed high values of Cu, Sn, Pb, Tl or Co, probably due to technological pollution. The other two outliers were tomatoes from Piedmont and Emilia-Romagna, both showing high values of As and REEs perhaps due to a not accurate industrial washing. As and REEs were highly correlated in tomatoes, probably because these elements were in some way bound in the related soils, but not in the other kinds of product as As probably volatilizes more easily than REEs during the heating steps of the production processes.

The new dataset was firstly checked to verify if, for each of the four kinds of product (tomato, juice, passata, paste) separately considered the regional origin was discriminable on the basis of isotopic and mineral contents. The creation of an LDA model *per* kind of product separately led to predictions of 88.46%, 91.03%, 95.15%, 92.98% for tomato, juice, passata and paste respectively (cross validation leave-one-out – LOO -, similar results were found upon leaving out 10% and 20% of the data in each iteration), as evident in Figure 1. The four individual LDA models lead to four different sets of significant variables (Fig. 2), showing remarkable differences in their order of importance. Considering a cut-off limit of 1, Cr, U, Gd, Cs, Tm, Eu, La, Ni and δ^{34}S, are found influential in all of the four sets, suggesting their usefulness as geographical origin markers. Each one of the previous models shows individual capability of tracing the regional origin. But the question is whether it is possible to develop a general model able to trace the origin of tomato products independently of their specific position along the transformation chain.

Considering the whole dataset, exploratory PCA (Principal Component Analysis) is performed in order to evaluate if regional origin, as well as type of product, year and month of harvest, affect isotopic, elemental and anionic compositions. On the basis of visual evaluation of the plots of the first two PCs, only regional groupings are detectable, whereas the other variables show random patterns in the score plots.

To assess discrimination efficiency for sample origin, a multivariate analysis of all the data was carried out by discriminant analysis (LDA - Linear Discriminant Analysis) (Fig. 3). The model was validated through a LOO cross-validation leading to a success rate of just over 95%. Again, leave-10% and leave-20% out crossvalidation gave the same result (1000 iterations) indicating that the model is sufficiently robust. Afterwards the LDA model was inspected to see which variables were more important in distinguishing the three Italian regions. The coefficients of the model, in particular the sums of the squared coefficients for LD1 and LD2, were considered (see Fig. 4). Based on the jumps in coefficient size visible in the plot, cut-off values of 0.3 and 0.5 were chosen. In the first case, seventeen variables were selected (Gd, La, Tl, Eu, Cs, Ni, Cr, Co, δ^{34}S, δ^{15}N, Cd, K, Mg, δ^{13}C, Mo, Rb and U), in the second case ten (Gd, La, Tl, Eu, Cs, Ni, Cr, Co, δ^{34}S and δ^{15}N). Using only the selected variables, LOO crossvalidation leads to over 95% accuracy.

It is interesting to note that three out of the four most important variables in the model are REEs. The potential use of lanthanides as soil tracers is still under discussion but results of previous works suggest their usefulness in food authentication,[48] especially in the case of heavy lanthanides (Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu).[44, 49] On the basis of the previous considerations on evaporation phenomena during the industrial processing that heavily affect these parameters, flattening the differences due to the geographical origin, and different to previous works on food authentication[34, 38, 42] δD and δ^{18}O are not included between the influential parameters. In contrast, LDA confirms the importance of δ^{15}N and δ^{34}S in tracing geographical origin.[31, 38]

CONCLUSIONS

Mineral and isotopic compositions can support the regional traceability of Italian tomato products (tomatoes, juices, passata, pastes). By applying linear discriminant analysis to seventeen of the most significant parameters (Gd, La, Tl, Eu, Cs, Ni, Cr, Co, δ^{34}S, δ^{15}N, Cd, K, Mg, δ^{13}C, Mo, Rb and U), the model allows to correctly classify over 95% of the samples according to the geographical origin irrespective of the technological processes applied. Type of product, as well as year and month of harvest, do not affect the mineral and isotopic compositions of tomatoes, juices, passata and pastes. It can be expected that these results will lead to the development of an analytical control procedure to check the geographical provenance of tomato products used in food industry.

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Table 1. Limits of detection, values in µg/kg

Element	Isotope	LOD	Element	Isotope	LOD
Li	7	0.75	Y	89	0.10
Be	9	0.05	Mo	98	0.25
B	11	60	Ag	109	0.60
Na	23	200	Cd	111	0.03
Mg	26	200	Sn	118	0.70
Al	27	150	Sb	121	0.10
P	31	50	Cs	133	0.40
K	39	3100	Ba	137	4.0
Ca	40	3000	La	139	0.15
V	51	1.3	Ce	140	0.15
Cr	52	5.1	Pr	141	0.15
Mn	55	130	Nd	146	0.10
Fe	56	515	Sm	147	0.05
Co	59	1.2	Eu	151	0.04
Ni	60	8.1	Gd	160	0.03
Cu	63	85	Dy	163	0.05
Zn	66	50	Ho	165	0.02
Ga	71	0.85	Tm	169	0.01
Ge	74	0.03	Yb	171	0.01
As	75	0.10	Ir	193	0.01
Se	78	0.30	Tl	205	0.40
Rb	85	45	Pb	206+207+208	1.60
Sr	88	20	U	238	0.01

Table 2: Results for NIST 1547 and BCR 100 measurements, values in mg/kg

	Peach Leaves NIST 1547							Beech Leaves BCR 100					
	Certified value	Uncertainty	Indicative value	Mean	Mean Recovery %	Std. Dev		Certified value	Uncertainty	Indicative value	Mean	Mean Recovery %	Std. Dev.
B	29	2		25	87%	4	B			28	25	90%	3
Na	24	2		25	106%	3	Na			0.24	0.24	99%	0.05
Mg	4320	80		4542	105%	610	Mg	878	17		932	106%	116
Al	249	8		197	79%	27	Al	435	4		366	84%	41
P	1370	70		1202	88%	188	P	1550	40		1248	81%	153
K	24300	300		26158	108%	2572	K	9940	200		10145	102%	776
Ca	15600	200		16153	104%	1299	Ca	5300	50		5649	107%	70
V	0.37	0.03		0.29	79%	0.04	V	-	-	-	-	-	-
Cr			1	0.8	82%	0.09	Cr	8	0.6		6	79%	0.6
Mn	98	3		87	89%	7	Mn			1330	1153	87%	59
Fe	218	14		210	96%	30	Fe			550	468	85%	79
Co			0.07	0.08	110%	0.01	Co	-	-	-	-	-	-
Ni	0.69	0.09		0.56	81%	0.09	Ni	-	-	-	-	-	-
Cu	3.7	0.4		3.3	90%	0.6	Cu			11.8	10	82%	1
Zn	17.9	0.4		17.2	96%	3.3	Zn			69	57	82%	6
As	0.06	0.02		0.05	87%	0.01	As			0.51	0.50	98%	0.07
Se	0.120	0.009		0.132	110%	0.022	Se			0.15	0.16	110%	0.02
Rb	19.7	1.2		19.3	98%	4.9	Rb			32	29	92%	4
Sr	53	4		51	96%	5	Sr			13.3	11	85%	1
Mo	0.060	0.008		0.049	81%	0.007	Mo			0.5	0.4	86%	0.1
Cd	0.026	0.003		0.022	85%	0.005	Cd			0.34	0.29	85%	0.03
Sn			<0.2	0.11	-	0.02	Sn	-	-	-	-	-	-
Sb			0.02	0.02	80%	0.001	Sb			0.36	0.30	82%	0.03
Cs	-	-	-	-	-	-	Cs			0.031	0.033	106%	0.005
Ba	124	4		109	88%	8	Ba	-		-	-	-	-
La			9	8.5	94%	0.5	La	-		-	-	-	-
Ce			10	9.4	94%	0.7	Ce	-		-	-	-	-
Nd			7	6.7	95%	0.6	Nd	-		-	-	-	-
Sm			1	1.0	102%	0.1	Sm	-		-	-	-	-
Eu			0.17	0.20	120%	0.02	Eu	-		-	-	-	-
Gd			1	1.0	101%	0.1	Gd	-		-	-	-	-
Yb			0.20	0.22	109%	0.02	Yb	-		-	-	-	-
Pb	0.87	0.03		0.71	81%	0.08	Pb			16	13	83%	1
U			0.015	0.012	82%	0.002	U	-		-	-	-	-

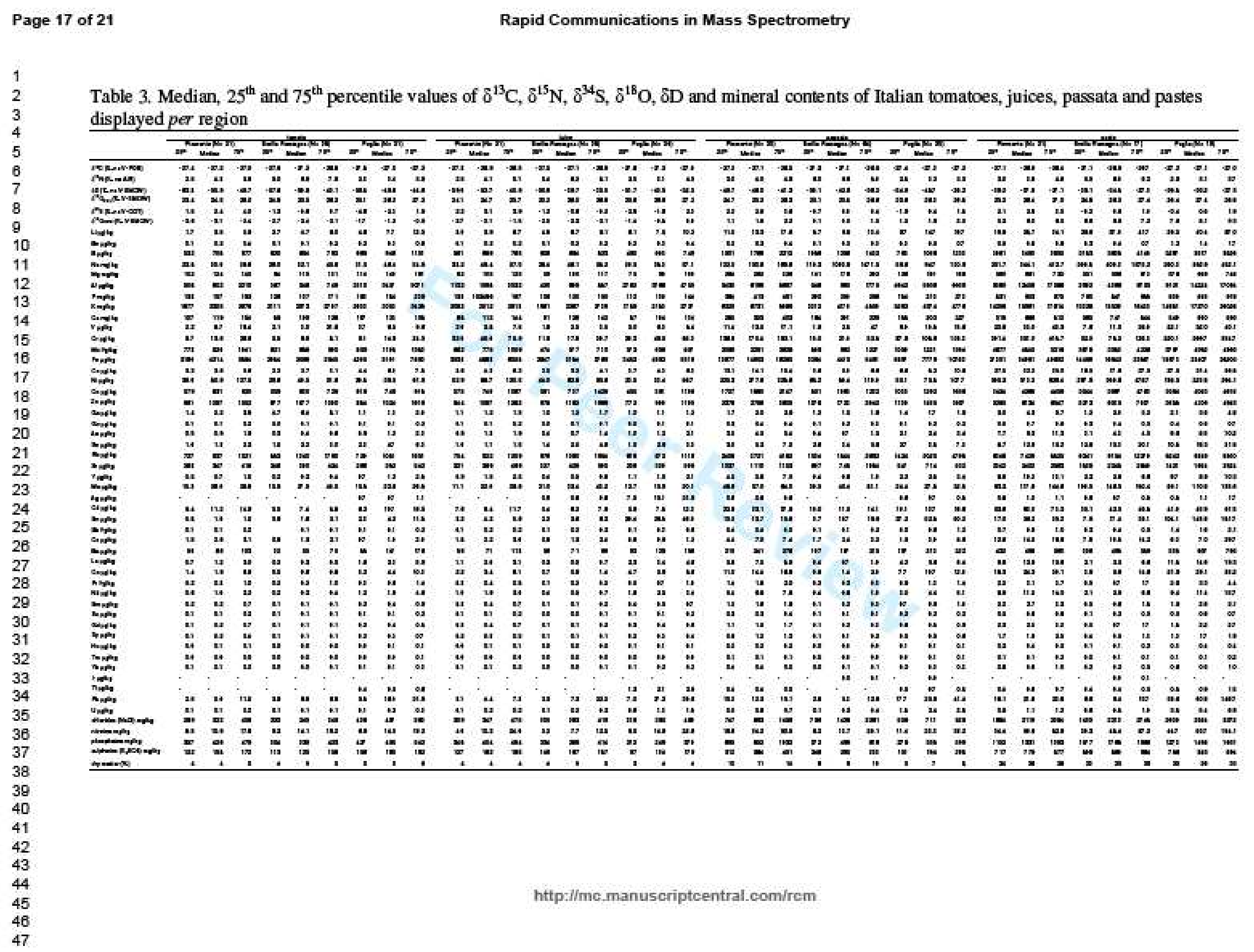

Table 3. Median, 25th and 75th percentile values of δ^{13}C, δ^{15}N, δ^{34}S, δ^{18}O, δD and mineral contents of Italian tomatoes, juices, passata and pastes displayed *per* region

Table 4. Significance of HSD for unequal N Tukey: groups with different letters are statistically different ($p < 0.001$)

	Tomato	Juice	Passata	Paste
δD	a	a	a	b
$\delta^{18}O_{bulk}$	a	ab	ab	b
$\delta^{18}O_{water}$	a	a	b	c
B	ab	b	ab	a
Cr	a	b	b	b
Zn	ab	b	a	a
Sr	a	b	b	ab
Ba	a	ab	b	ab
phosphates	b	b	a	a
sulphates	a	c	b	bc
chlorides	a	a	b	a

Figure 1. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia per kind: scatterplot of the first two canonical variables.

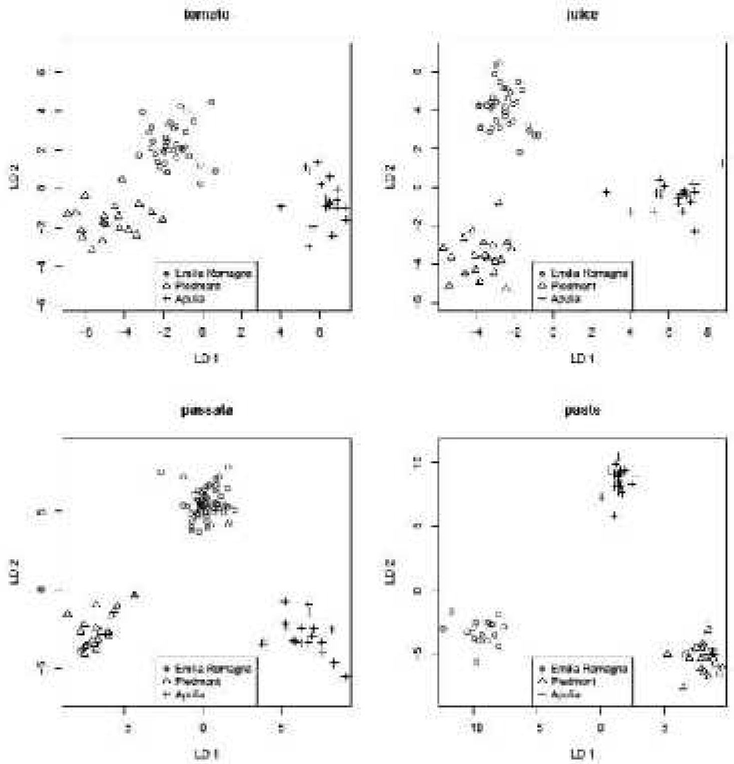

Figure 1. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia per kind: scatterplot of the first two canonical variables.
143x163mm (96 x 96 DPI)

Figure 2. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia *per* kind: coefficients of the model.

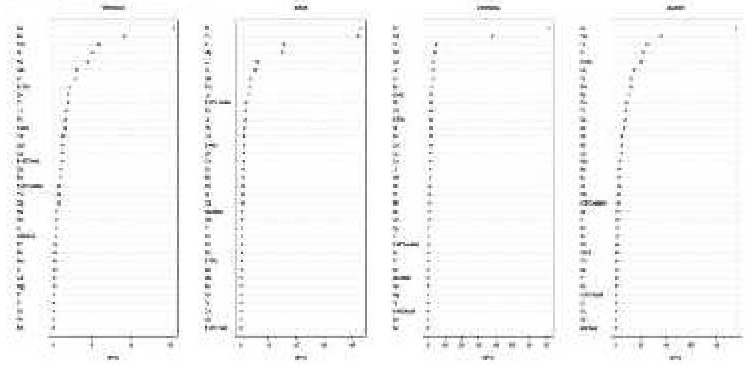

Figure 2. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia *per* kind: coefficients of the model.
143x83mm (96 x 96 DPI)

Figure 3. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia: scatterplot of the first two canonical variables.

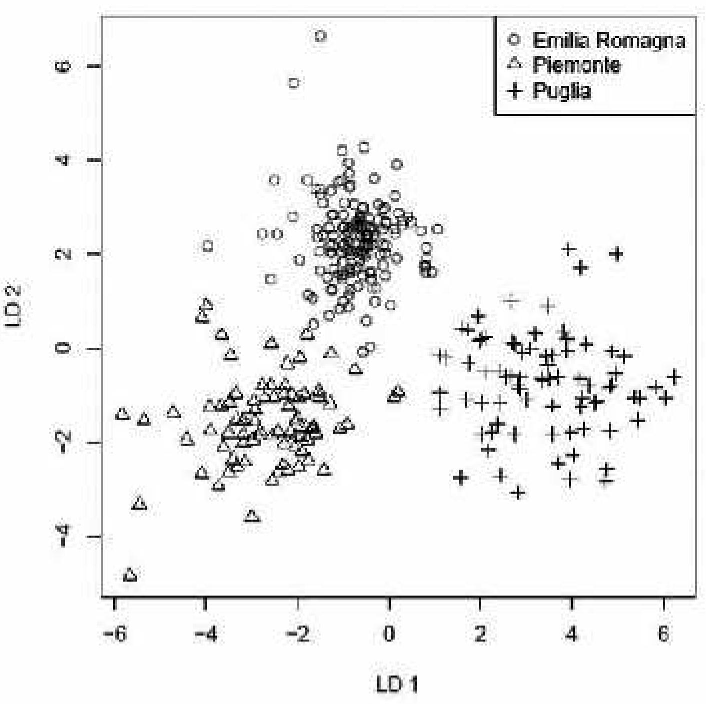

Figure 3. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia: scatterplot of the first two canonical variables.
143x167mm (96 x 96 DPI)

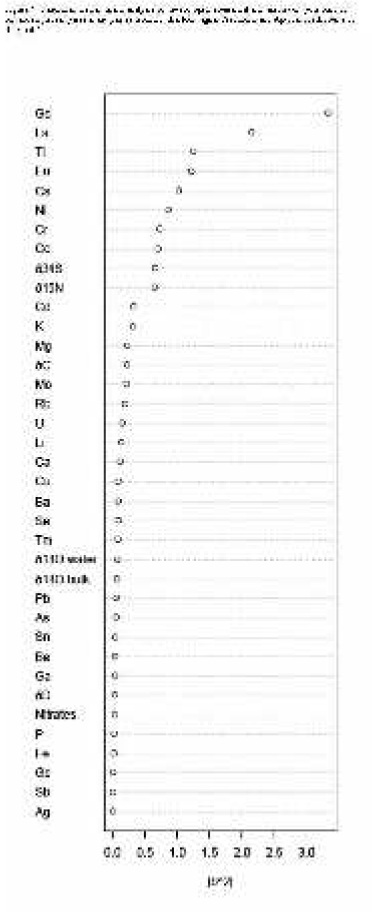

Figure 4. Canonical discriminant analysis of the isotopic, elemental and anion compositions of tomatoes, juices, passata and pastes from Emilia-Romagna, Piedmont and Apulia: coefficients of the model.
143x356mm (96 x 96 DPI)

Results

δD and $\delta^{18}O_{bulk}$ values in tomatoes and derivatives were reported for the first time. They were shown to be significantly correlated in tomatoes, juice and paste but not in passata. The relationships found had an intercept similar to those previously found in Italian olive oils (Chapter 4).

To assess discrimination efficiency in terms of sample origin, multivariate analysis of the whole dataset was carried out, regardless of food processing technology, using discriminant analysis adopting the most significative variables (Gd, La, Tl, Eu, Cs, Ni, Cr, Co, $\delta^{34}S$, $\delta^{15}N$, Cd, K, Mg, $\delta^{13}C$, Mo, Rb and U). The model obtained led to over 95% of samples being correctly reclassified into the production site in crossvalidation.

To conclude elemental and isotopic composition can assist with the regional traceability of Italian tomato products (tomatoes, juice, passata, paste) and it may be expected that these results will lead to the development of an analytical control procedure for checking the geographical provenance of tomato products usable in the food industry.

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CHAPTER 7 CONCLUSIONS

In this thesis the usefulness of stable isotope ratios and elemental composition as markers for food authenticity was extensively verified.

Specifically, following the development of a simple and effective preparation method and subsequent analysis of elemental composition, the isotopic and elemental composition of extra virgin olive oils was investigated. Firstly a dataset with the isotopic values and elemental profiles of Italian extra virgin olive oils was created and validated, allowing the distinction of some macro areas (mainly North versus South Italy). Verification of a ‘coast effect’ in the D/H and $^{18}O/^{16}O$ values of olive oils from the Adriatic and Tyrrhenian coasts made it possible to improve discrimination of samples from Central Italy. The isotope ratios and elemental composition of olive oils collected in eight European sites with different climatic and geological characteristics (Trentino, Tuscany, Sicily, Algarve, Carpentras, Barcelona, Chalkidiki, Lakonia) were shown to be related respectively to geographical and climatic factors and to the geological characteristics of the site where the plants were grown. The statistical model subsequently developed offered good geographical traceability. The model developed could be used to verify the authenticity of commercial samples, becoming a tool for ensuring compliance with European law.

Furthermore, $\delta^{13}C$ was shown to be a tool which can be used to distinguish squalene and squalane obtained from olive oil from same products obtained from shark liver oil. Consequently $\delta^{13}C$ analysis can be suggested as an official way of detecting whether any batch of commercial squalene or squalane and of squalane within cosmetic formulations has come from shark liver or olive oil.

The same analytical approach applied to seven types of Italian cheese from alpine regions (Asiago, Fontina, Montasio, Vezzena, Puzzone, Spressa and Toma), led to the development of a statistical model that can be proposed as a suitable tool for detection of mislabelling, useful for verifying the authenticity of commercial samples. This approach could be particularly helpful in the case of dairy products with special regulatory status, such as PDO products, or products commanding a price premium based on their provenance.

Finally, the usefulness of isotope ratios and elemental composition for the purposes of traceability was verified for Italian tomato products (tomatoes, juice, passata and paste) along the production chain. The statistical model developed allowed good differentiation of products from Emilia-Romagna, Apulia and Piedmont, independently of the technological process they had been subjected to. With a view to the future, once it has been verified that the model could be successfully extended to other European and extra-European countries, it may be expected that these results will lead to the development of an analytical control procedure checking on the geographical provenance of tomato products used in the food industry.

The analytical approach applied throughout this thesis proved to be highly reliable, as it was successfully applied to different premium products and in general it was confirmed that the isotopic or elemental composition of commodities reflects the geographical, climatic or geological composition of the site of provenance. This assumption, rarely verified experimentally, is the premise for each traceability study.

Currently elemental composition and isotope ratios analysis definitely offer one of the most promising hypothesis-driven approaches for establishing the authenticity of premium products. This is further highlighted by the fact that in the last thirty years some official methods have been based on some of these parameters. For example, as adulteration of wine through the addition of inexpensive commercial sugars is accompanied by the addition of l-malic acid in order to maintain the sugar acid ratio and dilution with water in order to maintain appropriate soluble solid concentration, and since the water used to dilute the wine is likely to be ground water, this leads to a decrease in the overall $^{18}O/^{16}O$ ratio of the water present in the wine. As a result determination of the $\delta^{18}O$‰ value of wine water is a routine test in wine control laboratories and is conducted as one of the European Wine Databank procedures (Regulation 555/2008/EC). Moreover, since 1977 the Association of Official Analytical Chemists - AOAC has used stable isotope ratios, in particular D/H and $^{13}C/^{12}C$, as authenticity markers for honey. In particular, the addition of cane or corn syrup to honey produces a deviation from the normal ratio between the $\delta^{13}C$‰ values of bulk honey and honey proteins (AOAC Official Method 998.12). Finally, very recently, the stable isotope ratios of bioelements and the elemental composition were recognised as authentication markers for Grana Padano cheese (EC 2009/C 199/11, amendment application pursuant to 510/2006/EC Regulation).

Furthermore, in the last few years stable isotope ratios and elemental composition have begun to be successfully applied to fields other than food authentication. In particular, stable isotope ratios can be used in paleoclimatology to reconstruct past environmental changes. For example, $\delta^{18}O$ values of foraminifera shells, that can constitute sediments of sea bed, vary according to temperature and $\delta^{18}O$ of water. Thus it is possible to select standard species of forminifera from sections through the sediment column, and by mapping the variation in oxygen isotopic ratio, deduce the temperature that the forminifera encountered during life. In forensic science, research suggests that the variations in certain isotope ratios in drugs derived from plant sources (*e.g.* cannabis, cocaine) can be used to determine the drug's continent of origin, whereas the elemental composition of particles and deformed bullets found at the crime scene can be compared with the elemental fingerprints of bullets found on suspects.

Despite the high potential of stable isotope ratios and elemental composition, it is important to mention some common limits and reservations that were highlighted during the drawing up of this thesis. Firstly, rapid profiling (screening) techniques (*e.g.* H NMR) and classical parameters (*e.g.* concentration of specific organic compounds) should be not overlooked, as they often provide invaluable additional information assisting with interpretation or allowing a preselection of samples for isotopic or elemental analysis. For example, the concentration of β-carotene in dairy products indicates whether the milk used in production was collected during periods of pasture feeding, ensuring that appropriate meteorological data is applied in the interpretation of $\delta^{18}O$ and δD data.

Therefore these analytical approaches are based on a database, in which the number of analysed samples has to be sufficient to truly reflect commercial samples.

Furthermore, it is extremely important to evaluate the accuracy and precision of the measurements in order to verify whether the differences found can be deemed to be statistically significant or not. This issue is especially important if the database is to be developed on an European scale for the purposes of enforcement, as in the case of the wine databank. In this case long-term reproducibility is also of paramount importance.

Finally, in the longer term, it is to be hoped that a greater understanding of how meteorological and geochemical signatures are transferred to premium products may reduce the need for expensive comparative databases of authentic commodities. Ultimately, this would allow the generation of isotopic and multi-element ‘maps’ for foods from different geographical locations, which could be incorporated into traceability systems.

Overcoming the need for comparative databases in food provenance determination will be the next extremely challenging task.

Overview of completed training activities

- Course on “Non-parametric statistics”, 2008, IASMA-FEM, S. Michele a/A, Italy
- Course on “Valutazione, controllo e gestione della qualità del processo e del prodotto”, 2008, University of Padua, Padua, Italy
- Seminar on “Case histories di nuovi processi”, 2008, University of Udine, Udine, Italy
- Seminar on “Il mercato mondiale del vino”, 2008, Scuola enologica di Conegliano, Conegliano Veneto, Italy
- Seminar on “Il comportamento dei consumatori verso gli alimenti transgenici”, 2008, Scuola enologica di Conegliano, Conegliano Veneto, Italy
- Seminars on “Luce di sincrotrone e tecnologie alimentari” and on “Microtomografia e tecnologie alimentari: applicazioni a Elettra”, 2008, University of Udine, Udine, Italy
- Course on “Banche dati ISI”, 2008, IASMA-FEM, S. Michele all’Adige, Italy
- Seminar on “La modellistica dei fenomeni di trasporto nell’ambito dell’ingegneria alimentare affrontata con strumenti computazionali”, 2008, University of Udine, Udine, Italy
- Stage to develop an analyitical method in GC-C-IRMS compound specific, 2008, University of East Anglia, Norwich, United Kingdom
- Participation at “9th Belgian Chemometrics Symposium”, 2008, Centre Wallon de Recherches Agronomiques, Gembloux, Belgium
- Course on the use of Statistica for Windows software, 2009, IASMA-FEM, S. Michele all’Adige, Italy
- Course on “Stable Isotope Course: An introduction to uses in ecology and plant physiology”, 2009, Technical University München, Germany
- Participation at “Isocompound 2009 - Compound specific isotopes in ecology and earth sciences”, 2009, Potsdam, Germany
- Participation at “Workshop di Chemiometria 2010”, 2010, Università del Piemonte Orientale, Alessandria, Italy
- Seminar “How *not* to lie with statistics”, 2010, IASMA-FEM, S. Michele all’Adige, Italy

Curriculum vitae

Personal information

First name(s) / Surname(s)	**Luana Bontempo**
E-mail	luana.bontempo@iasma.it
Nationality	Italian
Date of birth	13/01/1976
Place of birth	Bolzano
Gender	Female

Education and training

Dates	2002 - 2004
Title of qualification awarded	Post-graduated specialization school degree
Principal subjects/occupational skills covered	Chemical Methodologies for Survey and Analysis (‘Metodologie Chimiche di Controllo e di Analisi’)
Name and type of organisation providing education and training	Dept. of Chemistry, University of Padua
Title of the thesis	‘La tecnica ICP-MS interfacciata al sistema di generazione degli idruri. Ottimizzazione strumentale e validazione della metodica nella matrice vino’, supervisor Prof. F. Magno, co-supervisor Dr. R. Larcher
Dates	October 1995 - April 2001
Title of qualification awarded	Master’s degree
Principal subjects/occupational skills covered	Pharmaceutical Chemistry and Technology
Name and type of organisation providing education and training	Dept. of Pharmacy, University of Padua
Title of the thesis	‘Caratterizzazione di alcune tipologie botaniche e geografiche di miele attraverso l’analisi multiisotopica ed elementare’, supervisor Prof. C. Grandi, co-supervisor Dr. G. Versini
Dates	1990-1995
Title of qualification awarded	High school leaving qualification
Principal subjects/occupational skills covered	Scientific studies
Name and type of organisation providing education and training	Liceo Scientifico A. Maffei, Riva del Garda

Scientific career/work experiences

2008-current	Researcher at the Fondazione Edmund Mach, formerly Istituto Agrario di San Michele all’Adige

November 2008	Visiting scientist at the University of East Anglia, UK (Dept. of Environmental Science)
April 2009	Visiting scientist at the Technical University München, D (Lehrstuhl für Grünlandlehre)
2001 - 2008	Fellowship at the Fondazione Edmund Mach, formerly Istituto Agrario di San Michele all'Adige

Current position

Dates	2008 - current
Occupation or position held	Researcher at the Quality, Nutrition and Traceability Department
Main activities and responsibilities	Research activity on traceability of premium products using stable isotope ratios
Name and address of employer	IASMA Fondazione Edmund Mach, via Mach 1, 38010 San Michele all'Adige (Trento)
Type of business or sector	Developing of methods for the analysis of stable isotope ratios of bioelements in several matrices. Application of isotopic analysis to food, aimed at characterising geographical origin and controlling and protecting quality and authenticity. Extension of the application field of stable isotope analysis towards research topics in other core fields of the Centre, such as limnology (hydrological dynamics), ecology (trophic relationships, degradation kinetics) and climate change (*e.g.* impact in isotopic composition of tree rings).

Research awards and fellowships

April 2010	Selection of the paper 'Stable isotope ratios of C and H to distinguish olive oil from shark squalene-squalane' to take part in the article-level PR program for Rapid Communications in Mass Spectrometry
12/06/2008	Award for the originality and validity of the scientific research at the First National Dairy Congress, Milan
25/04/2008	Award for the second best poster at the congress 'Lost without TRACE', Torremolinos Spain

Positions of responsibility and achievements

2000-2004	Regional project MIROP, Metodi innovativi di rintracciabilità di origine e di processo a tutela di produzioni lattiero-casearie tipiche locali, funded by Provincia Autonoma di Trento; fellowship for FEM
2005-2009	FP6 European project TRACE, Tracing the origin of food, N°FP6-2003-FOOD-2-A 006942; fellowship from 2005 to 2006 and then involved researcher for FEM
2006-2009	National project BIOMARKERS, New Markers for characterisation of organic fruits, DM 91461/06-09-2005, funded by Italian Ministry of Food, Agriculture and Forestry; involved researcher for FEM
2006-2010 2010-2013	Project for the traceability of the Grana Padano cheese, funded by the Consortium for the protection of Grana Padano cheese; involved researcher for FEM

2007-2008	National project 'Qualità, percezione della qualità e tracciabilità del prodotto nei sistemi ovini da carne', funded by Ministero dell'Istruzione, dell'Università e della Ricerca; principal investigator for FEM
2006-2009	National project ProAlpe, 'I terroir delle Alpi per la caratterizzazione e la difesa delle produzioni casearie d'alpeggio', funded by Ministero dell'Istruzione, dell'Università e della Ricerca; principal investigator for FEM
2008-2009	Project for the traceability of the Parmigiano Reggiano cheese, funded by the Regione Emilia Romagna; involved researcher for FEM
2007-2010	Collaboration with Italian Ministry of Food, Agriculture and Forestry for the creation and validation of isotopic databanks concerning several commodities and verification of authenticity of commercial products; involved researcher for FEM

Invited lecturers and seminars

04/10/2007	Bontempo L. 'Caratterizzazione dei prodotti biologici e tracciabilità dei prodotti alimentari', Thermo Congress, Milan.
17/04/2008	Bontempo L. 'Analisi isotopiche per la caratterizzazione di carne e prodotti lattiero-caseari', Autenticità e tracciabilità degli alimenti mediante tecniche chimico-fisiche, Università Cattolica del Sacro Cuore, Piacenza.
03/11/2009	Bontempo L. 'Isotopic and elemental data for tracing the origin of European olive oils', Workshop on determining the geographical origin of food: TRACE elements and isotopic patterns in food verification, Prague.
19-20/10/2010	Bontempo L. 'Rapporti tra isotopi stabili di bioelementi', Terroir delle Alpi per la caratterizzazione e la difesa delle produzioni casearie d'alpeggio, Torino

Publications

ISI papers

1 Nicolini, G., Larcher, R., Pangrazzi, P., Bontempo L. (2004). Changes in the contents of micro- and trace-elements in wine due to winemaking treatments. Vitis - Journal of Grapevine Research, 43: 41-45.

2 Camin F., Bontempo L., Heinrich K., Horacek K., Kelly S.D., Schlicht C., Thomas F., Monahan F., Hoogewerff J., Rossman A. (2007). Multi-element (H,C,N,S) stable isotope characteristics of lamb meat from different European regions. Analytical & Bioanalytical Chemistry, 389: 309-320. DOI 10.1007/s00216-007-1302-3

3 Camin F., Perini M., Colombari G., Bontempo L., Versini G. (2008). Influence of dietary composition on the carbon, nitrogen, oxygen and hydrogen stable isotope ratios of milk. Rapid Communications in Mass Spectrometry, 22: 1690-1696. DOI: 10.1002/rcm.3506

4 Bontempo L., Camin F., Larcher R., Nicolini G., Perini M., Rossmann A. (2009). Discrimination of Tyrrenian and Adriatic Italian olive oils using H, O, and C stable isotope ratios. Rapid Communications in Mass Spectrometry, 23: 1043-1048. DOI: 10.1002/rcm.3968

5 Perini M., Camin F., Bontempo L., Rossmann A., Piasentier E. (2009). Multielement (H, C, N, O, S) stable isotope characteristics of lamb meat from different Italian regions. Rapid Communications in Mass Spectrometry, 23:2573-2585. DOI: 10.1002/rcm.4140

6 Camin F., Larcher R., Perini M., Bontempo L., Bertoldi D., Gagliano G., Nicolini G., Versini G. (2010). Characterisation of authentic Italian extra-virgin olive oils by stable isotope ratios of C, O and H and mineral composition. Food chemistry, 118: 901–909. DOI:10.1016/j.foodchem.2008.04.059

7 Camin F., Larcher R., Nicolini G., Bontempo L., Bertoldi D., Perini M., Schlicht C, Schellenberg A., Thomas F., Heinrich K., Voerkelius S., Horacek M., Ueckermann E, Froeschl H., Wimmer B., Heiss G., Baxter M., Rossmann A., Hoogewerff J. (2010). Isotopic and elemental data for tracing the origin of European olive oils. Journal of Agricultural and Food Chemistry, 58, 570–577. DOI: 10.1021/jf902814s.

8 Schellenberg A., Chmielus S., Schlicht C., Camin F., Perini M., Bontempo L., Heinrich K., Kelly S.D., Rossmann A., Thomas F., Jamin E., Horacek M. (2010). Multielement Stable Isotope Ratios (H, C, N, S) of Honey from different European Regions. Food Chemistry, 121, 770-777. DOI:10.1016/j.foodchem.2009.12.082

9 Camin F., Bontempo L., Ziller L., Piangiolino C., Morchio G. (2010). Stable isotope ratios of C and H to distinguish olive oil from shark squalene-squalane. Rapid Communications in Mass Spectrometry, 24, 1810–1816. DOI: 10.1002/rcm.4581

10 Camin F., Perini M, Bontempo L., Fabroni S., Faedi W., Magnani S., Tabillio M.R., Musmeci S., Rossmann A., Kelly S., Rapisarda P. (2011). Potential isotopic and chimical markers for characterising organic fruits. Food Chemistry, 125, 1072–1082. DOI:10.1016/j.foodchem.2010.09.081

11 Bontempo L., Camin F., Manzocco L., Nicolini G., Wehrens R., Ziller L., Larcher R.. Traceability along the production chain of Italian tomato products on the basis of stable isotopes and mineral composition. Rapid Communications in Mass Spectrometry. DOI:10.1002/rcm.4935. (In press)

12 Bertoldi D., Bontempo L., Larcher R., Nicolini G., Voerkelius S., Lorenz G.D., Ueckermann H., Froeschl H., Baxter M.J., Hoogewerff J., Brereton P. Survey of the chemical composition of 571 European bottled mineral waters. Journal of Food Composition and Analysis. DOI:10.1016/j.jfca.2010.07.005. (In press)

13 Goitom-Asfaha D., Quétel C., Thomas F., Horacek M., Wimmer B., Heiss G., Dekant C., Deters-Itzelsberger P., Hoelzl S., Rummel S., Brach-Papa C., Van-Bocxstaele M., Jamin E., Baxter M., Heinrich K., Bertoldi D., Bontempo L., Camin F., Larcher R., Perini M., Kelly S., Rossmann A., Schellenberg A., Schlicht C., Froeschl H., Hoogewerff J., Ueckermann H. Combining isotopic signatures of n(87Sr)/n(86Sr) and light stable elements (C, N, O, S) with multi-elemental profiling for the authentication of provenance of European cereal samples. Journal of Cereal Science. DOI:10.1016/j.jcs.2010.11.004. (In press)

14 Bontempo L., Larcher R., Camin F., Hoelzl S., Rossmann A., Nicolini G. Elemental and isotopic characterisation of typical Italian alpine cheeses. International Dairy Journal. DOI:10.1016/j.idairyj.2011.01.009. (In press)

Not ISI papers

1 Nicolini G., Larcher R., Bontempo L. (2003). Composizione di base e profilo minerale di spumanti classici italiani. L'Enologo, 39: 111-115.

2 Nicolini G., Larcher R., Bontempo L. (2003). Caratterizzazione della composizione di base e del contenuto di elementi in micro-quantità ed in tracce in spumanti italiani elaborati con il metodo classico. Rivista di Viticoltura ed Enologia, 56: 29-44.

3 Nicolini G., Larcher R., Bontempo L. (2004). Micro- and trace-element composition of tannins used in winemaking. Journal of Commodity Science, 43: 21-29.

4 Poznanski E., Cavazza A., Schiavon S., Camin F., Gasperi F., Fasoli S., Framondino V., Bontempo L., Carlin S., Nicolini G., Larcher R., Biasioli F., Versini G. (2004). L'irripetibilità di un formaggio. Nostrani a confronto. Caseus, 9: 4-7.

5 Larcher R., Nicolini G., Pangrazzi P., Bontempo L., Russo S., Stocchetti R., Versini G., Magno F. (2006). Microelements and wine. Vignevini, 33: 108-114.

6 Bagnaresi P., Moschella A., Camin F., Bontempo L., Perini M., Parisi B., Ranalli P. (2007). Identificazione di marker bio-molecolari e metabolici specifici per la tracciabilità delle filiere biologiche di barbabietola da zucchero e pomodoro da industria. Agroindustria, 6: 145-152.

7 Colombari G., Zapparoli G.A., Araldi F., Migliorati L., Buttasi C., Perini M., Bontempo L., Camin F. (2008). Influenza di dosi crescenti di *Zea mays* sui rapporti isotopici di carbonio e azoto di razioni unifeed e latte. Scienza e tecnica lattiero-casearia, 59: 97-112.

8 Bontempo L., Camin F., Perini M. (2008). Characterising natural tomato passata by means of the isotopic ratio of oxygen. Annual report IASMA Research Centre, 29.

9 Camin F., Bontempo L., Perini M. (2008). Chemical analytical strategies to protect and promote organic commodities. Annual report IASMA Research Centre, 21-22.

10 Camin F., Larcher R., Bontempo L., Perini M., Bertoldi D. , Nicolini G. (2009). Geographical traceability of Italian and European extra-virgin olive oils. Annual report IASMA Research Centre, (2008): 21-22.

11 Camin F., Perini M., Bontempo L., Giongo L. (2009). Multi-element (H, C, N, O) stable isotope characterization of blueberries. In: IX International vaccinium symposium: Corvallis, Oregon, July 13-16, 2008 (editor K.E. Hummer). Leuven: ISHS. (Acta Horticulturae 810). 2: 697-704. ISBN: 978-90-66057-41-8

12 Giongo L., Vrhovsek U., Gasperi F., Endrizzi I., Palmieri L., Saviane A., Merola S., Camin F., Perini M., Bontempo L., Danek I., Krol K., Mladin P., Mattivi F. (2009). A three-year highbush blueberry survey in different European locations for the fresh and processing markets. In: IX International vaccinium symposium: Corvallis, Oregon, July 13-16, 2008 (editor K.E. Hummer). Leuven: ISHS. (Acta Horticulturae 810). 2: 887-894. ISBN: 978-90-66057-41-8

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