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METODI ISOTOPICI E MODELLI PER LA TRACCIABILITA' DEI PRODOTTI ANIMALI

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LIST OF PUBLICATIONS

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F. Camin*, L. Bontempo, M. Perini, E. Piasentier. Stable Isotope Ratio Analysis for assessing the authenticity of food of animal origin. Trends in Food Science & Technology. *Under review*

L. Bontempo, **F. Camin**, L. Ziller, L. Biondi, M.G. D'Urso, V. Vasta, G. Luciano (2016) Variations in stable isotope ratios in lamb blood fractions following dietary changes: a preliminary study. RAPID COMMUNICATIONS IN MASS SPECTROMETRY. 30, 170–174.

I. Mekki, **F. Camin**, S. Smeti, H. Hajji, M. Mahouachi, E. Piasentier, N. Atti Determining the geographical origin of Tunisian indigenous lamb using stable isotope ratio and fatty acid content, *in submission*

F. Camin, A. Tonon, L. Ziller, A. Sepulcri, M. Perini, L. Bontempo, F. Tulli, E. Tibaldi, M. Galeotti, E. Piasentier. Stable isotope ratios of H, C, O, N and S for the geographical traceability of the Italian rainbow trout (Oncorhynchus mykiss). Journal of Agriculture and Food Chemistry. *Submitted*

F. Camin, D. Bertoldi, A. Santato, L. Bontempo, M.Perini, L. Ziller, A. Stroppa, R. Larcher (2015) Validation of methods for H, C, N and S stable isotopes and elemental analysis of cheese: results of an international collaborative study. Rapid Commun. Mass Spectrom. 2015, 29, 415–423

ABSTRACT

The main elemental constituents (H, C, N, O, 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, ^{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.

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 memorised 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 and poultry, milk, butter, cheese, fish and shellfish.. In the case of the hard PDO 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.

With this work I tried to fill in some of the research gaps found reviewing the literature on the application of the analysis of the stable isotope ratios of bioelements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$), for the traceability of animal products, in particular meat, dairy and fish.

In detail, the research aimed at:

- studying the variation of stable isotope ratios in blood fractions of lamb following dietary changes,
- detecting the efficacy of IRMS for tracing lamb production systems in four regions of the north west of Tunisia
- investigating the ability of the isotopic analysis to discriminate and trace the geographical origin of rainbow trout,
- validating through a collaborative study the methods for analysing the isotopic and mineral profile of cheese,
- characterising the isotopic and mineral profile of Mozzarella di Bufala PDO cheese.

We found that $\delta^{13}C_{plasma}$, $\delta^{18}O_{plasma}$ and $\delta^{34}S_{plasma}$ of lamb were different 7 days after an abrupt switch from a pasture- to a concentrate-based diet. $\delta^{15}N_{plasma}$ and the isotope ratios of blood erythrocytes were not different on the different sampling dates and were not affected by the change of diet over a 14 days period. Therefore the combination of isotopic ratios of plasma and erythrocytes represents a good tool for deducing the dietary background of lambs. Indeed, erythrocytes were shown to be suitable for verifying whether the animal was actually pasture-raised

and could merit a higher price, while the analysis of plasma could detect very short finishing periods in previously grazed animals.

The efficacy of IRMS for tracing lamb production system was investigated in four regions of the north west of Tunisia, characterized by herbaceous pasture. The isotopic profiles of the Tunisian lamb meat types provided useful signatures for a satisfactory traceability of the lamb meat samples produced in Tunisia (sensitivity = 1.00). However, the differences between them were not sufficient and systematic to be validated by an external set of samples including 10 Italian lamb types.

For investigating the ability of the isotopic analysis to trace the geographical origin of fish, we measured the isotopic ratios of H, C, N, O and S in defatted fillet and the extracted lipid fraction from farmed rainbow trout reared in different Italian farms and with two types of feeds: high fish content (HF) and low fish content (LF) feeds. The aim was to investigate the ability of isotopic analysis to discriminate and trace the geographical origin of trout from two regions of northern Italy (Friuli Venezia Giulia and Trentino), also according to the type of feed. We found that the C, N and S isotope ratios of feed and fillet were highly positively correlated among and between each other, and negatively correlated with the δ^2H and $\delta^{18}O$ of feed and with the δ^2H_{fat} of fillet, which were in their turn highly interrelated. The δ^2H and $\delta^{18}O$ of fillet were interrelated and positively correlated with $\delta^{18}O_{water}$. In comparison with other variables, the $\delta^{18}O_{fat}$ of fillet showed less significant correlation with the other isotopic parameters. Good discrimination was found between trouts according to the type of feed and with the origin.

For validating the isotopic and elemental analytical methods, an international collaborative study based on blind duplicates of 7 hard cheeses was performed according to the IUPAC protocol and ISO Standards 5725/2004 and 13528/2005. The H, C, N and S stable isotope ratios of defatted cheese determined using IRMS and the content of Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba, Re, Bi, U in cheese after acid microwave digestion using ICP-MS were analysed in 13 different laboratories. The average standard deviations of repeatability (sr) and reproducibility (sR) were defined for both the isotopic and the mineral variables. The validation data obtained here can be submitted to the standardisation agencies to obtain official recognition for the methods, which is fundamental when they are used in commercial disputes and legal debates. This is very important for PDO cheeses, such as the Parmigiano Reggiano and Grana Padano, which cost more than double the generic similar cheeses and must be protected against mislabelling.

A first evaluation of the isotopic and mineral data measured in buffalo milk and corresponding Mozzarella di Bufala Campana PDO, highlights the importance of the $\delta^{18}O$ and δ^2H values of casein, as well as of the content in Rb for tracing the origin of these products.

SUMMARY

1. INTRODUCTION

1.1 Food authenticity

Determining the authenticity of foods means uncovering misdescription 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 (e.g. 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), which came into force in January 2005, and defines 'food and feed traceability'. For bovines, since 2000 EU law has 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 1 April 2015, the labelling 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 Specialities Guarantee (TSG), European laws EC N. 510/2006 and 1151/2012 require protection against the mislabelling. 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 non-targeted food samples to provide proof of origin and prevent deliberate or accidental undeclared, unpermitted admixture to food samples.

Stable isotope ratios analysis, measured using Isotope Ratio Mass Spectrometry (IRMS), has taken on increasing importance in determining the authenticity of food of animal origin for producers and control agencies.

1.2 Stable isotope ratio variability in animals

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

1.2.1 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 vegetal 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 ($\delta^{13}C$: -12 ÷ -14‰) than C3 plants ($\delta^{13}C$: -23 ÷ -30‰) (Camin et al., 2004; Crittenden et al., 2007; Knobbe et al., 2006; Molkentin & Giesemann, 2007, 2010; Molkentin, 2009; Camin, Perini, Colombari, Bontempo, & Versini, 2008)

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

As reported by Tieszen, Boutton, Tesdahl, & Slade (1983), the $\delta^{13}C$ value in animals increased by about 1‰ according to the mean isotopic value of their diets. In detail, the fractionation of $\delta^{13}C$ between diet and tissue was estimated to be +1.9‰ for muscle and 1.3‰ for intra-muscular lipid (Harrison et al., 2011). Bloomfield, Elsdon, Walther, Gier, & Gillanders (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 et al. (2004) confirmed the significant ^{13}C depletion of lipids due to isotopic fractionation during oxidation of piruvate to acetic CoA, but also enrichment of muscle, hair, liver, blood and plasma. The order of $\delta^{13}C$ values was hair > muscle > liver > kidney fat. Tieszen et al. (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 non ruminants was teeth > hair > brain > muscle > diet > liver, kidney > heart > lung > breath CO2. Recently, some authors have tried to 'validate' muscle turnover rates and discrimination factors to ensure greater accuracy in interpreting stable isotope data. Harrison and colleagues (2010 and 2011) carried out two 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 two different energy allowances. Small, albeit significant, differences were detected in tissue carbon turnover in the Longissimus dorsi muscle whereas inter-muscular comparison showed similar C half-lives for most of the muscles analyzed (Harrison et al., 2010). Furthermore, it was found that the energy allowance had a significant impact on intra- and inter-muscular C turnover (Harrison et al., 2010), so that C half-lives of muscle were determined to be 75.7 and 91.6 days for animals receiving high and low energy allowances, respectively (Harrison et al., 2011).

Finally one study (Svensson et al., 2014) investigated the differences in $\delta^{13}C$ and $\delta^{15}N$ determined in muscle and gill in fish and found a strong correlation between the two 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.

1.2.2 HYDROGEN AND OXYGEN

The H and O isotopic composition of animal tissue is strongly correlated with that of drinking water (Podlesak et al., 2008). In particular, $\delta^{2}H$ and $\delta^{18}O$ of hair keratin were found to be related to those of water from the same location (Ehleringer et al., 2008), and the same was found for $\delta^{2}H$ of cheese casein (Camin et al., 2012). This relationship is important because $\delta^{2}H$ and $\delta^{18}O$ in the global water cycle vary predictably, decreasing from low-latitude, low-elevation coastal regions to inland, high-latitude, mountainous regions (Bowen, Ehleringer, Chesson, Stange, & Cerling, 2007).

Animal body intrinsic water derives 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 emission of urine, water in faeces, sweat and breath water, milk and, mainly, breath CO_2, which is highly enriched in ^{18}O through equilibration with water. The

average $\delta^{18}O$ value of the body water of most domestic animals is about 3 ± 1‰ more positive than that of the drinking water. The correlation between the $\delta^{18}O$ values of animal body water and drinking water depends on species, drinking and respiration rates (Krivachy, Rossmann, & Schmidt, 2015), season, farm, breed and the physiological condition of the animal (Abeni and colleagues 2015). Furthermore, the correlation between $\delta^{18}O$ and $\delta^{2}H$, and the correlation between these isotope ratios in body water and in drinking water is related to diet, but in the specific case of carnivores this expectation is not met. It follows that attempts to assign meat to geographical origin exclusively on the basis of the $\delta^{18}O$ value of squeezed tissue water were not very promising (Krivachy et al., 2015).

Furthermore, organically bound oxygen, in specific cases, could 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 ± 1‰ for hydroxyl groups, to +23‰ for the carbonyl groups) (Krivachy et al., 2015).

Harrison and colleagues (2011) determined diet-muscle and diet-intramuscular lipid fractionations 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 intra-muscular lipid. In the same study, drinking water was found to be the main source of muscle O and thus of $\delta^{18}O$ variation.

Hydrogen and oxygen integration in the different tissues through metabolic processes causes considerable isotopic fractionation. For example, Tuross, Warinner, Kirsanow, & Kester (2008) reported that proteinaceous materials clustered in the most $\delta^{2}H$-enriched range, e.g. collagen mandible (-63±8‰), and in the most $\delta^{2}H$-depleted range, blood (-128±7‰) and muscle (-137±7‰). Fat materials had the most depleted $\delta^{2}H$ value (-284±12‰).

As for animal 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, Valenzuela, O'Grady, Cerling, T.E., & Ehleringer, 2010). When cows eat fresh herbage containing water enriched in ^{18}O due to evapotranspiration, milk $\delta^{18}O$ increases considerably (Bontempo et al., 2012; Abeni et al., 2015).

1.2.3 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 are almost exclusively herbivorous (e.g.

cows, sheep), and therefore the isotopic composition of plant feed is the most important factor in N variability.

Atmospheric N_2 is the ultimate source of all natural N-containing compounds, containing about 0.4% ^{15}N. It 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, hydrogen and oxygen. It moves from the atmosphere through various plants and microbes, and occurs in a variety of reduced and oxidized forms (Werner & 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 et al., 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‰) (Bateman, Kelly, & Woolfe, 2007). Intensive use of organic fertilizers can therefore cause significant enrichment in the ^{15}N of nitrogen compounds in soil (Bateman, Kelly, & Jickells, 2005).

The $\delta^{15}N$ values in plants are generally correlated with those of nitrates and ammonia in soils. Uptake does not cause any substantial fractionation (Werner & 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 et al., 1995).

Findings regarding $\delta^{15}N$ are widely used in ecological studies to determine the trophic levels of animals (Kurle & Worthy, 2002; Post, 2002) and humans (Hedges & Reynard, 2007); enrichment has been shown to be approximately +3‰ per trophic level (Kurle & Worthy, 2002).

This stepwise enrichment of $\delta^{15}N$ is also used to establish patterns in breastfeeding in modern humans (Fuller et al., 2006). Analysis of $\delta^{15}N$ also enables researchers to explore the existence of nutritional stress during starvation (Fuller et al., 2005) or pregnancy (Fuller et al., 2006). More recently, Bloomfield and colleagues (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 & Epstain (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 et al. 2006).

1.2.4 SULPHUR

The S source of any animal tissue is the sulphur contained in plants. Natural factors influencing $\delta^{34}S$ values in terrestrial plants are the abundance of heavy sulphides in the soil, but also aerobic or anaerobic growing conditions (Rubenstein & 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, sulphate aerosol deposition over forage in coastal areas (sea-spray effect) (Krouse & Mayer, 2000).

Terrestrial and marine plants can be differentiated using $\delta^{34}S$ (Rubenstein & 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 & Fry, 1987). Fractionation of sulphur in marine habitats is mainly caused by sulphate-reducing bacteria (Thode, 1991). In a study on Inuit diets, Buchardt, Bunch, & Helin (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.

The sulphur trophic shift between animals and their diet was estimated at between 0‰ and +1‰ (Harrison et al., 2011; Krivachy et al., 2015), although small differences could be found among the different animal organs due to the differing presence of methionine, cysteine and proteoglykans. 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 sulphate esters proteoglykans, contained in the connective tissue, are the only S-containing compounds whose biosynthesis involves significant sulphur isotope fractionation in animals.

1.3 Stable isotope ratios measurement in animal matrices

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

In food authenticity studies, isotopic analysis of meat has usually been undertaken on defatted muscle and/or fat (e.g. Bahar et al., 2005). Fractions of fish subjected to analysis generally concern the otolith, which provides a record of data over the life of the fish and the defatted fish fillet

(Curtis, Stunz, Overath, & Vega, 2014; Moreno-Rojas, Tulli, Messina, Tibaldi, Guillou, 2008). In the case of dairy products, the bulk product, the defatted cheese or the extracted casein are the main matrices analyzed (Bontempo, Lombardi, Paoletti, Ziller, & Camin, 2012; Camin et al., 2012; Crittenden et al., 2007; Manca et al., 2001). Some studies have carried out compound specific $\delta^{13}C$ and $\delta^{2}H$ analysis of individual fatty acids (Molkentin, 2013; Ehtesham, Baisden, Keller, Hayman, Van Hale, & Frew, 2013a; Ehtesham, Hayman, McComb, Van Hale, & Frew, 2013b). Analysis of the SIRs of H, C, N, S in cheese casein has recently been validated through an international collaborative study according to the IUPAC protocol (Camin et al., 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 (e.g., cellulose nitration, DeNiro & Epstein, 1976). Another option is to experimentally calculate the fraction of exchangeable H atoms in an equilibrating material with water vapors of different isotopic compositions. The difference between the total $\delta^{2}H$ values of an unknown equilibrated with two distinct water vapors could then be used to calculate the contribution of exchangeable H atoms (Chesson, Podlesak, Cerling, & Ehleringer, 2009). For example, Chesson and colleagues found that this fraction at 25°C averaged 9% in keratin, up to 17‰ in cut hair, and 12% in muscle tissue. 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 unknowns are simultaneously calibrated with ambient water vapor under identical conditions prior to isotopic analysis. Post-analysis, the measured total $\delta^{2}H$ values for the reference material(s) are compared with the defined non-exchangeable $\delta^{2}H$ value, and the calculated difference is used to remove the effect of exchangeable H atoms on the $\delta^{2}H$ value of the unknowns. This method is rapid, but requires reference materials which match the matrix as closely as possible, at least in terms of the fraction of exchangeable hydrogen. At the moment, however, only two organic reference materials are available and recognized for the determination of $\delta^{2}H$ (USGS 42 - Tibetan human hair, and USGS 43- Indian human hair). To bypass this snag, Wassenaar and Hobson (2003) used three calibrated keratin working standards (chicken feather - CFS, cow hoof – CHS, and bowhead whale baleen - BWB-II) and the comparative equilibration method to correct the effects of moisture on exchangeable hydrogen. Other authors have used a casein with an assigned value of -113‰ for calibration (Auerswald et al., 2011; Camin et al., 2007).

1.4 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.

1.4.1 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 (e.g. BSE spread due to the use of feeds of animal origin), and on economic grounds (e.g. 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 corn-fed (EEC Regulation 1906/90), chicken must be fed a diet containing at least 50% (w/w) corn for the greater part of the fattening period. Rhodes and colleagues (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 colleagues (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 et al., 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 et al., 2012) or in combination with other isotope ratios ($\delta^{2}H$ and $\delta^{34}S$; Osorio, Moloney, Schmidt, & Monahan F.J., 2011a) or parameters related to different kinds of diet (silage, C3 or C4 concentrates, pasture, etc.) (Osorio et al., 2013). On the basis of $\delta^{13}C$ and $\delta^{34}S$ of adipose and liver tissue, it was also possible to characterize meat from PDO Iberian swine, because of their diet based on acorns (Gonzales-Martin et al., 1999) and to differentiate swine of different breeds receiving different diets (acorns or feed) (González-Martín, González Pérez, Hernández Méndez, & Sánchez González, 2001).

In the case of lambs, stable isotope ratios have always been applied in comparisons between pasture- and stall-feeding. Devincenzi, Delfosse, Andueza, Nabinger, & Prache (2014), using only

$\delta^{15}N$ muscle, and Prache and colleagues (2009), using a combination of $\delta^{15}N$ muscle and d-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 colleagues (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 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 et al., 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 discriminate lamb types from different countries with the same feeding regime. Subsequently, Boner and Förstl (2004) showed that beef samples could be differentiated 'globally' (Argentina vs. 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 et al., 2007; Perini, Camin, Bontempo, Rossmann, & Piasentier, 2009), and between beef from several European and non-European countries (Osorio, Moloney, Schmidt, & Monahan, 2011b). Heaton, Kelly, Hoogewerff, & Woolfe (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 colleagues (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 et al., 2010).

The only application of geographical origin to poultry (in addition to beef) was done by Franke, Hadorn, Bosset, Gremaud, & Kreuzer (2008), who distinguished samples according to their country of origin using $\delta^{18}O$ of water extracted from meat.

Stable isotope ratios have also been used to determine if a product was organically or conventionally farmed. This was first done in 2004 when Boner and Forstl found that a $^{13}C/^{12}C$ value above –20‰ appeared to be the limit for 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 colleagues (2005) showed that beef reared in the USA 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 et al. (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 Gonzales-Martin, 2001, Longobardi and colleagues (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, Camin, del Pulgar, & Piasentier (2013), who investigated the stable isotope ratios ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) 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.

1.4.2 Dairy products

As for meat, also for dairy products the main reason for applying SIRA is 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 two different geographical areas in southern Italy (Brescia, Caldarola, Buccolieri, Dell'Atti, & Sacco, 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 et al., 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 et al., 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 et al., 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 et al., 2010) and with $\delta^{2}H$ of bulk milk powder and milk fatty acid (Ehtesham et al., 2013a), it was possible to predict the geographical origin of milk produced in the United States and New Zealand. The multivariate statistical combination of $\delta^{2}H$ and $\delta^{13}C$ of four fatty acids (c4:0, c14:0, c16:0, c18:1) and bulk milk powder was found effective in distinguishing samples from the North and South Islands of New Zealand (Ehtesham et al., 2013b). Organic milk was discriminated from conventional milk in Korea (Chung, Park, Yoon, Yang, & Kim, 2014) and Germany (Kaffarnik, Schröder, Lehnert, Baars, & Vetter, 2014; Molkentin & Giesemann, 2007, 2010; Molkentin, 2009) 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). Molketin 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, yoghurt and low-fat milk (Molkentin, 2013).

With respect to cheese, the stable isotope ratios of C and N of casein were able to distinguish between Pecorino (sheep's milk) cheeses produced in Sardinia and those produced in Sicily and Apulia (Manca et al., 2001), and between buffalo mozzarella originating from two areas of Southern Italy (Brescia, Monfreda, Buccolieri, & Carrino, 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 et al., 2004), Emmental cheeses from Finland, Brittany and Savoy (Pillonel et al., 2003), and Sardinian Peretta cheese from competitors produced in Northern Europe (Manca et al., 2006). Pillonel et al. (2003) for the first time combined the stable isotope ratios of bioelements ($\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$) 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 $\delta^{18}O_{glycerol}$; Bontempo et al., 2011; $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, $\delta^{34}S$ of casein and Sr, Cu, Mo, Re, Na, U, Bi, Ni, Fe, Mn, Ga, Se, and Li; Camin et al., 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 recognised 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).

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 et al., 2000).

1.4.3 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 (e.g. recently: Abrantes, Johnston, Connolly, & Sheaves, 2014). As regards the authenticity of seafood products, three 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 colleagues (Aursand, Mabon, & Martin, 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 four fatty acids and D/H of fish oils assigned all the oils to the correct group. Subsequently, Dempson & Power (2004) used carbon and nitrogen stable isotope ratios to distinguish farmed from wild Atlantic salmon, and found that aquaculture salmon was consistently more significantly enriched in $\delta^{13}C$ but depleted in $\delta^{15}N$ compared with wild fish. Afterwards, four other studies (Busetto et al., 2008; Fasolato et al., 2010; Oliveira, Bastos, Claudino, Assumpção, & Garcia, 2014; Serrano, Blanes, & Orero, 2007) similarly used $\delta^{13}C$ and $\delta^{15}N$ with or without other analyses and techniques (e.g. fatty acid profiles and chemical composition) to differentiate between different wild and farmed sea and freshwater fish species (i.e. 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 (e.g. 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 et al. (2014) combined $\delta^{13}C$ and $\delta^{18}O$ isotope ratios and seven trace elements (Ba, B, Mn, K, Rb, Na, Sr) determined in otoliths of spotted

seatrouts, and their results showed that otolith chemistry could be a powerful tool for discriminating hatchery-reared from wild spotted seatrout.

Other authors (Bell et al., 2007; Morrison et al., 2007; Thomas et al., 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 colleagues (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 "heavier" in wild sea bass than in farmed sea bass. On the other hand, Thomas et al. (2008) found that the two parameters, $\delta^{15}N_{choline}$ and $\delta^{18}O_{oil}$, were particularly effective in discriminating between authentic wild and farmed salmon.

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

Up to now, only one study (Turchini, Quinn, Jones, Palmeri, & Gooley, 2009) has investigated the use of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ to discriminate between different intensive farms in different geographical areas (with respect to Murray cod). These researchers found that $\delta^{13}C$ and/or $\delta^{15}N$ clearly linked fish to a specific commercial diet, while $\delta^{18}O$ 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 even fewer recently published papers dealing with the application of these techniques to the geographical origin of seafood for traceability purposes. Ortea & Gallardo (2015) considered possible differentiation between wild and farmed shrimps, different species, and different geographical origins, using $\delta^{13}C$ and $\delta^{15}N$ with or without elemental composition. The use of SIRs alone gave satisfactory results in all three cases, although far better results were obtained using a combination of both techniques. Carter, Tinggi, Yang, & Fry (2015) determined $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ in the shells, chitin, and meat of Australian prawns and Asian prawns imported into Australia (Carter et al., 2015) and 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 two components and therefore only one of them needs to be analyzed.

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2. OBJECTIVES

The main objective of this research was to fill in some of the research gaps found reviewing the literature concerning the application of the analysis of the stable isotope ratios of bioelements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$), for the traceability of animal products, in particular meat, dairy and fish.

In detail the research focused on:

- For animal/meat:
    - investigate the variation of stable isotope ratios in blood fractions of lamb following dietary changes
    - determine the efficacy of IRMS for tracing lamb production systems in four regions of the north west of Tunisia
- For fish:
    - investigate the ability of isotopic analysis to discriminate and trace the geographical origin of trout
- For dairy products:
    - Validate through a collaborative study the methods for analysing the isotopic and mineral profile of cheese
    - Define the isotopic and mineral profile of Mozzarella di Bufala PDO cheese.

3. CHAPTER 1 - Variation of stable isotope ratios in blood fractions of lambs following dietary changes

3.1 Introduction

The animal husbandry system can heavily affect lamb meat quality and therefore influence the consumer rating. In particular, meat from animals raised on pasture is darker in colour and is also considered to be healthier than meat from animals fed concentrate in stalls, due to the higher content of omega-3 fatty acids, conjugated linoleic acid and oleic acid[1].

Isotopic methods have been successfully used for more than twenty years to analyse the trophic relationships in food networks and have been applied to both wild and farmed animals[2]. The stable isotope ratio patterns in herbivores are a result of the interaction between ecological, physiological and biochemical processes, and the isotopic composition of animal tissues reflects that of the diet in a predictable manner[3]. The carbon, nitrogen, oxygen and sulphur isotopic ratios of the diet vary in different degrees according to photosynthetic and nitrogen cycles, pedological and geological characteristics of soils, agricultural practices and geographical origin. Furthermore, due to the diversity in their turnover rates, the different animal tissues integrate this information over different temporal and spatial scales. As a consequence, stable isotope ratios of animal tissues can be used with varying degrees of success to verify the diet and/or the change of diet in animals[4-7]. However, in the specific case of pasture-fed lambs there is one 'hurdle' that needs to be verified, as in meat production it is common practice to make use of finishing diets based on concentrates, even for those animals previously raised on pasture[8].

The easy collection of blood makes this tissue an ideal substrate to trace the diet of an animal before slaughtering. Only a few studies have investigated stable isotope ratios in lamb blood and considered their variation following a diet switch. In particular, Norman et al. [9] studied the variation in the integration time of dietary carbon in plasma and faeces in sheep fed with C3 or C4 plants for a period of 2-3 weeks. Martins et al. [10] determined the half-life of $\delta^{13}C$ in whole blood of lambs fed with leguminous plants or corn silage and found that the 167 days of the experiment were not sufficient to reach the isotope equilibrium level. Lately, Biondi et al. [8] determined $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values in plasma and erythrocytes from lambs raised on different diets for 89 days. They observed that plasma $\delta^{13}C$ and $\delta^{18}O$ values were different in exclusively pasture-fed animals, compared to those finished in stalls for 14 and 37 days.

In this study we wished to take an in-depth look at these observations, investigating the variations in the four isotopic variables ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$) in the plasma and erythrocytes of lambs

whose diet was switched from pasture to concentrate in the last 14 days before slaughter. To our knowledge, the isotopic ratio of $\delta^{34}S$ has never been included in similar studies analysing blood fractions; however, it may provide additional information because it is affected by different factors, as compared to the other ratios, such as the geology of the area, the atmospheric deposition of sulphate on the forages in coastal areas as well as from anthropogenic factors (industrial emissions from power plants, traffic, mining activities)[11]. The aim of the study was to verify whether the measurement of isotopic ratios in blood fractions can detect a brief variation in lambs' diet.

3.2 Material and Methods

Animal management and sampling

This study is part of a broader experiment in which stable isotope ratios were analysed in the muscle and blood components of lambs raised on four different diets[8]. The experimental lambs were born and raised in a farm located in Calabria (southern Italy, 38°38'N, 16°04'E) from December 2008 to March 2009. In this study, ten male Italian Merino lambs were fed maternal milk and received a commercial weaning concentrate starting from the 35th day of age. At 60±10 days of age, lambs were weaned and then were gradually adapted to the experimental pasture-based diet; the adaptation period lasted 18 days. During the 89-day experimental period the animals were fed exclusively at pasture for 75 days (non-fertilized, 80% Gramineae and 20% Leguminosae, with a C3 metabolism). Subsequently, for the last 14 days, the animals were housed in stalls and abruptly switched to a diet based on concentrated feed (64% barley, 34% chickpea and 2% mineral and vitamin mix) and natural pasture hay in a proportion of 80 : 20 (as fed).

Blood samples were taken in the morning, before feeding time, on days 1, 2, 3, 4, 5, 6, 7 and 14 following the diet change. Day 1 represents the first day of stall feeding; however, considering that blood was sampled before feed supplying, the blood collected on day 1 reflects the pasture diet of the previous 75 days of grazing. Blood samples were taken individually from the jugular vein, collected in tubes and immediately centrifuged (3000 x g, 15 min, 4°C) to separate plasma and erythrocytes [8]. All the experimental procedures were approved by the University of Catania and were in accordance with national regulations concerning animal care and use.

Feed samples used during the experimental period (pasture, hay and concentrate) were collected and analysed as well [8].

Sample preparation

Blood fractions were stored in Eppendorf vials at -20°C until analysis[9]. Blood erythrocytes and plasma samples for the analysis of $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ isotopic ratios were freeze-dried for

48 h to remove water and were stored at room temperature in small glass sealed bottles put in a vacuum desiccator with P_2O_5, pending analysis. Another subsample of plasma, destined for the analysis of $^{18}O/^{16}O$ isotopic ratio, was stored at -20°C.

Stable isotope ratios analysis

The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios in feeds (≈1.5mg) and blood fractions (≈0.5mg) were measured simultaneously using an Isotope Ratio Mass Spectrometer (DELTA V, Thermo Scientific, Germany) following total combustion in an elemental analyser (EA Flash 1112, Thermo Scientific). $^{34}S/^{32}S$ was determined using IRMS (Vario Isotope Cube, Elementar Analysensysteme GmbH, Hanau, Germany) in plasma (≈1.6mg) and blood erythrocytes (≈2mg). The $^{18}O/^{16}O$ ratio in feed (≈0.8 mg) was measured using an Isotope Ratio Mass Spectrometer (DELTA XP, Thermo Scientific) coupled with a Pyrolyser (TC/EA, high temperature conversion elemental analyzer, Thermo Scientific). Further details of the analytical procedures adopted are reported in previous papers[8,12]. The $^{18}O/^{16}O$ ratio in plasma was determined in 2ml of defrosted samples after equilibration with CO_2 using an ISOPREP 18 (VG Isotech, Middlewich, UK) online preparation system that allows CO_2/H_2O equilibration, interfaced with an Isotope Ratio Mass Spectrometer (SIRA II, VG Isogas Middlewich, UK), according to the water equilibration method described for wine in the OIV method (MA-AS2-12: R2009). In particular Isoprep18 allows the equilibration of oxygen exchange between sample water and an ultrapure CO_2 with a known oxygen isotope ratio at 25°C for 5 hours. 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).

The values were expressed in δ (=$[(R_{sample} - R_{standard})/ R_{standard}]$[13], where R is the ratio between the heavier isotope and the lighter one) with reference to international standards: Vienna-Pee Dee Belemnite (VPDB) for $\delta^{13}C$, Air for $\delta^{15}N$, Vienna Standard Mean Ocean Water (VSMOW) for $\delta^{18}O$, Vienna Canyon Diablo Troilite (VCDT) for $\delta^{34}S$. For further details see the previous paper by Biondi et al. [8]. For the calculation of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$, a casein and a water working standards calibrated against international reference materials were used. In the specific L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA) were used for $^{13}C/^{12}C$, L-glutamic acid USGS 40 for $^{15}N/^{14}N$, V-SMOW (IAEA) and benzoic acid (IAEA-601) for $^{18}O/^{16}O$ in water and in casein, respectively. The $\delta^{34}S$ values were calculated against barium sulphates IAEA-SO-5 and NBS 127

(IAEA) through the creation of a linear equation.Furthermore for $\delta^{13}C$, around 10% of the samples were also analysed calculating data in comparison to three international standards: glutamic acid USGS 40 (IAEA), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA), and a calibration curve was constructed as suggested by IUPAC [12] (Brand et al., 2014), obtaining the difference with data normalised using a single standard, always lower than 0.2‰.

The uncertainty of measurements (calculated as two standard deviations obtained by analysing the same sample ten times on different days and by different operators) was < 0.1 ‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ and < 0.4‰ for $\delta^{34}S$.

Statistical analysis

The data were processed using the statistical software package Statistica 9 (StatSoft Inc., Tulsa, OK, USA).

The repeated measures ANOVA test was applied to each variable to identify statistical differences between the groups. Specifically, the test on $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values included the days after the diet change and the blood component (plasma or erythrocytes) as fixed experimental factors, and the days after the diet change x blood component interaction. Data on the $\delta^{18}O$ values in plasma were analysed with a monofactorial repeated measures ANOVA test, including the days from the diet change as a fixed factor. Pairwise comparisons were performed using Tukey's HSD (Honestly Significantly Different) test.

3.3 Results and discussion

In Table 3.1 the distribution of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values determined in plasma and blood erythrocytes and $\delta^{18}O_{plasma}$ values are presented according to the sampling days (from day 1 to day 14 following the dietary switch from pasture to concentrate). In the same Table 3.1 only the groups that resulted highly statistically different at Tukey's HSD test ($p<0.001$) are indicated. $\delta^{18}O_{plasma}$ values ranged between -3.0 and 1.5‰. $\delta^{13}C$ and $\delta^{15}N$ had similar value spans for both plasma and blood erythrocytes ($\delta^{13}C$: from -26.1 ‰ for both fractions to -24.0 ‰ and -23.6 ‰ for plasma and erythrocytes respectively; $\delta^{15}N$: from 5.9 ‰ for both fractions to 7.5 ‰ for plasma and 6.9 ‰ for erythrocytes). In contrast, the $\delta^{34}S$ values of plasma had a wider range of values (4.8 – 7.4 ‰) than the $\delta^{34}S$ of erythrocytes (5.3 – 6.3 ‰).

ANOVA analysis (Table 3.1) demonstrated that both the sampling day and the blood fraction were shown to have a highly statistically significant effect on $\delta^{15}N$ and $\delta^{34}S$ values ($p<0.001$), whereas the interaction between them was significant only in the case of $\delta^{34}S$ ($p<0.001$). $\delta^{13}C$ values were

affected by the sampling date ($p<0.05$) and sampling day x blood fraction interaction was also found ($p<0.01$). In order to examine this aspect more closely, Tukey's HSD test was performed to verify whether there were statistically significant differences between plasma and erythrocytes on each sampling day, and then between sampling days in each blood fraction.

Stable isotope ratios differences in blood fractions

The mean values of $\delta^{15}N$ and $\delta^{34}S$ in plasma were higher than those measured in erythrocytes basically over all the sampling days, confirming what highlighted by ANOVA analysis (Table 3.1): the blood fraction was shown to have a highly statistically significant effect on $\delta^{15}N$ and $\delta^{34}S$ ($p<0.001$). The mean differences between plasma and erythrocytes ratios over all the sampling days were 0.6 ‰ for $\delta^{13}C$, 0.3 ‰ for $\delta^{15}N$ and 0.8 ‰ for $\delta^{34}S$.

Considering the isotope ratio values in plasma and erythrocytes within each day of sampling, the $\delta^{34}S$ of plasma and erythrocytes were shown to be different from each other in the first three days following the diet switch, whereas the values became not statistically differentiable on other sampling days (Table 3.1). Indeed, for $\delta^{34}S$ values the mean daily difference plasma-erythrocytes decreased from the first to the last sampling day (from 1.6 ‰ to 0.6 ‰) as emerged in the Tukey test. For $\delta^{13}C$ and $\delta^{15}N$ values, the differences between the two blood fractions within the day of sampling were much more limited and only 'hinted at', as a statistical difference was detected for both only on the first sampling day ($p<0.05$). For $\delta^{13}C$, the found data were in agreement with the previous findings of Biondi et al. [8], who found lower $\delta^{13}C$ values in plasma than in erythrocytes in pasture-fed lambs, whereas in other lambs (stall-fed, grazed and switched from pasture to stalls for 37 or 14 days before slaughter), the value was always higher in plasma than in erythrocytes.

A comparison with other published studies is not possible, as data from Martins et al. [10] pertained to $\delta^{13}C$ values in whole blood, whereas Norman et al.[9] analysed exclusively $\delta^{13}C$ variations in plasma but not in erythrocytes.

Stable isotope ratios differences on different sampling days

In this study, the diet change occurring on the first day led to a change in the isotopic composition of the ingested diet. The mean values of pasture were: $\delta^{13}C$ -30.5 ‰, $\delta^{15}N$ 1.1 ‰, $\delta^{18}O$ 22.5 ‰ and $\delta^{34}S$ 6.3 ‰ and those of the administered mixture concentrate/hay 80 : 20, calculated using a simple proportion (δX_{conc} *0.8 + δX_{hay}*0.2) were $\delta^{13}C$ -25.9 ‰, $\delta^{15}N$ 2.6 ‰, $\delta^{18}O$ 27.9 ‰ and $\delta^{34}S$ -2.8 ‰ (Table 3.2). In particular, the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values of feed supplied in stalls

were higher (by 4.6‰ for $\delta^{13}C$, 1.5‰ for $\delta^{15}N$ and 5.4 ‰ for $\delta^{18}O$) whereas $\delta^{34}S$ values were lower (by 9.1‰) as compared to pasture.

The trend for the mean values of stable isotope ratios (Table 3.1) observed in the blood fractions showed that $\delta^{34}S_{plasma}$ and $\delta^{18}O_{plasma}$ values decreased throughout the experimental period (absolute difference between day 1 and day 14 values: 1.6‰ for $\delta^{34}S_{plasma}$ and 3.4‰ for $\delta^{18}O_{plasma}$), whereas $\delta^{13}C_{plasma}$ values increased (difference of 1.1‰). $\delta^{15}N_{plasma}$ and all the isotope ratios determined in blood erythrocytes seemed not to be affected by the change in diet and consequently did not show any particular trend (differences: 0.6‰ for $\delta^{15}N_{plasma}$, 0.4‰ for $\delta^{13}C_{erythrocytes}$, 0.3‰ for $\delta^{15}N_{erythrocytes}$ and $\delta^{34}S_{erythrocytes}$). ANOVA analysis (Table 3.1) showed that the sampling day affected $\delta^{13}C$ ($p<0.05$) and $\delta^{15}N$, $\delta^{34}S$ and $\delta^{18}O_{plasma}$ ($p<0.001$) values and therefore data collected on the different sampling days were compared with each other (Table 3.1). $\delta^{13}C_{plasma}$ and $\delta^{34}S_{plasma}$ values were significantly different 7 days after the abrupt change from pasture to stall feeding ($p<0.001$), while $\delta^{18}O_{plasma}$ values showed a very fast decrease being significant lower after only 3 days from the abrupt diet change. In contrast, $\delta^{15}N_{plasma}$ values were less affected by the sampling date and only after 14 days were $\delta^{15}N_{plasma}$ values different from those found on day 1 ($p<0.01$). A possible explanation for these results might be related to the fact that the nitrogen isotopic signature of the two diets was more similar (absolute difference of 1.5‰) as compared to $\delta^{13}C$ and $\delta^{34}S$ (absolute difference of 4.6‰ and 9.1‰).

Plasma $\delta^{13}C$ and $\delta^{34}S$ values changed according to the change in the isotopic composition of the diet ($\delta^{13}C$ increased and $\delta^{34}S$ decreased). The $\delta^{13}C_{plasma}$ values trend found in this experiment showed a significant variation seven days after the diet switch. This is in agreement with the results found by Norman et al.[9]. Specifically, these authors found that $\delta^{13}C_{plasma}$ values could be used to differentiate animals fed 100% C3 or C4 diets just three days after the switch. But, as well, they found that $\delta^{13}C_{plasma}$ values were not suitable for use to determine the proportion of C4 plants used in the diet of lambs because a time period of 18 days after diet change was not sufficient for equilibration of this parameter. The two studies thus reinforced each other, as the results are comparable despite the fact that they were conducted using totally different diets. The novelty of the results obtained in the present trial is due to the fact that both pasture and concentrate were from C3 plants.

The ^{13}C values presented in the paper by Martins et al. [10] were determined in whole blood and cannot therefore be directly compared with the results of this study. Nevertheless, we estimated the theoretical isotopic values of whole blood, using the values measured for the individual blood fractions. Considering that plasma makes up about 55% of the blood and erythrocytes account for

most of the remaining 45% [14], and by applying a simple proportion ($\delta X_{blood} = \delta X_{plasma} * 0.55 + \delta X_{erythrocytes} * 0.45$) theoretical whole blood values were calculated for the eight days of sampling. For the first and last days of sampling the calculated data were -25.2 and -24.8‰ for $\delta^{13}C$, 6.8 and 6.3‰ for $\delta^{15}N$ and 6.4 and 5.4‰ for $\delta^{34}S$ (data not shown). Therefore, the differences between the initial and final dates calculated for whole blood were more limited, particularly for $\delta^{13}C$ and $\delta^{34}S$, than those determined in plasma (0.4 instead of 1.1‰ for $\delta^{13}C$, 0.5 instead of 0.6‰ for $\delta^{15}N$ and 1.0 instead of 1.5‰ for $\delta^{34}S$). ANOVA analysis was applied to this 'new' dataset of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values for whole blood, to verify whether the statistical differences between sampling days were maintained. No statistically significant differences were found for $\delta^{13}C$ values, whereas statistically significant differences were confirmed for $\delta^{15}N$ ($p<0.05$) and $\delta^{34}S$ ($p<0.001$). The 'recalculated' data on the carbon of blood confirmed the conclusions of Martins et al. [10] that this parameter determined in animals fed with C3 plants did not reach isotopic equilibration within 14 days following a diet switch, due to a longer carbon turnover, and thus is substantially indicative of former diet.

The $\delta^{18}O$ values of feed when switching from one diet to another during this study increased by 5.4‰. In contrast, the $\delta^{18}O$ determined in plasma decreased by 3.4‰ from the first to the last sampling day. As plasma is made up of more than 90% water, it is logical to expect that $\delta^{18}O_{plasma}$ values are more related to ingested water than to the $\delta^{18}O$ values of feed. The decrease in the values of $\delta^{18}O$ in blood plasma could be explained by the fact that fresh herbage, which contains water enriched in ^{18}O [15], was eliminated from the lambs' diet in the 14 days of the experimental trail and a higher ingestion of tap drinking water compared to the previous period probably occurred.

The isotope ratios of blood erythrocytes were not statistically different over the different sampling days and were therefore not affected by the diet change over a 14 day period. These results are in agreement with our previous findings[8]; indeed, comparison of the pre-slaughter blood isotopic values of the pasture group, which grazed for the whole trial, and the group abruptly switched from pasture to stall feeding for the last 14 days (the same group for which we are studying the kinetics of variations in depth here) showed significant differences for plasma and no differences for erythrocytes.

The difference in plasma and erythrocyte behaviour is probably due to the different turnover rate for the constituents of the two fractions. Plasma is made up of water and proteins, whose composition probably changes on a 'day by day' basis, according to the substances absorbed from the diet,

whereas erythrocytes live in the blood circulation for about 100 to 120 days [14] and this is probably the minimum time required to reach an isotopic equilibrium in the erythrocyte fraction.

We can conclude that the chance of detecting a very short finishing period (14 days) by analysing stable isotope ratios in blood is strongly dependent on two factors: i) the difference in the isotopic characteristics of the two diets and ii) the blood fractions: a period of 14 days was not long enough to affect the isotopic signature of blood erythrocytes, still affected by the isotope composition of the previous diet, in contrast to the observations regarding plasma. The different kinetics of the variations observed in erythrocytes and plasma could be strongly dependent on the different turnover rate of these two blood fractions, as pointed out by Phillips and Eldridge[16].

3.4 Conclusions

This study shows that plasma and erythrocytes have different kinetics in terms of stable isotope ratio variations in lambs. In particular, the isotopic values of erythrocytes were shown not to be affected by the diet change in the 14 days following the switch, whereas plasma isotopic variation occurred within few days of the switch (after 3 for $\delta^{18}O$ values and 7 days for $\delta^{13}C$ and $\delta^{34}S$ values).

This different behaviour could be a useful tool in traceability studies. Indeed, our results clearly suggest that the isotopic ratios of blood erythrocytes can be considered insensitive to short-term diet shifts. Indeed, in previously pasture raised lambs, erythrocytes maintained the isotopic signature of pasture despite the short finishing period with concentrate and hay feed. On the contrary, the carbon, oxygen and sulphur isotopic ratios in plasma responded promptly to diet changes. Therefore, erythrocytes may be suitable for verifying whether the animal was actually pasture-raised before a finishing period on concentrates. Additionally, the analysis of plasma could detect very short finishing periods on concentrate-based diets after a previous pasture-based diet. Analysis of both plasma and erythrocytes could represent a good tool for validation and could be therefore a valid alternative to muscle for authentication of the animal production system.

3.5 References

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Table 3.1: Mean and standard deviation values for the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ of plasma and blood erythrocytes and the $\delta^{18}O$ of plasma in lambs whose diet was switched from pasture to concentrate. Significantly different mean values between sampling days in plasma samples (Tukey's HSD test, $p<0.001$) are indicated with different superscript letters. Erythrocytes showed no highly statistical differences between sampling days ($p < 0.001$). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. F indicates the fraction factor (blood plasma vs. erythrocytes), D indicates the day of sampling factor, F x D indicates the interaction between day of sampling and fraction factors.

Sampling day - D		1		2		3		4		5		6		7		14		ANOVA p values		
Blood fraction - F		Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	F	D	F x D
$\delta^{13}C$	Mean	-25.5^{a}	-24.9	-25.5^{a}	-25.0	-25.2^{ab}	-25.1	-25.2^{ab}	-25.1	-25.2^{ab}	-25.1	-24.9^{abc}	-24.5	-24.9^{bc}	-25.2	-24.4^{c}	-25.2	-	*	**
	Std.Dev.	0.4	0.7	0.3	0.7	0.3	0.7	0.2	0.7	0.4	0.7	0.7	1.0	0.3	0.7	0.2	0.7			
$\delta^{15}N$	Mean	6.9	6.6	6.8	6.6	6.7	6.5	6.7	6.5	6.7	6.4	6.5	6.5	6.8	6.4	6.3	6.2	***	***	-
	Std.Dev.	0.3	0.2	0.3	0.2	0.3	0.2	0.3	0.3	0.4	0.2	0.2	0.2	0.4	0.2	0.3	0.2			
$\delta^{34}S$	Mean	6.8^{a}	5.8	6.7^{ab}	5.8	6.7^{ab}	5.7	6.3^{ab}	5.8	6.2^{ab}	5.7	6.1^{abc}	5.7	6.0^{bc}	5.7	5.3^{c}	5.5	***	***	***
	Std.Dev.	0.4	0.2	0.2	0.3	0.2	0.3	0.5	0.2	0.4	0.2	0.5	0.3	0.4	0.3	0.6	0.2			
$\delta^{18}O$	Mean	0.9^{a}	-	0.6^{ab}	-	-0.4^{bc}	-	-0.8^{c}	-	-1.0^{c}	-	-1.4^{cd}	-	-1.4^{cd}	-	-2.5^{d}	-		***	
	Std.Dev.	0.2	-	0.3	-	0.7	-	0.6	-	0.5	-	0.5	-	1.2	-	0.4	-			

Table 3.2: $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ of feed administered to lambs whose diet was switched from pasture to concentrate.

	$\delta^{13}C$	$\delta^{15}N$	$\delta^{18}O$	$\delta^{34}S$
Pasture	-30.5	1.1	22.5	6.3
Adm. Mix. conc./hay 80 : 20	-25.9	2.6	27.9	-2.8

4. CHAPTER 2- **Determining the geographical origin of Tunisian indigenous lamb using H, C, N, O and S stable isotope ratio**

4.1 Introduction

In Mediterranean countries, lamb is widely appreciated by consumers; it represents the basic protein source for people in developing countries (Gürsoy, 2006). Meat quality appears to be strongly affected by the animal feeding system (Priolo et al., 2001; Renerre, 1990). The main reported differences are in subcutaneous fat (Prache & Theriez, 1999), meat colour (Ådnøy et al., 2005), carcass fatness (Atti & Abdouli, 2001; Joy et al., 2008) and fatty acid composition (Ådnøy et al., 2005; Aurosseau et al., 2007; Hajji et al., 2016). The three main components in eating quality – tenderness, juiciness and flavour – can also vary due to production factors (Rousset-Akrim, et al., 1997, Wood et al., 2008). The farming system, namely the combination of the geographical, orographic, climatic, social, historical and cultural conditions in the reference area and production factors such as feeding regime, husbandry techniques, animal breed and category, gives rise to specific lamb types distinguished by particular meat characteristics that consumers may identify and appreciate (Sañudo et al., 2007). The farming system could thus result in a "Protected Designation of Origin" for specific meat, associated by consumers with higher quality and healthier foods (Hermansen, 2003). On the other hand, there is a growing enthusiasm among consumers for high quality food with a clear regional identity. The reasons for this vary from certain specific culinary and sensory qualities, or purported health benefits associated with regional products, to decreased confidence in the quality and safety of food produced outside their local region (Kelly, Heaton & Hoogewerff, 2005). Authentication and objective food information are major demands from consumers (Monin, 1998). Of the analytical approaches that can be used for the authentication of meat products, stable isotope ratio analysis is well-known as an accurate method (Piasentier et al., 2003; Franke et al., 2005; Camin et al., 2007; Perini et al., 2009) although in the field of food control, there are currently no official methods for multi-element stable isotope analysis of meat (Camin et al., 2007). Stable isotope ratios provide an analytical tool for confirming meat origin, as there are region-specific patterns in the environmental isotopic ratios (Piasentier et al., 2003; Franke et al., 2005). Dietary components have typical isotopic "signatures" determined by climate (ratios of H and O), vegetation composition (C), feed type (C, N), crop production practices (N), and proximity to the sea (S) (Moloney et al., 2009). Isotopes can therefore be used to detect dietary differences and origin.

This experiment is part of a project focusing on aspects related to the regional origin of lamb from Tunisia. The research aims to evaluate analytical techniques and chemical profiles useful for

authentication and determining the origin of lamb, which is one of the first fresh meat products with the potential to be recognised with a Protected Designation of Origin in Tunisia. The main objective of this work was to study the traceability of meat from lambs coming from various locations in the north of Tunisia, grazing on herbaceous pasture or woody pasture and with or without concentrate and forage supplements. These objectives were achieved by developing classification models through the use of isotope ratio mass spectrometry (IRMS).

4.2 Materials and methods

Animals, diets and experimental design

The study was carried out at four sites in north-western Tunisia (Table 4.1). The farming systems are characterised by different kinds of pasture, mountainous terrain covered with woody pasture (WP) and plains dominated by herbaceous pasture (HP). In the Ain Draham (AD) and Fernana (F) systems, pasture grazing takes place virtually throughout the year, with bushes and shrubs dominated by cork oak (Quercus suber), while the kermes oak (Quercus coccifera) covers relatively small areas. The shrubs are represented by Arbutus unedo, Calycotum villosa, Erica arborea, Myrtus communis, Pistacia lentiscus and Phillyrea angustifolia. Lambs are given supplements of oak acorn, some commercial concentrates, barley and oat hay. The Amdoun (AM) and Joumine (J) sites are characterised by HP, comprising a herbaceous stratum dominated by Gramineae, on which the lambs graze with their dams; as a supplement to pasture, the flocks receive commercial or farm concentrate, green barley, oats, hay and wheat straw.

At all sites, lambing occurred in December and lambs of mixed breeds (Barbarine and Queue Fine de l'Ouest) and their crosses were reared with their dams until they reached weaning weight. Eight male lambs from each site were provided by four farms deemed to be highly representative of the area for a meat quality study.

Table 4.1. Sites of lamb production and their dietary calendar during the follow-up period. The percentage contribution to the monthly diet of the different feedstuffs is reported in brackets.

Region	December	January	February	Mars
Aïn Draham Lat. North: 36°46'36.35" Long.: 8°40'49.75" Elevation: 756 m Sea distance: 18.54 km	Forest plants (100)	Forest plants (40) Straw (15) Barley + Acorn (20) Triticale (10)	Forest plants (40) Straw (15) Barley +Acorn (25) Triticale (10)	Forest plants (70) Barley + Acorn (20) Herbaceous (10)
Fernana Lat. North: 36°36'46.66" Long.: 8°36'41.28" Elevation: 553 m Sea distance: 36.52 km	Forest plants (40) CC (20) Straw (25) Acorn (15)	forest plants (40) Straw+ CC (20) Barley (20) Acorn (20)	forest plants (40) straw (15) Barley (20) Acorn (25)	Forest plants (40) Acorn + barley (20) herbaceous legume (40)
Joumine Lat. North: 36°55'23.21"	FC (55) clover hay +oat	FC (50) clover hay +oat	FC (50) clover hay + oat	Green barley green oats (50)

Long.: 9°23'13.49" Elevation: 335 m Sea distance: 58.97 km	straw (25) eucalaptus + olive twig (20)	straw (40) eucalaptus + olive twig (10)	straw (40) Grass (10)	clover hay (25) Barley (25)
Amdoun Lat. North: 36°45'54.88" Long.: 9°05'59.52" Elevation: 315 m Sea distance: 41.37 km	Barley (35) FC (45) clover + hay-oat (20)	Barley (25) FC (35) clover hay +oat straw (40)	Barley (35) FC (35) clover hay +oat straw (30)	Green barley (30) green oats (30) clover hay (20) barley (20)

Farmer concentrate = Barley + wheat bran + faba beans. CC=corn+ wheat bran + soy + faba beans +Barley

Slaughter and sampling procedures

The lambs were transported to a commercial slaughterhouse located 120 km from the farms and then slaughtered after an overnight period without feed but with free access to water. Their body weight (BW) was recorded just before slaughtering (Table 4.2). All the procedures employed in this study (transport and slaughtering) meet ethical guidelines and adhere to Tunisian legal requirements in accordance with Law no. 2005-95 (18 October 2005).

The longissimus dorsi (thoracis + lumborum) (LD) muscles of both sides of the lamb carcasses were separated for meat quality analysis. The left and right muscles from each lamb were divided into eight samples; one of them were frozen at -20°C for IRMS analysis.

Table 4.2. Growth performance and carcass traits of lambs in the various feeding systems.

	AD-WP	F-WP	J-HP	AM-HP	*p*	SEM
Age at slaughter (d)	150a	148a	106b	118b	0.01	3.78
Slaughter body weight (kg)	23.9b	28.5a	22.1bc	20.2c	0.01	1.08

The LD meat samples were minced and freeze-dried in a lyophilizer (freeze-drier) then homogenized with a suitable grinder and freeze-dried again. The resulting dry powder was fractionated into crude fat (Fat), by extraction with petroleum ether for 6 h in a Soxhlet apparatus, and defatted dry matter (Protein), essentially protein. Afterwards the protein and fat fractions were stored in an appropriate container until measurement. Measurement of the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of protein and fat fractions was carried out as described by Perini et al. (2009). The values were expressed in δ‰ against international standards, calculated against working in-house standards and calibrated against international reference materials, as reported by the same authors. To measure the $\delta^{2}H_{Protein}$values, we adopted the method employed for Italian lambs (Perini et al., 2009) in order to obtain comparable results. For measurement of the $^{34}S/^{32}S$ ratios, we used an elemental analyser (EA Flash 1112 Thermo Finnigan, Bremen, Germany) connected to an isotope ratio mass spectrometer (Delta plus XP mass spectrometer, Thermo Finnigan). The protein sample (□2.5 mg) was burned at 1000 °C in a quartz tube filled from the bottom with quartz wool (2 cm),

elemental copper (14 cm), copper oxide (5 cm) and quartz wool (1 cm). The water was removed using a glass trap filled with Mg $(ClO_4)_2$. The isotopic values were calculated against international reference materials: IAEA-SO-5 ($\delta^{34}S = +0.5‰$) and NBS 127 ($\delta^{34}S = +20.3‰$), through the creation of a linear equation. The uncertainty (2σ) of measurements was <0.3‰ for $\delta^{13}C$ and $\delta^{15}N$ analysis, and respectively <3‰, <0.6‰ and <0.8‰ for the $\delta^{2}H$, $\delta^{18}O$ and $\delta^{34}S$ values.

Statistical analysis

Univariate analysis was performed using SPSS for Windows (v. 7.5.21; SPSS Inc., Chicago, IL, USA), while multivariate analysis was performed using the Unscramble X, 10.3 package (CAMO Software AS., Oslo, Norway).

Discriminant PLS (PLS-DA; Chevallier, Bertrand, Kohler, & Courcoux, 2006) was applied to check the efficacy of the isotope and/or FA profile of lamb, to discriminate the farming systems according to their characteristics, namely the geographical origin and feeding regime of the lambs. Specifically, the PLS-DA model was built between the isotope or the FA matrixes (X) and the farming system matrix (Y), which was made by defining a dummy variable for each combination of origin and pasture type considered. Moreover, PLS-DA was applied to discriminate Tunisian from Italian lamb types, according to their IR profile. The published data (Perini et al., 2009) from a comparable investigation on various Italian lamb types, carried out using the same methodological approach adopted for the Tunisian lamb types, were taken into account.

The optimum number of PLS components was estimated using random cross-validation. The significance of IR/FA predictors was evaluated using Martens' uncertainty test. Classification performance was assessed in the validation phase in terms of sensitivity, specificity and total accuracy (Kjeldahl & Bro, 2010; Sokolova & Lapalme, 2009) estimated at a given threshold or cut-off limit. Specifically, adopting a cut-off value of 0.5, samples with a predicted Y-value of over 0.5 were recognised as belonging to one class, whilst those with predicted Y-values lower than 0.5 were predicted as belonging to the other class (Barbin, Sun & Su, 2013).

4.3 Results and discussion

Stable Isotope ratios

The IR profiles of lamb produced at the four locations in north-west Tunisia are shown in Table 4.3. All the IRs, except $\delta^{18}O_{Fat}$, were significantly influenced by the geographical origin of the meat. However, only in the case of $\delta^{34}S_{Protein}$ did the pasture type significantly affect the IR value (9.7‰ vs. 6.9‰ for WP and HP respectively; $p<0.001$), as highlighted in Figure 4.1, summarising the

isotopic values in relation to the feeding regime. This means that IR variability between sites is higher than that between pasture types.

A clear example of this can be seen in the $\delta^{13}C$ values of both protein and the more depleted fat fractions (DeNiro & Epstein, 1977), which did not differ for the pasture systems. $\delta^{13}C$ values instead showed statistically significant differences, although numerically low (0.6 – 1.2‰) between sites. These differences are probably linked to the forage/concentrate ratio in the lamb diet and to C4 corn intake (González-Martin, González-Pérez, Hernández Méndez, & Sánchez González, 2001; Perini, Camin, Sánchez del Pulgar & Piasentier, 2013). As expected, the $\delta^{13}C$ values were low in comparison with those observed by Perini et al. (2009) in Italian lamb types, because of the absence of C4 plants in their diet (Camin et al., 2007).

Meat from Amdoun, produced from lambs grazing on herbaceous pasture with their dams, was characterised by the highest $\delta^{15}N_{Protein}$ and the lowest $\delta^{2}H_{Fat}$ (10.2‰ and -200.3‰ respectively). Meat from Joumine, although also produced on grazing herbaceous pasture, instead showed the opposite results for the two isotope ratios (5.6‰ and -187.9‰ for $\delta^{15}N_{Protein}$ and $\delta^{2}H_{Fat}$ respectively). The WP systems showed intermediate values. The differences between Amdoun and Joumine, characterised by a similar humid climate and HP, may result from differences in agricultural practices, particularly the organic fertilisation largely used in Amdoun, which increases the $\delta^{15}N$ of forage (Laursen et al., 2013). Moreover, in comparison with AM-HP (Table 1), J-HP breeders feed their flocks with significant amounts of legumes, namely clover, which is known to have a low ^{15}N level in its nitrogen compounds (Laursen et al., 2013). Furthermore, they usually wean their lambs earlier than AM lambs, which are kept with the ewes until slaughter, and higher $\delta^{15}N$ are expected in suckling animals (Perini et al., 2009).

Within the same woody pasture system, the meat obtained from the area at lower altitude, Fernana, had higher $\delta^{2}H_{Protein}$ and $\delta^{18}O_{Protein}$ (-81.1‰ and 16.4‰ respectively) than meat produced in the area at higher altitude, Ain Draham (-86.2‰ and 15.4‰ respectively). These isotopes are responsible for the environmental water signature (Bowen et al., 2005). Amdoun and Joumine, at lower altitude than both WP locations but more distant from the sea, produced lamb with $\delta^{2}H_{Protein}$ and $\delta^{18}O_{Protein}$ values not statistically different from those for meat from Ain Draham.

Of the SIRs examined, sulphur provided the clearest lamb type discrimination. Similar results were obtained in a study carried out on Italian lamb types (Perini et al., 2009). The basis of Italian-lamb-type variability was not feed dependent. The upper end of the $\delta^{34}S_{Protein}$ range was represented by two lamb types (9.5‰ and 9.2‰), reared on the Mediterranean islands of Sicily and Sardinia at 5 and 10 km from the sea respectively. The two woody pasture systems in Tunisia, Fernana and Ain Draham in particular, are also the closest to the sea. Thus, the high ^{34}S content would seem to be a

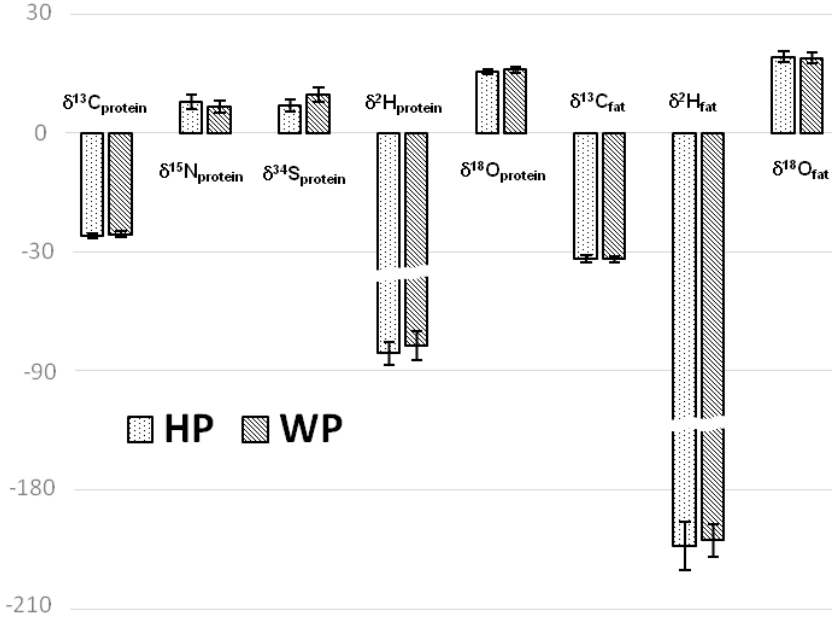
"coastal" signal, resulting from the influence of so-called sea-spray. Sea-spray sulphate is deposited as an aerosol over pasture and crops, in decreasing amounts with increasing distance from the sea. The marine signature has already been described in lamb and other products from animals reared in regions located near the sea (Perini et al., 2009).

Table 4.3. Isotopic ratio values (‰) of protein and fat of lamb meat from four farming systems located in four areas of Northwest Tunisia, Ain Draham (AD) and Fernana (F) characterized by woody pasture (WP), Amdoun (AM) and Joumine (J) characterized by herbaceous pasture (HP).

	AD-WP	F-WP	J-HP	AM-HP	*p*	SEM
$\delta^{13}C_{Protein}$	-26.1^{c}	-25.1^{a}	-26.2^{c}	-25.6^{b}	.000	.08
$\delta^{15}N_{Protein}$	7.1^{b}	6.3^{bc}	5.6^{c}	10.2^{a}	.000	.15
$\delta^{34}S_{Protein}$	10.0^{a}	9.4^{a}	7.5^{b}	6.3^{b}	.000	.21
$\delta^{2}H_{Protein}$	-86^{b}	-81^{a}	-85^{b}	-86^{b}	.002	.49
$\delta^{18}O_{Protein}$	15.4^{bc}	16.4^{a}	15.0^{c}	15.8^{ab}	.002	.11
$\delta^{13}C_{Fat}$	-32.5^{b}	-31.3^{a}	-32.3^{b}	-31.1^{a}	.000	.10
$\delta^{2}H_{Fat}$	-192^{b}	-194^{b}	-188^{a}	-200^{c}	.000	.56
$\delta^{18}O_{Fat}$	18.6	19.0	19.3	19.0	.814	.25

^{a,b,c}: $p<0.05$.

Figure 4.1. Isotopic ratio values (‰) of protein and fat fractions of lamb meat from Northwest Tunisia according to the grazing regime, woody pasture (WP) or herbaceous pasture (HP).



Among the stable isotope ratios examined, sulphur provided the clearest lamb type discrimination. Similar results were obtained in a study carried out on Italian lamb types (Perini et al., 2009). The basis of Italian-lamb-type's variability was not feed dependent. The upper end of the $\delta^{34}S_{Protein}$ range was represented by two lamb types (9.5‰ and 9.2‰), reared in the Mediterranean islands of Sicily and Sardinia, respectively 5 km and 10 km from the sea. Even the two woody pastures systems of Tunisia, Fernana and, particularly, Ain Draham, are the closest to the sea. Thus, the high ^{34}S content would seem to be a "coastal" signal, resulting from the influence of so-called sea-spray. Sea-spray sulphate is deposited as an aerosol over pasture and crops, in decreasing amounts with the increasing distance from the sea. The marine signature has already been described in lamb meat and other products from animals reared in regions located near the sea (Perini et al., 2009).

Traceability of Tunisian lamb farming systems

A PLS-DA model was considered to trace the four Tunisian lamb farming systems, based on the isotopic profile of meat (X-matrix). Four dummy variables (Y-matrix) were then defined, one for each regional farming system, i.e. AD-WP, F-WP, J-HP and AM-HP.

The predictions for the random coefficients (with 8 segments) in the cross-validation phase for the three-factor PLS discriminant model (R^2_V) were 0.48, 0.54, 0.71 and 0.71 for AD-WP, F-WP, J-HP and AM-HP local production systems respectively. The total variance explained by the model was 88.9% for meat isotope ratios (X variables) and 69.2% for lamb types (Y variables). To avoid over-fitting and considering the consistent structure of the model, only significant predictors were taken into account. The correlation loadings of the isotope ratios for lamb (X variables) and the four local systems (Y variables) were plotted in Figures 4.2.a and Figure 4.2.b respectively, along factors 1 and 2, and along factors 1 and 3.

Four IRs were included in the model, $\delta^{13}C_{Fat}$, $\delta^{15}N_{Protein}$, $\delta^{34}S_{Protein}$ and $\delta^{18}O_{Protein}$. All of them were located within the outer ring of the Figure 4.2.a plot; the same thing happened for two of the four regional lamb types, namely AM-HP and F-WP. The last two, AD-WP and J-HP, were located in the outer ring of the Figure 4.2.b plot. This confirms that the three components in the model explained at least 50% of the original co-variance for the isotope ratios and local lamb farming systems. The three-dimensional space resulting from the combination of the two plots may thus be considered as a viable visualisation of the major orthogonal relationship between and within the two sets of variables, i.e. IRs and lamb types. In line with the univariate results already discussed, multivariate analysis provided the following main output. AM-HP loaded in the negative half of factor 1, in close relation with $\delta^{15}N_{Protein}$, which specifically discriminated AM-HP lamb. A similar case was that of F-WP lamb, with $\delta^{18}O_{Protein}$ in the positive half of factor 2. The remaining two

meats loaded in the positive half of factor 1, opposite $\delta^{13}C_{Fat}$, and discriminated along factor 3. Specifically, as shown in Figure 4.2.b, AD-WP segregated due to a high $\delta^{34}S_{Protein}$value and J-HP due to a low $\delta^{15}N_{Protein}$value.

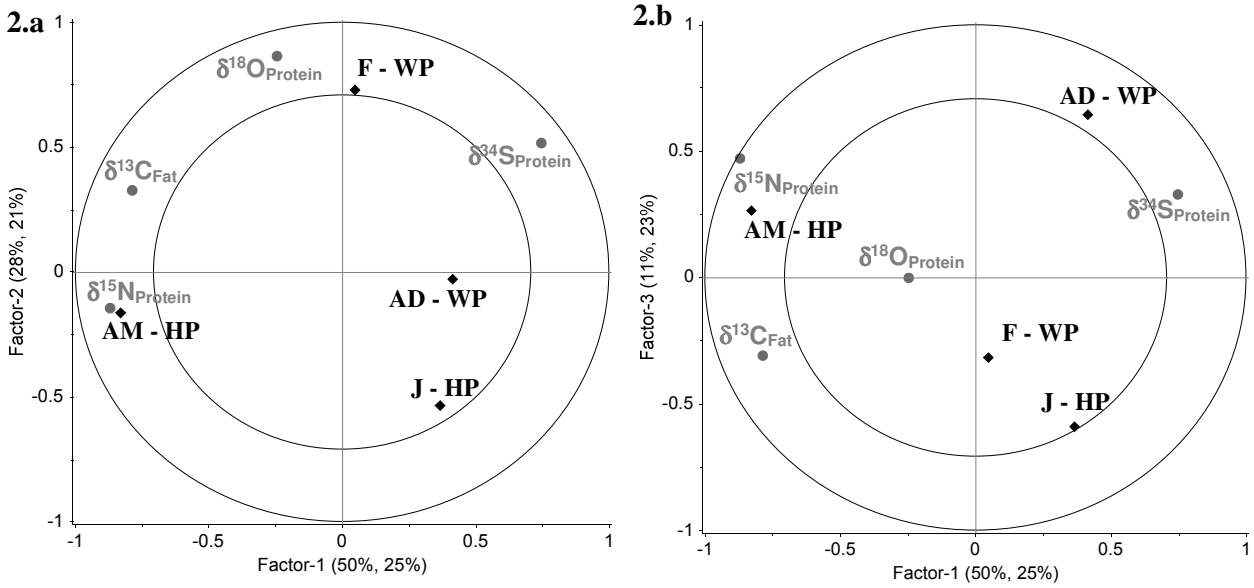


Figure 4.2. Correlation loadings on factors 1 and 2 (1.a, on the left) and factors 1 and 3 (1.b, on the right) of a PLS-DA model for the classification of lamb farming systems according to the breeding location: Ain Draham (AD) and Fernana (F) characterized by woody pasture (WP), Amdoun (AM) and Joumine (J) characterized by herbaceous pasture (HP) (Y-matrix), based on their isotope ratios profile (X-matrix).

The full cross-validation results of the PLS-DA model are shown in Table 4.4, together with evaluation of prediction performance. The quality measurements of each binary discriminant model are built from the confusion matrix, the records of which correctly and incorrectly recognised samples from each local farming system. True positives are samples of one selected farming system, correctly predicted; false negatives are samples of one selected farming system, incorrectly predicted to be from other farming systems; true negatives are samples of other farming systems, correctly predicted to be from another farming system; false positives are samples of other farming systems, incorrectly predicted to be from the selected one. The quality of the whole model was calculated as a multiclass overall classification (Sokolova & Lapalme, 2009).

Table 4.4. Results for classification of regional lamb farming system according to isotope ratios of lamb meat (full cross-validation phase).

	Classes of binary discrimination				Multiclass
	AD-WP	F-WP	J-HP	AM-HP	overall classification[1]
Confusion matrix (no of meat samples)					
true positive (tp_i)	6	5	8	8	
false negative (fn_i)	2	3	0	0	
true negative (tn_i)	23	24	23	21	
false positive (fp_i)	1	0	1	3	
Performance evaluation[1]					
sensitivity	0.75	0.63	1.00	1.00	0.84
specificity	0.96	1.00	0.96	0.88	0.95
(average) accuracy	0.91	0.91	0.97	0.91	0.92

[1] : $Sensitivity_i = tp_i/(tp_i+fn_i)$; $Sensitivity_\mu = \Sigma\, tp_i/\Sigma\, (tp_i+fn_i)$;
$Specificity_i = tn_i/(fp_i+tn_i)$; $Specificity_\mu = \Sigma\, tn_i/\Sigma\, (fp_i+tn_i)$;
$Accuracy_i = (tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)$; Average accuracy $= \Sigma\, [(tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)]\ /4$.

The discriminant multiclass model showed moderate sensitivity (84%). Five meat samples were misclassified as false negatives because they were regarded as being produced outside the correct farming system, namely the Fernana and Ain Draham areas, characterised by woody pasture. The specificity of the model was higher than sensitivity (95%). The highest number of false positives occurred for the Amdoun area. Indeed, three samples, although produced in F-WP and J-HP, were incorrectly identified as originating from the AM-HP system. The overall performance of the multiclass model, expressed in terms of average accuracy, was relatively satisfactory and stood at 92%.

To complete the validation of IR performance to trace lamb from Tunisian farming systems and to check the consistency of the database structure, external IRMS data were included in the multivariate analysis. Specifically, we considered a study on Italian lamb types carried out in the same laboratory as this study, using the same methodological approach adopted for Tunisian lamb types (Perini et al., 2009). The Italian study focused on the $^{2}H/H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ of defatted dry matter (protein) and on the $^{2}H/H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$ of the fat fraction in meat

samples from ten lamb types reared in seven Italian regions, following different feeding regimes. The average IR values of the Italian lamb types were retained as published by Perini et al. (2009). A full data set, comprising the IR profile of the 32 individual Tunisian lamb samples and the ten Italian lamb types produced throughout the country from the northern alpine region of Trentino to the southern Mediterranean island of Sicily was thus built. The aim was to verify whether authentic Tunisian products could be correctly classified to the four different farming systems and discriminated from lamb produced in various Italian regions, as an example of commercial meat from other geographical areas.

For this purpose, a PLS-DA model for multiclass discrimination was considered, defining five dummy variables, one for each Tunisian farming system, namely AD-WP, F-WP, J-HP and AM-HP, and one for the Italian lamb types. The estimated three-factor model, including five significant predictors ($\delta^{13}C_{Fat}$, $\delta^{13}C_{Protein}$, $\delta^{15}N_{Protein}$, $\delta^{34}S_{Protein}$ and $\delta^{2}H_{Protein}$), provided very low sensitivity values for binary classification of the three farming systems (data not shown). Indeed, the model allowed the correct identification of only one lamb sample from the AD-WP system (sensitivity equal to 0.13) and three samples from F-WP (0.38 sensitivity), while no true positive sample was classified for the J-HP system. Thus the differences in the IR profiles of the Tunisian lamb types evaluated allowed satisfactory discrimination between them, but were not sufficiently wide and systematic to be validated by adding an external set of samples to the classification model. The effect of environmental and husbandry factors on the IR variability of the main bio-elements was not specific and regular enough to provide consistent and steady classification of the four Tunisian farming systems. However, a larger scale geographical signature discriminating lamb from the Amdoun area, the rest of north-west Tunisia and Italy appeared to be workable. A PLS-DA model based on this hypothesis was thus built. Figure 4.3 and Table 4.5 summarise the main results of the two-factor classification model, including five significant predictors ($\delta^{13}C_{Fat}$, $\delta^{13}C_{Protein}$, $\delta^{15}N_{Protein}$, $\delta^{34}S_{Protein}$ and $\delta^{2}H_{Protein}$).

The bi-plot in Figure 4.3 demonstrates that Tunisian and Italian lamb samples were distinguishable according to both feed and geographical isotopic signatures. Country discrimination was expressed along factor 1, which accounted for a large percentage of the original variance of both X and Y variables (48% and 43% respectively). Meat from the two countries differs firstly due to the carbon isotope ratio in both protein and fat fractions. The lack of corn in Tunisian diets was probably the main factor, accounting for the lower ^{13}C content in meat from the North African country. Secondly, meat from different countries differed in terms of the deuterium content in the protein fraction, due to the predictable variation in $\delta^{2}H$ in the global water cycle according to longitude, elevation, coastal distance and latitude (Bowen et al., 2005). The two false negative Italian lamb

types (Table 4.5), close to factor 1 and marked by a ring in Figure 4.3, came from Sicily, near Tunisia, from animals fed on forage diets. This result is a risk for Sicilian shepherds, with some of their products potentially being incorrectly regarded as not original. In contrast, the purchasing of local lamb by Italian consumers would be assured by the absence of false positives in the binary classification of Italian lamb types as compared to Tunisian meat.

Figure 4.3. Bi-plot of lamb meat samples scores and loadings of their isotope ratio predictors, from a PLS-DA model of multiclass classification considering 32 Tunisian samples and 10 Italian lamb meat types. The Tunisia samples were produced in the Northwest region, height in Amdoun (AM-HP) farming systems and the other 24 in three sites [Ain Draham (AD) Fernana (F) characterized by woody pasture (WP), and Joumine (J) characterized by herbaceous pasture (HP)] representing the rest of the region. The Italian lamb types were produced throughout the country, from Trentino to Sicily, as described by Perini et al. (2009). The samples incorrectly classified, both false positive and negative, are marked by a ring.

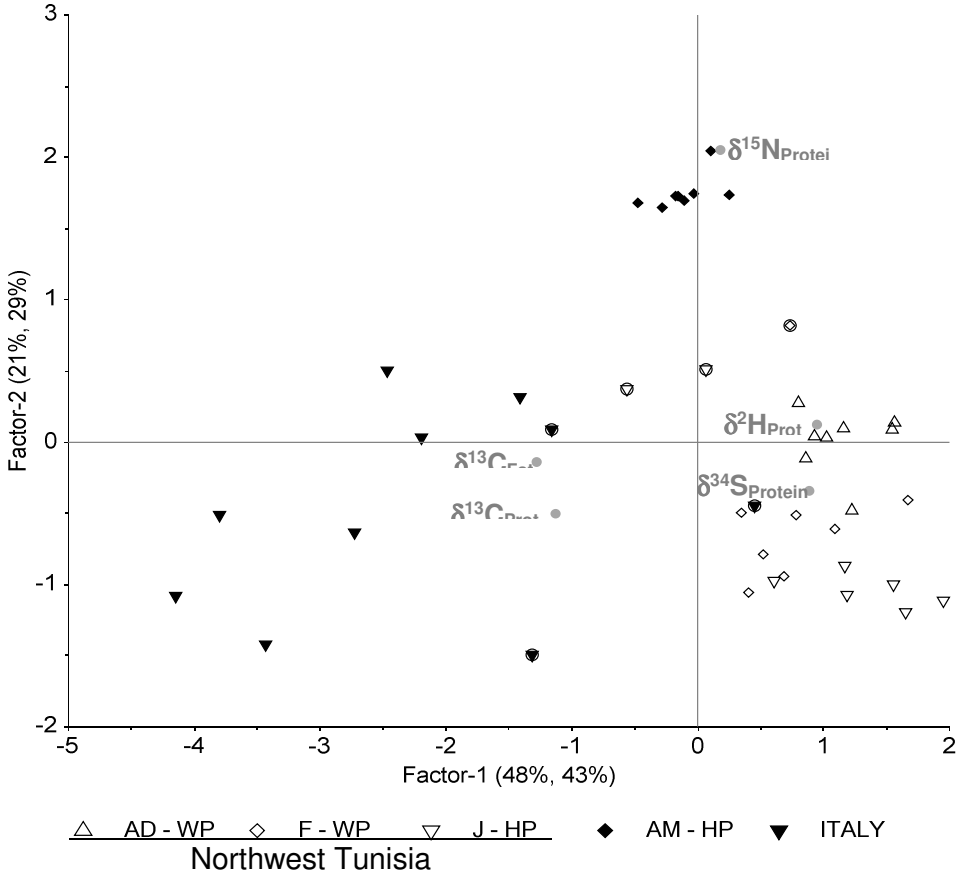


Table 4.5. Results for the classification of lamb meat produced in northwest Tunisia and Italy according to the isotope ratios profile (random cross-validation phase).

	Classes of binary discrimination			Multiclass overall classification[1]
	AM-HP	Northwest Tunisia	Italy	
Confusion matrix (no of meat samples)				

true positive (tp_i)	8	22	8	
false negative (fn_i)	0	2	2	
true negative (tn_i)	33	16	32	
false positive (fp_i)	1	2	0	
sensitivity	1.00	0.92	0.80	0.90
specificity	0.97	0.89	1.00	0.96
(average) accuracy	0.98	0.90	0.95	0.94

[1] : $Sensitivity_i = tp_i/(tp_i+fn_i)$; $Sensitivity_\mu = \Sigma\, tp_i/\Sigma\, (tp_i+fn_i)$;
$Specificity_i = tn_i/(fp_i+tn_i)$; $Specificity_\mu = \Sigma\, tn_i/\Sigma\, (fp_i+tn_i)$;
$Accuracy_i = (tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)$; Average accuracy $= \Sigma\, [(tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)]\ /3$.

As regards Tunisian meat, based on the isotope ratio profile, it would appear possible and reliable to distinguish only lambs reared in the Amdoun area from north-west Tunisia. Indeed, the bi-plot in Figure 4.3 shows that, along factor 2, due to its low $\delta^{34}S_{Protein}$ and very high $\delta^{15}N_{Protein}$ values, the AM-HP farming system was clearly set apart from the rest of north-west Tunisia. Only one sample from Fernana was erroneously regarded as produced in Amdoun (false positive), with very high accuracy (0.98) in the binary classification performance of this farming system. The rest of the north-west region of Tunisia should be regarded as a single lamb-producing area, the IR profile of which does not allow reliable discrimination of sub-units and small scale farming systems. The evaluation performance of binary classification of lamb from this breeding area was the poorest (average accuracy 0.90), indicating a lack of internal homogeneity. Indeed, two samples from Joumine were not regarded as originally from north-west Tunisia (false negatives), while two Italian lamb meat types were designed as Tunisian (false positives).

4.4 Conclusions

The first application of the IRMS for tracing the farming systems of the northwest Tunisia gives promising results for both a large scale discrimination of the North West of Tunisia as a whole geographic region of lamb production and a small scale classification of individual regional farming systems. In particular, it appeared reliable distinghishing on the basis of its IR profile the lamb meat produced in Amdoun area, characterized by a humid climate, large use of organic manure to fertilize forage crops and fallows grazed by flocks comprising lambs suckling their dams until slaughtering.

However, the number of samples for calibration must be increased for improving the robustness of the model, the validation of which must be tested collecting external observations, other than those with which it was defined.

The IRMS did not prove to be able to provide deep and systematic signatures of lamb meat for tracing the individual farming systems of the North West of Tunisia that may be instead sucesfully discriminated as a whole geograpical region of lamb production.

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5. CHAPTER 3- Stable isotope ratios of H, C, O, N and S for the geographical traceability of the Italian rainbow trout (Oncorhynchus mykiss)

5.1 Introduction

Analysis of the stable isotope ratios of bioelements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) is increasingly applied to trace the geographical origin of animal products, such as cheese (Bontempo et al., 2012; Camin et al., 2012; Bontempo et al., 2011), meat (Piasentier et al., 2003; Camin et al., 2007; Silva et al., 2014;) and meat products (Perini et al., 2013), honey (Schellenberg et al., 2010; Kropf et al., 2010; Chesson et al., 2011; Bontempo et al., 2016), etc. The applications are based on the fact that isotopic ratios in naturally occurring compounds have different values depending on latitude, altitude, distance from the sea, temperature, humidity, rainfall and the geological characteristics of the area of provenance, the fertilisation practices adopted and the species of plant from which they originate (Perini et al., 2009). With regard to fish, isotopic analysis has been used extensively to study trophic dynamics in aquatic environments, especially in the ecological field, also in relation to spatial and temporal variations (e.g. Reid et al., 2013; Logan et al., 2013). Other applications include reconstruction of fish migration routes (e.g. Trueman et al., 2013; Durbec et al., 2013), water paleotemperature (Vanhove et al., 2013) or assessment of the anthropogenic impact on aquatic systems (Borderelle et al., 2009). The parts of fish subjected to analysis are generally the otolith, which provides a record of data over the period of life of the fish, and defatted fish fillet.

Few papers have dealt with the use of isotopic analysis to determine the authenticity of fish. $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios have made it possible to differentiate Atlantic cod from cod of lower quality and price (Oliveira et al., 2011), trout fed on plant feed from those fed with animal feed (Moreno-Rojas et al., 2008) and wild from farmed fish (Santana et al., 2010; Fasolato et al. 2010; Busetto et al., 2008, Thomas et al., 2008, Bell et al., 2007, Morrison et al., 2007). Turchini et al. (2009) investigated the use of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ to discriminate between intensively farmed freshwater Murray cod originating from different farm systems (indoor recirculating, outdoor floating cage, and flow through systems) in different geographical areas, finding that $\delta^{13}C$ and/or $\delta^{15}N$ clearly linked fish to a specific commercial diet, while $\delta^{18}O$ linked fish to a specific water source. Thus, the combination of these isotopes can distinguish between fish originating from different farms. A recent paper published in Japanese (Isshiki et al., 2014) evaluated stable isotope analysis as a potential tool for discriminating the geographic origin of fish. The authors concluded that stable isotope analysis of carbon, nitrogen and oxygen is useful for discriminating Japanese eel

products from Chinese ones. Moreover, investigating $\delta^{13}C$ and $\delta^{15}N$ of three commercial fish, *viz.* mackerel, yellow croaker and pollock, originating from various countries, Kim and collaborators (Kim et al.,2015) found that apart from species-dependent variation in isotopic values, marked differences were also observed with respect to the country of origin. Finally, using the $\delta^{13}C$, $\delta^{15}N$ or $\delta^{13}C$ and $\delta^{2}H$ of fillet in combination with multi-element composition, Ortea and Gallardo (2015) Carter et al. (2015) attained correct classification of shrimp and prawn samples according to their actual origin and concluded that these methodologies should be considered for studies regarding seafood product authenticity.

Tracing the geographical origin of food products such as fish is very important, because the consumer is increasingly interested in buying local products, due to the assumed higher quality and eco-compatibility, and is willing to add value to products with certified origin, e.g. PDO (Protected Designation of Origin) or PGI (Protected Geographical Indication). In the case of fish and shellfish, 12 PDO and 25 PGI denominations have been registered so far by the European Union (DOOR database "Database of Origin and Registration", http://ec.europa.eu/agriculture/quality/schemes/index_en.htm, data for October 2014. Furthermore, European law (Commission Regulation (EC) No 2065/2001) regarding fishery and aquaculture products states that 'The requirements governing consumer information, in particular as regards the commercial designation and method of production of a species, and the area in which it is caught, should be specified." In particular, it is necessary to indicate whether a product was 'caught' or 'farmed' and the catch area or 'in the case of farmed products, a reference to the Member State or third country in which the product undergoes the final development stage'. Lastly, as an animal product fish is subject to EU Regulation 178/2002, which requires the traceability of food or food-producing animals to be established at all stages of production, processing, and distribution.

In this study, for the first time, we measured the isotopic ratios of H, C, N, O and S in defatted fillet and the extracted lipid fraction from farmed rainbow trout reared in different Italian farms, to investigate the ability of isotopic analysis to discriminate and trace the geographical origin of trout from Friuli Venezia Giulia (FVG) and the province of Trento (TN), also according to the type of feed.

The work focuses particularly on trout from these two Italian areas, because here trout farmers are interested in promoting fish products of assured and verifiable local origin, besides providing consumers with a high quality production protocol (Galeotti, 2014).

5.2 Materials and Methods

Samples

20 farms were taken into account. 10 of them were located in two northern Italian areas, namely Friuli Venezia Giulia region (FVG) and Trentino district (TN), whereas the others 10 in other Italian regions (Veneto, Piemonte, Lombardia, Toscana, Marche, Umbria) and were identified as ITALY.

A total of 130 fish, 20 feed and 20 water samples from the rearing tanks were considered (**Table 5.1**). From 6 to 8 samples of fish and 1 sample of feed and water were collected in each farm.

Before collecting the fish, it was checked that their biomass had increased by more than half in that farm. The collected feed had been dispensed to trout for at least the previous 2 months.

Table 5.1 Description of the sampling plan

Province	Region	N. of farms	N. of fish collected	N. of feed collected	N. of water collected	Latitude range	Longitude range
Udine	Friuli Venezia Giulia	3	24	3	3	45.84.40,26" - 45.91.08,21"	12.96.52,07" - 13.33.00,22"
Pordenone	Friuli Venezia Giulia	2	16	2	2	45.96.21,00" - 45.97.14,04"	12.59.27,40" - 12.75.39,41"
Trento	Trentino Alto Adige	5	30	5	5	45.49.08,32" - 46.11.30,10"	10.32.42,95" - 11.35.39,66"
Perugia	Umbria	1	6	1	1	42.87.91,42"	13.00.01,73"
Macerata	Marche	2	12	2	2	42.90.65,68" - 43.18.95,33"	12.89.16,72" - 13.03.16,54"
Lucca	Toscana	1	6	1	1	44.04.22,59"	10.56.03,92"
Massa	Toscana	1	6	1	1	44.25.16,49"	10.02.47,87"
Treviso	Veneto	3	18	3	3	45.38.42,01" - 45.52.41,11"	11.57.47,64" - 12.25.21,27"
Novara	Piemonte	1	6	1	1	45.42.34,55"	08.80.71,85"
Pavia	Lombardia	1	6	1	1	45.38.92,72"	08.82.67,53"
Total		20	130	20	20		

The fish samples were frozen and kept in the freezer until analysis. We analysed the defatted fish and extracted fat for each sample. The feed samples were pulverised with a domestic grinder.

Separation of fillet and fat from fish

Once lyophilised (Heto freeze dryer, Analytical Control De Mori, Milan, Italy) and finely ground (with a windmill blade at 4000 rpm x 10 sec), the sample was extracted using petroleum ether at 40 ° -60 ° (Soxhlet Extraction, AOAC 2000, Method 991.36, Crude Fat in Meat and Meat Products, in a SER 148 extraction apparatus - Model Velp scientifica, Italy). The residue containing fat after removal of the solvent was suspended in 4 ml of hexane and transferred into vials with screw cap (fat fraction of filet). The defatted fillets were freeze-dried and also transferred into vials with screw cap (protein fraction). In some samples we quantified the residual fat content after extraction, without finding any measurable quantity. Afterwards the protein and fat fractions (after evaporating hexane) were stored in an appropriate container in a vacuum desiccator until measurement.

IRMS analysis

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ isotopic ratios in fish fractions and feed were measured using an Isotope Ratio Mass Spectrometer (Isoprime, Manchester, UK) after combustion of the sample (Isotope Vario, Elementar, Bremen, Germany).

The $^{18}O/^{16}O$ ratio in water was determined after equilibration of water with CO_2 with a known content of ^{18}O ($C^{16}O_2 + H_2{}^{18}O = C^{16}O^{18}O + H_2{}^{16}O$), using an isotopic mass spectrometer (Sira II - ISOGAS VG, Fisons, Rhone, Milan, Italy) interfaced with a CO_2 compensator (Isoprep 18, VG-Fisons).

Measurement of $^{2}H/^{1}H$ and $^{18}O/^{16}O$ in fish fractions and feed was carried out with a Delta Plus XP IRMS (ThermoFinnigan, Bremen, Germany) connected to a TC/EA pyrolyzer (ThermoFinnigan).

Samples were introduced into the pyrolysis/combustion column via the autosampler, equipped with a suitable cover, where dry conditions were ensured by flushing nitrogen continuously over the samples.

The values are expressed according to the IUPAC protocol (Brand, Coplen, Vogl, Rosner & Prohaska, 2014).

The δ^2H and $\delta^{18}O$ values of defatted fillet and feed were calculated against CBS (Caribou Hoof Standard δ^2H = -197 ± 2 ‰ and $\delta^{18}O$ = +2.4 ±0.1 ‰) and KHS (Kudu Horn Standard, δ^2H = -54 ± 1 ‰ and $\delta^{18}O$ = +21.2 ± 0.2 ‰) through the creation of a linear equation and adopting the comparative equilibration procedure (Wassenaar & Hobson, 2003). We used these two keratinous standards because of the absence of any international organic reference material with a similar matrix to ours.

The $\delta^{18}O$ of fat was calculated against a working in-house standard (commercial olive oil) calibrated against benzoic acid-601 (IAEA-International Atomic Energy Agency, Vienna, Austria)., As recently suggested (Brand, Coplen, Vogl, Rosner and Prohaska, 2014), beside the olive oil standard (calibrated against NBS-22), we used a second standard for δ^2H with different δ^2H values (magnesium stearate for the FIRMS FT method, δ^2H value: -228‰).

For $\delta^{13}C$ and $\delta^{15}N$, the samples were analysed using a single working standard for normalisation, calibrated against NBS-22 fuel oil (IAEA-International Atomic Energy Agency, Vienna, Austria), IAEA-CH-6 sucrose for $\delta^{13}C$ and USGS 40 (U.S. Geological Survey, Reston, VA, USA) for both $\delta^{13}C$ and $\delta^{15}N$ and potassium nitrate IAEA-NO_3 for $\delta^{15}N$. We did not use a calibration curve for $\delta^{13}C$ as suggested by IUPAC (Brand et al., 2014) because as we used a single standard with a value similar to that of the samples, the data determined using a single-anchoring point or two-three anchoring points were not significantly different (Camin et al., 2015).

The $\delta^{34}S$ values were calculated against barium sulphates IAEA-SO-5, NBS 127 (IAEA) and a calibrated protein working standard through the creation of a linear equation.

One control sample was routinely included in each analytical run to check system performance and we obtained very repeatable results over the 2 month running period.

Measurement uncertainty, expressed as one standard deviation when measuring a sample 10 times, was ≤ 2‰ for $\delta^{2}H$, 0.3‰ for $\delta^{34}S$ and for $\delta^{18}O$ and 0.2‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ in the water.

Statistical analysis

Univariate analysis was performed using SPSS for Windows (v. 7.5.21; SPSS Inc., Chicago, IL, USA), while multivariate analysis was performed using The Unscrambler X, 10.3 package (CAMO Software AS., Oslo NORWAY).

Non parametric tests (Kruskall–Wallis and multiple bilateral comparison) were applied because of the unequal numbers of samples per group and the not always normal distribution (Soliani, 2003).

The relationship between pairs of IRs was analysed with Spearman's rho coefficient (Rho) and linear regression analysis. The multivariate relationship between the whole isotope profile of fish fillets (X-matrix comprising 8 IRs) and that of their feed and rearing water (Y-matrix, 6 IRs) was investigated using partial least square regression (PLS2).

Discriminant PLS (PLS-DA; Chevallier, Bertrand, Kohler, & Courcoux, 2006) was then applied to check the efficacy of the isotope profile of fillet or feed in discriminating the geographical origin of fish or the nature of the feed type. In particular, the PLS-DA model was built between the isotope matrixes (X) and the geographic origin or feed type matrix (Y), which was created by defining a dummy variable for each origin and feed type considered.

Finally, a PLS-DA model was proposed for tracing trout, considering the geographic location of farms and the feed type together. With this aim, the fillet's isotope data was divided into two sets, the training set being used for model calibration and the test set for validation. An overall 65–35% division was applied to the isotope dataset. All farms were included and represented in both the training and test sets. In each farm five out of eight fillet samples, in the FVG region, or four out of six in the other districts, were chosen in a random way to build a training set of 85 samples out of 130, specifically for calibration.

The optimum number of PLS components was estimated using full cross-validation. The significance of IR predictors was evaluated with Marten's uncertainty test. Classification performance was assessed in the validation phase in terms of sensitivity, specificity and total accuracy (Kjeldahl & Bro, 2010; Sokolova & Lapalme, 2009), estimated at a given threshold or cut-off limit. In particular, adopting a cut-off value of 0.5, samples with a predicted Y-value greater

than 0.5 were identified as belonging to one class, whilst those with predicted Y-values lower than 0.5 were predicted as belonging to the other class (Barbin, Sun & Su, 2013).

5.3 Results and discussion

Isotope ratio value and correlation

The literature states that the isotopic ratios of C, N and S in fish depend on those of their diet, whereas the isotopic ratios of H depend on those of the aquatic environment (Gerdeaux and Dufour, 2001). It also specifies that factors such as the size of the fish, its trophic level, species and the fillet defatting procedure can have an influence (Sotiropoulos et al., 2004; Nakazawa et al., 2010; Sweeting et al. 2007; Soto et al., 2011). In this study we considered a single species of fish of uniform size and used the same fillet defatting procedure, so the latter factors should not have an influence. In any case, regardless of the species, the size of the fish and water temperature, the isotopic ratio of H in the otolith and muscle of fish of 11 different species was shown to be correlated with the same ratio in the water (Whitledge et al. , 2006). This is very important for the purpose of tracing the origin of fish, because the isotopic composition of water is related to geographical factors such as latitude, altitude and distance from the sea (Clark and Fritz, 1997; Bowen et al., 2005)

Table 5.2 shows the average values for the δ^2H, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ of defatted fillet, δ^2H, $\delta^{18}O$, $\delta^{13}C$ of fish fat, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, δ^2H, $\delta^{18}O$ of feed and $\delta^{18}O$ of tank water for each farm.

Table 5.2: Mean and standard deviation of isotopic values for the protein and fat of fish fillet, feed and tank water for each farm (farm number in brackets) and type of finishing feed used (low fish content: LF, or high fish content: HF).

		Protein filet					Fat filet				Feed					Water
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	δ^2H	$\delta^{18}O$	$\delta^{13}C$	δ^2H	$\delta^{18}O$	Type	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	δ^2H	$\delta^{18}O$	$\delta^{18}O$
PN1 (1)	$\bar{x}$	-20.4	11.3	11,3	-122.3	11.5	-25.6	-201.4	15.8	**HF**	-23.8	8.8	12.1	-129.0	20.4	-7.9
	SD	0.05	0.15	0.19	1.34	0.33	0.13	4.12	0.75							
PN2 (2)	$\bar{x}$	-21.9	10.0	9,9	-125.5	12.1	-26.5	-197.0	15.8	**HF**	-24.8	6.7	9.1	-109.4	19.5	-8.3
	SD	0.10	0.17	,12	1.43	.26	0.07	1.71	0.45							
UD1 (3)	$\bar{x}$	-20.5	12.8	11,9	-120.1	12.0	-26.3	-199.7	15.5	**HF**	-24.1	11.1	11.9	-120.1	19.9	-8.3
	SD	0.08	0.06	,16	1.78	0.59	0.05	2.98	0.27							
UD2 (4)	$\bar{x}$	-21.5	12.5	12,1	-126.8	11.4	-27.1	-187.1	16.3	**HF**	-24.5	8.3	12.1	-118.9	19.4	-7.9
	SD	0.08	0.20	,17	1.66	0.30	0.15	3.05	0.26							
UD3 (5)	$\bar{x}$	-21.0	12.5	11,1	-123.2	13.1	-26.1	-194.3	16.6	**HF**	-23.7	10.8	12.0	-107.9	20.2	-6.8
	SD	0.08	0.14	,24	1.08	0.29	0.15	2.51	0.44							
TN1 (6)	$\bar{x}$	-22.4	9.9	9,7	-127.4	10.2	-27.8	-191.0	16.2	**LF**	-26.3	3.8	4.9	-89.5	23.4	-9.6
	SD	0.24	0.65	,51	2.28	1.23	0.79	10.41	0.63							

TN2 (7)	x̄	-21.9	11.2	11,4	-131.2	9.6	-27.3	-185.2	15.6	**HF**	-24.9	6.4	9.4	-92.6	21.8	-10.4
	SD	0.08	0.13	,24	1.71	1.04	0.60	9.27	1.06							
TN3 (8)	x̄	-21.8	11.1	10,8	-128.9	10.4	-26.7	-195.1	15.2	**HF**	-24.4	8.4	10.8	-99.2	21.4	-9.3
	SD	0.10	0.20	,18	0.83	.54	1.04	10.25	1.23							
TN4 (9)	x̄	-21.9	11.2	10,8	-129.1	10.3	-26.8	-190.0	16.1	**HF**	-25.0	6.8	9.8	-110.0	20.6	-9.6
	SD	0.15	0.31	,31	1.17	0.38	0.18	2.14	1.03							
TN5 (10)	x̄	-21.5	11.5	10,9	-129.7	9.0	-26.4	-180.8	16.0	**HF**	-24.5	7.5	9.6	-101.9	20.1	-10.5
	SD	0.08	0.30	,38	2.01	0.97	0.15	6.79	0.95							
PG (11)	x̄	-22.9	8.1	5,5	-127.3	9.4	-28.5	-179.0	16.9	**LF**	-25.8	4.4	4.0	-87.6	24.4	-8.6
	SD	0.31	0.15	,32	2.13	0.62	0.15	6.16	.86							
MC1 (12)	x̄	-23.0	7.9	5,7	-126.4	10.2	-28.3	-174.5	17.2	**LF**	-25.6	4.0	4.6	-98.5	22.9	-8.5
	SD	0.27	0.28	,69	1.56	0.61	0.16	5.66	0.72							
MC2 (13)	x̄	-22.9	8.4	6,2	-126.6	9.8	-28.5	-172.3	17.3	**LF**	-25.9	4.2	3.9	-81.6	24.7	-9.4
	SD	0.36	0.41	,56	1.10	0.43	0.34	6.80	0.95							
LU (14)	x̄	-21.2	8.6	6.6	-121.5	11.2	-26.8	-187.8	17.9	**LF**	-26.3	4.2	4.1	-95.8	23.4	-7.8
	SD	0.78	0.28	0.53	2.36	0.53	0.82	2.33	1.47							
MS (15)	x̄	-21.8	10.7	10.1	-120.5	11.6	-26.9	-192.2	16.4	**HF**	-25.3	6.1	7.4	-122.6	18.1	-7.7
	SD	0.13	0.30	0.63	1.40	0.36	0.16	1.82	0.73							
TV1 (16)	x̄	-21.8	11.5	11.2	-126.6	11.1	-27.1	-187.2	16.8	**HF**	-24.7	4.6	10.9	-107.2	20.9	-9.2
	SD	0.15	0.28	0.12	3.54	0.22	0.08	2.79	1.67							
TV2 (17)	x̄	-21.7	9.2	10.2	-124.2	10.4	-27.5	-188.8	18.8	**HF**	-24.1	5.6	7.6	-112.7	21.4	-8.7
	SD	0.30	0.56	0.74	1.11	0.36	0.79	3.22	0.28							
TV3 (18)	x̄	-22.4	8.2	7.7	-126.3	11.3	-28.8	-180.2	16.1	**LF**	-25.7	5.1	8.4	-102.1	24.1	-8.8
	SD	0.22	0.23	0.10	1.75	0.56	0.17	0.44	0.64							
NO (19)	x̄	-21.9	10.6	10.5	-126.4	9.7	-27.0	-185.9	17.1	**HF**	-24.8	6,7	9.0	-109.7	21.7	-9.4
	SD	0.05	0.09	0.12	1.91	0.48	0.06	1.45	0.98							
PV (20)	x̄	-21.5	10.2	10.1	-126.1	10.2	-27.0	-188.9	17.0	**HF**	-23.7	5.6	8.6	-111.0	21.4	-9.1
	SD	0.15	0.28	0.43	1.23	0.311	0.08	2.92	1.09							

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of feed ranged from -26.3 to -23.7‰, from 3.8 to 11.1‰ and from 3.9 to 12.1 ‰ respectively. Low $\delta^{13}C$ values probably indicate the absence of C4 plants (Hulsemann et al., 2015) and higher $\delta^{15}N$ values are due to a higher amount of animal protein including haemoglobin (Beltran et al., 2009), whereas higher $\delta^{34}S$ is due to the presence of sea components (Krivachy et al., 2015).

Comparing the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of fillet protein with the isotopic values of feed, we found average increases of 3.1 ‰ (from 2.2 to 5.1 ‰), 3.8 ‰ (from 1.7 to 6.9‰) e 1.1 ‰ (from -0.9 to +4.8‰) respectively. With regard to the $\delta^{13}C$ of fish fat, we found an average variation of -2.2‰ as compared to diet values. Lipids were isotopically depleted in comparison to proteins and carbohydrates, due to kinetic isotope effects that occur during the conversion of pyruvate to acetyl coenzyme A in lipid synthesis (DeNiro and Epstein, 1977). Here we found an average homogenous depletion of –5.3 ‰ in fat compared to defatted muscle, in line with the findings of other authors in other fish species (e.g. Kiljunen et al., 2006). With regard to $\delta^{15}N$, the increase in defatted fillet essentially agrees with the results found for rainbow trout (+2.6 to 5.3, Beltran et al., 2009).

As expected, $\delta^{2}H_{fat}$ was always lower than $\delta^{2}H_{protein}$ determined in defatted matter (mean difference of 63‰) whereas $\delta^{18}O_{fat}$ values were always higher than $\delta^{18}O_{protein}$, with a mean difference of 5.7‰, as observed in other animals (Tuross et al., 2008; Perini et al., 2009, 2013) confirming that the hydrogen and oxygen stable isotopes in protein and lipids are incorporated according to different biosynthetic pathways involving isotope fractionation (Hobson, Atwell, & Wassenaar, 1999, Schmidt, Werner, & Rossmann, 2001).

To better understand the relationship between the isotope ratios (IRs) of fillet and of feed and tank water, PLS2 regression was carried out between the two matrixes. The two series of data were closely related. The four-factor PLS model accounted for 90% (77% in validation) of the original variance of fillet isotope ratios, and 67% (63% in validation) of the original variance of the isotope ratios of feed and tank water.

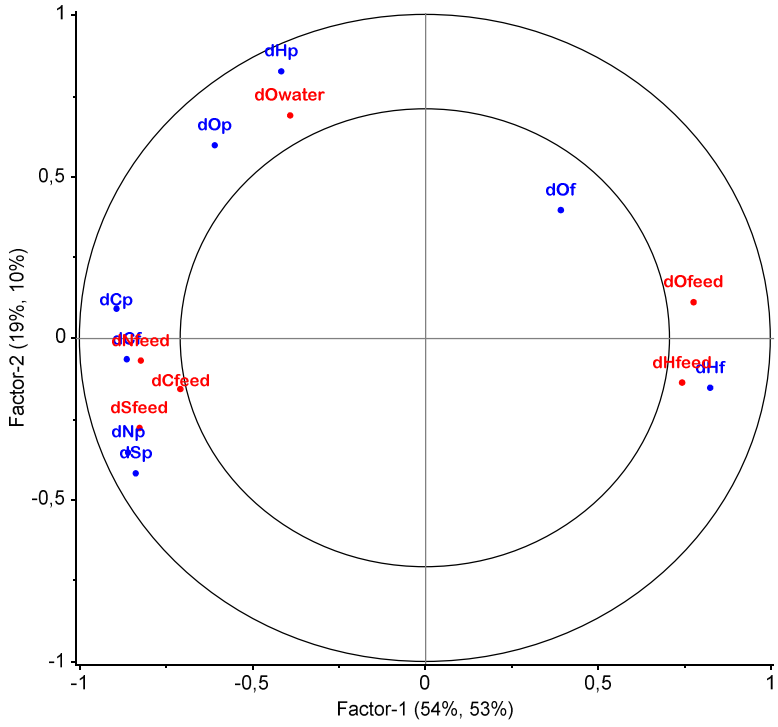

Figure 5.1. Correlation loadings of the isotope ratios of trout fillet (X-matrix) and feed and tank water (Y-matrix) on the two first factors of a PLS2 regression model.

The correlation loadings of the processed X variable (isotope ratios of trout fillet) and Y variable (isotope ratios of feed and tank water) along factor 1 and factor 2 are plotted in **Figure 5.1.** All the IRs were located within or very close to the outer ring of the plot, indicating that the first two components of the model explained at least 50% of the original variance of both X and Y isotope ratios. The two dimensional plot of **Figure 5.1** may thus be considered a viable visualisation of the

significant correlations between the IRs of fillet, feed and tank water. IRs close to each other have a high positive correlation, while those in opposing quadrants have a tendency to be negatively correlated. All the feed IRs lying close to the first component may thus be regarded as representing the trophic factor. Of the trophic component, $\delta^{13}C_{feed}$, $\delta^{15}N_{feed}$ and $\delta^{34}S_{feed}$ loaded near to each other in the left half, very close to $\delta^{13}C_{fat}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$ and $\delta^{34}S_{protein}$. On the opposite side, the positive half of the trophic factor loaded $\delta^{2}H_{feed}$ and $\delta^{18}O_{feed}$, close to each other and to $\delta^{2}H_{fat}$. We can conclude that the C, N and S isotope ratios of feed and fillet were highly positively correlated among and between each other, and negatively correlated with the $\delta^{2}H$ and $\delta^{18}O$ of feed and the $\delta^{2}H_{fat}$ of fillet, which were in their turn highly interrelated. The linear relationships between $\delta^{13}C$ and $\delta^{15}N$ in defatted matter and feed ($\delta^{13}C_{protein} = 0.4458 * \delta^{13}C_{feed}$ -10.709; $\delta^{15}N_{protein} = 0.4654 * \delta^{15}N_{feed} + 7.372$) were similar to those found by Turchini et al. (2009).

The H and O isotope ratios of protein fillet loaded near to each other and the O isotope ratio of tank water. $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$ of fillet were thus interrelated and positively correlated with $\delta^{18}O_{water}$. Overall, these IRs were located close to the 2nd component, which may be regarded as the geographic factor in the PLS model, accounting for IR relationships. Indeed, the interaction between terrestrial location and the isotopic signature of environmental water is well-known (Bowen et al., 2005). Other authors (Soto et al., 2013) have already demonstrated the influence of the isotopic signature of environmental water on the $\delta^{18}O$ and $\delta^{2}H$ of fish tissue protein. In particular, Soto et al. (2013) hypothesised that fish only assume hydrogen from feed and environmental water, whereas oxygen is also absorbed from the O_2 dissolved in water. Differently from Turchini et al. (2009), we did not find a perfect linear relationship between $\delta^{18}O_{protein}$ and $\delta^{18}O_{water}$. However, while Turchini and colleagues considered four farms located in different places in Australia covering more than 10 degrees latitude, this study covered a more restricted area in central-northern Italy, including around 2° latitude. A highly significant ($p<0.001$) and similar ($\delta^{18}O_{protein} = 0.9419 * \delta^{18}O_{water} + 19.041$) relationship to that reported by the Australian team was nevertheless highlighted for Italian trout farms.

In comparison with other variables, the $\delta^{18}O_{fat}$ of fillet showed less significant correlation with the other IRs. $\delta^{18}O_{fat}$ loaded in the first quadrant, in an intermediate position between the trophic and the geographic components of the model, probably influenced by both feed (significant Rho with $\delta^{13}C_{protein}$, -.193 p=.03; $\delta^{15}N_{protein}$,-.342 $p<0.001$; $\delta^{34}S_{protein}$, -.359 $p<0.001$; $\delta^{13}C_{fat}$, -.284 p=0.001; $\delta^{15}N_{feed}$, -.399 $p<0.001$; $\delta^{34}S_{feed}$, -.408 $p<0.001$; and with $\delta^{2}H_{fat,}$ 0.406 $p<0.001$ and $\delta^{18}O_{feed}$, 0.300 p=0.001) and environmental (significant Rho with $\delta^{18}O_{protein}$: -.225; p=0.01) factors. To our knowledge, only one paper has reported data on the $\delta^{18}O$ of fat extracted from fish tissue (Morrison

et al., 2007). However, these authors examined only its ability to distinguish wild and farmed gilthead, without any comment on the relationship between $\delta^{18}O_{fat}$ and other IRs.

Sources of isotope ratio variability

Trout feed formulation relies on the use of complete feeds, with fish meal and fish oil representing nearly ideal sources of dietary energy and nutrients for a long time in the past. However, due to rising market prices and sustainability issues linked to the limited availability of fishery–based raw materials, in recent years the use of such commodities in commercial trout feed has been substantially reduced and mostly replaced by oils and protein-rich raw materials of vegetable origin. Interviews with farmers and analysis of the compulsory detailed list of feed ingredients for each commercial preparation allowed the identification of two types of feeds for the finishing phase in the experimental farms: high fish content (HF) and low fish content (LF) feeds, as described in **Table 5.2**. In the first feed type, fish meal and oil supplied more than 50% of the overall protein and lipid content, with a wide variety of ingredients (Tibaldi et al., 2014), whereas vegetable protein and particularly seed-extracted oils made up the bulk of protein and lipids supplied by the LF type feeds.

PLS-DA analysis was carried out to verify the possibility of discriminating between the two levels of vegetable ingredient inclusion, according to the isotope ratio profile of: (1) feed, first PLS-DA model, and (2) fillets, second model. For this purpose, a dummy Y-variable of 1 was assigned to HF feed and a variable of 0 to LF feed.

The results of the first PLS-DA classification model (full cross validation method) were excellent. The coefficients of prediction were 0.92 and 0.91 for calibration (R^2_C) and validation (R^2_V) respectively. The root mean square error (RMSE) was 0.13 for both calibration and validation, after three latent variables. In the validation phase, all the 14 HF feeds were correctly predicted (sensitivity, or true positive rate, i.e. number of HF samples correctly detected by the model divided by the total number of HF samples =1.00), as well as all six LF feeds (specificity, or true negative rate, i.e. number of LF samples correctly detected by the model divided by the total number of LF samples =1.00), with overall correct classification (accuracy) of 100%. All the five predictors included in the model ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$) were significant for discriminating the feed type (Marten's uncertainty test).

The same classification model was applied to the fillet isotope data to check its efficacy in predicting the nature of trout diet. The results were satisfactory, although not as good as those previously described. The proportion of original variance of the fillet IR profile predicted by the PLS-DA model was R^2_C =0.78 and R^2_V =0.75, with RMSEs respectively equal to 0.21 and 0.23,

after five latent variables. In the validation phase, all but one of the 94 fillets fed with HF feed were correctly predicted (sensitivity =0.99), whilst 6 fillets were misclassified as from LF fed trout (specificity =0.83), with a total accuracy of 94.6% (**Figure 5.2**).

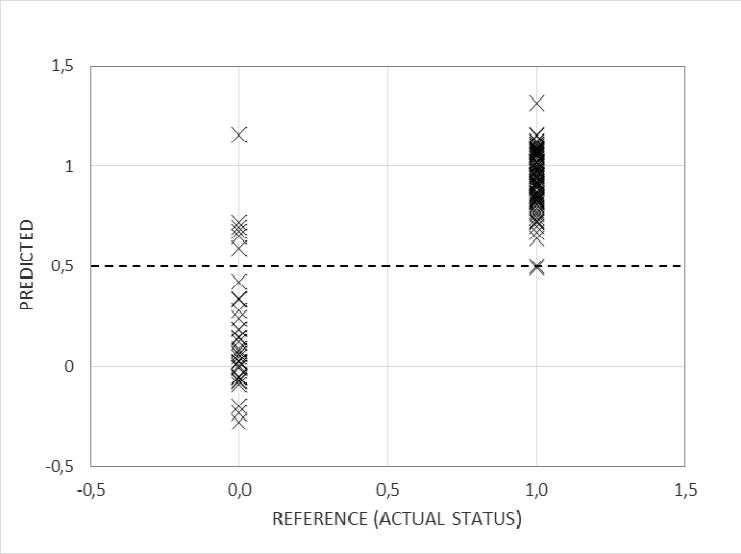

Figure 5.2. Prediction of feed type (validation phase) provided to the trout, based on PLS-DA regression using the isotope ratio profile of the fillets for a two-group model: 0 (low fish content) and 1 (high fish content).

The highlighted differences in the prediction efficacy of the two models were probably due to the influence of factors other than the feed isotopic profile on the fillet IRs. This condition is clearly highlighted in the first lines of **Table 5.3,** which summarises the mean values of the isotopic ratio profiles as affected by feed type. The IR of each feed differed significantly, according to the nature of the ingredients used for the formulation of the complete diets. HF feeds, with a high level of ingredients of animal and marine origin, showed higher $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values in comparison with LF feeds, including much higher levels of ingredients of plant origin, confirming the literature (Beltran et al., 2009; Camin et al., 2010; Krivachy et al., 2015).

Unlike feed IR, $\delta^{18}O_{water}$ was the same for the two groups of trout farms using different types of feed. The isotopic signatures of feed and tank water in the experimental farms were thus independent. Consequently and as already suggested, in addition to the nature of the feed the geographical location of the trout farm also contributed to the isotopic signature of the fillets, through the tank water composition. This condition accounted for the different profiles of fillets from the farm groups using feeds characterised by the two kinds of ingredients (**Table 5.3**). Indeed, while $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{13}C_{fat}$ and $\delta^{2}H_{fat}$ followed the same significant trend as the

homologue elements across feed types, IRs more dependent on the environmental water signature, i.e. $\delta^2H_{protein}$, $\delta^{18}O_{protein}$ and $\delta^{18}O_{fat}$, did not differ between feed types.

Table 5.3. IR profile of fillets, feed and tank water in relation to the nature of feed and the geographical location of farms.

	Protein fillet					Fat fillet			Feed					Water
	$\delta^{13}C$	δ^2H	$\delta^{18}O$	$\delta^{15}N$	$\delta^{34}S$	$\delta^{13}C$	δ^2H	$\delta^{18}O$	$\delta^{13}C$	δ^2H	$\delta^{18}O$	$\delta^{15}N$	$\delta^{34}S$	$\delta^{18}O$
Feed type														
High fish	-21.5^A	-125.8	10.9	11.2^A	10.9^A	-26.7^A	-191.0^B	16.4	-24.5^A	-110.9^B	20.5^B	7.4^A	10.0^A	-8.8
Low fish	-22.5^B	-125.9	10.4	8.5^B	6.9^B	-28.1^B	-180.8^A	16.9	-25.9^B	-92.5^A	23.8^A	4.3^B	5.0^B	-8.8
MSE	0.14	0.76	0.25	0.23	0.25	0.15	1.54	0.21	0.11	2.22	0.23	0.40	0.40	0.23
Origin														
FVG	-21.1^a	-123.6^A	12.0^A	11.8^a	11.2^a	-26.3^a	-195.9^B	16.0^B	-24.2	-117.1^b	19.9^b	9.1^A	11.4^A	-7.8^A
TN	-21.9^b	-129.3^B	9.9^B	11.0^a	10.7^a	-27.0^{ab}	-188.4^{AB}	15.8^B	-25.0	-98.6^a	21.5^{ab}	6.6^B	8.9^{AB}	-9.9^C
ITALY	-22.1^b	-125.2^A	10.5^B	9.3^b	8.4^b	-27.6^b	-183.7^A	17.1^A	-25.2	-102.9^a	22.3^a	5.1^B	6.8^B	-8.7^B
MSE	0.14	0.53	0.16	0.27	0.40	0.16	1.47	0.15	0.18	2.51	0.38	0.33	0.53	0.14

A,B,C: $p<0.01$ a,b: $p<0.05$.

On the bottom line of **Table 5.3**, the analysed IRs were averaged and compared according to the three main geographical areas of interest for this experiment, i.e. the Friuli Venezia Giulia region (FVG, 5 farms), the province of Trento (TN, 5 farms) and the rest of central-northern Italy (ITALY, 10 farms). The trout from the first two areas needed to be discriminated using the isotopic signature. $\delta^{18}O_{water}$ was clearly different ($p<0.01$) in the various geographical areas. Significant differences regarding the geographical signature also occurred for $\delta^2H_{protein}$, $\delta^{18}O_{protein}$ and $\delta^{18}O_{fat}$.

The feed isotope profile also showed significant differences for the three origins, indicating that the surveyed farms, randomly sampled to represent the commercial situation of the various trout production areas, did not use the two types of feed to the same extent. Indeed, as highlighted in **Table 5.2**, HF feed dominated in FVG and prevailed in the TN area, while HF and LF feeds occurred equally in Italian farms generally. The trend and significance of the differences in origin in the fillet isotopic profile did not completely overlap those for feed and tank water. Individual fish variability probably influenced this outcome, together with the effect of interaction between trophic and environmental factors.

Model discriminating trout reared in farms in the Friuli Venezia Giulia region and the province of Trento

Following on from the above observations, a PLS-DA model was proposed for tracing trout, based on the isotopic profile of fillets (X-matrix), considering the geographic location of farms and the feed type provided for trout finishing together. Four dummy variables (Y-matrix) were then defined, one for each of the controlled sources of variability in the fillet IR profile, i.e. FVG, TN,

ITALY-HF and ITALY-LF classes. FVG and TN origin was assigned independently of the feeding formulation. Indeed, the aim of the study was to check whether it was possible to distinguish fillets from trout farmed in these two geographical areas. They were sampled for efficiency, representing local farms interested in promoting fish products of assured and verifiable origin. The other Italian trout farms were instead divided into two classes, according to the nature of feed: ITALY-HF and ITALY-LF. This made it possible to check the possibility of discriminating fillets from trout fed with feedstuff of mainly animal or plant origin. A supervised approach was adopted, dividing fillet samples into two datasets, for calibration and for validation (85 and 45 samples respectively) randomly extracting 4 and 2 (5 and 3 in FVG) samples per dataset from every farm.

The coefficients of prediction for the six-factor PLS discriminant model (R^2_C) were 0.71, 0.62, 0.46 and 0.88 respectively for the FVG, TN, ITALY-HF and ITALY-LF classes, with total explained variance of 96.5% for fillet isotope ratios (X variables) and 66.7% for discriminant classes (Y variables). The number of factors required suggests possible incorporation of noise in the model. The weighted regression coefficients of the various IRs for each class of fillets are plotted in **Figure 5.3**, to summarise the relationship between the fillet IR predictors and a given trout origin. Having included all the X variables in the same scale through standardisation, the weighted regression coefficients show the relative importance of each isotope ratio as a tracer of trout origin. IRs with a large regression coefficient play an important role in the regression model; a positive coefficient shows a positive link with the response, while a negative coefficient shows a negative link. Predictors with a small coefficient are negligible. We also assessed the significance of each IR as a predictor for varying trout origin using Marten's uncertainty test. **Figure 5.3** shows that $\delta^{15}N_{protein}$ and $\delta^{18}O_{protein}$ contributed most to tracing trout from FVG farms, while $\delta^{34}S_{protein}$ played a significant role in discriminating ITALY farms finishing fish farming with feed containing high levels of plant ingredients. As expected, fillets from the latter farms were characterised by a negative correlation with $\delta^{34}S$, its value being lower in feed with a lower fish content, as already shown in **Table 5.3**. Both classes, FVG and ITALY-LF, were discriminated thanks to the concurrence of trophic and geographical signatures; this condition is highlighted by the simultaneous significance of isotope ratios related to the influence to both feed ($\delta^{15}N_{protein}$ or $\delta^{34}S_{protein}$) and tank water ($\delta^{18}O_{protein}$) in the discriminant model. TN origin was predictable using fillet isotope ratios, $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$, mainly attributable to the geographical signature. Specifically, TN origin was negatively correlated to both $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$, i.e. it was discriminable by the relatively low values of these IRs (**Table 5.3**). This condition, also observed in lamb meat (Perini et al., 2009), is due to the continental and altitudinal gradient of the province of TN, which is located in a northern Alpine mountainous area in Italy (see **Table 5.1**). The low $\delta^{18}O$

values of environmental and tank water (**Table 5.3**) were to some extent transferred to the δ^2H and $\delta^{18}O$ of fish fillets. Farms located in central-northern Italy using feed formulated with foodstuffs of predominantly fish origin (ITALY-HF), needed the concurrence of all the bio-elements but one, the $\delta^{13}C$ of both fillet fractions, in order to be traced. This result was probably influenced by the wide range of ingredients used to formulate this type of complete diet. In a recent survey regarding 16 commercial feeds of this type, Tibaldi et al. (2014) highlighted the presence of 23 different ingredients.

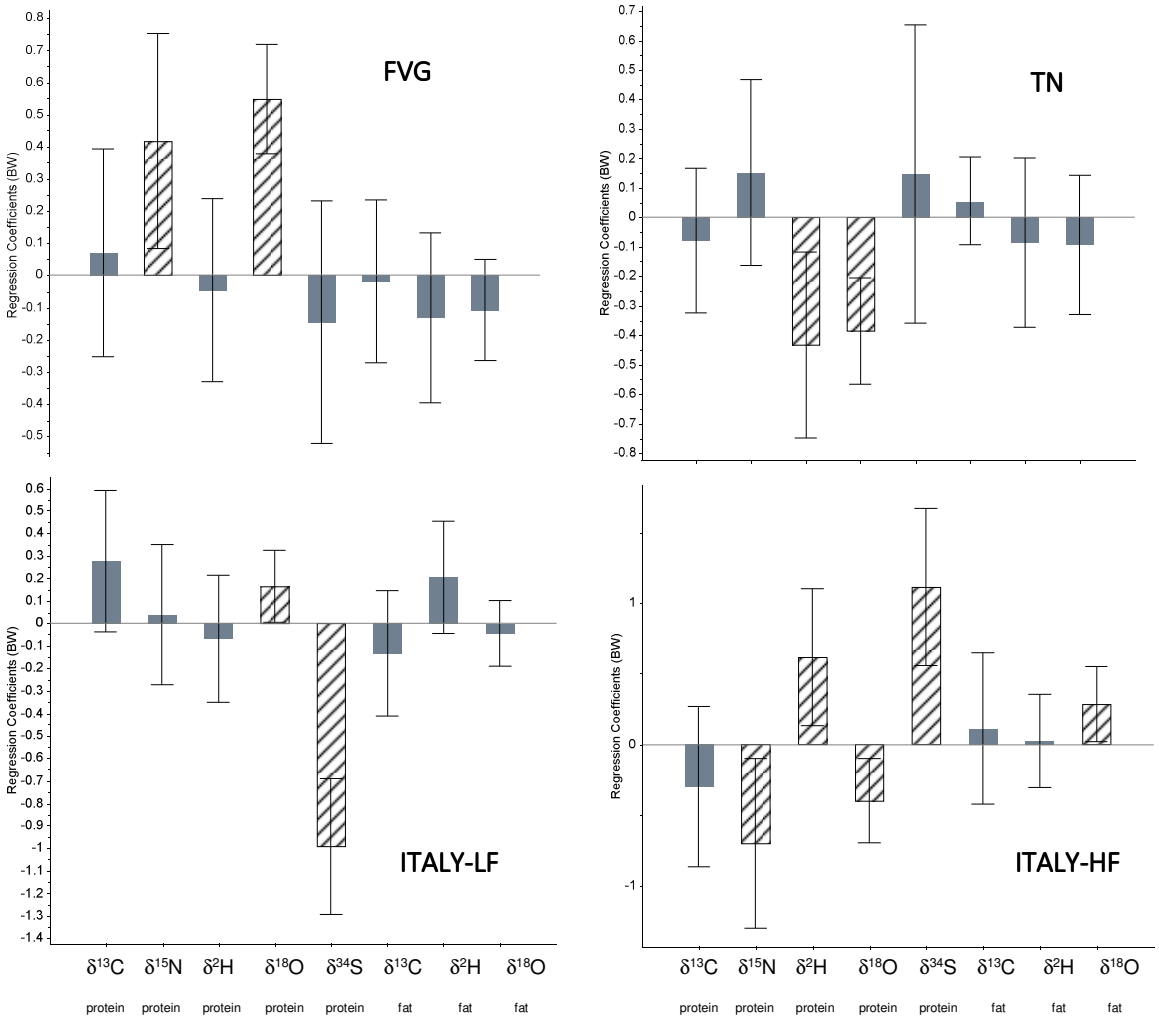

Figure 5.3. Weighted regression coefficients (BW) of the various isotope ratios for each class of fillet origin (FVG, TN, ITALY-LF and ITALY-HF) from a six-factor discriminant PLS model. IRs significant ($P < 0.05$, Marten's uncertainty test) for discriminating the various classes are highlighted using bars with diagonal lines.

The results of applying the calibrated PLS-DA model to the validation dataset are reported in **Table 5.4**, together with evaluation of prediction performance. Measurements of the quality of each binary classification model come from the confusion matrix, which records correctly and incorrectly recognised examples for each class of origin. True positives are samples of one selected origin, correctly predicted; false negatives are samples of one selected origin, incorrectly predicted to be of other origin; true negatives are samples of other origin, correctly predicted to be of other origin; false negatives are samples of other origin, incorrectly predicted to be from the selected origin. The quality of the whole model was calculated as a multiclass overall classification (Sokolova & Lapalme, 2009).

Table 5.4. Trout origin classification results according to the isotope ratios of fillets

	Binary discrimination classes				Multiclass overall classification[1]
	FVG	TN	ITALY-LF	ITALY-HF	
Confusion matrix (no. of validation samples)					
true positive (tp_i)	15	7	10	7	
false negative (fp_i)	0	3	0	3	
true negative (tn_i)	26	34	35	35	
false positive (fp_i)	4	1	0	0	
Performance evaluation[1]					
sensitivity	1.00	0.70	1.00	0.70	0.87
specificity	0.87	0.97	1.00	1.00	0.96
(average) accuracy	0.91	0.91	1.00	0.93	0.94

[1] : $Sensitivity_i = tp_i/(tp_i+fn_i)$; $Sensitivity_\mu = \Sigma\, tp_i/\Sigma\, (tp_i+fn_i)$;

$Specificity_i = tn_i/(fp_i+tn_i)$; $Specificity_\mu = \Sigma\, tn_i/\Sigma\, (fp_i+tn_i)$;

$Accuracy_i = (tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)$; Average accuracy $= \Sigma\, [(tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)]\ /4$.

The average accuracy of the discriminant multiclass model was 94%, a satisfactory result overall that however needs to be more detailed. The best performance was provided by the model for binary classification of ITALY-LF trout (100% accuracy), which allowed correct discrimination of all the samples finished in central-northern Italian farms with low-fish content feeds (sensitivity = 1.0) and did not misclassify any trout otherwise finished as ITALY-LF (specificity = 1.0). The ITALY-HF discriminant model achieved the second best accuracy percentage (93%), avoiding any false positive identification of trout (specificity = 1.0) but misclassifying three out of ten true ITALY-HF fillets (false negatives), which approached (0.489 to 0.496) without touching the predictive Y-value of 0.5, to be identified as processed from trout finished in central-northern Italy with high-fish content feeds (sensitivity = 0.7).

The lowest evaluation performance was that of FVG and TN binary classification models (accuracy of both = 91%), which aimed to discriminate solely the geographical origin of fish, from either the

Friuli Venezia Giulia region or the province of Trento respectively, regardless of finishing feed. Although having the same percentage, the performance of the two binary territorial models hides different classification outcomes and practical consequences. The FVG model correctly classified true regional trout (sensitivity = 1.0), indeed all the test samples from FVG (15 trout fillets randomly harvested from five regional farms) were identified as produced in FVG (true positive). However, this highly satisfactory sensitivity was achieved at the partial expense of the classification specificity (0.87) of the model, which also identified four out of 30 samples (13%) produced in extraterritorial farms as regional (false positive). The TN binary model attained the opposite classification outcome, associating high specificity performance (0.97, i.e. very few false positives) with relatively low sensitivity (0.7, i.e. many false negatives). In trout farms that do not use local feed, geographical traceability based on the stable isotopic profile seems less achievable than in other animal industries and specifically ruminant production chains, where local forage is significantly and widely included in diets and thus contributes significantly to the geographical signature (Piasentier et al., 2003; Perini et al., 2009; Camin et al., 2012).

The classification performance of the FVG model could probably be seen to be more satisfactory than that of the TN model for a producers' association aimed at bringing members together in a project ensuring traceability. In this situation, it is more important to avoid the risk of unjustified penalties against members honestly marketing genuine original trout and have a discriminant model that erroneously identifies products as not locally produced (false negatives), rather than to favour the ability of a classification model to avoid any risk of not distinguishing false positive samples. On the other hand, the latter is a more consumer-oriented solution that better protects the customer against fraud, namely the risk of buying trout fillets produced outside the declared geographical area. We might regard this choice as a dilemma for the local fish farming industry interested in promoting products of assured and verifiable origin.

5.4 Conclusions

The experiment provides effective evaluation of IRMS traceability models in the trout industry, quantifying the contribution of geographical and trophic signatures at various levels of interaction for the first time.

Classification model performance should be improved and overfitting reduced by increasing the size of dataset, individually validating the binary models, which should only include significant predictors.

The local industry aiming to ensure the origin of trout products must take into account the powerful influence of feed signature. Different pathways may be considered and evaluated, such as territorial

specialist complete feed formulation and/or the inclusion of individual marker ingredients, e.g. corn gluten feed from xantofil-free white varieties, which though avoiding any interference with fillet pigmentation may significantly change $\delta^{13}C$ values. Finally, other natural fillet tracers, such as trace mineral elements or strontium could be included in the discriminant model, as has already taken place for other foods of animal origin, such as PDO Parmesan.

5.5 References

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6. CHAPTER 4- Validation of methods for isotopic and elemental analysis of cheese

6.1 Introduction

European laws EC N. 510/2006 and 1151/2012 require protection against the mislabelling of foods with Protected Geographical Indications (PGI), Protected Designations of Origin (PDO) and Traditional Specialities Guarantee (TSG). According to a recent report by the Italian authority responsible for supervising the agro-food market [1], the number of these protected products is increasing year by year, reaching a total number of 1137 at the end of 2012. Italy is the country with the highest number of designations (250) involving a sales turnover of around 8.5 billion euro. In first place in Italy in terms of sales there are 2 PDO hard cheeses, renowned all over the world: Grana Padano and Parmigiano Reggiano.

Nevertheless, several other hard cheeses without any certified origin designation [2] are available on the market, sometimes with a wholesale price which is less than half the price of the two PDO cheeses. Especially when the cheese is sold in grated or shredded form, easy profits can be made by dishonest producers by fraudulently labelling common non-PDO cheeses with the 2 most famous designations.

In order to protect both consumers and honest producers from mislabelling fraud, it is desirable to develop objective and effective methods capable of identifying the origin of the cheese used to prepare pre-packed grated or shredded products, when the usual check on the original branded PDO logo on the rind is impossible. This is the most likely explanation for the remarkable wealth of papers on methods considered suitable for characterising the unique nature of Grana Padano and Parmigiano Reggiano, also in comparison with non-PDO products (around 150 papers in the last 10 years in Scopus)

A recent paper [2] showed that by combining the stable isotope ratios of H, C, N and S and the elemental profile of cheese it was possible to trace the origin of seven types of European hard cheeses and specifically to identify PDO Parmigiano Reggiano cheese from non-PDO imitations. Moreover, isotopic analysis has been officially adopted since 2011 as a reference method for verifying the authenticity of PDO Grana Padano cheese (EU Reg 584/2011).

The methods for analysing H, C, N and S stable isotope ratios using Isotope Ratio Mass Spectrometry (IRMS) and the elemental profile using Inductively Coupled Plasma– Mass Spectrometry (ICP-MS) are described in several papers in the scientific literature [2-7] but they are not backed up by collaborative studies, as required nowadays by all official method standardisation bodies. This is necessary to make validated and officially recognised methods [e.g. European

Committee for Standardisation (CEN), AOAC International] available in any commercial disputes and legal debates.

This paper presents the results of a collaborative study coordinated by the Fondazione E. Mach (Italy) and sponsored by the Consortium for the Protection of the Grana Padano PDO to validate these methods. The study was organised and performed according to the International Union of Pure and Applied Chemistry (IUPAC) protocol [8] and International Organisation for Standardisation (ISO, Geneva, Switzerland) Standards 5725/2004 and 13528/2005. The isotopic ratios of H, C, N and S were considered, whereas for elemental analysis the 13 elements Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba, Re, Bi, U were taken into account. They were indeed found to be the most effective in tracing the origin of both Grana Padano and Parmigiano Reggiano, and the analytical methods proposed guaranteed repeatable results, at least on an intra-laboratory scale [2].

Twenty laboratories were initially involved in the collaborative study, but only 13 of them completed the entire procedure, sending in the results (Table 6.1). Three laboratories performed both isotope and elemental analysis, 7 only isotopic analysis and 3 only elemental analysis.

6.2 Materials and methods

Samples

The samples were grated and homogenised directly by the Consortium for the Protection of Grana Padano, divided into 50 g portions and vacuum packed in plastic bags. Starting from 7 different commercial cheeses, three of them were grated and freeze-dried in duplicate (cheese: 1, 2, and 3; label: A, C, F, H, L, and O), while four were only grated in duplicate (4, 5, 6 and 7; B, D, E, G, I, M, N, and P) in order to have 7 pairs of blind duplicates (A-H, B-I, C-L, D-M, E-N, F-O, and G-P; Table 6.2). Samples 3 and 7 were mixture of 50% of cheese 1 with 50% of cheeses 5 and 2 respectively. This procedure complies with the requirements of the IUPAC internal harmonized protocol for collaborative studies [8], which asks for more than five ‘materials’ (i.e. different matrix/sample pairs). The laboratories involved in the isotope analysis were also provided with a sample of casein to be used as a standard for $^{2}H/^{1}H$ analysis, whereas those performing elemental analysis were given of a solution of Indium (1.6 mg/L) as internal standard (see method below).

Methods

The analytical protocol (described below) based on the methods reported in Camin et al. (2012) [2], was sent to the participants, but some laboratories adopted modified approaches. The method details, instrument model/producer and standards used by the participants are summarised in Table 6.1.

Stable isotope analysis

Fat elimination from cheese

The fresh cheeses (samples B, D, E, G, I, M, N, P) were lyophilised.

4 g of freeze-dried cheese was extracted 3 times with 30 mL of a petroleum ether : diethyl ether (2:1) mixture, homogenising with an Ultraturrax device (11500 rpm for 3 minutes) and using a centrifuge (e.g. 4100 rpm for 6 min) to separate the ether from the residue. A Soxhlet extractor was used as an alternative to extract fat. After lipid extraction, the skimmed cheese was warmed to 40 °C to remove any possible residual ether. Then the residue was washed twice with 20 mL of water, centrifuging each time (e.g. 4100 rpm for 3 min). The residue, made up mainly of casein, was lyophilised and conserved at room temperature until analysis.

IRMS analysis

Each sample was measured twice and the mean value was considered. The isotope ratios were expressed in δ ‰ relative to VPDB (Vienna – Pee Dee Belemnite) for $\delta^{13}C$, Air-N_2 for $\delta^{15}N$ and VCDT (Vienna - Canyon Diablo Troilite) for $\delta^{34}S$ and were calculated against international reference materials, which are specified for each laboratory in Table 6.1. Data were normalised with different approaches and using different types and numbers of international reference materials, as specified for each laboratory in Table 6.1. Some of them used a calibration curve (in the case of two or three reference materials used) as suggested by IUPAC [9], while others normalised the data against a single reference material. This is why $\delta^{13}C$ data were not expressed in relation to the VPDB – LSVEC scale.

For $\delta^{2}H$, the values were corrected against a working standard casein (assigned $\delta^{2}H$: -113 ‰) supplied to participants, using the preparation procedure described by Wassenaar and Hobson [10], i.e. leaving samples and standards to equilibrate with laboratory air moisture for at least 96 h. We are aware that δ-value two-point calibration is the method of choice for the normalisation of data (IUPAC indications, [9]) especially in the case of $\delta^{2}H$ measurements. Unfortunately, due to the absence of any international organic reference material with a similar matrix (for similar exchange in exchangeable Hs) and casein samples with $\delta^{2}H$ VSMOW values sufficiently different to provide an appropriately wide ^{2}H abundance value range for meaningful scale calibration, single point anchoring mode was used here. Fortunately, the $\delta^{2}H$ values of the samples do not have a wide range and are close to those of the standard (from around -120 to -90 ‰). However, the $\delta^{2}H$ values indicated here cannot be related to a VSMOW-SLAP normalised scale.

Mineral element analysis

All the materials used for the sample preparation and analysis were washed with 5% HNO_3 and

rinsed with ultrapure water (Milli-Q® water, 18.2 MΩ, Sartorius, Goettingen, Germany) before use.

Microwave mineralisation:

About 0.5 g (fresh or freeze-dried) of the sample was mineralised in a closed vessel in a microwave oven digester after the addition of ultrapure HNO_3, ultrapure H_2O_2, ultrapure water and internal standard solution (In, supplied to all participants). The digested samples were transferred into vials and the microwave vessel was rinsed with one or more small aliquots of ultrapure water that were then added to the sample. A blank sample (reagents without sample) was mineralised in each analytical batch. More details about the procedure adopted by each lab are given in Table 6.1.

ICP-MS analysis

Analyses of Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba, Re, Bi and U were carried out in the labs using ICP-MS or ICP-OES (Optical emission spectrometry) (Table 6.1), if necessary after dilution. Results for fresh cheeses were expressed as dry matter (105°C) after analysis of moisture by each laboratory at the time of sample preparation.

The intra-laboratory accuracy of the analytical process was assured using an on–line internal standard solution prepared following the procedure in use in each laboratory.

Considering the results of different laboratories and the techniques used during preparation and analysis, the detection limit (DL) ranged between 0.1 and 0.7 μg/kg for Li, 0.0001 and 0.006 g/kg for Na, 0.5 and 60 μg/kg for Mn and Fe, 4 and 40 μg/kg for Cu, 0.1 and 20 μg/kg for Se and Sr, 0.1 and 2 μg/kg for Rb, 0.1 and 5 μg/kg for Mo, 1 and 5 μg/kg for Ba, 0.038 and 1 μg/kg for Re, 0.01 and 6.8 μg/kg for Bi and 0.068 and 2 μg/kg for U.

Statistical evaluation of the data

Statistical calculations were then performed according to the IUPAC protocol [8]. Outliers were removed in the following way: a loop of Cochran tests for removal of laboratories with the highest variance, single and pair value Grubbs tests for individual or paired individual outliers, then returning to the Cochran test etc., keeping a proportion of outliers <2/9. For the comparison between laboratories using different normalization methods for isotope ratio values a paired-t-test and a U-Mann Whitney tests were performed (Statistica v. 9, StatSoft Italia sr, Padua, Italy).

6.3 Results and Discussion

Stable isotope analysis

As regards isotopic analysis, 10 laboratories presented their results and none of them was eliminated as a technical outlier, although some of them did not apply the exact protocol. This means that the use of other extraction devices (soxhlet extractor instead of the matching ultraturrax-

centrifuge) or even solvents (water/dichloromethane, 2:1 and acetone instead of petroleum ether: diethyl ether, 2:1) does not have a significant impact on the variability of the isotopic ratios and can be used as an alternative.

The data received from the participants are presented in the Supporting information (S 1) . All the laboratories reported $\delta^{13}C$ values, 9 $\delta^{15}N$, 7 $\delta^{2}H$ and 5 $\delta^{34}S$ values. The minimum of five laboratories supplying valid results required in the IUPAC harmonised protocol for collaborative studies on complex methods [8] was satisfied for all the elements. The data, especially for $\delta^{13}C$ and $\delta^{2}H$, cover most of the range of variability found for European hard cheeses [2]. The standard deviations of repeatability (sr) and reproducibility (sR) for each sample were calculated considering only the valid results of the blind duplicates (see Data Elaboration section) according to the ISO Standard 5725 and the IUPAC protocol [8]. A summary of the results of these calculations is presented in Table 6.3.

In general, the sr and sR values obtained for all materials are comparable, despite the fact that some samples were fresh and others freeze-dried. It follows that the freeze-drying process does not have any significant effect on isotopic variability.

For $\delta^{13}C$ the average standard deviation of repeatability (sr) was 0.1 ‰ and that of reproducibility (sR) was 0.2 ‰. Despite the wide range of values (from -26 to -18 ‰), the sr and sR values were very similar for the samples, except for sample 7, which had a slightly higher but still acceptable value. For all the samples, sr and sR were comparable or even lower to those previously observed for wine ethanol (OIV 312-06), honey bulk and protein (AOAC 998.12) and fruit juice sugar and pulp (ENV 12410, ENV 13070), for which the stable isotope analysis has been validated since the 1990s.

For $\delta^{15}N$, $\delta^{2}H$ and $\delta^{34}S$, the average standard deviations of repeatability (sr) were 0.1 ‰, 2 ‰ and 0.4 ‰, whereas those of reproducibility (sR) were 0.3 ‰, 3 ‰ and 0.6 ‰. For these elements, validated methods do not yet exist. In any case, the sr and sR values are comparable with those found in the literature for several food matrices, including dairy products [2-7].

As described in the materials and methods section, the different laboratories used from one to three reference materials for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, according to their internal working protocols. As the use of one standard is not recommended by IUPAC (especially for $\delta^{13}C$) due to possible scale span problems, ANOVA/Tukey and U-Mann Whitney tests were performed on the data. The labs were grouped according to the number of standards used for data elaboration and it was checked if there were statistically significant differences between the groups. No statistically significant differences were highlighted ($p<0.05$) so it could be deduced that in this particular case, using a single standard

with a value similar to that of the samples, the data determined using a single-anchoring point or two-three anchoring points were not different.

The results of the two samples 3 and 7, prepared by mixing 50% of cheese 1 with 50% of cheeses 5 and 2 respectively have been evaluated in comparison with the expected values, calculated by adding the values of the original samples multiplied per 0.5. This mixing protocol was already used in the literature for isotopic methods [11]. The differences between the measured and the expected values for $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{34}S$ (Table 6.4) were always below the repeatability limits (expressed as .2.8 x sr [8])

Mineral element analysis

Six laboratories presented their results and none of them was eliminated as a technical outlier, although some of them did not apply the exact digestion protocol (Table 6.1).

In general, the elemental concentration ranges of the samples in this study (Table 6.5) were consistent with those of commercial hard cheeses [2, 7, 12]. The content of Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba and Bi was found to be over the detection limit (DL) in at least one sample in all the 6 laboratories, only in 5 labs for U and in 3 for Re. Due to instrumental method limitations, Re was not provided by three labs. For this last element, always present in these cheeses at levels below 1 µg /kg, no statistical processing was possible.

For data processing, values less than DL were not considered. The data provided by the participating laboratories are presented in the Supporting information and the statistics in Table 6.5.

For Li the same laboratory was discarded as an outlier for all samples by applying Cochran and Grubbs tests. The average standard deviations of repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R), calculated using data provided by the other 5 labs, were 10 and 28% respectively, ranging between 3 and 28% for r and between 20 and 41% for R.

For Na one laboratory for sample 5 was considered as an outlier. The average RSD_r and RSD_R values were 2 and 13%, with a min-max range of 1-3% and 8-15%.

For Mn one laboratory for samples 1 and 3 and two for samples 4 were discarded as outliers. The average RSD_r and RSD_R values calculated on 6 valid samples were 10% (min-max: 3-19%) and 13% (10-17).

For Fe the same laboratory was discarded as an outlier in all samples. The average RSD_r and RSD_R values were 11 and 23%, with RSD_r sr ranging from 4 and 30% and RSD_R from 15 to 44%.

For Cu one laboratory for each sample of 1, 4, 5, 6 and 7 was eliminated as an outlier. The average RSD_r was 7%, ranging between 1 and 19%, while the average and RSD_R was 19%, ranging between 11 and 28%.

For Se one laboratory for samples 1, 2, 4 and 7 was discarded. The RSD_r value varied from 2 to 22%, with an average of 10%, while RSD_R varied from 9 to 30 %, with an average of 19%.

For Rb one laboratory for samples 4, 5 and 6 was eliminated. The average RSD_r was 3%, ranging between 1 and 5%, while average RSD_R was 12%, ranging between 10 and 14%.

For Sr one laboratory for samples 2, 5 and 6 was discarded as an outlier. The average RSD_r was 4%, ranging between 1 and 9%, while the average and RSD_R was 12%, ranging between 10 and 16%.

For Mo one laboratory was eliminated for samples 3, 6 and 7. The average RSD_r and RSD_R values were 5% and 17%, ranging from 2 to 10% and from 11 to 23% respectively.

For Ba one laboratory was eliminated for samples 2, 5 and 7. The average RSD_r was 3%, ranging from 1 to 7% while the average and RSD_R was 9%, ranging from 4 to 19%.

For Bi only for samples 3, 5 and 6 did a sufficient number of laboratories provide results higher than DL. For sample 6 one laboratory was eliminated as an outlier after the application of statistical tests. On the basis of the only two valid samples, RSD_r varied between 3 and 6% and RSD_R between 12 and 13%.

For U less than 5 laboratories (see supporting information S 2) provided results higher than DL for samples 4 and 6 and one laboratory was discarded as an outlier for sample 5. Considering the 4 valid samples, RSD_r ranged between 5 and 10% and RSD_R between 13 and 26%.

For Re statistical processing was not possible, nevertheless the data seems to be reasonably consistent.

For Li and Fe the same laboratory was discarded as an outlier. This could be due to the presence of a matrix interference not removed or not correctly subtracted, or to a calibration error.

Regarding the quantification of elements usually reported in the literature for hard cheeses, at trace concentration (such as Se, Re, Bi and U) the ICP-MS analysis is recommendable for the higher sensitivity of this technique.

The use of fresh cheeses (instead of lyophilized cheeses) requires particular attention during the preparation of samples that must be carefully homogenized before mineralization This seems particularly important for Rb and Cu determination, that on fresh cheeses (samples 4-5-6-7) generally shows a worse intra-lab repeatability (on blinded duplicates) than on lyophilised samples.

The obtained results of RSD_r and RSD_R were consistent with the precision of replicate of intra-laboratory analysis reported for ICP-OES determination of elements in cheddar cheese which was < 15% for Ba, Mn, Mo, Na and Sr, 16% for Cu and 19% for Fe (Food and Drug Administration, FDA, Elemental analysis manual, section 4.4; www.fda.gov) and for ICP-OES (Inductively Coupled Plasma–Optical Emission Spectrometer) and ICP-MS analysis on different cheeses

reported by Bontempo et al [7] that was < 10% for all the determined elements with the exception of Mn (11%) and Fe (18%).

Table 5.6 gives the mean results and expected values for samples 3 and 7, calculated after the removal of outliers. Mean values were calculated when there were at least 3 valid laboratories for the samples. For Re an uncertainty of 28% (that is the maximum obtained in this study) was considered. Generally the differences between the results and the expected values were not statistically significant (t-test, p>0.05), except for Mn, Ba and Re for sample 7 (p= 0.043, p=0.003 and 0.031 respectively).

6.4 Conclusions

An international collaborative study was organised according to the IUPAC protocol and ISO Standards 5725/2004 and 13528/2005 to determine the performance or validation data of methods for the isotopic and elemental analysis of cheese.

The average standard deviations of repeatability (sr) and reproducibility (sR) were 0.1 and 0.2 ‰ for $\delta^{13}C$, 0.1 and 0.3 ‰ for $\delta^{15}N$, 2 and 3 ‰ for $\delta^{2}H$ and 0.4 and 0.6 ‰ for $\delta^{34}S$. These are comparable to or even better than those found in official methods for other food matrices and in the literature. The average RSD_r and RSD_R for the analysis of Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba, Bi and U ranged between 2 and 11% and between 9 and 28%. They are slightly higher than those reported in official methods for other foods, probably due to the matrix effect, but consistent, in regard to precision, with methods reported by FDA and in the literature for cheese. For Re it was not possible to determine validation data. The robustness of both the isotopic analysis was very good. For mineral elements the differences between the results and the expected values were not statistically significant, except for Mn, Ba and Re in one sample.

Different lipid extraction methods and mineralization procedures do not have a significant impact on the variability of the data. For elements at trace concentration (such as Se, Re, Bi and U) the ICP-MS analysis is recommendable for the higher sensitivity. For mineral elements analysis, the use of fresh cheeses (instead of lyophilized cheeses) requests particular attention during the preparation of samples that must be carefully homogenized before mineralization

The performance data obtained here can be submitted to standardisation agencies to obtain official recognition of the methods. This is necessary when these methods are used to verify the mislabelling of PDO cheese in commercial disputes or legal debates. The $\delta^{2}H$ analysis of casein is an exception, because suitable reference standards are not yet available.

6.5 References

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Table 6.1: list of participants. (I) = isotope analysis; (E) = elemental analysis.

Laboratory	Contacts	Instrument	Standard [9]*	Method
FEM, Research and Innovation Centre – Italy	Luca Ziller (I)	DELTA V and EA Flash 1112, Thermo Scientific; DELTA XP and TC/EA, Thermo Scientific; Isoprime and Vario Isotope, Elementar	USGS 40, NBS-22, IAEA-CH-6 for $\delta^{13}C$; USGS 40 for $\delta^{15}N$; IAEA-SO-5 and NBS 127 for $\delta^{34}S$; NBS-22 and casein for $\delta^{2}H$	petroleum ether: diethyl ether (2:1), ultraturrax device and centrifuge
	Luana Bontempo (E)	ICP-MS Agilent 7500ce	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with 2mL HNO_3 and 1mL H_2O_2
FEM, Technology Transfer Centre – Italy	Daniela Bertoldi (E)	ICP-MS Agilent 7500ce	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with 4 mlLHNO_3 and 2mL H_2O_2
EUROFINS – France	Freddy Thomas (I)	DELTA V Advantage and EA Flash 1112, Thermo Scientific; Isoprime and EuroPyrOH, Elementar	NBS 22 and IAEA-CH-6, for $\delta^{13}C$; IAEA-N1 and IAEA-N2 for $\delta^{15}N$ NBS22 and IAEA-CH-7 for $\delta^{2}H$	petroleum ether: diethyl ether (2:1), ultraturrax device and centrifuge
ISOLAB – Germany	Andreas Rossmann (I)	Isoprime, Vario Cube EL CNS simultaneous, Vario EL III Pyrolysis	NBS-22 for $\delta^{13}C$; IAEA-N1, IAEA-N2 for $\delta^{15}N$; IAEA-S1 for $\delta^{34}S$; NBS-22 and casein for $\delta^{2}H$,	petroleum ether: diethyl ether (2:1), centrifuge
Anti-Fraud Department - Ministry of Agricultural, Food and Forestry Policy (ICQRF-MIPAAF) – Italy	Fabio Fuselli, Rosamaria Marianella (I)	DELTA V and EA Flash HT 2000, Thermo Scientific	USGS 40, NBS-22, IAEA-CH-6 for $\delta^{13}C$; USGS 40 for $\delta^{15}N$	water/dichloromethane (2:1) and acetone, ultraturrax device and centrifuge.
Max Rubner-Institut (MRI) – Germany	Joachim Molkentin (I)	DELTA^{plus} XL and Flash EA 1112 Series, Thermo Scientific	IAEA-CH-3, IAEA-CH-6, NBS 22 for $\delta^{13}C$; IAEA-N1, IAEA-N2 for $\delta^{15}N$	petroleum ether: diethyl ether (2:1), ultraturrax device and centrifuge
Agroisolab GmbH – Germany	Markus Boner, Claudia Erven (I)	EA (Carlo Erba) with IRMS (Optima Isochrom). High temperature pyrolysis with IRMS (IsoPrime); EA (Eurovector) with IRMS (Optima)	IAEA-CH-6, IAEA-CH-7 for $\delta^{13}C$; IAEA-N1, IAEA-N2 for $\delta^{15}N$; IAEA-S1, IAEA-S2, IAEA-S3 for $\delta^{34}S$	soxhlet
Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) – Germany	Meylahn, Klaus (I)	Delta xp, Thermo Electron	USGS 40 for $\delta^{13}C$ and for $\delta^{15}N$; IAEA-S1 for $\delta^{34}S$; casein for $\delta^{2}H$	petroleum ether, soxhlet
	Meylahn, Klaus (E)	ICP-MS Agilent 7500ce	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with 4 mL HNO_3 and 2mL H_2O_2 (Internal standard not used)
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) – Germany	Antje Schellenberg (I)	DELTA V and EA Flash HT 2000, Thermo Scientific; Delta XL Plus and TC/EA; Thermo Scientific	USGS 41, USGS 40, IAEA-CH-6 for $\delta^{13}C$; IAEA-N2, IAEA-N1, USGS 40 for $\delta^{15}N$, NBS 127, IAEA SO5, IAEA-S1 for $\delta^{34}S$; casein for $\delta^{2}H$	petroleum ether: diethyl ether (2:1), soxhlet
	Peter Fecher (E)	ICP-OES Varian 750 for Mn, Fe, Cu, Sr, Ba; Flame AAS ContrAA 600 Analytic Jena for Na; ICP-MS Element 2 Thermo for other elements	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with 4mL HNO_3
University of Bari -	Francesco	DELTA V and EA Flash HT 2000,	USGS 40 for $\delta^{13}C$ and	petroleum ether:

Italy	Longobardi (I)	Thermo Scientific	$\delta^{15}N$; casein for $\delta^{2}H$	diethyl ether (2:1), ultraturrax device and centrifuge
Laboratorio Arbitral Agroalimentario - Spain	Mercedes Rupérez Cuenca (I)	Delta Plus (Thermo Electron, Bremen, Germany) and Carlo Erba (Milan, Italy) NC 2500 elemental analyzer (EA)	NBS-22 for $\delta^{13}C$	petroleum ether: diethyl ether (2:1), ultraturrax device and centrifuge
Chelab - Italy	Giampaolo Perinello, Marzia Tonellato (E)	ICP-OES Perkin Elmer optima 4300 DV and ICP-MS Perkin Elmer elan DRC II	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with HNO_3 and H_2O_2
Universidad Nacional de Córdoba - Argentina	Magdalena Monferran, Daniel A. Wunderlin (E)	ICP-MS Agilent 7500cx	NIST 3104a, 3106, 3114, 3126a, 3129a, 3132, 3134, 3152a, 3145a, 3149, 3153a, 3164, 3143, 682, 3124a.	microwave digestion with 4 mL HNO_3 and 2mL H_2O_2 (Internal standard, Pd)

***NIST: National Institute of Standards and Technology, Gaithersburg, MD, USA**
NBS: National Bureau of Standards, now NIST
IAEA: International Atomic Energy Agency, Vienna, Austria
USGS: U.S. Geological Survey, Reston, VA, USA

Table 6.2: sample description

Code	Cheese	Status	Type of cheese
A	1	freeze-dried	Hard cheese from Italy 1 (PDO)
B	4	fresh	Hard cheese from Italy 2 (PDO)
C	2	freeze-dried	Hard cheese from Lithuania (no –PDO)
D	5	fresh	Hard cheese from Italy 3 (no –PDO)
E	6	fresh	Hard cheese from Germany (no –PDO)
F	3	freeze-dried	50% cheese 1 + 50% cheese 5
G	7	fresh	50% cheese 1 + 50% cheese 2
H	1	freeze-dried	duplicate of A
I	4	fresh	duplicate of B
L	2	freeze-dried	duplicate of C
M	5	fresh	duplicate of D
N	6	fresh	duplicate of E
O	3	freeze-dried	duplicate of F
P	7	fresh	duplicate of G

Table 6.3: Summary of statistics for $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{34}S$

$\delta^{13}C$ / ‰							
sample description	1	2	3	4	5	6	7
number of valid results	10	10	10	10	10	10	10
number of replicates	2	2	2	2	2	2	2
mean	-19.13	-25.85	-18.54	-22.15	-18.00	-21.37	-22.63
sr	0.07	0.06	0.08	0.06	0.06	0.06	0.18
sR	0.15	0.13	0.17	0.12	0.20	0.18	0.26
$\delta^{15}N$ / ‰							
sample description	1	2	3	4	5	6	7
number of valid results	9	9	9	9	9	9	8
number of replicates	2	2	2	2	2	2	2
mean	4.88	4.80	5.31	5.50	5.71	6.13	4.83
sr	0.16	0.20	0.06	0.32	0.06	0.06	0.06
sR	0.30	0.27	0.31	0.48	0.33	0.28	0.32
$\delta^{2}H$ / ‰							
sample description	1	2	3	4	5	6	7
number of valid results	6	6	6	6	6	6	7
number of replicates	2	2	2	2	2	2	2
mean	-106.8	-122.3	-106.8	-116.4	-107.8	-107.2	-112.1
sr	2.8	1.5	0.5	2.2	1.1	2.2	3.0
sR	3.3	2.7	3.2	3.3	2.0	3.0	4.9
$\delta^{34}S$ / ‰							
sample description	1	2	3	4	5	6	7
number of valid results	5	5	5	5	5	5	5
number of replicates	2	2	2	2	2	2	2
mean	5.24	5.66	4.93	3.77	4.82	5.02	5.40
sr	0.46	0.49	0.15	0.52	0.26	0.49	0.47
sR	0.67	0.47	0.46	0.70	0.52	0.46	0.56

Table 6.4: Comparison between the mean values of samples 3 and 7 and the expected values

	3			7		
	measured	expected	difference	measured	expected	difference
$\delta^{13}C$ / ‰	-18.5	-18.6	0.1	-22.6	-22.5	0.1
$\delta^{15}N$ / ‰	5.3	5.3	0.0	4.8	4.8	0.0
$\delta^{2}H$ / ‰	-107	-108	1	-112	-115	3
$\delta^{34}S$ / ‰	4.9	5.0	0.1	5.4	5.5	0.1

Table 6.5: Summary of statistics for mineral elements

Li µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	5	5	5	5	5	5
number of replicates	2	2	2	2	2	2	2
mean	4.99	6.45	4.99	5.18	5.59	3.58	5.91
sr	0.49	0.24	0.28	0.24	0.85	0.99	0.20
sR	2.03	1.27	1.51	1.14	1.23	1.29	1.61
Na g/kg							
sample description	1	2	3	4	5	6	7
number of valid results	6	6	6	6	5	6	6
number of replicates	2	2	2	2	2	2	2
mean	9.31	10.15	9.29	8.44	9.66	12.24	10.70
sr	0.24	0.22	0.20	0.14	0.08	0.20	0.12
sR	1.35	1.32	1.22	1.25	0.73	1.81	1.31
Mn µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	6	5		6	6	6
number of replicates	2	2	2		2	2	2
mean	254	385	288		354	420	364
sr	8	12	22		60	81	31
sR	26	39	26		56	73	59
Fe µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	5	5	5	5	5	5
number of replicates	2	2	2	2	2	2	2
mean	2460	2882	2489	2015	2397	2298	2888
sr	91	102	197	249	314	684	143
sR	379	508	575	877	376	622	524
Cu µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	6	6	5	5	5	5
number of replicates	2	2	2	2	2	2	2
mean	9618	616	5372	5791	1276	1013	5689
sr	156	12	486	70	244	85	475
sR	1671	127	936	624	361	273	709
Se µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	5	6	5	6	6	5
number of replicates	2	2	2	2	2	2	2
mean	204	177	252	245	295	263	217
sr	5	16	20	17	56	58	7
sR	29	29	34	21	80	80	48
Rb µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	6	6	6	5	5	5	6
number of replicates	2	2	2	2	2	2	2
mean	1023	825	966	1363	964	1060	1004
sr	25	41	35	20	35	27	23

sR	106	79	115	183	116	152	127
Sr µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	6	5	6	6	5	5	6
number of replicates	2	2	2	2	2	2	2
mean	5258	8010	6062	7755	7211	7029	7266
sr	70	108	83	669	217	532	365
sR	549	950	770	926	820	1148	908
Mo µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	6	6	5	6	6	5	5
number of replicates	2	2	2	2	2	2	2
mean	175	142	180	335	196	108	175
sr	6	3	4	29	20	3	5
sR	20	24	19	67	45	22	28
Ba µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	6	5	6	6	5	6	5
number of replicates	2	2	2	2	2	2	2
mean	1164	1287	1063	1722	1078	1677	1402
sr	30	12	29	120	23	118	23
sR	101	70	80	147	104	322	56
Bi µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results			6		5		
number of replicates			2		2		
mean			14.63		29.00		
sr			0.83		0.87		
sR			1.93		3.34		
U µg/kg							
sample description	1	2	3	4	5	6	7
number of valid results	5	5	5				5
number of replicates	2	2	2				2
mean	0.78	0.34	1.26				0.61
sr	0.07	0.03	0.06				0.06
sR	0.12	0.09	0.16				0.14

Table 6.6: Comparison between the mean values of samples 3 and 7 and the expected value
* p <0.05, ** p<0.01, t-test ; n.d. not determined

	3			7		
	measured	expected	difference	measured	expected	difference
Li µg/kg	4.99	5.29	0.30	5.91	5.72	0.19
Na g/kg	9.29	9.48	0.20	10.70	9.73	0.97
Mn µg/kg	288	304	16	364	319	44*
Fe µg/kg	2489	2429	60	2888	2671	217
Cu µg/kg	5372	5447	75	5689	5117	572
Se µg/kg	252	250	3	217	191	26
Rb µg/kg	966	993	28	1004	924	80
Sr µg/kg	6062	6235	173	7266	6553	712

Mo µg/kg	180	176	4	175	158	17
Ba µg/kg	1063	1121	58	1402	1226	177**
Re µg/kg	0.158	0.181	0.023	0.173	0.128	0.045*
Bi µg/kg	14.63	n.d.	n.d.	n.d.	n.d.	n.d.
U µg/kg	1.26	1.25	0.01	0.60	0.60	0.05

7. CHAPTER 5- Preliminary study for characterising the mineral and isotopic profile of the Mozzarella di Bufala Campana PDO

7.1 Introduction

Mozzarella di Bufala Campana (MBC) is an Italian protected designation of origin (PDO) cheese produced particularly in Campania in the province of Caserta and Salerno, and partially in other regions: Latium, Puglia and Molise (Decreto Ministero delle Politiche Agricole, Alimentari e Forestali del 11/2/2008, Modifica del disciplinare di produzione della denominazione "Mozzarella di Bufala Campana", registrata in qualità di denominazione origine protetta in forza del regolamento CE n. 1107 del 12 giugno 1996). Mozzarella di Bufala Campana is a pasta filata cheese product that is made from whole buffalo milk and an acid whey starter.

The high consumer's demand of this cheese, the limited amount of available buffalo's milk, and the high market price, make this cheese a remunerative target for either adulterated or false PDO sales. Consequently, it is necessary to develop rapid and accurate methods to verify its authenticity, and protect both the consumers and the honest producer. As described in the previous chapters, the stable isotope ratio analysis of bioelements combined with the trace element analysis is one of the most powerful technique for detecting the authenticity of PDO cheeses.

In this work we present the mineral and isotopic profile of 38 authentic samples of buffalo milk and of the corresponding samples MBC.

7.2 Material and Methods

Samples

76 authentic samples of buffalo milk were collected together with 76 corresponding MBC in 20 dairy farms located in the area of production of the PDO Mozzarella di Bufala Campana. The first sampling was made in summer 2015, whereas the second one was completed in December 2015. The sampling plane was defined within the PhD project, and is summarised in **Table 7.1**

Table 7.1: Description of the samples

Province	Dairy farms	Replicates for milk and cheese	Seasons	Total
Caserta	10	2	2	40
Salerno	7	2	2	28
Latina/Frosinone	1	2	2	4
Foggia	1	2	2	4
Totale				76

Only the 38 samples of the first season have been submitted to the analysis at the moment.

Methods

The methods used for the analysis are described in chapter 6.2.

In few words, for isotopic analysis, milk was acidified to precipitate casein. The precipitate, as well as the cheese, after lyophilisation, were extracted with petroleum ether, diethyl ether mixture, and the residue, made up mainly of casein, was lyophilised and submitted to IRMS analysis of the isotopic ratio of H, C, N, O and S. The isotopic ratio of O was analysed also in the milk water.

The mineral profile was analysed on cheese and milk after acid digestion. The following 61 elements were detected: Li, Be, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Mo, Pd, Ag, Cd, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Nd, Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb, Lu, Hf, Re, Ir, Pt, Au, Hg, Tl, Pb, Bi, Th, U. The content of Bi, Au, Pt, Ir, Re, Ag, As, Ge, Ga, Ti, V were not taken into account because it was under the detection limit for most of the samples.

7.3 Results and discussion

In **Table 7.1** mean, median, std dev, min and max for the different variables of the different samples, grouped for matrix (milk/cheese) and origin (province) are shown.

Comparing the values of milk with those of cheese, the content of Mg, K, Co, Rb, Hg, B, Mo, Lu, Cs, $\delta^{18}O$ and $\delta^{2}H$ of casein are significantly ($p>0.05$) higher in milk, whereas Na, Fe, Zn, Se and U were significantly higher in Mozzarella.

The higher content of some metals in Mozzarella can be due to the technological contribution of the dairy manufacturing or to process of concentration, whereas the higher content in milk can be due to the fact that these elements are less soluble in organic cheese matrix (fat and protein). The lower level of $\delta^{18}O$ and $\delta^{2}H$ in mozzarella casein respect to milk casein confirms what found previously (Bontempo et al.,) and needs in depth study for understanding the contribution of tap water added during cheese production For the authors, there did not seem to be a reasonable explanation for the fact that cheese and milk $\delta^{18}O$ were shown to be statistically different (mean value: 10.5‰ *vs* 11.5‰). Further studies are necessary to confirm and explain these findings through experimentation.

Table 7.1: mean, median, std dev, min and max for the different variables of the different samples, grouped for matrix (milk/cheese) and origin (province).

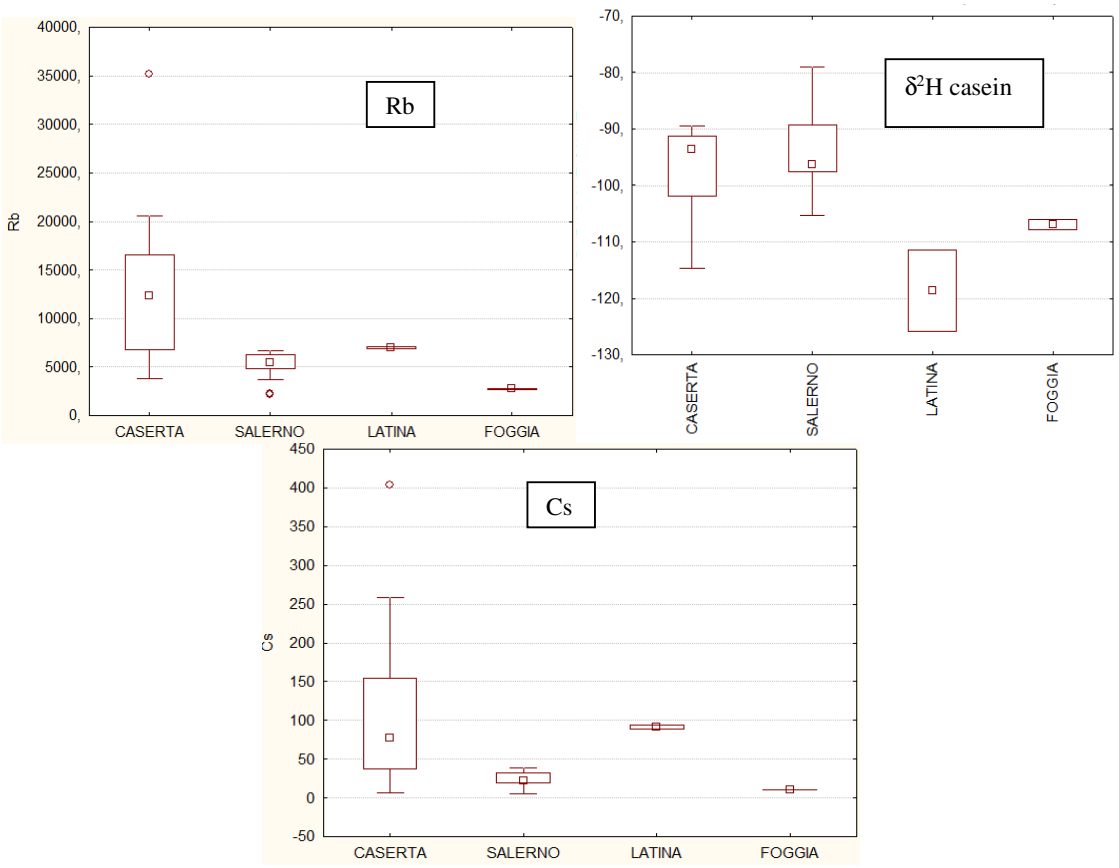
province	matrix	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	$\delta^{18}O$ casein	$\delta^{2}H$	$\delta^{18}O$ water	Li	Be	B	Na	Mg	Al	P	K	Ca	Cr	Mn	Fe	Co	Ni	Cu	Zn	Se	Rb	Sr	Y	Mo	Pd
CASERTA	MILK	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
		-19,6	6,3	3,1	15,1	-97	-4,5	16,0	1,1	942	2244	1013	808	4900072	5573	9571	31,7	306	2093	20,3	24,2	377	26004	75,9	12620	5695	0,5	265	0,4
		-19,4	6,3	3,5	15,3	-94	-4,6	13,3	0,2	966	2191	1026	622	4813132	5550	9413	17,2	268	2059	20,5	16,4	385	27055	70,1	12306	5508	0,3	251	0,3
		-25,9	5,3	-1,5	13,8	-115	-5,5	6,4	-0,2	566	1906	910	191	4083950	4997	8888	2,1	157	1358	13,6	8,3	162	16372	31,3	3838	3182	0,1	221	0,2
		-17,2	8,2	8,4	16,3	-90	-3,6	47,3	3,8	1252	2646	1124	3791	5808745	6496	10703	89,8	753	3250	26,6	56,8	638	32369	125,9	35163	11335	1,8	358	1,2
		-20,4	5,8	1,9	14,3	-102	-5,0	10,1	-0,1	820	2018	973	500	4440252	5403	9142	7,0	204	1708	17,5	11,8	267	25852	54,2	6804	4332	0,3	240	0,3
		-18,3	6,4	4,0	15,7	-91	-4,2	15,4	3,5	1066	2461	1042	845	5352855	5774	9966	52,7	340	2260	23,0	35,6	420	28428	103,1	16593	6250	0,5	279	0,4
		2,0	0,7	2,0	0,8	8	0,6	11,1	1,7	181	239	52	763	534904	336	559	28,9	149	489	3,7	16,5	117	4369	28,4	7228	2016	0,4	39	0,2
	MOZZARELLA	20	20	20	20	20	-	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
		-19,4	6,4	3,1	12,2	-133	-	13,1	0,4	181	4042	375	1928	5475980	378	8925	119,7	477	3456	13,3	40,1	600	50942	179,2	755	6578	1,1	186	0,2
		-19,4	6,4	3,6	12,2	-133	-	12,2	0,2	188	3990	372	1495	5386187	353	8981	70,2	380	3274	13,0	23,2	560	51788	173,0	671	6421	0,9	189	0,2
		-22,1	5,6	-0,1	11,7	-137	-	4,9	0,0	94	2001	222	301	3332284	195	3493	24,1	198	2472	9,2	8,3	223	22132	103,3	154	1684	0,4	120	0,1
		-17,5	7,8	5,4	12,7	-128	-	29,9	1,1	234	7782	499	7233	6799445	634	11283	675,6	1660	6584	17,3	352,2	1389	67474	274,1	1831	11531	2,6	285	0,5
		-20,1	5,9	2,7	12,0	-135	-	10,4	0,2	173	2929	337	1123	5091247	286	7851	34,4	296	2681	11,5	15,3	428	45242	149,2	385	4812	0,7	152	0,2
		-18,4	6,8	3,8	12,3	-132	-	14,7	0,7	205	4739	405	2474	6162877	467	10354	121,3	500	4049	15,5	29,1	603	56749	211,1	1093	8089	1,3	211	0,2
		1,3	0,6	1,4	0,3	2	-	5,8	0,3	38	1416	68	1515	837130	123	1774	153,7	332	985	2,3	74,7	308	9953	51,9	497	2577	0,5	42	0,1
SALERNO	MILK	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
		-20,6	6,2	2,9	15,5	-95	-4,4	12,3	1,1	774	2052	990	692	4698780	5568	9208	68,6	228	2043	21,8	39,7	385	23795	93,9	5197	4602	0,3	255	0,3
		-20,0	6,4	3,0	15,5	-96	-4,5	9,8	-0,1	731	2019	1005	509	4611972	5616	9176	29,9	222	1984	22,8	18,1	359	24866	76,5	5476	4710	0,2	251	0,3
		-24,0	5,5	0,6	14,6	-105	-5,5	7,1	-0,2	500	1643	792	294	3932925	4858	7758	3,7	179	1437	16,3	8,1	146	16759	54,0	2206	3210	0,2	203	0,2
		-17,8	7,0	4,1	16,7	-79	-3,5	23,7	3,5	1088	2567	1199	2577	5346019	6286	10998	433,3	301	3075	25,8	257,1	603	28572	240,0	6719	6097	1,0	334	0,4
		-22,6	5,7	2,7	14,8	-98	-4,9	8,9	-0,2	641	1874	961	371	4309980	5379	8300	10,6	203	1748	19,8	10,8	325	21561	61,9	4870	3604	0,2	227	0,3
		-18,6	6,7	3,6	16,1	-89	-3,7	15,1	3,4	910	2258	1020	650	5219515	5901	9847	58,4	259	2230	24,1	37,8	513	27151	93,7	6273	5224	0,3	273	0,4
		2,3	0,5	1,1	0,7	7	0,7	5,2	1,8	181	261	101	607	467369	465	1016	113,0	33	415	3,0	64,9	131	4203	53,7	1490	904	0,2	34	0,1
	MOZZARELLA	14	14	14	14	14	-	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
		-20,6	6,5	3,1	12,4	-134	-	6,8	0,1	127	4675	431	1653	5658208	376	10042	59,8	340	3028	14,5	21,3	620	54143	231,7	297	6270	0,7	191	0,2
		-20,2	6,5	3,2	12,3	-134	-	5,7	0,0	124	4478	414	922	5497484	381	9359	52,5	328	2793	14,2	19,4	601	53825	201,1	312	5904	0,5	189	0,2
		-22,6	5,8	0,8	11,7	-140	-	3,7	0,0	91	3176	359	569	4829141	251	8668	20,5	250	2147	12,5	14,8	411	40965	120,4	129	3244	0,3	147	0,1
		-18,6	7,2	4,4	13,2	-131	-	15,7	0,4	177	6620	573	6278	7167973	481	14021	134,8	469	5334	18,6	39,9	841	63945	465,7	451	9765	2,3	256	0,2
		-22,2	6,1	2,9	11,9	-136	-	4,5	0,0	106	3721	381	764	5284897	320	9087	38,4	291	2300	13,4	16,8	477	47854	185,0	205	4231	0,4	155	0,1
		-19,4	6,9	3,7	12,9	-133	-	7,6	0,1	138	5433	448	1234	6003680	445	10635	75,9	406	3270	14,6	23,6	778	59301	252,4	390	9246	0,9	222	0,2
		1,5	0,5	1,1	0,5	2	-	3,5	0,1	28	1163	62	1813	624244	74	1467	29,7	66	1036	1,8	7,0	157	6780	90,4	100	2302	0,6	36	0,0
LATINA	MILK	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		-21,3	7,2	3,6	14,2	-119	-4,6	7,6	-0,2	567	2073	1083	361	4883738	5625	9941	29,0	235	1763	19,6	20,8	430	27369	137,0	6978	3832	0,2	284	0,3
		-21,3	7,2	3,6	14,2	-119	-4,6	7,6	-0,2	567	2073	1083	361	4883738	5625	9941	29,0	235	1763	19,6	20,8	430	27369	137,0	6978	3832	0,2	284	0,3
		-21,5	7,1	3,6	13,5	-126	-4,6	7,0	-0,2	541	2057	1054	261	4711584	5534	9492	22,2	230	1741	19,2	18,3	426	26224	136,6	6844	3741	0,1	275	0,3
		-21,2	7,4	3,7	14,8	-112	-4,6	8,2	-0,2	593	2088	1112	461	5055892	5715	10390	35,8	240	1786	20,0	23,3	434	28515	137,3	7113	3923	0,2	294	0,3
		-21,5	7,1	3,6	13,5	-126	-4,6	7,0	-0,2	541	2057	1054	261	4711584	5534	9492	22,2	230	1741	19,2	18,3	426	26224	136,6	6844	3741	0,1	275	0,3
		-21,2	7,4	3,7	14,8	-112	-4,6	8,2	-0,2	593	2088	1112	461	5055892	5715	10390	35,8	240	1786	20,0	23,3	434	28515	137,3	7113	3923	0,2	294	0,3
		0,2	0,2	0,0	0,9	10	0,0	0,8	0,0	36	22	41	142	243463	128	634	9,6	7	32	0,6	3,5	6	1620	0,5	190	129	0,1	13	0,0
	MOZZARELLA	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		-20,2	6,1	3,5	11,7	-136	-	4,4	0,1	128	7649	411	1287	5064053	334	9978	335,4	365	3861	15,2	196,2	423	47811	277,5	598	5881	0,7	219	0,2
		-20,2	6,1	3,5	11,7	-136	-	4,4	0,1	128	7649	411	1287	5064053	334	9978	335,4	365	3861	15,2	196,2	423	47811	277,5	598	5881	0,7	219	0,2
		-21,2	5,5	3,2	11,6	-139	-	4,1	0,1	100	6511	356	1187	4499253	255	9532	41,6	338	2419	11,4	11,4	391	47238	254,2	319	4413	0,6	144	0,1
		-19,2	6,8	3,8	11,8	-134	-	4,6	0,1	156	8787	467	1386	5628852	414	10423	629,2	393	5302	19,0	381,0	455	48384	300,9	876	7349	0,7	294	0,3
		-21,2	5,5	3,2	11,6	-139	-	4,1	0,1	100	6511	356	1187	4499253	255	9532	41,6	338	2419	11,4	11,4	391	47238	254,2	319	4413	0,6	144	0,1
		-19,2	6,8	3,8	11,8	-134	-	4,6	0,1	156	8787	467	1386	5628852	414	10423	629,2	393	5302	19,0	381,0	455	48384	300,9	876	7349	0,7	294	0,3
		1,4	0,9	0,5	0,1	4	-	0,4	0,0	40	1609	79	141	798747	112	630	415,5	39	2039	5,4	261,3	46	810	33,1	393	2076	0,0	106	0,1
FOGGIA	MILK	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		-19,5	6,5	2,3	14,7	-107	-4,3	30,4	2,1	862	2282	1042	872	5105218	6041	10060	34,3	257	2106	23,9	13,9	326	29795	85,2	2746	8501	0,6	243	0,5
		-19,5	6,5	2,3	14,7	-107	-4,3	30,4	2,1	862	2282	1042	872	5105218	6041	10060	34,3	257	2106	23,9	13,9	326	29795	85,2	2746	8501	0,6	243	0,5
		-19,5	6,4	2,2	14,6	-108	-4,4	28,8	-0,2	830	2099	995	761	4691588	5832	9631	6,7	257	1837	20,3	13,6	311	28658	73,1	2719	8139	0,4	242	0,2
		-19,4	6,5	2,3	14,7	-106	-4,1	32,1	4,3	894	2464	1089	983	5518848	6249	10490	62,0	257	2375	27,4	14,3	341	30932	97,3	2773	8863	0,8	245	0,7
		-19,5	6,4	2,2	14,6	-108	-4,4	28,8	-0,2	830	2099	995	761	4691588	5832	9631	6,7	257	1837	20,3	13,6	311	28658	73,1	2719	8139	0,4	242	0,2
		-19,4	6,5	2,3	14,7	-106	-4,1	32,1	4,3	894	2464	1089	983	5518848	6249	10490	62,0	257	2375	27,4	14,3	341	30932	97,3	2773	8863	0,8	245	0,7
		0,0	0,1	0,1	0,1	1	0,2	2,3	3,2	45	258	66	157	584961	295	608	39,1	0	381	5,1	0,5	21	1607	17,1	38	512	0,3	2	0,3
	MOZZARELLA	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		-20,2	6,7	3,2	11,0	-132	-	12,4	0,1	242	8263	279	1295	4025528	232	6383	54,3	353	2399	10,8	24,4	467	52127	173,1	110	7281	0,5	108	0,1
		-20,2	6,7	3,2	11,0	-132	-	12,4	0,1	242	8263	279	1295	4025528	232	6383	54,3	353	2399	10,8	24,4	467	52127	173,1	110	7281	0,5	108	0,1
		-21,2	6,5	2,5	10,6	-133	-	11,6	0,1	235	7884	265	1062	3656470	226	5629	46,6	315	2386	9,5	23,9	431	48122	167,7	107	6199	0,5	104	0,1
		-19,3	6,9	3,8	11,4	-131	-	13,1	0,1	248	8642	292	1529	4394586	237	7137	62,0	391	2413	12,1	24,9	503	56132	178,5	113	8363	0,5	112	0,2
		-21,2	6,5	2,5	10,6	-133	-	11,6	0,1	235	7884	265	1062	3656470	226	5629	46,6	315	2386	9,5	23,9	431	48122	167,7	107	6199	0,5	104	0,1
		-19,3	6,9	3,8	11,4	-131	-	13,1	0,1	248	8642	292	1529	4394586	237	7137	62,0	391	2413	12,1	24,9	503	56132	178,5	113	8363	0,5	112	0,2
		1,3	0,3	0,9	0,6	1	-	1,0	0,0	9	536	19	330	521927	7	1066	10,9	53	19	1,8	0,7	51	5664	7,6	4	1531	0,0	6	0,0

province	matrix		Cd	Sn	Sb	Te	Cs	Ba	La	Ce	Pr	Nd	Nd	Sm	Eu	Gd	Gd	Dy	Ho	Er	Tm	Yb	Lu	Hf	Hg	Hg	Tl	Pb	Th	U
CASERTA	MILK	N	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
		Mean	0,5	9,5	0,3	-0,1	105,9	1106	0,7	1,4	0,2	1,1	0,5	0,1	0,1	0,1	0,4	0,1	0,0	0,1	0,0	0,1	54,7	7,7	1,3	2,5	2,3	10,9	7,1	0,7
		Median	0,5	7,5	0,2	0,0	77,1	1086	0,5	1,1	0,1	0,9	0,4	0,1	0,1	0,1	0,3	0,1	0,0	0,1	0,0	0,0	51,4	3,6	1,2	2,5	1,7	10,9	3,5	0,4
		Min	0,3	1,4	-0,1	-0,5	6,0	770	-0,1	0,1	0,0	0,5	-0,1	0,0	0,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	5,2	1,5	0,7	1,4	0,0	2,0	0,9	-0,1
		Max	0,8	23,9	1,2	0,1	404,1	1652	4,1	7,8	0,9	3,5	2,5	0,6	0,3	0,5	0,8	0,3	0,0	0,2	0,1	0,2	118,8	62,7	1,9	3,2	7,2	22,3	42,8	2,1
		25%	0,4	4,1	0,0	-0,1	37,6	911	0,4	0,9	0,1	0,8	0,2	0,0	0,1	0,1	0,2	0,1	0,0	0,0	0,0	0,0	26,0	2,8	1,0	2,2	1,0	6,4	2,8	0,3
		75%	0,6	12,1	0,3	0,0	153,8	1190	0,7	1,3	0,2	1,3	0,6	0,2	0,2	0,2	0,5	0,1	0,0	0,1	0,0	0,1	70,8	6,1	1,6	2,8	3,3	14,2	7,0	0,8
		Std.Dev.	0,1	6,7	0,3	0,1	96,8	234	0,9	1,6	0,2	0,7	0,5	0,1	0,1	0,1	0,2	0,1	0,0	0,0	0,0	0,0	35,4	13,5	0,4	0,5	1,9	5,4	9,8	0,7
	MOZZARELLA	N	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
		Mean	0,3	12,4	0,5	0,0	8,4	1886	1,5	2,7	0,3	1,4	1,1	0,2	0,1	0,2	0,2	0,2	0,0	0,1	0,0	0,1	4,9	1,7	0,2	0,4	0,8	15,1	2,3	13,6
		Median	0,3	12,4	0,4	0,0	5,8	1746	1,3	2,3	0,2	1,3	1,0	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	5,8	1,1	0,2	0,4	0,7	15,9	1,9	7,3
		Min	0,2	3,2	0,2	0,0	1,2	569	0,3	0,4	0,0	0,3	0,2	0,1	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	-5,3	0,4	0,1	0,2	0,5	6,6	0,8	2,8
		Max	0,7	29,4	0,8	0,0	33,8	3891	2,9	5,4	0,7	2,7	2,4	0,5	0,2	0,4	0,5	0,3	0,1	0,2	0,1	0,3	10,4	9,9	0,3	1,1	1,4	24,4	4,8	49,5
		25%	0,3	4,9	0,4	0,0	2,7	1486	1,1	1,9	0,2	1,0	0,8	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	3,5	0,7	0,2	0,3	0,6	10,9	1,4	5,6
		75%	0,4	17,6	0,5	0,0	11,1	2011	2,1	3,4	0,4	1,8	1,4	0,3	0,1	0,3	0,3	0,2	0,1	0,1	0,0	0,2	7,4	1,6	0,2	0,5	0,9	17,8	3,3	24,4
		Std.Dev.	0,1	7,3	0,2	0,0	7,8	766	0,7	1,4	0,2	0,6	0,6	0,1	0,0	0,1	0,1	0,1	0,0	0,0	0,0	0,1	4,0	2,1	0,1	0,2	0,3	4,7	1,2	12,6
SALERNO	MILK	N	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
		Mean	0,5	10,0	0,0	-0,1	23,2	1039	0,6	1,1	0,1	1,1	0,4	0,1	0,1	0,1	0,4	0,1	0,0	0,1	0,0	0,1	68,5	2,6	1,3	2,2	1,0	6,5	5,1	0,4
		Median	0,5	10,6	0,0	-0,1	22,1	1070	0,4	0,8	0,1	0,9	0,3	0,1	0,1	0,1	0,4	0,1	0,0	0,1	0,0	0,1	62,3	2,1	1,2	2,2	1,1	6,8	2,3	0,2
		Min	0,2	1,8	-0,1	-0,5	5,3	697	0,1	0,4	0,1	0,5	0,0	0,0	0,1	0,1	0,1	0,0	0,0	0,0	0,0	0,0	32,0	1,0	0,7	1,4	0,0	2,1	0,5	0,0
		Max	0,8	30,2	0,2	0,2	38,1	1496	2,2	4,1	0,5	2,0	1,5	0,3	0,2	0,4	0,7	0,2	0,0	0,2	0,1	0,1	116,0	6,4	1,8	3,0	2,9	14,9	33,9	2,1
		25%	0,4	2,4	0,0	-0,2	19,8	809	0,3	0,6	0,1	0,8	0,2	0,1	0,1	0,1	0,3	0,0	0,0	0,0	0,0	0,0	46,4	1,8	1,0	1,7	0,2	2,7	2,0	0,0
		75%	0,7	12,6	0,1	0,0	32,3	1142	0,5	1,0	0,1	1,1	0,4	0,1	0,2	0,1	0,5	0,1	0,0	0,1	0,0	0,1	96,7	3,5	1,6	2,4	1,3	9,5	4,0	0,6
		Std.Dev.	0,2	7,4	0,1	0,1	10,7	251	0,6	1,0	0,1	0,4	0,4	0,1	0,0	0,1	0,2	0,0	0,0	0,1	0,0	0,0	28,6	1,4	0,4	0,5	0,8	4,0	8,6	0,6
	MOZZARELLA	N	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
		Mean	0,3	12,5	0,3	0,0	1,7	1971	1,3	2,3	0,3	1,3	1,0	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	5,0	1,0	0,2	0,4	0,6	11,7	2,1	4,6
		Median	0,3	6,2	0,3	0,0	1,5	1976	0,7	1,1	0,1	0,9	0,6	0,1	0,1	0,1	0,1	0,1	0,0	0,1	0,0	0,1	6,9	0,8	0,2	0,4	0,6	9,8	1,3	4,4
		Min	0,2	3,6	0,1	0,0	0,4	1034	0,5	0,8	0,1	0,6	0,3	0,1	0,0	0,1	0,1	0,1	0,0	0,0	0,0	0,0	-6,0	0,5	0,1	0,3	0,4	5,4	0,6	2,2
		Max	0,4	35,4	0,5	0,0	4,0	3337	5,2	9,8	1,1	4,3	3,7	0,7	0,2	0,7	0,7	0,4	0,1	0,3	0,0	0,2	10,0	1,9	0,3	0,6	0,8	37,8	7,4	9,0
		25%	0,3	4,8	0,2	0,0	0,9	1505	0,6	1,0	0,1	0,7	0,4	0,1	0,1	0,1	0,1	0,1	0,0	0,0	0,0	0,0	-0,3	0,6	0,2	0,3	0,5	7,4	1,0	3,0
		75%	0,4	23,4	0,4	0,0	2,1	2189	0,9	1,5	0,2	1,0	0,7	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	9,2	1,4	0,3	0,5	0,6	13,6	1,6	5,6
		Std.Dev.	0,1	11,0	0,1	0,0	1,1	647	1,5	2,9	0,3	1,2	1,1	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	5,3	0,5	0,0	0,1	0,1	8,2	2,2	1,9
LATINA	MILK	N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		Mean	0,6	10,9	0,1	-0,2	91,4	1303	0,6	1,1	0,2	1,1	0,5	0,2	0,1	0,1	0,3	0,0	0,0	0,1	0,0	0,0	44,0	1,6	1,7	2,3	1,3	10,5	1,2	0,1
		Median	0,6	10,9	0,1	-0,2	91,4	1303	0,6	1,1	0,2	1,1	0,5	0,2	0,1	0,1	0,3	0,0	0,0	0,1	0,0	0,0	44,0	1,6	1,7	2,3	1,3	10,5	1,2	0,1
		Min	0,5	10,4	0,0	-0,4	89,1	1266	0,2	0,3	0,1	0,8	0,1	0,1	0,1	0,1	0,3	0,0	0,0	0,0	0,0	0,0	41,6	1,3	1,6	2,1	1,3	10,2	1,2	0,1
		Max	0,6	11,4	0,1	0,1	93,6	1340	1,0	1,8	0,2	1,5	0,8	0,2	0,1	0,1	0,3	0,0	0,0	0,1	0,0	0,0	46,3	1,8	1,7	2,6	1,4	10,9	1,2	0,1
		25%	0,5	10,4	0,0	-0,4	89,1	1266	0,2	0,3	0,1	0,8	0,1	0,1	0,1	0,1	0,3	0,0	0,0	0,0	0,0	0,0	41,6	1,3	1,6	2,1	1,3	10,2	1,2	0,1
		75%	0,6	11,4	0,1	0,1	93,6	1340	1,0	1,8	0,2	1,5	0,8	0,2	0,1	0,1	0,3	0,0	0,0	0,1	0,0	0,0	46,3	1,8	1,7	2,6	1,4	10,9	1,2	0,1
		Std.Dev.	0,1	0,7	0,1	0,4	3,2	52	0,6	1,0	0,1	0,5	0,5	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	3,3	0,4	0,1	0,4	0,1	0,5	0,0	0,1
	MOZZARELLA	N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		Mean	0,4	13,3	0,4	0,0	6,7	2582	1,1	1,9	0,2	1,2	0,9	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	9,8	1,1	0,3	0,5	0,7	10,7	2,1	2,3
		Median	0,4	13,3	0,4	0,0	6,7	2582	1,1	1,9	0,2	1,2	0,9	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	9,8	1,1	0,3	0,5	0,7	10,7	2,1	2,3
		Min	0,3	10,4	0,3	0,0	3,4	2155	1,1	1,9	0,2	1,1	0,8	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	9,4	0,9	0,3	0,4	0,5	9,3	1,9	1,6
		Max	0,5	16,3	0,4	0,0	10,0	3008	1,1	1,9	0,2	1,2	1,0	0,2	0,1	0,2	0,2	0,1	0,1	0,1	0,0	0,1	10,2	1,2	0,3	0,5	0,8	12,1	2,2	2,9
		25%	0,3	10,4	0,3	0,0	3,4	2155	1,1	1,9	0,2	1,1	0,8	0,2	0,1	0,2	0,2	0,1	0,0	0,1	0,0	0,1	9,4	0,9	0,3	0,4	0,5	9,3	1,9	1,6
		75%	0,5	16,3	0,4	0,0	10,0	3008	1,1	1,9	0,2	1,2	1,0	0,2	0,1	0,2	0,2	0,1	0,1	0,1	0,0	0,1	10,2	1,2	0,3	0,5	0,8	12,1	2,2	2,9
		Std.Dev.	0,1	4,1	0,1	0,0	4,6	603	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5	0,2	0,1	0,0	0,2	2,0	0,2	0,9
FOGGIA	MILK	N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		Mean	0,5	4,3	0,5	-0,1	10,3	1216	0,7	1,6	0,2	1,4	0,6	0,2	0,2	0,3	0,6	0,1	0,0	0,1	0,0	0,1	62,8	7,6	1,5	2,5	0,8	9,1	27,4	0,7
		Median	0,5	4,3	0,5	-0,1	10,3	1216	0,7	1,6	0,2	1,4	0,6	0,2	0,2	0,3	0,6	0,1	0,0	0,1	0,0	0,1	62,8	7,6	1,5	2,5	0,8	9,1	27,4	0,7
		Min	0,4	1,9	0,1	-0,2	10,1	1197	0,6	1,4	0,2	1,1	0,6	0,2	0,2	0,2	0,4	0,0	0,0	0,1	0,0	0,1	37,6	1,3	1,3	1,6	0,7	3,4	1,7	0,3
		Max	0,5	6,6	1,0	0,0	10,6	1234	0,7	1,8	0,2	1,7	0,7	0,2	0,2	0,3	0,9	0,2	0,0	0,1	0,0	0,1	88,0	14,0	1,7	3,5	0,9	14,8	53,2	1,1
		25%	0,4	1,9	0,1	-0,2	10,1	1197	0,6	1,4	0,2	1,1	0,6	0,2	0,2	0,2	0,4	0,0	0,0	0,1	0,0	0,1	37,6	1,3	1,3	1,6	0,7	3,4	1,7	0,3
		75%	0,5	6,6	1,0	0,0	10,6	1234	0,7	1,8	0,2	1,7	0,7	0,2	0,2	0,3	0,9	0,2	0,0	0,1	0,0	0,1	88,0	14,0	1,7	3,5	0,9	14,8	53,2	1,1
		Std.Dev.	0,1	3,3	0,6	0,2	0,3	26	0,1	0,2	0,0	0,4	0,1	0,0	0,0	0,1	0,4	0,1	0,0	0,1	0,0	0,0	35,6	9,0	0,3	1,3	0,2	8,1	36,4	0,6
	MOZZARELLA	N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
		Mean	0,2	4,5	0,2	0,0	0,6	1987	0,8	1,4	0,1	1,1	0,7	0,1	0,1	0,1	0,2	0,1	0,0	0,1	0,0	0,1	11,8	0,5	0,2	0,3	0,4	8,8	1,3	2,3
		Median	0,2	4,5	0,2	0,0	0,6	1987	0,8	1,4	0,1	1,1	0,7	0,1	0,1	0,1	0,2	0,1	0,0	0,1	0,0	0,1	11,8	0,5	0,2	0,3	0,4	8,8	1,3	2,3
		Min	0,2	3,6	0,2	0,0	0,5	1831	0,8	1,3	0,1	1,0	0,6	0,1	0,1	0,1	0,2	0,1	0,0	0,1	0,0	0,1	10,6	0,5	0,2	0,3	0,4	5,8	1,2	2,2
		Max	0,2	5,4	0,3	0,0	0,6	2143	0,8	1,4	0,1	1,2	0,7	0,1	0,1	0,1	0,3	0,1	0,0	0,1	0,0	0,1	13,0	0,5	0,2	0,4	0,5	11,9	1,4	2,4
		25%	0,2	3,6	0,2	0,0	0,5	1831	0,8	1,3	0,1	1,0	0,6	0,1	0,1	0,1	0,2	0,1	0,0	0,1	0,0	0,1	10,6	0,5	0,2	0,3	0,4	5,8	1,2	2,2
		75%	0,2	5,4	0,3	0,0	0,6	2143	0,8	1,4	0,1	1,2	0,7	0,1	0,1	0,1	0,3	0,1	0,0	0,1	0,0	0,1	13,0	0,5	0,2	0,4	0,5	11,9	1,4	2,4
		Std.Dev.	0,0	1,3	0,0	0,0	0,0	221	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,7	0,1	0,0	0,1	0,0	4,4	0,2	0,2

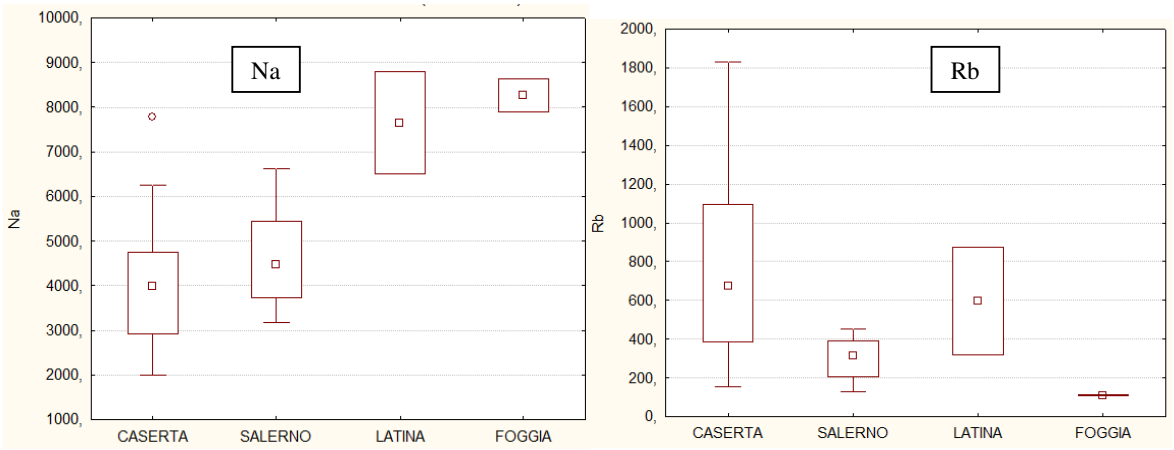
Within milk, the most significant variables for differentiating the 4 different provinces are · ^{2}H of casein, Rb and Cs (Figures 7.1), and within Mozzarella, are · ^{18}O of casein, Be, B, Na and Rb (Figure 7.2).

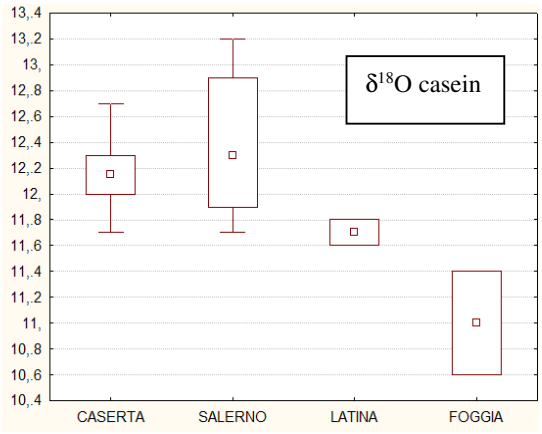
· ^{18}O and · ^{2}H of casein and the content of Rb are confirmed to be the most significant variables for discriminating the geographical origin of dairy products (Camin et al., 2012).

Figures 7.1: Milk: box-plot of the values of · ^{2}H of casein, Rb and Cs grouped for provinces.



Figures 7.2: Mozzarella di Bufala Campana: box-plot of the values of · ^{18}O of casein, Be, B, Na and Rb grouped for provinces.





Final conclusions will be drafted when also the resuts of the samples of the second season will be available.

7.1 References

L. Bontempo, G. Lombardi, R. Paoletti, L. Ziller, F. Camin H, C, N and O stable isotope characteristics of alpine forage, milk and cheese International Dairy Journal, 2012, 23, 99–104.

F. Camin, R. Wehrens, D. Bertoldi, L. Bontempo, L. Ziller, M. Perini, G. Nicolini, M. Nocetti, R. Larcher. Can H, C, N and S stable isotopes and mineral profiles objectively guarantee the authenticity of grated hard cheeses? *Anal. Chim. Acta* **2012**, *711*, 54.

8. CONCLUSIONS

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 memorised 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 and poultry, milk, butter, cheese, fish and shellfish.

Sometimes isotopic analysis has also 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, with the production rules indicating SIRA as one of the analytical methods to adopt to assess the authenticity of cheese.

In this work we found that the combination of isotopic ratios of plasma and erythrocytes represents a good tool for deducing the dietary background of lambs. Moreover we found that IRMS is efficient for tracing lamb production system in four region of north west of Tunisia, characterized by herbaceous pasture. We also evaluated the capability of isotopic analysis for tracing the origin and feed of farmed rainbow trout reared in different Italian farms and with two types of feeds: high fish content and low fish content feeds. Furthermore we were able to validate the methods for analysing the isotopic and mineral composition of cheese. Finally we found that for characterising the Mozzarella di Bufala Campana PDO, $\delta^{18}O$ and $\delta^{2}H$ values of casein, and the content in Rb are the best candidates for protecting this product from mislabelling.

As general conclusion, we can state that a large dataset, comprising samples from wide and representative ranges of geographical, seasonal, dietary and production conditions, must be created in order to create a reliable reference system also usable in legal cases to check the authenticity of animal products Moreover, the analytical methods should be recognised by certification bodies. Such a robust system can only be created with the involvement and funding of PDO protection consortia, producers' associations or official bodies.

9. **Main activities**

Oral presentation at the following congresses:

2012

- EU-China Forum on Technologies Used in Food Safety and Product Authentication, Beijing, Cina
- Spettrometria di massa isotopica - Giornate di studio ed incontro. S. Michele all'Adige, Trento
- Congresso Lattiero-Caseario, Milano
- Convegno Espositivo Il Latte, Parma

2013

- Forensic Isotope Ratio Mass Spectrometry Conference, Montreal, Canada
- LA CONTRAFFAZIONE DEI PRODOTTI E SEGNI DISTINTIVI: STRUMENTI DI PREVENZIONE E DI TUTELA, Camera di Commercio di Trento (Metodi analitici per contrastare la contraffazione dei prodotti alimentari).Trento

2014:

- 105th AOCS Annual Meeting & Expo, San Antonio, Texas
- 1st IMEKOFOODS - Metrology Promoting Objective and Measurable Food Quality and Safety, Rome, Italy.

2015

- second FOOD INTEGRITY CONFERENCE: "Assuring the integrity of the food chain: food authenticity research priorities and funding opportunities", Bilbao, Spagna
- Convegno Massa 2015, Alghero

Teaching activities

2012, 2013, 2014: seminar at the National School of Analytical and Bioanalytical Methodologies in Mass Spectrometry, Parma

ISI Papers

1. Camin F, Wehrens R, Bertoldi D., Bontempo L., Ziller L, Perini M, Nicolini G, Nocetti M, Larcher R. (2012) H, C, N and S stable isotopes and mineral profiles to objectively guarantee the authenticity of grated hard cheeses. Analytica Chimica Acta 711 54– 59,
2. Bontempo L, Lombardi G, Paoletti R, Ziller L, Camin F (2012) H, C, N and O stable isotope characteristics of alpine forage, milk and cheese. International Dairy Journal 23 99-104 10.1016/j.idairyj.2011.10.005
3. Perini M, Camin F, Piasentier E (2013) Sources of variability of H, C, O, N and S stable isotope ratio in dry-cured ham, Food Chemistry, 136, 3–4, 1543–1550
4. Biondi L, D'Urso M. G., Vasta V., Luciano G., Scerra M., Priolo A., Ziller L., Bontempo L., Camin F. (2013) Stable isotopes ratios for tracing feeding systems changes in lamb. Animal. 7/9, 1559-1566.
5. Bontempo L., Ceppa F., Ziller L., Pedrini P., Hobson K.A., Wassenaar L.I., Camin F. Comparison of methods for stable isotope ratio (δ13C, δ15N, δ2H, δ18O) measurements of feathers. Methods in Ecology and Evolution, Methods in Ecology and Evolution. Volume 5, Issue 4, pages 363–371.

6. Katryna A. van Leeuwen, Paul D. Prenzler, Danielle Ryan, Federica Camin. (2014) Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry for Traceability and Authenticity in Food Stuffs and Beverages. Comprehensive Reviews in Food Science and Food Safety. COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY Volume: 13 Issue: 5 Pages: 814-837 Published: SEP 2014
7. A Fadda, R Rawcliffe, B M Padedda, A Lugliè, N Sechi, F Camin, Luca Ziller, Marina Manca. (2014) Spatio-temporal dynamics of C and N isotopic signature of zooplankton: an annual study on a man-made reservoir in the Mediterranean Region. Ann. Limnol. - Int. J. Lim. 50 , 279–287
8. Federica Camin, Daniela Bertoldi, Alessandro Santato, Luana Bontempo, Matteo Perini, Luca Ziller, Angelo Stroppa, Roberto Larcher. 2015 Validation of methods for H, C, N and S stable isotopes and elemental analysis of cheese: results of an international collaborative study. RAPID COMMUNICATIONS IN MASS SPECTROMETRY,Volume 29, Issue 5, 15 March 2015, Pages: 415–423, DOI: 10.1002/rcm.7117
9. Fabio Abeni, Francesca Petrera, Maurizio Capelletti, Aldo Dal Prà, Luana Bontempo, Agostino Tonon, Federica Camin. 2015. Hydrogen and oxygen stable isotope fractionation in body fluid compartments of dairy cattle according to season, farm, breed, and physiological stage Plose one, 10 (5), e0127391