Stable Isotope Ratio Analysis for Assessing the Authenticity of Food of Animal Origin

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Abstract: The main elemental constituents (H, C, N, O, and S) of bio-organic material have different stable isotopes (^{2}H, ^{1}H; ^{13}C, ^{12}C; ^{15}N, ^{14}N; ^{18}O, ^{17}O, ^{16}O; ^{36}S, ^{34}S, ^{33}S, and ^{32}S). Isotopic ratios can be measured precisely and accurately using dedicated analytical techniques such as isotope ratio mass spectrometry (IRMS). Analysis of these ratios shows potential for assessing the authenticity of food of animal origin. In this review, IRMS analysis of food of animal origin and variability factors related to stable isotope ratios in animals are described. The study also lists examples of application of stable isotope ratio analysis to meat, dairy products, fish, and shellfish and emphasizes the strengths and weaknesses of the technique. Geographical, climatic, pedological, geological, botanical, and agricultural factors affect the stable isotope ratios (SIR) of bio-elements, and SIR variations are ultimately incorporated into animal tissue through eating, drinking, breathing, and exchange with the environment, being recorded in the resulting foods. SIR analysis was capable of determining geographical origin, animal diet, and the production system (such as organic/conventional or wild/farmed) for pork, beef, lamb, poultry, milk, butter, cheese, fish, and shellfish. In the case of the hard PDO (protected designations of origin) cheeses Grana Padano and Parmigiano Reggiano it is also used in real-life situations to assess the authenticity of grated and shredded cheese on the market.

Keywords: authenticity, dairy products, fish, meat, stable isotope ratios

Food Authenticity

Determining the authenticity of foods means uncovering mislabeling of foods not meeting the requirements for legally adopting a particular name, substitution with cheaper but similar ingredients, undeclared processes, and/or extension of food using adulterants, incorrect origin (such as geographical), species, or production method. Nowadays, the objective assessment of food authenticity is of paramount importance as consumers come into daily contact with a wide variety of foods. Indeed, globalization means that more and more foods are traded around the world. Traceability has thus become a cornerstone of the EU's food safety policy, a risk-management tool which enables the food industry or 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 means of ensuring that foods are of a high quality and safe to be eaten when they reach the consumer.

This need led the European Union to institute a Traceability Regulation (178/2002/EC). It came into force in January 2005 and defines "food and feed traceability." For bovines, the EU law has since 2000 provided for a traceability system and mandatory indication of the origin and place of slaughter on the label (EU Reg. 1760/2000 and 653/2014). Furthermore, starting from April 1, 2015, the labeling of meat obtained from swine, sheep, goats, and poultry must also contain indication of the Member State or third country in which animal rearing and slaughter took place, and at each stage of meat production and distribution food business operators must have in place and use an identification and registration system for traceability (Commission Implementing Regulation EU 1337/2013). Moreover, for foods with Protected Geographical Indications (PGI), Protected Designations of Origin (PDO), and Traditional Specialties Guarantee (TSG), European laws EC N. 510/2006 and 1151/2012 require protection against mislabeling. Although in the majority of cases paper traceability and livestock tagging systems can guarantee the geographical origin of foods on sale in the retail market, the food industry urgently needs methods to screen no targeted food samples to provide proof of origin and prevent deliberate or accidental undeclared unpermitted admixture to food samples.

Among the exploitable techniques (Danezis and others 2016) stable isotope ratios analysis (SIRA), measured using isotope ratio mass spectrometry (IRMS), has taken on increasing importance in determining the authenticity of foods of animal origin for producers and control agencies.

Stable Isotope Ratio Variability in Animals

In bio-organic material, the main elemental constituents (H, C, N, O, and S) have different stable isotopes (^{2}H, ^{1}H; ^{13}C, ^{12}C;

[15]N,[14]N; ^{18}O,[17]O,[16]O; [36]S, [34]S, [33]S, and [32]S). Although isotopes have the same number of electrons and chemically behave in a similar way, the energy they need to undergo physical changes, as for breaking old and forming new bonds, may be different for various isotopes of the same element (Wagner 2005). This effect which has its origin in the slightly different physical properties of isotopes, is called fractionation and it is at the basis of the variability of the isotope ratios in nature.

The stable isotope ratios $^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$ are precisely and accurately analyzed using dedicated techniques such as IRMS (Hölzl and others 2004). The isotopic composition is denoted in delta, according to Brand and Coplen (2012) and the delta values are multiplied by 1000 and are expressed in units "per mil" (‰).

The main factors affecting variability of the 5 isotopic ratios in animals ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$) are discussed in detail in order to clarify their applications in terms of verifying the authenticity of animal-based foods.

Carbon

The $\delta^{13}C$ of different animal tissues (muscle or lipid fraction) is highly influenced by the composition of the animal's diet, normally consisting of different plant species.

The ranges of $\delta^{13}C$ values in plants differ according to the kind of photosynthetic cycle (C3, C4), due to the different isotopic discrimination capabilities of the carboxylase enzymes involved in CO_2 fixation, in addition to different CO_2 concentrations. Maize is a C4 plant with a higher ^{13}C content ($\delta^{13}C$: from −14‰ to −12‰) than C3 plants ($\delta^{13}C$: from −30‰ to−23‰) (Camin and others 2004, 2008; Knobbe and others 2006; Crittenden and others 2007; Molkentin and Giesemann 2007, 2010; Molkentin 2009)

Bahar and others (2005) showed that when shifting cattle from a C3 diet to a C4 diet, based on an incremental % of maize, the $\delta^{13}C$ increased in defatted dry muscles and in its lipid fraction. Camin and others (2008) observed the same trend in milk (casein and lipid). Each 10% increase in the maize content of the diet corresponded to a 0.7‰ to 1.0‰ shift in the $\delta^{13}C$ of casein. Also, urine showed the same behavior as milk, with a value about 2‰ higher (Knobbe and others 2006).

As reported by Tieszen and others (1983), the $\delta^{13}C$ value in animals increased by about 1‰ according to the mean isotopic value of their diets. The increase is confirmed by Post (2002) that reported a $\Delta\delta^{13}C = 0.39\pm1.3‰$ and by McCutchan and others (2003) with $\Delta\delta^{13}C = 0.4\pm0.4‰$. In detail, the fractionation of $\delta^{13}C$ between diet and tissue was estimated to be +1.9‰ for muscle and 1.3‰ for intramuscular lipid (Harrison and others 2011). Bloomfield and others (2011) tested the effects of temperature and diet on tissue turnover rates and discrimination of ^{13}C in black bream (*Acanthopagrus butcheri*) and found that the trophic discrimination of $\delta^{13}C$ could vary from 1‰ to 4‰ depending on conditions.

De Smet and others (2004) confirmed the significant ^{13}C depletion of lipids due to isotopic fractionation during oxidation of pyruvate to acetic CoA, but also enrichment of muscle, hair, liver, blood, and plasma. The order of $\delta^{13}C$ values was hair > muscle > plasma > blood > liver > kidney fat. Tieszen and others (1983) showed that tissues with high metabolic rates (such as blood and liver) have a higher C turnover than tissues with slower metabolic rates (such as muscle and bone) and that the order of $\delta^{13}C$ values for nonruminants was teeth > hair > brain > muscle > diet > liver, kidney > heart > lung > breath CO_2. Recently,

some authors have tried to "validate" muscle turnover rates and discrimination factors to ensure greater accuracy in interpreting stable isotope data. Harrison and others (2010, 2011) carried out 2 studies to investigate whether all muscles, or locations within a muscle, and various tissues (muscle, muscle lipids, and lipids) have the same isotopic composition; the diet of lambs was switched from a control diet to an isotopically distinct experimental diet supplied at 2 different energy allowances. Small, albeit significant, differences were detected in the tissue carbon turnover in the *longissimus dorsi* muscle, whereas inter-muscular comparison showed similar C half-lives for most of the muscles analyzed (Harrison and others 2010). Furthermore, it was found that the energy allowance had a significant impact on intra- and intermuscular C turnover (Harrison and others 2010), so that C half-lives of muscle were determined to be 75.7 and 91.6 d for animals receiving high and low energy allowances, respectively (Harrison and others 2011).

Finally, 1 study (Svensson and others 2014) investigated the differences in $\delta^{13}C$ and $\delta^{15}N$ determined in muscle and gill of fish and found a strong correlation between the 2 tissues for $\delta^{13}C$, indicating that both could be used to determine long-term feeding or migratory habits of fish, whereas a slight difference between these tissues was found in bulk $\delta^{15}N$, suggesting different isotopic turnover rates or different compositions of amino acids.

Nitrogen

The principal source of N for tissue protein synthesis in herbivorous animals is plant feed, and in carnivorous animals it is other animals. Farm animals, such as cattle and sheep, are almost exclusively herbivorous, and, therefore, the isotopic composition of plant feed is the most important factor in N variability.

The $\delta^{15}N$ values in plants are generally correlated with those of nitrates and ammonia in soils, which derive from atmospheric N_2 containing about 0.4% ^{15}N. Air nitrogen is transformed through physical processes and the activity of microorganisms into inorganic (nitrates, ammonia) and organic forms (amino acids, proteins) that are present and available in the soil. The natural cycle of nitrogen in the environment is relatively complex compared with carbon, as it moves from the atmosphere through various plants and microbes and occurs in a variety of reduced and oxidized forms (Werner and Schmidt 2002).

According to the extent of each of these processes, which are mainly affected by depth of soil, kind of vegetation, and climate, the $\delta^{15}N$ values of soils can vary considerably, generally falling between −10‰ and +15‰. In particular, water stress and nearness to the sea can lead to ^{15}N enrichment in the soil (Heaton and others 1997). The main factor affecting $\delta^{15}N$ in cultivated land is fertilization practices. Synthetic fertilizers, produced from atmospheric nitrogen via the Haber process, have $\delta^{15}N$ values between -4 and +4‰, whereas organic fertilizers are enriched in ^{15}N, ranging between +0.6‰ and +36.7‰ (manure between +10‰ and +25‰) (Vitòria and others 2004; Bateman and others 2007). Intensive use of organic fertilizers can, therefore, cause significant enrichment in the ^{15}N of nitrogen compounds in soil and plants (Bateman and others 2005).

Uptake of soil nitrogen by the plant does not cause any substantial fractionation (Werner and Schmidt 2002), whereas enzymatic reactions, such as nitrate reduction or transamination, significantly affect isotopic composition (Yoneyama 1995). Leguminous and nitrogen-fixing plants are an exception, as they can fix nitrogen directly from the air, having $\delta^{15}N$ values around 0‰ (Yoneyama and others 1995).

For carnivores, an enrichment in $\delta^{15}N$ of approximately $+3‰$ per trophic level has been observed (Kurle and Worthy 2002; McCutchan and others 2003). Thus $\delta^{15}N$ is widely used in ecological studies to determine the trophic levels of animals (Kurle and Worthy 2002; Post 2002) and humans (Hedges and Reynard 2007); This stepwise enrichment of $\delta^{15}N$ is also used to establish patterns in breastfeeding in humans (Fuller and others 2006). Analysis of $\delta^{15}N$ also enables researchers to explore the existence of nutritional stress during starvation (Fuller and others 2005) or pregnancy (Fuller and others 2006). More recently, Bloomfield and others (2011) determined $\delta^{15}N$ in black bream (*Acanthopagrus butcheri*) and found that temperature and diet affected bulk tissue $\delta^{15}N$ turnover and discrimination factors, with increased turnover and smaller discrimination factors at warmer temperatures. This means that trophic discrimination for $\delta^{15}N$ and $\delta^{13}C$ can differ significantly from those typically used in food-web analyses.

Different tissues have different levels of fractionation. De Niro and Epstein (1981) found that in mice fed the same diet the $\delta^{15}N$ isotopic values differed in these tissues: brain > liver > hair > muscle > kidney. The $\delta^{15}N$ of milk protein was 4‰ higher than that of urine protein (Knobbe and others 2006).

Hydrogen and oxygen

The sources of H and O in animals and animal tissues are drinking water and food, and in the case of O, also air (Hobson and Koehler 2015). δ^2H and $\delta^{18}O$ in water vary predictably with geographical origin, decreasing from low-latitude, low-elevation coastal regions to inland, high-latitude, mountainous regions (Bowen and others 2007). In plants, the main components of feed, 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 undergoes isotopic fractionation during evapotraspiration processes, which are affected by temperature and relative humidity, leading to an enrichment of the heavier isotopes. It is therefore to be expected that growing regions with relatively low humidity, where the rate of evaporation from the leaf is higher, will be characterized by vegetal water and plant materials with relatively enriched δ^2H and $\delta^{18}O$ values (Martin and others 1986).

Animal's body intrinsic water is derived from drinking water, intrinsic water in the food, and oxidation water originating from organically bound oxygen and atmospheric O_2 converted in the respiratory chain. Water loss occurs through the emission of urine, water in faeces, sweat and breath water and milk. The average $\delta^{18}O$ value of the body water of most domestic animals is about $3 \pm 1‰$ more positive than that of the drinking water (Krivachy and others 2015). The correlation between the $\delta^{18}O$ values of the animal's body water and drinking water depends on species, drinking, and respiration rates (Krivachy and others 2015), season, farm conditions, breed, and the physiological condition of the animal (Abeni and others 2015).

As regards animals' body water, the $\delta^{18}O$ of milk water records the isotopic composition of drinking water and water intake from fresh forage, with minor deviations due to the contribution of food and atmospheric oxygen to body water (Chesson and others 2010). When cows eat fresh herbage containing water enriched in ^{18}O due to evapotranspiration, then milk $\delta^{18}O$ increases considerably (Bontempo and others 2012; Abeni and others 2015).

In the case of animal tissue, the H and O isotopic composition of animal tissue is also strongly correlated with that of drinking water (Podlesak and others 2008). In particular, the δ^2H and $\delta^{18}O$ of human hair keratin were found to be related to those of

water from the same location (Ehleringer and others 2008), and the same was found for the δ^2H of cheese casein (Camin and others 2012).

Hydrogen and oxygen integration in the different tissues through metabolic processes causes considerable isotopic fractionation. Harrison and others (2011) determined diet-muscle and diet-intramuscular lipid fractionation following an experimental diet switch in lambs, and found them to be 44.0‰ and 0‰ for H and O in muscle and 172.7‰ and 11.5‰ in intramuscular lipid. In the same study, drinking water was found to be the main source of muscle O and thus of $\delta^{18}O$ variation.

Tuross and others (2008) reported that proteinaceous materials clustered in the most δ^2H-enriched range, such as collagen mandible (–63±8‰), and in the most δ^2H-depleted range, blood (–128±7‰) and muscle (–137±7‰). Fat had the most depleted δ^2H value (–284±12‰).

As in the case of organically bound oxygen, compounds can exchange oxygen atoms with the surrounding water, particularly in the case of carbonyl and carboxyl groups, as well as esters, amides, hydroxyl groups of phenols, hydroxy fatty acids, steroids and hydroxyproline. This exchange follows different routes, depending on the functional group involved, and therefore leads to different $\Delta^{18}O$ fractionation (from $+7 \pm 1‰$ for hydroxyl groups to $+22‰$ for the carbonamide group of protein and $+28‰$ for the ester group of triglycerides) (Schmidt and others 2001, Krivachy and others 2015).

Sulfur

The S source of any animal tissue is the sulfur contained in plants.

Natural factors influencing $\delta^{34}S$ values in terrestrial plants are the abundance of sulfides in the soil, but also aerobic or anaerobic growing conditions (Rubenstein and Hobson 2004), underlying local bedrock (igneous or sedimentary, acid or basic), active microbial processes in the soil, fertilization procedures, and atmospheric deposition, such as, and mainly, sulfate aerosol deposition over forage crops in coastal areas (sea-spray effect) (Krouse and Mayer 2000).

Terrestrial and marine plants can be differentiated using $\delta^{34}S$ (Rubenstein and Hobson 2004). Commonly found $\delta^{34}S$ values for terrestrial plants range from $-5‰$ to $+22‰$, with most plants ranging between $+2‰$ and $+6‰$. The $\delta^{34}S$ values of marine plants usually range from $+17‰$ to $+21‰$ (Peterson and Fry 1987). Fractionation of sulfur in marine habitats is mainly caused by sulfate-reducing bacteria (Thode 1991). In a study on Inuit diets, Buchardt and others (2007) showed that S stable isotope ratios are a very effective way of estimating the relative proportions of the Inuit diet originating from terrestrial and marine sources.

Of the stable isotope ratios, $\delta^{34}S$ is the least studied in animal science and food authentication.

The sulfur trophic shift between animals and their diet was estimated at between 0‰ and $+1‰$ (Harrison and others 2011; Krivachy and others 2015) and small differences could be found among the various animal organs due to the differing presence of methionine, cysteine, and proteoglycans. Indeed, the first organic S-containing product in animals is cysteine, the precursor of all other S-containing compounds, which are all depleted in ^{34}S relative to the precursor. Furthermore, the sulfate esters proteoglycans, contained in the connective tissue, are the only S-containing compounds whose biosynthesis involves significant sulfur isotope fractionation in animals. (Richards and others 2003; Tanz and Schmidt 2010; Florin and others 2011).

Measurement of Stable Isotope Ratios in Animal Matrices

In food authenticity studies, isotopic analysis of meat has usually been undertaken on defatted muscle and/or fat as in the case of the study by Bahar and others (2005) because lipids typically have lower $\delta^{13}C$ and δ^2H values than muscle (protein). The contribution of varying amounts of lipids to "bulk" measurement of meat could thus have an impact on data interpretation. The fat/lipid fraction can then be measured separately. Fractions of fish subjected to analysis generally concern the otolith (Whitledge and others 2006; Vanhove and others 2011), which provides a record of data over the life of the fish, and the defatted fish fillet (Moreno-Rojas and others 2008; Curtis and others 2014). In the case of dairy products, the bulk product, the defatted cheese or the extracted casein are the main matrices analyzed (Manca and others 2001; Crittenden and others 2007; Bontempo and others 2012; Camin and others 2012). Some studies have carried out compound-specific $\delta^{13}C$ and δ^2H analyses of individual fatty acids (Ehtesham and others 2013a,b; Molkentin 2013). Analysis of the SIRs of H, C, N, and S in cheese casein has recently been validated through an international collaborative study according to the IUPAC protocol (Camin and others 2015).

Proteinaceous material has a fraction of the H atoms which exchange with atmospheric water vapor, leading to potentially erroneous results unless controlled. Some materials can be easily derivatized to a form without labile H atoms such as cellulose nitration (DeNiro and Epstein 1976). Another option is to experimentally calculate the fraction of exchangeable H atoms in an equilibrating material using water vapors with a different isotopic compositions. The difference between the total δ^2H values of a sample, equilibrated with 2 distinct water vapors, could then be used to calculate the contribution of exchangeable H atoms (Wassenaar and Hobson 2000). Wassenaar and Hobson (2003) developed a more rapid method, called the comparative equilibration technique, based on the principle of identical treatment: calibrated reference materials and samples are simultaneously calibrated with ambient water vapor under identical conditions before isotopic analysis. After analysis, the measured δ^2H values of the reference materials are compared with the known nonexchangeable δ^2H value, and the calculated difference is used to normalize the δ^2H value of the samples, as it removes the effect of exchangeable H atoms. The equilibration can be performed at room temperature (Chesson and others 2009) or at a higher temperature to speed up the process (Wassenaar and Hobson 2003). Lately a new technological solution has been proposed in order to make this step more automated (Wassenaar and others 2015), and a new system for conducting the exchange step online through the use of an ad hoc carousel was presented (Uniprep®, Eurovector SpA, Italy).

The comparative equilibration method is effective, but it requires reference materials which match the matrix as closely as possible, at least in terms of the fraction of exchangeable hydrogen. For example, Chesson and others found that at 25 °C this fraction averaged 9% in keratin, up to 17‰ in cut hair, and 12% in muscle tissue. At the moment, however, only few organic reference materials are available and recognized for determination of δ^2H by the United States Geological Survey (USGS, http://isotopes.usgs.gov/lab/referencematerials.html): USGS 42, Tibetan human hair (δ^2H: −78.5‰), USGS 43, Indian human hair (−50.3‰), CBS, Caribou Hoof Standard (−197‰ and KHS, Kudu Horn Standard (−54.1‰). Some authors have used a casein with an assigned value of −113‰ for calibration (Camin and others 2007; Auerswald and others 2011).

Particular attention should be paid when analysing δ^2H and $\delta^{18}O$ of nitrogen-bearing compounds. Some authors (Gehre and others, 2015; Nair and others 2015) have reported that the classic HTC (high-temperature conversion) technique to produce H_2 from organic materials using high temperatures in the presence of glassy carbon is not suitable for these types of compounds and suggest adding chromium to the reaction zone to avoid or suppress hydrogen cyanide (HCN) formation and improve the yield to 100 %. One problem that can affect $\delta^{18}O$ determination is the overlapping of the N_2 peak with the subsequent CO peak (Coplen and others 2012; Hunsinger and others 2012). For this reason, control of peak separation must be carried out, and in the event of overlapping, longer columns (for example, 1.2 m) should be used.

Some authors have analyzed H and O ratios in animal tissue water, also evaluating the effect of storage (Boner and Forstel 2004, Thiem and others 2004, Franke and others 2008, Horacek and others 2010)

Authenticity of Foods of Animal Origin

SIRA of various combinations of bio-elements has been largely applied for assessing the authenticity of animal products. In this paragraph an updated overview of the studies regarding meat, dairy products, and fish is provided.

Meat

Different motivations have led many researchers to investigate possible applications of stable isotope ratios to meat traceability. The first application, naturally and directly derived from ecological studies, has been the reconstruction of animal diet for health and safety reasons (spread of BSE [Bovine Spongiform Encephalopathy] spread due to the use of feeds of animal origin), and on economic grounds (for example, meat from animals raised in the stable claimed to be from more valuable grazing animals, or the protection of premium PDO/PGI/TSG products). The majority of these studies have used $\delta^{13}C$ and/or $\delta^{15}N$ according to the specific diet that has to be traced back (C3/C4 plants, legumes, pasture/stall breeding). In order to comply with EU legislation concerning the marketing of poultry labeled as maize-fed (EEC Regulation 1906/90), chicken must be fed a diet containing at least 50% (w/w) maize for the greater part of the fattening period. Rhodes and others (2010) found $\delta^{13}C$ of protein to be a reliable marker of the dietary status of the chickens and set a threshold value of −22.5 $\delta^{13}C$‰ as indicating that the minimum content of 50% maize in the diet was observed. On the other hand, Coletta and others (2012) compared $\delta^{13}C$ and $\delta^{15}N$ determined on meat from free-range and barn animals and found significantly higher $\delta^{15}N$ in the former than in the latter, probably due to ingestion of animal protein. Satisfactory results have emerged from investigations of the proportion of C4 plant material in beef cattle using the $\delta^{13}C$ values of different tissue samples (DeSmet and others 2004). In particular, $\delta^{13}C$ and $\delta^{15}N$ determined in muscle, lipids, and hair were found to be effective in reflecting the diet administered to the cattle, either alone (Yanagi and others 2012) or in combination with other isotope ratios (δ^2H and $\delta^{34}S$; Osorio and others 2011a) or parameters related to different kinds of diet (silage, C3 or C4 concentrates, pasture, and soon) (Osorio and others 2013). On the basis of $\delta^{13}C$ and $\delta^{34}S$ of adipose and liver tissues, it was also possible to characterize meat from PDO Iberian swine, because of their diet based on acorns (Gonzalez-Martin and others 1999) and to differentiate swine of different breeds receiving different diets (acorns or feed) (González-Martín and others 2001).

In the case of lambs, stable isotope ratios have always been applied in comparisons between pasture- and stall-feeding. Devincenzi and others (2014), using only $\delta^{15}N$ muscle, and Prache and others (2009), using a combination of $\delta^{15}N$ muscle and δ-cadinene in perirenal fat and plasma carotenoid concentration, clearly separated pasture-fed lambs from lambs fed on high levels of alfalfa indoors. Recently, Biondi and others (2013) determined $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$ in lamb plasma, erythrocytes, and muscle, and they found that determination of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios in plasma were the most powerful variables for tracing back the change in the finishing period from herbage- to concentrate-based C3-based diets.

Another issue that led to the application of stable isotope ratios to meat is geographical origin, for verification of the authenticity of PDO/PGI/TSG products, and for implementation of controls in the event of pandemic diseases. The first study on this topic (Piasentier and others 2003) determined the $\delta^{13}C$ and $\delta^{15}N$ of fat and protein in lambs reared in Italy, France, Spain, UK, Greece, and Iceland. These ratios made it possible to separate lamb types from different countries with the same feeding regime. Subsequently, Boner and Forstel (2004) showed that beef samples could be differentiated 'globally' (Argentina compared with Germany) using $\delta^{2}H$ and $\delta^{18}O$ of meat water, and "locally" (various German farms) using $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ of defatted meat. Other authors have determined $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{34}S$ on defatted meat with and without the lipid fraction. Using this approach, significant differences were found between the multi-element isotope ratios of lamb samples from different European regions (Camin and others 2007; Perini and others 2009), and between beef from several European and non-European countries (Osorio and others 2011b). Heaton and others (2008) determined C and N isotope ratios of defatted dry mass and H and O of the corresponding lipid fractions in beef and found that the mean $\delta^{2}H$ and $\delta^{18}O$ values of beef lipid correlated well with latitude of production region. Furthermore, by combining stable isotope ratios with elemental composition they differentiated areas on a broad geographical scale (Europe, South America, and Australasia). Bong and others (2010) determined $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ in defatted beef meat and achieved generally successful discrimination even though there was some overlap among samples of different global origins circulating on the Korean markets. Finally, the combination of $\delta^{13}C$ and $\delta^{15}N$ in defatted meat, fat, and hair, was successfully used to trace cattle diet and origin in Chinese beef (Guo and others 2010).

The application of geographical origin to poultry (in addition to beef) was done by Franke and others (2008) who distinguished samples according to their country of origin using $\delta^{18}O$ of water extracted from meat and by Rees and others (2016) who combined SIRA and trace elements.

Stable isotope ratios have also been used to determine if a product was organically or conventionally farmed. This was first done when Boner and Forstel (2004) found that a $^{13}C/^{12}C$ value above $-20‰$ appeared to be the limit for European organic farming, as organic cattle fodder consists mainly of C3 plants while the use of C4 plants is more usual in conventional cattle farming. Subsequently, Schmidt and others (2005) showed that beef reared in the United States and Brazil was isotopically different from northern European beef due to the different proportions of C3 and C4 plants in the cattle diets, and that the combination of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ separated organically and conventionally farmed Irish beef. Finally, Bahar and others (2008) investigated the seasonal isotopic ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) composition of organic and conventional beef and found that $\delta^{13}C$ of conventional beef presented a seasonal positive shift between December and June, whereas organic beef was less variable and significantly more negative; $\delta^{15}N$, on the other hand, was roughly invariable throughout the year in conventional samples, whereas in organic beef it was more variable and always significantly lower.

A minor application concerns the characterization of a particular breed. Beside the work of Gonzalez-Martin and others (2001), Longobardi and others (2012) determined $\delta^{13}C$ and $\delta^{15}N$ together with other parameters (conventional parameters, elemental composition, metabolites) to distinguish the highly valuable Garganica kid goat meat.

Finally, a multipurpose study was carried out by Perini and others (2013) who investigated the stable isotope ratios ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$) of dry-cured ham in defatted dry matter, marbling, and subcutaneous fat fractions, taking different sources of variability into account (origin, pig genotype, feeding regime, and ham-seasoning). The isotopic composition of meteoric water and the dietary abundance of C4 plants distinguished Italian PDO hams from Spanish hams; in addition, the different treatments within the regional batches generated promising differences in SIR, potentially useful for tracing the whole ham production system, including the processing procedure.

Dairy products

The main reason for utilizing SIRA for dairy products is similar to that for meat, to trace origin and production system of premium products, using different combination of stable isotope ratios.

$\delta^{13}C$ and $\delta^{15}N$ of milk were used to distinguish between 2 different geographical areas in southern Italy (Brescia and others 2003). $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$, together with the isotopic ratio of Sr ($^{87}Sr/^{86}Sr$) in skimmed milk, combined with $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ of casein, were shown to have good potential for determining the geographical origin of dairy products produced in Australia and New Zealand (Crittenden and others 2007). Milk water $\delta^{18}O$ was able to differentiate higher-value French lowland sites (<500 m altitude) from upland sites (>700 m altitude), but high variability related to sampling period may mask this discrimination capability (Engel and others 2007). The $\delta^{18}O$ and $\delta^{2}H$ of milk water and $\delta^{13}C$ and $\delta^{15}N$ of milk have recently provided an initial insight into the isotopic variability of buffalo milk and cheese from the Amazon basin in Brazil (Silva and others 2014). Based on the relationship of the isotopic value of local precipitation with the $\delta^{18}O$ and $\delta^{2}H$ of milk water (Chesson and others 2010) and with $\delta^{2}H$ of bulk milk powder and milk fatty acid (Ehtesham and others 2013a), it was possible to predict the geographical origin of milk produced in the U.S. and New Zealand. The multivariate statistical combination of $\delta^{2}H$ and $\delta^{13}C$ of 4 fatty acids (c4:0, c14:0, c16:0, and c18:1) and bulk milk powder was found effective in distinguishing samples from the North and South Islands of New Zealand (Ehtesham and others 2013b). Recently, Ehtesham and others (2015) found that $\delta^{2}H$ composition of bovine milk is affected not only by $\delta^{2}H$ of drinking water but also by grass fatty acids and that there is a significant correlation between the $\delta^{2}H$ of milk and feed fatty acids. Scampicchio and others (2015) combined $\delta^{13}C$ and $\delta^{15}N$ values with mid- (MIRS) and near-infrared spectroscopy (NIRS) data, as well as the fatty acid profile to distinguish milk samples from North and South Tyrol in Austria and other European countries.

Organic milk was discriminated from conventional milk in Korea (Chung and others 2014) and Germany (Molkentin and Giesemann 2007, 2010; Molkentin 2009; Kaffarnik and others

2014) based on $\delta^{13}C$ of protein in combination with other isotopes ($\delta^{15}N$) or other analytical parameters (fatty acid, α-linolenic acid, phytanic acid diastereomer ratios). Molkentin (2013) suggested α-linolenic acid (+0.5 %), $\delta^{13}C$ (−26.5 ‰), and $\delta^{15}N$ (+5.5 ‰) thresholds for organic milk, which were found generally applicable to German processed dairy products, such as soft and semi-hard cheeses, butter, cream, sour cream, buttermilk, yogurt, and low-fat milk. Similarly Erich and others (2015) combined chemometric analysis ^1H NMR-, ^{13}C NMR-spectroscopy data with $\delta^{13}C$ and α-linolenic acid content to improve the differentiation of organic and conventional milk (raw, pasteurized, and ultraheat treated).

McLeod and others (2015) determined $\delta^{13}C$, $\delta^{15}N$, and the concentrations of some trace elements in goat milk powder. The chemical fingerprint obtained allowed to verify the origin of the product even, in some cases, at the manufacturer or factory level.

With respect to cheese, the stable isotope ratios of C and N of casein were able to distinguish between Pecorino (sheep milk) cheeses produced in Sardinia and those produced in Sicily and Apulia (Manca and others 2001), and between buffalo mozzarella originating from 2 areas of Southern Italy (Brescia and others 2005). Together with the isotopic ratios of other bioelements (S and H in casein, and C and O in glycerol), this analytical approach has made it possible to differentiate between European cheeses from France, Italy, and Spain (Camin and others 2004), Emmental cheeses from Finland, Brittany, and Savoy (Pillonel and others 2003), and Sardinian Peretta cheese from competitors produced in Northern Europe (Manca and others 2006). Pillonel and others (2003) for the first time combined the stable isotope ratios of bioelements ($\delta^{13}C$, δ^2H, and $\delta^{15}N$) and of heavy elements ($^{87}Sr/^{86}Sr$) with the elemental profile to authenticate Emmental-type cheeses produced in Switzerland, Germany, France, Austria, and Finland. More recent papers show that the combination of stable isotope ratios and elemental data (Ba, Ca, K, Mg, Rb, $\delta^{13}C_{casein}$, $\delta^{15}N_{casein}$, and $\delta^{18}O_{glycerol}$ in Bontempo and others 2011; $\delta^{13}C$, δ^2H, $\delta^{15}N$, $\delta^{34}S$ of casein and Sr, Cu, Mo, Re, Na, U, Bi, Ni, Fe, Mn, Ga, Se, and Li in Camin and others 2012) improves discrimination between Alpine PDO cheeses and Parmigiano Reggiano DOP cheese on the one hand and non-PDO hard cheeses imported into Italy on the other. The reliability and efficiency of this approach has also been recognized by the protection consortia for PDO Grana Padano and Parmigiano Reggiano cheese, which since 2000 have created a huge reference databank for PDO and non-PDO hard cheeses and since 2011 have officially adopted stable isotope ratio analysis to verify the authenticity of grated and shredded products on the market (EU Regulation 584/2011). Stevenson and others (2015) combined H,O,N,C stable isotope ratios with $^{87}Sr/^{86}Sr$ data for cheese, milk, animal feed, and soil from 6 artisanal cheese producers in Quebec, Canada, and demonstrated the potential of isotope geochemistry in linking land/terroir to agricultural produce. Recently a paper (Capici and others 2015) demonstrated that $\delta^{13}C$ and $\delta^{15}N$ isotope values of fat and defatted fraction have different values between each other depending on if a cheese was produced with raw milk or with pasteurized milk thus allowing differentiation between these 2 kinds of product.

In the case of butter, stable isotope ratio determination of C, N, O, and S and of Sr of butter from several European countries and from outside the EU enable the regional provenance of butter to be reliably detected (Rossmann and others 2000).

Fish and shellfish

With regard to fish and seafood in general, isotopic analysis has been extensively used, especially in the field of ecology to study trophic dynamics in aquatic environments and in relation to spatial and temporal variations (for example, Abrantes and others 2014). As regards the authenticity of seafood products, 3 factors are at play, possibly related to fraud and defining traceability as established by EU law (EC Reg. 2065/2001): production method (wild or farmed, sea or freshwater), geographical origin, and biological species. Despite its potential, few papers have dealt with the use of isotopic analysis to determine the authenticity of seafood, especially when compared to the amount of research on, for example, meat and dairy products. Most of these papers focus on differentiating wild from reared products. The first application of this kind dates back to 2000, when Aursand and others (2000) determined δ^2H and $\delta^{13}C$ and fatty acid composition to study different kinds of fish oils and lipids extracted from muscle tissue of wild and farmed salmon. These authors showed that a classification analysis of 4 fatty acids and D/H of fish oils assigned all the oils to the correct group. Subsequently, Dempson and Power (2004) used carbon and nitrogen stable isotope ratios to distinguish farmed from wild Atlantic salmon, and they found that aquaculture salmon was consistently more significantly enriched in $\delta^{13}C$ but depleted in $\delta^{15}N$ compared with wild fish. Afterwards, 4 other studies (Serrano and others 2007; Busetto and others 2008; Fasolato and others 2010; Oliveira and others 2014) similarly used $\delta^{13}C$ and $\delta^{15}N$ with or without other analyses and techniques (fatty acid profiles and chemical composition) to differentiate between different wild and farmed sea and freshwater fish species (turbot, sea bass, cod, cachara fish, gilthead sea bream). There was not always agreement over the relative depletion or enrichment of $\delta^{13}C$ and $\delta^{15}N$ in wild and farmed fish determined in these studies due to factors closely linked to the behaviors of the species examined and their diets (such as origin and type of protein consumed, or metabolic turnover related to the scarcity, or abundance of food). Trembaczowski (2012) introduced determination of $\delta^{34}S$ of muscles and scales coupled with $\delta^{13}C$ to identify the production method of trout specimens from Polish rivers and pond farms. Recently, Curtis and others (2014) combined $\delta^{13}C$ and $\delta^{18}O$ isotope ratios and 7 trace elements (Ba, B, Mn, K, Rb, Na, and Sr) determined in otoliths of spotted seatrouts, and their results showed that otolith chemistry could be a powerful tool for differentiating hatchery-reared from wild spotted seatrout.

Other authors (Bell and others 2007; Morrison and others 2007; Thomas and others 2008) investigated variability within the stable isotope ratios in various lipidic fractions (raw oil, fatty acids, glycerol, choline) and used them to distinguish between wild and farmed sea bass, sea bream, and Atlantic salmon from different geographical origins in Europe, North America, and Tasmania. In particular, Bell and others (2007) found that bulk $\delta^{13}C$ of the total lipid fraction denoted a highly significant difference between farmed and wild sea bass, with farmed fish being isotopically lighter than wild fish, probably related to different dietary inputs. The measured $\delta^{13}C$ values of individual fatty acids yielded some significant differences, such as $\delta^{13}C$ values of 16:0, 18:0, 16:1n-7, 18:1n-9, and 18:1n-7 being more positive in wild sea bass than in farmed sea bass. On the other hand, Thomas and others (2008) found that the 2 parameters, $\delta^{15}N_{choline}$ and $\delta^{18}O_{oil}$, were particularly effective in discriminating between authentic wild and farmed salmon.

Moreno-Rojas and others (2008) found differences in $\delta^{13}C$ and $\delta^{15}N$ values of farmed rainbow trout fillets fed diets based on plants (pea protein concentrate and wheat gluten) or fishmeal proteins, with depleted values in fish fed exclusively on the plant diet.

Up to now, only 1 study (Turchini and others 2009) has investigated the use of δ^{13}C, δ^{15}N, and δ^{18}O to discriminate between different intensive farms in various geographical areas (with respect to Murray cod). These researchers found that δ^{13}C and/or δ^{15}N clearly linked fish to a specific commercial diet, while δ^{18}O linked fish to a specific water source, thus the combination of these ratios can be useful in distinguishing between fish from different farms.

There are also fewer recently published papers dealing with the application of these techniques to the geographical origin of seafood for traceability purposes. Ortea and Gallardo (2015) considered possible differentiation between wild and farmed shrimps, different species, and different geographical origins, using δ^{13}C and δ^{15}N with or without elemental composition. The use of SIRs alone gave satisfactory results in all 3 cases, although far better results were obtained using a combination of both techniques. Carter and others (2015) determined δ^{2}H, δ^{18}O, δ^{13}C, and δ^{15}N in the shells, chitin, and meat of Australian prawns and Asian prawns imported into Australia (Carter and others 2015) and they found strong differences between prawns of different geographical origins for all the parameters. Furthermore, the isotopic composition of all the elements showed a strong correlation between meat and chitin, indicating that equivalent information can be obtained from these 2 components and, therefore, only one of them needs to be analyzed.

Concluding Remarks

Geographical, climatic, pedological, geological, botanical, and agricultural factors affect the stable isotope ratios of bioelements in nature, and isotopic variations are ultimately incorporated into animal tissue throughout eating, drinking, breathing, and exchange with the environment, being memorized in the resulting foods. As a consequence, the stable isotope ratios analysis of H, C, N, O, and S has shown high potential for determining geographical origin, animal diet, and the production system (organic/conventional) for pork, beef, poultry, milk, butter, cheese, fish, and shellfish.

Isotopic analysis has sometimes been used in real–life situations to detect the authenticity of foods of animal origin. This is the case of the hard cheeses Grana Padano and Parmigiano Reggiano PDO: starting from 2000 a huge number of authentic samples have been collected by the consortia, and subjected to analysis of stable isotope ratios and elements (Camin and others 2015) to create a reference system called databank and a random forest statistical model (Camin and others 2012). To evaluate the authenticity of commercial samples, their values are compared with the limits computed on the basis of the databank and are evaluated in terms of fit with the statistical model. If the values falls outside the authenticity limits and the model does not recognize the cheese as PDO, the sample is declared not to be authentic. At this point the consortium can suspend the PDO license for 6 months and asks for a fee. Moreover, the producer can be prosecuted in court.

The main drawback in application of the technique to real–life situations is the expense of creating a reference databank for each product, in terms of both time and financial costs. Indeed, a large data set, comprising samples from a wide and representative range of geographical, seasonal, dietary and production conditions is needed. Involvement and funding from PDO protection consortia, producers' associations or official bodies is therefore recommended.

Acknowledgment

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