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TESI DI DOTTORATO DI RICERCA

Stable isotope ratio analysis for the traceability of raw and cured meat

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ABSTRACT

The effect of different factors on the stable isotope ratio (SIR) variability of the most important bioelements ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$), measured using IRMS (Isotopic Ratio Mass Spectrometry), was evaluated in different types of tissues, including pig and ovine muscles, muscle lipids and lipid fractions.

To evaluate the effect of tissue turnover on the different isotope ratios, in a diet-switch experiment, 28 lambs were switched to an isotopically distinct diet and raised on two different energy allowances (high energy allowance (HEA) with a target weight gain of 150 g/day or low energy allowance (LEA) with a target weight gain of 50 g/day).

Determination of multiple stable isotope ratios (δ^2H, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{34}S$) in ovine muscle indicated diet-muscle fractionations prior to the diet-switch to be -44.0‰ +1.9‰ and 0‰ for H. C and S respectively. The muscle sampled from animals on the HEA recorded higher $\delta^{13}C$ values than muscle from animals on the LEA (p = 0.0003). The $\delta^{18}O$ values in ovine muscle were largely influenced by the water ingested while most of the H used to build ovine muscle tissue depend on the feed rather than the drinking water. The discrimination between intra muscular lipid and diet was estimated to be -172.7, -1.3‰ and -11.5‰ for H, C and O respectively. The C half-lives of muscle were determined to be 75.7 and 91.6 days for animals receiving the high and low EA respectively and it is comparable with S half-lives measured in ovine LD. However, the estimated half-lives of lipids were too long to be used for tracking short term dietary changes in lambs and probably also in other meat animals.

In a second experiment the stable isotope ratios of bioelements in defatted dry matter and marbling and subcutaneous fat fractions were assessed in 86 ham samples belonging to six different types, with the aim of ascertaining the effect of origin and the production system on 11 isotopic ratios. The ham types were obtained from pigs reared in three regions, examining one different production factor in every location, at two levels of expression: pig genotype (local breed vs. industrial hybrid) in Friuli (Italy), pig feeding regime (Bellota vs. Campo) in Extremadura (Spain) and ham seasoning time (mid vs. end) in Emilia (Italy). The isotopic composition of meteoric water and the dietary abundance of C4 plants made it possible to distinguish Italian PDO from Spanish hams. Higher $\delta^{15}N$ values in local breeds than in

industrial hybrids might indicate relatively more nitrogen excretion, due to a higher ratio between protein degradation and protein synthesis.

The Strecker degradation of sulfur amino acids taking place during seasoning and generating dimethyl disulfide and other similar volatile compounds can explane δ^{34}S isotopic fractionation showed to compare the End-seasoned Italian hams vs mid-seasoned ones.

The contrasting treatments within the regional batches generated promising differences in SIR, potentially useful for tracing the whole ham production system, including the processing procedure.

In a third experiment the stable isotope ratios of five bio-elements - H, O, C, N and S - were analysed in the protein and fat muscle fractions of sixty beef carcasses from zebu cattle reared in an tropical African country (Cameroon) with the scope of evaluating the effect of this specific origin on the different isotopic ratios. Zebu beef from Cameroon was shown to have a specific isotope profile, characterised by lower δ^{13}C, δ^{2}H and δ^{18}O values than those reported for other areas around the world, as a consequence of the almost exclusive use of tropical C4 pasture grass for cattle feed and the geographic and climatic gradient in the isotope composition of precipitation. The isotopic composition of the fat fraction of muscle, and in particular the δ^{2}H and δ^{13}C values of fat, was significantly affected by the subcutaneous fat colour. The zebu with a white subcutaneous fat ("white type") showed a clear tendency to be more enriched in ^{2}H isotopes and more depleted in ^{13}C isotopes than the "yellow type" , while the "cream type" presented an intermediate condition. These trends are correlated with fat composition. Within the Cameroon, multi-element analysis gave promising results for tracing the regional origin of beef and some aspects of the cattle breeding system, such as the nutritional status of animals.

On the basis of the results of this study all of the considering factors can have effect on the different isotope ratios but with different weight. Origin and type of diet outweigh turnover and technical process of products, even if they must be carefully considered to prevent possible mistake during the results evaluation.

Keywords: stable isotope ratios, IRMS, ham, lamb, beef, origin, traceability, authenticity

LIST OF PUBLICATIONS

This thesis is based on the following papers,

Harrison, SM; Schmidt, O; Moloney, AP; Kelly, SD; Rossmann, A; Schellenberg, A; Camin, F; **Perini, M**; Hoogewerff, J; Monahan, FJ. (2011). Tissue turnover in ovine muscles and lipids as recorded by multiple (H, C, O, S) stable isotope ratios. *Food Chemistry, 124,* 291-297. http://dx.doi:10.1016/j.foodchem.2010.06.035

Perini, M; Camin, F; Del Pulgar, JS; Piasentier, E.(2012). Effect of origin, breeding and processing conditions on the isotope ratios of bioelements in dry-cured ham. *Food Chemistry*, *136* (2013), 1543-1550. http://dx.doi.org/10.1016/j.foodchem.2012.06.084.

Perini, M; Camin, F; Piasentier, E. Isotope ratios of bioelements for inferring beef origin and zebu feeding regime in Cameroon. *Manuscript*

LIST OF ABBREVIATIONS

Non-technical

EC	European Commission
EU	European Union
UK	United Kingdom
TRACE	European Project *Tracing the origin of food*

Technical

AIR	AIR - atmospheric air used as an international standard for which the $^{15}N/^{14}N$ ratio is precisely known and is defined as 0 ‰ on the $\delta^{15}N$‰ scale.
ASP	Aspartate
ATP	Adenosine Triphosphate
BSE	Bovine Spongiform Encephalopathy
CAM	Crassulacean acid metabolism
CDA	Canonical Discriminant Analysis
CDT	Canyon Diablo Troilite - natural sulfur deposit used as an international standard for which the $^{34}S/^{32}S$ ratio is precisely known and is defined as 0 ‰ on the $\delta^{34}S$‰ scale
CoA	Coenzyme A
EA	Elemental Analyser
IAEA	International Atomic Energy Association
IRMS	Isotope Ratio Mass Spectrometer
MAL	Malate

MBM	Bovine Meat and Bond Meal
MWL	Meteoric Water Line
NADP	Nicotinamide Adenosine Diphoshate
OOA	Oxaloacetate
PDB	Pee Dee Belemnite - Calcium carbonate used as an international standard for which the $^{13}C/^{12}C$ ratio is precisely known and is defined as 0 ‰ on the $\delta^{13}C$‰ scale.
PEP	Phosphoenolpyruvate
PGA	Phosphoglycerate
PGAL	3-Phospho-glyceraldehyde
R	Ratio
RuDP	Ribulose-1,5-diphosphate
SIRA	Stable Isotope Ratio Analysis
V-SMOW	Vienna-Standard Mean Ocean Water - ocean water used as an international standard for which the $^{18}O/^{16}O$ ratio is precisely known and is defined as 0 ‰ on the $\delta^{18}O$‰ scale.
(ox)	Oxidase
(re)	Reductase
‰	Parts *per* thousand (*per* mil)
D/H	The ratio of the isotope of hydrogen with atomic mass 2 (deuterium) to the isotope of hydrogen with atomic mass 1.
δD‰, δD‰ₛₘₒw	"Delta deuterium *per* mil". The D/H ratio expressed relative to the international standard Vienna-Standard Mean Ocean Water.
$^{13}C/^{12}C$	The ratio of the isotope of carbon with atomic mass 13 to

	the isotope of carbon with atomic mass 12
$\delta^{13}C‰$, $\delta^{13}C‰_{PDB}$	"Delta carbon-13 *per* mil". The $^{13}C/^{12}C$ ratio expressed relative to the international standard Pee Dee Belemnite.
$^{15}N/^{14}N$	The ratio of the isotope of nitrogen with atomic mass 15 to the isotope of nitrogen with atomic mass 14.
$\delta^{15}N‰$, $\delta^{15}N‰_{AIR}$	"Delta nitrogen-15 *per* mil". The $^{15}N/^{14}N$ ratio expressed relative to the international standard AIR.
$^{18}O/^{16}O$	The ratio of the isotope of oxygen with atomic mass 18 to the isotope of oxygen with atomic mass 16.
$\delta^{18}O‰$, $\delta^{18}O‰_{V-SMOW}$	"Delta oxygen-18 *per* mil". The $^{18}O/^{16}O$ ratio expressed relative to the international standard Vienna-Standard Mean Ocean Water.
$^{34}S/^{32}S$	The ratio of the isotope of sulfur with atomic mass 34 to the isotope of sulfur with atomic mass 32.
$\delta^{34}S‰$, $\delta^{34}S‰_{CDT}$	"Delta Sulfur-34 *per* mil". The $^{34}S/^{32}S$ ratio expressed relative to the international standard CDT.

CHAPTER I: INTRODUCTION

1.1 FOOD AUTHENTICITY

1.1.1 Meat Traceability

According to EU law, "traceability" means the ability to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing and distribution. (Regulation EC/178/2002). In the last few years, the importance of meat traceability has increased in order to prevent the spread of animal diseases or potential human epidemics in specific areas (BSE, foot-and-mouth disease, avian influenza). Traceability has thus become a landmark of the EU's food safety policy, as it is an effective risk-management tool which enables the food industry or authorities to withdraw or recall products identified as dangerous.

Due to the increasing complexity and length of the food chain and many recent food scares, consumer awareness of food has increased rapidly, along with the need for new tools making it possible to ensure that all food products served to consumers have a high standard of quality and are safe to be eaten.

In April 2004 the TRACES programme, a community system for managing the movement of animals, was established to meet this new need for greater transparency as regards food origin.

1.2 USING THE STABLE ISOTOPE RATIOS OF BIO-ELEMENTS TO TRACE ANIMAL PRODUCTS

Different methods can be used to trace animal products, such as genotyping or electronic tagging, but stable isotope ratio analysis (SIRA) is one of the most promising. SIRA provides a considerable amount of information about the geographical origin of food (Boner & Förstel, 2004; Stöckigt *et al.*, 2005; Camin *et al.*, 2007; Guo, Wei, Pan, & Li, 2008; Nakashita *et al.*, 2008), the farming system used to rear animals (Boner & Förstel, 2004; Schmidt et al. , 2005) and the seasonal movement patterns of wild animals (Hobson, 1999; Cerling et al., 2004a). Furthermore, it can also be effective in identifying the different diets of animals.(González-Pérez, et al., 1999; Piasentier et al. 2003; Cerling et al. 2004b; Schmidt *et al.*, 2005; Balasse & Ambrise, 2005; Bahar *et al.*, 2008).

1.2.1 Stable isotopes

In bio-organic material, the main elemental constituents (H, C, N, O, S) have different stable isotopes (D,H; $^{13}C,^{12}C$; $^{15}N,^{14}N$; $^{18}O,^{17}O,^{16}O$; ^{36}S, ^{34}S, ^{33}S, ^{32}S). The main ones are lighter isotopes, as shown in Table 1, which gives their mean abundance. The natural isotopic composition of organic compounds shows fluctuations around these mean values, and these variations, even if measured in terms of ppm, can be calculated precisely and accurately using dedicated analytical techniques such as Isotope Ratio Mass Spectrometry (IRMS).

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

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

*= Cretaceous marine fossil, *Belemnitella americana*, from the Pee Dee Formation in South Carolina (USA)

**= A FeS meteorite

1.2.2 Measurement of stable isotope ratios with IRMS

Mass spectrometry is the principal means for measuring the isotopic ratio of light "bioelements" (Hölzl et al. 2004). Every mass spectrometer consists of three basic elements: an ion source, a mass analyser and an ion detector (Kelly, 2003; Scrimgeour & Robinson, 2004). The gaseous sample (H_2, N_2, CO_2, and SO_2) enters the ion source through a narrow capillary where it is then ionised. The ions needed for ionisation are reproduced by a hot filament usually made of rhenoim or thoriated tungsten (Scrimgeour & Robinson, 2004). The

sample ions generated are then accelerated by a series of electrode "lenses" before entering the mass analyser (Kelly, 2003).

In the mass analyser, the ions are deflected by either a permanent magnet or an electromagnet (Kelly, 2003). The radius of deflection depends on the mass-to-charge-ratio, where ions with the same ratio experience the same deflection and heavy ions are less deflected than light ions. This deflection focuses the ions into several beams that finally enter the ion detector.

In the ion detector, each of these beams is detected separately using a Faraday-cup (Kelly, 2003; Scrimgeour & Robinson, 2004). The voltage produced via the discharge of the ion in the cup is then amplified and transformed into digital output, which is then sent to a computer. Different resistors in the ion detector can be adjusted for different beam intensities, so that all beams can be detected in the same voltage range (Scrimgeour & Robinson, 2004).

Measurements are reported in δ‰ in comparison to international reference standards (Table 1), according to the equation:

$$\delta\text{\textperthousand} = \frac{R_{sample} - R_{s\tan dard}}{R_{s\tan dard}} \cdot 1000$$

Where R is the ratio between heavier and lighter isotopes.

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

The kinetic effect leads to greater mobility and lower bound strength and consequently less activation energy for lighter isotopes, which weigh less.

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

Isotopic fractionation can also be caused by an alteration in the equilibrium of the reaction, such as an instantaneous change in temperature, the removal of a reactant or a reaction product. This kind of fractionation (of non equilibrium, like enzymatic reactions) leads to the enrichment of a particular isotopic species but without pre-arranged rules.

Later on, the factors affecting the variability of the isotopic ratios investigated in this thesis ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) will be exhaustively discussed, in order to clarify their possible applications.

1.2.3 Animal tissues for SIRA analysis

In the last few years several studies have been conducted in order to evaluate the best animal fractions for SIRA analysis. All of these have been undertaken on two types of tissue samples, incremental tissues such as hair (Ayliffe et al., 2004; Cerling et al., 2004b), nail (Fraser et al., 2006; Fuller et al., 2006) and claws; and integrating tissues including muscles (Bahar et al., 2008; Schmidt et al., 2005), liver (Caut et al., 2008; Perga & Gerdeaux, 2005) and blood (Hobson et al., 1996; Miller et al., 2008). These types of tissues can both provide a range of different information. Incremental tissues are more sensible to "dietary changes"; therefore they are fundamental in detecting changes in animals' feeding habits occurring in the last few days or months before slaughter. Integrating tissues are more stable and play a key role in determining the standard/daily diet followed for several months or year prior to slaughter.

1.2.4 Fractionation and half-lives

Although isotopes have the same number of electrons and in principle behave in a similar way, the energy they need to undergo physical changes, e.g. breakage of old and formation of new bonds, may be different for various isotopes of the same element (Kelly, 2003; Wagner, 2005). This effect is called fractionation and has its origin in the slightly different physical properties of isotopes. Fractionation between any two chemical species is described by the following equation (Karasov & Martinez del Rio, 2007).

$$\alpha_{A-B} = \frac{R_A}{R_B} = \frac{(1000 + \delta_A)}{(1000 + \delta_B)}$$

In this equation, α_{A-B} is the fractionation while R_A and R_B are the ratios of the two species in delta (δ). It can generally be stated that more energy is needed to get a heavier isotope involved in any physical or chemical change (Fry, 2006; Kelly, 2003).

These fractionations can lead to enrichment or depletion of animal tissue, depending on its diet. Therefore, this diet-tissue discrimination Δ_{A-B} ($\Delta_{A-B}= \delta_A-\delta_B$; Karasov & Martinez del Rio, 2007) needs to be experimentally determined to correctly interpret the results obtained by SIRA (Ayliffe et al., 2004; Gannes et al., 1997).

The half-life of a tissue describes the spread of a tissue's turnover (Fry, 2006). It can be defined as the time it takes to replace half of the tissue's dietary signature with the signature of the new diet (MecAvoy et al., 2006). Since the half-life depends on a tissue's metabolic rate, bone has a significantly longer half-life than blood (Dalerum & Angerbjorn, 2005). As for diet-tissue discrimination, half-life needs to be determined through controlled studies such as diet switch experiments (Gannes et al., 1997).

1.3 CARBON

1.3.1 Diet effect

The $\delta^{13}C$ of different animal tissues (muscle or lipid fraction) is deeply influenced by the composition of animal diet, normally based on different vegetal species.

These show different $\delta^{13}C$ ranges according to the kind of photosynthetic cycle (C_3, C_4 or CAM) because of the different isotopic discrimination capability of the carboxylase enzymes involved in CO_2 fixation, in addition to the different CO_2 concentration in plants.

1.3.1.1 The Calvin Cycle

The Calvin cycle is the primary mechanism for CO_2-fixation described by Calvin. It is characteristic of plants from cold-temperate areas (*e.g.* tomatoes, potatoes, beetroot, wheat, rice, oats, barley, rye, soyabean, grapes, oranges, apples) and is called C_3, as CO_2 is fixed in intermediate products with three atoms of carbon. Atmospheric CO_2 gets into leaf' parenchyma cells through the stomata and dissolves in the cytoplasm (Hatch and Slack, 1966; Smith and Epstein, 1971). There it binds to ribulose-1,5-diphosphate (RuDP) giving rise to two molecules of phosphoglycerate (PGA) that are subsequently reduced to

phosphoglyceraldehyde (PGAL), the first sugar of photosynthesis (Figure 1) (Taiz and Zeiger, 1998). Five out of the six new PGAL molecules are used to synthesise more RuDP via a series of complex reactions driven by ATP (not shown in Figure 1). The sixth molecule of PGAL may be used to synthesise glucose (usually regarded as the end product of photosynthesis) via combinations and rearrangements. The glucose may subsequently be used to synthesise complex carbohydrates such as sucrose, structural materials such as cellulose, or energy storage compounds such as starch and lipids (Ting, 1982). Although the initial reaction of CO_2 with RuDP produces the overriding ^{13}C isotope effect associated with this species, there are many other factors which contribute to the final $\delta^{13}C$‰ value of plant material, such as, temperature, fertilisation, salinity, CO_2 concentration, light intensity and photorespiration (O'Leary, 1981). The interplay of all of these factors results in $\delta^{13}C$‰ values between –22‰ to -34‰ for 80% to 90% of plants which utilise the C_3 pathway (Krueger and Reesman, 1982).

Figure 1. Synthesis of carbohydrate by the Calvin (C_3) cycle. Each molecule of CO2 combines with one molecule of ribulose-1,5-diphosphate (RuDP, a 5 carbon sugar) to form a hypothetical unstable 6 carbon activated complex. This immediately splits into two molecules of phosphoglycerate (PGA, a C3 molecule). PGA is phosphorylated by ATP and then reduced by NADPre to form phosphoglyceraldehyde (PGAL, a C3 sugar). Five out of six new PGAL molecules are used to synthesise more RuDP via a series of complex reactions driven by ATP (not shown here). The sixth molecule of PGAL may be used to synthesise glucose (adapted from Ting, 1982; O'Leary, 1988; Taiz and Zeiger, 1998).
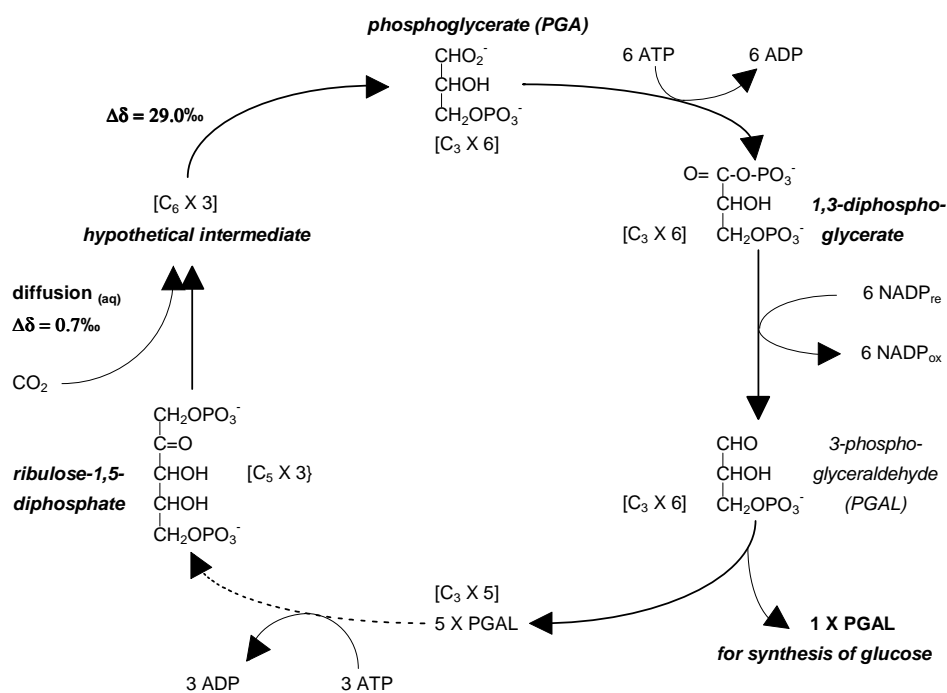
1.3.1.2 The Hatch-Slack Cycle

The mechanism of carbon fixation used by these plants was described by Hatch and Slack in the late 1960s as the C_4 pathway (Hatch and Slack, 1970). Hatch-Slack plants are able to utilise CO_2 concentrations as low as 0.1 ppm (while the C_3 mechanism does not operate at atmospheric concentrations of less than approximately 50 ppm). Hatch-Slack plants can perform photosynthesis in conditions of high temperature, intense light, low humidity, low CO_2 and high O_2 concentrations.

CO_2 is obtained from two sequential carboxylation reactions shown in Figure 2.

Figure 2. *The Hatch-Slack pathway of C_4 photosynthesis*. Each molecule of CO_2 combines with one molecule of phosphoenolpyruvate (PEP, a 3-carbon compound) to form a 4 carbon compound oxaloacetic acid (OAA). OAA is then reduced by NADPre to form malate (MAL, a C_4 acid) or animated to form aspartate (ASP, a C_4 acid). The acid is then oxidised by NADPox to form a C_3 compound and CO_2. The C_3 compound is converted into PEP, pyruvate or alanine (not shown here) and then into PEP by the action ATP. The CO_2 is fed into the Calvin cycle where it is used to synthesise glucose (adapted from Ting, 1982; O'Leary, 1988; Taiz and Zeiger, 1998).
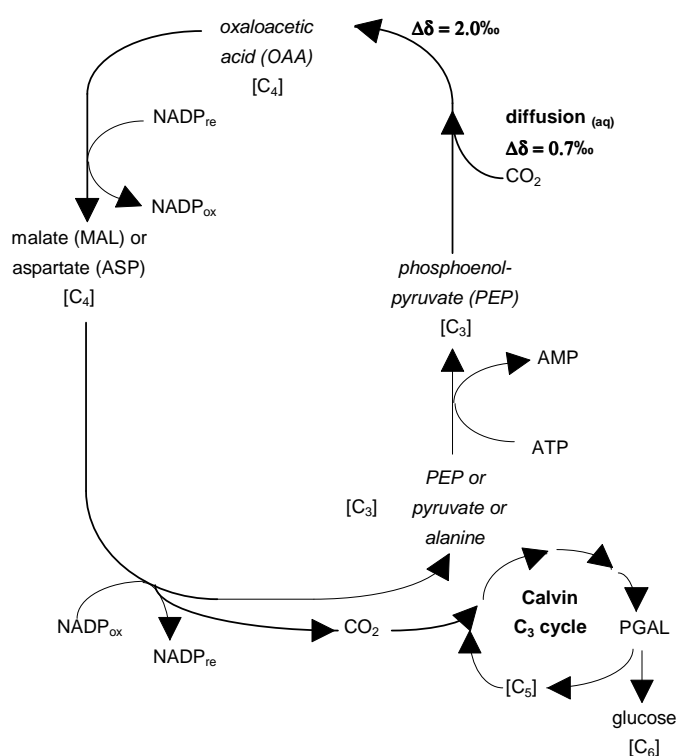
When CO_2 first enters the leaf stomata it combines with a 3-carbon compound phosphoenolpyruvate (PEP) to form oxaloacetate (OOA), a 4-carbon acid which is the origin of the term C_4, synonymous with the Hatch-Slack cycle (Hall &Rao, 1999). This first stage proceeds with much smaller fractionation as compared to the C_3 cycle, $\Delta\delta \sim 2\text{‰}$ (O'Leary, 1981).

OAA is then rapidly reduced by NADPre to form malate (MAL, a C_4 acid) or animated to form aspartate (ASP, a C_4 acid). These acids are transported deeper into the C_4 plant leaves. The acids are then oxidised by NADPox to form a C_3 compound and CO_2. The C_3 compound is converted into PEP, pyruvate or alanine and then into PEP by the action of ATP. The CO_2 feeds into the Calvin cycle where it is used to synthesise glucose as in Figure 1. It is important to note that although the C_3 carboxylase enzyme shows extensive ^{13}C isotope fractionation, it is *not expressed* in the Hatch-Slack photosynthetic pathway. This is because the pre-fixation of CO_2 by carboxylation of PEP is an irreversible reaction. This results in relatively enriched $\delta^{13}C\text{‰}$ values for C_4 plants between -10‰ and –14‰ (Winkler, 1984).

C_4 plants dominate in the tropics (Stryer, 1995) and include sugar cane, corn, sorghum, millet and some types of pasture grasses.

1.3.1.3 CAM-plants

During the night, *Crassulacean Acid Metabolism (CAM)* can absorb CO_2, by storing high concentrations of organic acids, in order to reduce the loss of precious water (Ting, 1982; Garrett & Grisham, 1999). CAM plants are generally cultivated in hot dry climates and usually have their stomata closed during the day and open at night. Consequently, the plant transpires and fixes CO_2 at night and this causes a reduction in starch and other storage glucans that energises the metabolism. However, if the climate during the day is relatively cold, the stomata may open and the plant will adopt direct C_3 metabolism of CO_2. When daytime temperatures are high the stomata remain closed to prevent water loss through transpiration (Krueger and Reesman, 1982). At night, when temperatures drop, the stomata opens and uses the C_4 metabolism of CO_2. The organic acids are then converted back into CO_2 for C_3 synthesis the day after. The metabolism adopted by CAM plants is therefore influenced by local climatic conditions and in extreme cases may be predominantly C_3 or C_4.

Consequently, the $\delta^{13}C‰$ value of CAM plants material varies widely between -30‰ and -12‰ (Winkler, 1984). Pineapple, vanilla and cacti are the most well known plants that use this metabolism.

The differences in $\delta^{13}C$ values mentioned above can be used to determine the type of feed consumed by animals. For example, interpretation of $\delta^{13}C$ values may show that cattle were reared with different types of diet: e.g. either conventionally or organically (Bahar et al., 2008; Boner & Forstel, 2004; Schmidt et al., 2005). In a study conducted by Gonzales-Martin et al. (1999) two different types of feeding (acorn feed vs barley + soy flour + wheat) were clearly defined with the $\delta^{13}C$ isotopic value of adipose tissue.

Bahar et al. (2005) showed that when shifting from a C_3 diet to a C_4 diet, based on an incremental % of maize, the isotopic ratio of carbon increases in defatted dry mass and in the lipid fraction. Camin et al. (2008) observed the same results in milk (casein and lipid). Each 10% increase in corn content in the diet corresponds to a 0.7–1.0‰ shift in the $\delta^{13}C$ of casein. Another animal product, urine, shows the same behaviour as milk, with a value about 2‰ higher (Knobbe et al., 2006).

Analysis of $\delta^{13}C$ also enables researchers to distinguish between terrestrial and marine feeding materials (Balasse et al., 2005; Handley & Raven, 1992), as marine plant material is enriched as compared to terrestrial plant material (Kelly, 2000).

1.3.2 Climatic effect

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

$\delta^{13}C$ also predictably varies according to the latitude in the northern hemisphere, with values becoming more depleted with increasing latitude, as a consequence of changes in the relative proportion of C_3 and C_4 plants.

1.3.3 Isotopic fractionation

As reported by Tieszen et al. (1983) the $\delta^{13}C$ value increased in animals by about 1‰ according to the mean isotopic value of its diets.

The results of De Smet et al (2004) showed a significant ^{13}C depletion of lipid, but also an enrichment of muscle, hair, liver, blood and plasma. The order of $\delta^{13}C$ values was hair>muscle>liver>kidney fat.

For example, as demonstrated by DeNiro and Epstain (1977), lipids are more depleted in ^{13}C than muscle, due to isotopic fractionation during the oxidation of piruvate to acetic CoA. Tieszen et al. (1983) showed how tissues with high metabolic rates (such as blood or liver) have a slower C turnover than tissues with slower metabolic rates (such as muscle or bone).

1.4 HYDROGEN AND OXYGEN

As demonstrated by Bowen et al. (2009), the δ^2H of meat protein is 70% influenced by diet and 30% by drinking water, and its value is higher than the lipid value. On the other hand, the $\delta^{18}O$ of meat protein depends 70% on water and 30% on diet. In this case we find a lower value than the lipid value.

1.4.1 Meteoric water effect

The δ^2H and $\delta^{18}O$ of drinking water reflect meteoric rainwater values, because the former normally comes from underground deposits supplied by precipitation.

The compositional variability of δ^2H and $\delta^{18}O$ in meteoric rain water is based on the cycle of evaporation from oceans and subsequent condensation in precipitation. The hydrogen and oxygen isotopic composition of oceanic water (from -1 to 0.7‰) (Clark and Fritz, 1997) approaches V-SMOW (0‰), the international reference standard for the measurement of δ^2H and $\delta^{18}O$ (Table 1), although it has changed considerably during different geological eras. The relationship between oxygen and hydrogen isotope patterns in the hydrosphere was first defined by Craig in 1961. The mean annual isotope ratios for hydrogen and oxygen in precipitation from regions as disparate as the Arctic, Antarctic, tropics and European and American continents all fall on the meteoric water line (MWL) (Dansgaard, 1964) described by the following equation:

$$\delta D‰ = 8 \, \delta^{18}O + 10$$

Precipitation occurs when humidity is 100%, so isotopic fractionation is mainly due to temperature. Oceanic vapour from subtropical areas moves to poles, becoming cooler and condensing in the form of precipitation, losing the heavier isotopes, which are concentrated in initial rainfall (Figures 3 and 4 show an example for $\delta^{18}O$). Consequently, latitude is a discriminating factor in hydrogen and oxygen isotopic fractionation.

Besides the 'latitude' effect, there is a 'continental' effect due to the distance from the sea, related to the vapour masses moving over continents (the closer the primary source of vapour, the more significant the decrease in δ^2H and $\delta^{18}O$ due to previous precipitation) and this causes precipitation on coasts isotopically richer than internal areas (mean reduction of -2.8‰/1000 km from the coast) (Förstel and Hutzen, 1984). Moreover, different altitudes inland lead to a reduction in ^2H and ^{18}O of about -0.15‰ to -0.5‰ *per* 100 metres elevation, because at higher altitude there is lighter vapour. Finally another deviation is due to seasonal trends; during summer there is an enrichment in ^{18}O and ^2H, especially inland.

Figure 3. Isotopic fractionation of a vapour mass in relation to temperature (Clark et al., 1997)
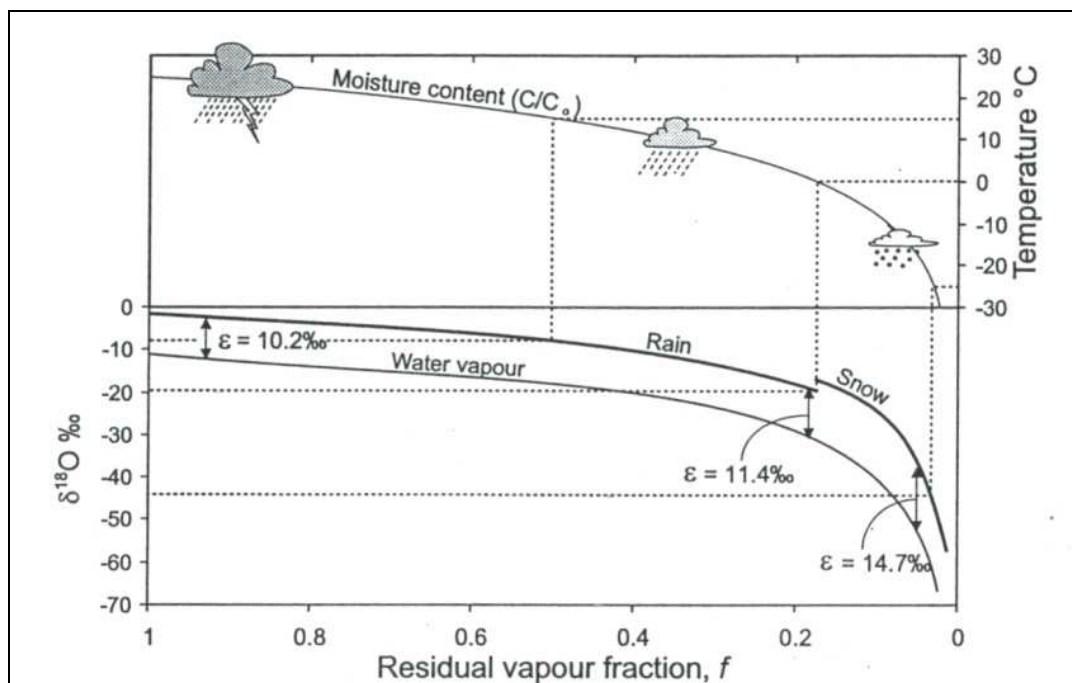
Figure 4. Distribution of mean $\delta^{18}O$ values in 1992 and 1993 precipitation (Rozanski, 1993)
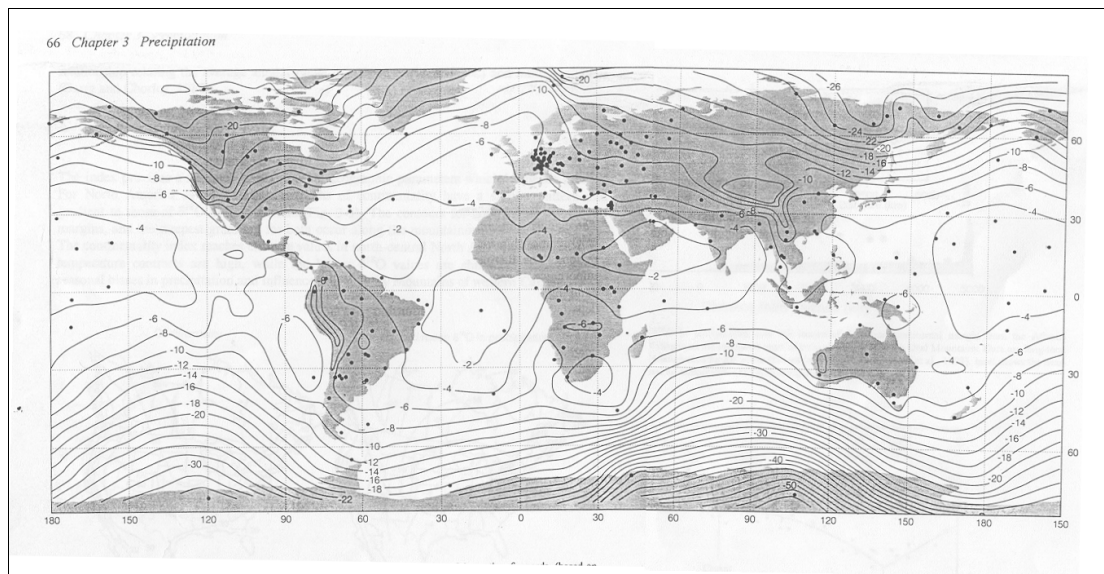
Ground water, and thus animal drinking water, has an isotopic composition related to the mean annual isotopic composition of precipitation water, and its δ^2H and $\delta^{18}O$ depend only on geographical factors (altitude, latitude, distance from the sea) but not on the season..

H and O SIRA may be used to determine the geographical origin of animal feed and human foods (Boner & Forstel, 2004; Camin et al., 2007; Franke et al., 2008; Hegerding et al., 2002; Kelly et al., 2005).

Ehleringer et al. (2008) showed a significant correlation between animal proteins and both the δ^2H and $\delta^{18}O$ isotopic values of rainfall. The same good correlation has also been detected in different animal products, such as cheese (Camin et al., 2012) and milk (Chesson et al., 2010).

1.4.2 Diet effect

Vegetal species are the main components of animal feed.

In plants, the isotopic composition of vegetal water is related to the water absorbed from the soil, so it is affected by the factors mentioned above. Furthermore, vegetal water in the leaf suffers isotopic fractionation during evapotraspiration processes, which are affected by temperature and relative humidity,, leading to an enrichment of the heavier isotopes. There

are no apparent differences in the degree of enrichment of deuterium and oxygen in the leaf water of plants utilising the Calvin (C_3), Hatch-Slack (C_4) or Crassulacean acid metabolism (CAM) photosynthetic pathways (Bricout, 1982). Therefore, it is to be expected that growing regions with relatively low humidity, where the rate of evaporation from the leaf is higher, will be characterised by plant materials with relatively enriched δ^2H and $\delta^{18}O$ values (Martin et al., 1986).

Relationships in terms of the variation of the δ^2H values of cellulose according to the δ^2H of leaf water and temperature (T) have been defined (Yapp and Epstein, 1982a; Yapp and Epstein, 1982b), where:

$\delta D‰$ cellulose $= 0.9\ \delta D‰$ leaf water $-22‰$

$\delta D‰$ cellulose $= 5.8\ T\ -\ 134‰$

To evaluate the contribution of the diet to the $\delta^{18}O$ isotopic value, Kornexl et al. 1997 measured this value in milk in different periods of the year. The results showed a rise in the $\delta^{18}O$ value when the animals were fed with grass instead of forage. Grass indeed contains enriched water, the result of evapotraspiration.

Balasse et al. (2003) were able to infer the seasonality of sheep birth and changes in sheep diet during the year, by analysing the O stable isotope ratios of teeth (Balasse et al. 2006)

1.4.3 Isotopic fractionation

Hydrogen and oxygen integration in the different tissues through metabolic processes causes considerable isotopic fractionation. For example, as reported by Tuross et al. (2008), proteinaceous materials clustered in the most δ^2H-enriched range, e.g. collagen mandible (-63±8‰) and in the most δ^2H-depleted range: blood (-128±7‰) (-137±7‰). Fat materials had the most depleted δ^2H value (-284±12‰).

Furthermore, metabolites show systematic shifts among the different groups of plant components, due to biochemically induced fractionation. The δ^2H value of protein does not differ significantly from carbohydrate (Winkler, 1984). However other carbohydrate reduction products such as ethanol, cholesterol and lipids are relatively depleted in deuterium (Bowen, 1966). The depletion of lipids can be further divided into two groups (Estep and Hoering, 1980). The δ^2H of triglycerdes generated by two-carbon precursors, in the fatty acid

biosynthetic pathway, is approximately –30 to –60‰. Whereas nonsaponifiable lipids, synthesised via the five-carbon isoprenoid pathway, are depleted by a further 80‰, resulting in–110 to –140‰ depletion relative to carbohydrate. It is important to note that these latter observations relate specifically to the δ^2H measurement of carbon-bound or non-exchangeable hydrogen atoms in plant materials. It does not apply to the labile hydrogen attached to oxygen in hydroxyl moieties that can readily exchange with water (Dunbar and Schmidt, 1984).

1.5 NITROGEN

1.5.1 Source of variability

The principal source of N for tissue protein synthesis is plant feed for herbivorous animals and other animals for carnivorous animals. Farm animals are almost exclusively herbivorous (e.g. cows, sheep); 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 (Stryer, 1995) and it contains about 0.4% ^{15}N. Through physical processes and the activity of microorganisms it is transformed into inorganic (nitrates, ammonia) and organic forms (aminoacids, proteins) that are present and available in the soil. The natural cycle of nitrogen in the environment is relatively complex as compared to other bio-elements: carbon, hydrogen, oxygen and sulphur. It moves from the atmosphere, through various plants and microbes and occurs in a variety of reduced and oxidised forms (Table 2).

According to the extent of each of these processes, which are mainly affected by the depth of soil, 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 lead to ^{15}N enrichment in the soil (Heaton et al., 1987). For cultivated lands, the main factor affecting $\delta^{15}N$ is fertilisation practices. Synthetic fertilisers, produced from atmospheric nitrogen *via* the Haber process, show $\delta^{15}N$ values between -4 and +4‰, whereas organic fertilisers are enriched in ^{15}N, ranging between +0.6 and +36.7‰ (manure between +10 and +25‰ (Bateman and Kelly, 2007). Intensive use of organic fertilisers can therefore cause a significant enrichment in the ^{15}N of nitrogen compounds in soil (Kreitler, 1975; Kelly and Bateman, 2005).

Table 2. Processes in the nitrogen cycle leading to isotopic fractionation

Process	Description of the process	Fractionation
Fixation	Natural process, either biological or abiotic, by which nitrogen (N_2) in the atmosphere is converted into ammonia: - through bacteria (*e.g.* through nitrogenise enzyme in legumes); - through physical processes producing high temperatures (*e.g.* lightening, fire) - through human activities (*e.g.* production of energy or fertilizers)	–3/+1‰ (data concerning legumes) (Fogel and Cifuentes, 1993)
Assimilation	Incorporation process of nitrogenous compounds (NO_x, NH_3) by microorganisms or plants. At the beginning nitrogen oxides are reduced to ammonia and subsequently integrated in organic matter.	Assimilation further incorporation of ^{14}N than ^{15}N, with a mean fractionation of –0.5‰ (Hübner,1986) that is negligible in plants.
Dissimilation	Metabolic reactions that use the assimilated nitrogen.	
Mineralization	Transformation of soil organic nitrogen in ammonia.	±1‰
Nitrification	Biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates	-12/-29‰
Volatilization	Volatilizzation reaction of ammonia as gas from soil to atmosphere (very marked in alkaline soil)	+20‰
Denitrification	Nitrate reduction that may ultimately produce molecular nitrogen	Enrichment in ^{15}N

1.5.2 Isotopic variability factors in plants

$\delta^{15}N$ values in plants are generally correlated to those of nitrates and ammonia in soils. They are therefore are affected by the same variability factors described above, but also by the isotopic fractionation involved in uptake and organic compound assimilation processes (Werner et al., 2002). Uptake does not cause any substantial fractionation, whereas enzymatic reactions, such as nitrate reduction or transamination, significantly affect isotopic composition (Yoneyama et al., 1995). Leguminous and nitrogen-fixing plants are an exception, as they can fix nitrogen directly from the air, showing $\delta^{15}N$ values around 0‰ (Yoneyama et al., 1995).

1.5.3 Information obtained from N SIRA

Analysis of $\delta^{15}N$ enables researchers to explore the existence of nutritional stress during starvation (Fuller et al., 2005) or pregnancy (Fuller at al., 2004).

1.5.4 Isotopic fractionation

$\delta^{15}N$ findings are widely used in ecological studies to determine the trophic level of animals (Kurle & Worthy, 2002; Minegawa & Wada, 1984; Post, 2002) and humans (Hedges & Reynard, 2007) and have shown enrichment of approximately +3‰ per trophic level (Minegawa & Wada, 1984).

This stepwise enrichment of $\delta^{15}N$ is also used to establish patterns in breastfeeding in modern humans (Fuller et al., 2006).

The different tissues have a different level of fractionation. De Niro & Epstain, 1981 demonstrated that mice fed the same diet had different $\delta^{15}N$ isotopic values in these tissues: brain > liver > hair > muscle > kidney. The $\delta^{15}N$ of milk protein showed a gap of 4‰ as compared to urine protein (Knobbe et al. 2006).

1.6 SULPHUR

1.6.1 Source of variability

The S source of any animal tissue is the sulphur contained in plants. Natural factors that influence $\delta^{34}S$ values in terrestrial plants are the abundance of heavy sulphides in the soil, but also aerobic or anaerobic growing conditions (Rubenstein & Hobson, 2004), underlying local bedrock (igneous or sedimentary, acid or basic), microbial processes active in soil, fertilisation procedures and atmospheric deposition (Krouse & Mayer, 2000), such as mainly sulphate deposition as an aerosol over forage in coastal areas (sea-spray effect) (Attendorn & Bowen, 1997).

The variation of ^{34}S in the terrestrial environment is shown in Figure 5.

The presence of any one sulphur isotope in preference to others will depend on mass fractionation and kinetic isotope effects both prior to, during and after formation of the substance. Thus, for example, anthropogenic SO_2 pollution can be correlated with its source (Winner et al., 1981) as can beach tar deposits, from sulphur isotope measurements (Hartman & Hammond, 1981).

In an analogous way to denitrification, bacteria metabolise the oxygen in sulphate to oxidise organic material, thereby producing isotopically light hydrogen sulphide (Schidlowski, 1982).

Figure 5. ^{34}S isotope variation ranges of sulphur pools (adapted from Winkler, 1984)

^{34}S isotope abundance (%)
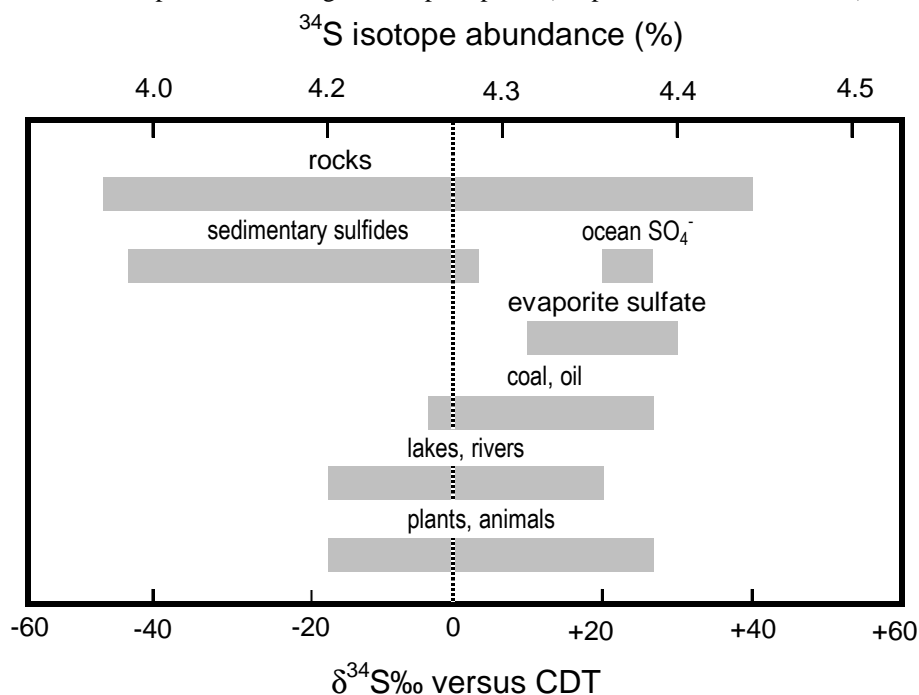
1.6.2 Information obtained from S SIRA

δ^{34}S can be used to differentiate terrestrial from marine plants (Kelly e al., 2005; Rubenstein & Hobson, 2004). Commonly found δ^{34}S values for terrestrial plants range from -5‰ to +22‰, with most plants ranging between +2‰ and +6‰. The δ^{34}S values of marine plants usually range from +17‰ to +21‰ (Peterson & Fry, 1987). The fractionation of sulphur in marine habitats is mainly caused by sulphate-reducing bacteria (Thode, 1991). In a study on Inuit diets, Buchardt et al.(2007) showed that S stable isotope ratios are a very effective way of estimating the proportions in the Inuit diet originating from terrestrial and marine sources. Several studies on the authenticity of foods (Boner & Fostel, 2004; Camin et al., 2007 Schmidt et al., 2005) have showed clearly that sea-spray accounts for the higher δ^{34}S values in meats. However, Bahar et al. (2008) and Molketin & Giesemann (2007) showed a clear seasonal pattern for δ^{34}S values in beef and milk respectively, for which both groups have some difficulties in finding an explanation. Furthermore, Gonzales-Martin et al. (2001) showed that it was possible to distinguish between the dietary regimes of Iberian swine and

therefore to distinguish between breeds using S SIRA. Finally, Nino-Torres et al. (2006) used analysis of the δ^{34}S on dolphin teeth to show that *Delphinus capensis* is a coastal feeder but that its feeding habits change with age, resulting in a higher trophic level later in life.

1.7 USING SIRA TO TRACE ANIMAL PRODUCTS: EXAMPLES OF APPLICATIONS

In 2003 Piasentier et al. published a first paper on SIRA and lamb meat traceability based on feeding habits and geographical origin. This article reported discrimination between the different origin of lamb (6 European countries) and 3 different feeding regimes (suckled milk, pasture and supplementation containing maize grain) based on the ^{13}C/^{12}C and ^{15}N/^{14}N in lamb meat (*longissimus thoracis*) protein and fat. The δ^{13}C of defatted dry protein and fat showed good discrimination between the three type of diets, with the lowest values in pasture samples and the highest value in the case of milk feeding. The different pedoclimatic conditions (humidity) justify the differences in the δ^{15}N isotopic value detected in samples with the same diets but of different geographical origin, such as Iceland and UK, or Spain and France.

As a consequence of the animal disease BSE (Bovine Spongiform Encephalopathy), the stable isotopic method was used to detect the content of MBM (bovine meat and bone meal) in the animal diet. Carrijo et al. (2006) showed good discrimination in boiler chickens fed a corn and soybean meal-based diet with an increasing level of MBM.

A significant contribution to improvement of multi-element stable isotopic analysis of meat (lamb, chicken and beef) was made by the European Project TRACE (*Tracing the origin of food*). The number of sampling sites (22) and samples (203) made it possible to evaluate the potential of different stable isotope ratios (C, N, O, H, S). Camin et al. (2007), comparing the δ^2H isotopic value of drinking water and defatted dry protein of lamb and confirming a good correlation (R^2=0.7). The correlation between the δ^2H and δ^{13}C of defatted protein showed the potential for discriminating between three macro-areas (UK + Ireland, Greece + South Italy and Alpine Regions). The different samples were allocated on the basis of the δ^2H isotopic value, with the higher values in the north of Europe. This study showed characteristic

behaviour for the δ^{34}S of defatted dry mass, making it possible to discriminate between the samples on the basis of geographical origin. The UK and Ireland had a higher δ^{34}S value, probably due to the sea-spray effect (see below) whereas a volcanic site (Sicily) showed a lower value.

To evaluate the effectiveness of stable isotopic ratio analysis in discriminating between lambs fed with herbage or concentrate, both obtained from C3 plants, Moreno-Rojas et al., (2008) analysed 34 samples of *Comisana* male lambs. The lambs, 45 days old, were fed three different diets: two C3 based (vetch *Vicia sativa ad libitum* and barley-based concentrate) and one C4 based (maize concentrate) and then slaughtered after 60 days. Wool, adipose tissue (perirenal fat) and muscle *Longissimus dorsi* from all lambs were sampled.

Tηε δ^{13}C of lipids and the δ^{13}C and δ^{15}N of meat and wool can discriminate between animals fed herbage or concentrate, both obtained from C3 plants. Wool δ^{15}N shows a value 1‰ lower than the δ^{15}N of meat. As the δ^{13}C and δ^{15}N values of wool can predict that of muscle, Moreno-Rojas et al., (2008) demonstrated that it is possible to authenticate animal-feeding systems without slaughtering the animals.

Heaton et al., (2008) tried to apply the isotopic method to beef samples originating from England, Ireland, Scotland, Brazil and the USA. Only with a δ^{13}C vs δ^{15}N plot was it possible to discriminate between European and extra-European samples. The Brazilian and USA beef samples, with a typical C4 based animal diet, were characterised by a δ^{13}C between -16‰ and -9‰, whereas European products had a δ^{13}C between -28‰ and -20‰. The δ^{18}O of the lipid fraction confirmed itself as a helpful resource for geographical traceability. The δ^{18}O values vary with the approximate latitude of the production region (R^2=0.76).

Perini et al. (2009) tried to check whether multi-element stable isotope analysis can be used to differentiate the regional origin of Italian lambs and to differentiate lambs on the basis of their diet. This study considered 160 lamb samples from six different Italian regions (Trentino, Friuli, Tuscany, Molise, Umbria and Sicily) fed four types of diets (only grass, grass + maize, hay + maize, milk). The δ^{13}C of defatted dry mass and lipid confirmed the ability to discriminate between C3 and C4 diets. Comparison between the δ^{13}C trend in proteins and lipids showed a significant difference between two samples in the oldest lambs, following a C3 diet before a diet-change. In this case, characterised by a change in diet from C3 to C4, we

detected a slower response in lipid as compared to protein. This is due to the different tissue turnover. δ^2H analysis discriminated between the different geographical origin of lambs, with the lowest value in the north (Trentino) and the highest in the south (Sicily). The $\delta^{18}O$ of lamb proteins and lipids depends on the type of diet. The highest values occur in the presence of grass containing vegetal water enriched with ^{18}O (evapotraspiration). When comparing samples from the same animals fed milk instead of grass, there was a significant difference due to the trophic level. $\delta^{15}N$ and $\delta^{34}S$ are correlated with pedoclimatic conditions and the underlying local bedrock. $\delta^{15}N$ showed the highest values in Sardinia, where the soil is very arid, whereas the lowest values were in Trentino. With $\delta^{34}S$ it is possible to differentiate between two different types of macroareas: regions more influenced by the sea-spray effect (Sardinia) and regions with volcanic soil (Sicily).

Guo et al. (2009) systematically determined the $\delta^{13}C$, $\delta^{15}N$, δ^2H and $\delta^{87}Sr$ of cattle tissue and tail hair from four major beef production sites in China. The results showed that the fingerprint of these indicators in cattle tissues from the different regions is unique. The $\delta^{13}C$ value in cattle tissues was effective in classifying beef from different regions, where cattle feed is significantly different in terms of the proportion of C_4 and C_3. The $\delta^{15}N$ value was effective in distinguishing cattle from farm land and pasture. The δ^2H value in cattle tissue was closely related to the geographical conditions and decreased at higher latitude. 92% of samples were correctly classified using the $\delta^{13}C$, $\delta^{15}N$ and δ^2H values.

Sun (2011) reported that there were significant differences in C, N and H stable isotopes in lamb tissues according to their geographical origin. The total correct classification rate of 88.9% was obtained according to the geographical origin by combining the $\delta^{13}C$, $\delta^{15}N$ and δ^2H values in lamb muscle.

Osorio et al. (2011a), by plotting only $\delta^{13}C$ values against $\delta^{15}N$ values, showed clear separation of beef from animals fed two different types of diet: grass-fed groups (animals raised at pasture for 12 months (P) or animals fed grass silage for 6 months, followed by 6 months at pasture (SiP)) from beef fed a cereal concentrate for 12 months (C) or an "intermediate" group fed grass silage for 6 months followed by 6 months of grass at pasture with a concentrate supplement (SiPC).

With canonical discriminant analysis (CDA) the percentage of correctly classified samples was 86.5% (Osorio *et al.*, 2011a). $\delta^{13}C$ and $\delta^{34}S$ values are the most significant parameter for classifying beef according to the feeding regimen, on the basis of muscle stable isotopic signatures.

In another study, Osorio et al. (2011b), used CDA applied to stable isotope data (H, C, N, S) to investigate the feasibility of discriminating Irish grass-fed beef from non- Irish beef raised in other European countries and the USA and Brazil.

85% of the 140 beef samples were correctly assigned to the country of origin, and 82.9% were cross-validated. All Irish grass-fed beef samples were correctly classified and then cross-validated.

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CHAPTER II: OBJECTIVES

The main objective of this research was to investigate the effect of different factors, such as tissue turnover, type of diet, geographical origin or breeding and processing conditions, on the stable isotope ratio variability of the most important bioelements (^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{18}O/^{16}O, ^{34}S/^{32}S), measured using IRMS (Isotopic Ratio Mass Spectrometry) in different types of tissues, including pig and ovine muscles, muscle lipids and lipid fractions.

This study was simultaneously conducted by other laboratories in order to extend geographical areas and tipologies of the animals indagated.

The specific strategies were:

1. analysis of the trend for the isotopic values of different bioelements during a diet switch;

2. evaluation of the δ^{18}O and δ^2H of samples (ham or beef) from different geographical areas;

3. evaluation of the trend for the isotopic values of different bioelements in animals with different breeding conditions.

In detail the research focused on:

− study of multi-element (H, O, C, N, S) stable isotope tissue turnover and diet-tissue discrimination in ovine muscle and its lipid classes and lipid fractions;

− study of the effect of various production factors in relation to both pig husbandry and thigh processing procedures on stable isotope ratio variability of the most important bioelements (^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{18}O/^{16}O, ^{34}S/^{32}S);

− evaluation of the efficacy of IRMS as a tool for tracing the geographical origin of dry-cured ham;

− characterisation and traceability of origin for beef from a country outside Europe (Cameroon).

CHAPTER III: TISSUE TURNOVER IN OVINE MUSCLES AND LIPIDS AS RECORDED BY MULTIPLE (H, C, O, S) STABLE ISOTOPE RATIOS

Abstract

Multiple stable isotope ratios (δ^2H, δ^{13}C, δ^{18}O and δ^{34}S) were measured in muscle, muscle lipids and lipid fractions collected from 28 lambs, subjected to a diet-switch and raised on two energy allowances (EAs), to determine tissue turnover and diet-tissue fractionation. The diet-muscle fractionations prior to the diet-switch were estimated to be −44.0‰, +1.9‰ and 0‰ for H, C and S, respectively, while the drinking water was demonstrated to be the main source of muscle O and thus δ^{18}O variation. The diet-intra-muscular lipid fractionations prior to the diet-switch were estimated to be −172.7‰, −1.3‰ and −11.5‰ for H, C and O, respectively. The C half-lives of muscle were determined to be 75.7 and 91.6 days for animals receiving the high and low EA, respectively. Extracting temporally resolved pre-slaughter dietary information from meat by analysing bulk muscle, muscle lipids and muscle lipid fractions appeared to be not practicable due to possible incomplete turnover of lipids.

3.1. Introduction

The recent findings of elevated levels of dioxin in Irish pork and of formula milk contaminated with melamine in China were just the latest in a series of scares to beset the food sector following the previous outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD) and avian influenza. These scares are largely responsible for raised consumer demands concerning clear origin labelling of food, especially meat (Food Standards Agency, 2001 and Kelly et al., 2005). However, analytical tools are needed to enable confirmation of country of origin of animal products and to verify the authenticity of foods. The potential of stable isotope ratio analysis (SIRA) in this regard has been demonstrated (Camin et al., 2007, Franke et al., 2008, Heaton et al., 2008 and Schmidt et al., 2005).

Measurements of multiple stable isotope ratios can be used to gain information about plant sources used as animal feed (Bahar et al., 2009 and Boner and Förstel, 2004), the proximity to the sea of farms on which animals were raised (Bol, Marsh, & Heaton, 2007) and the latitude of the country of origin (Camin et al., 2007 and Heaton et al., 2008). However, it is necessary to determine the diet-tissue fractionation for C (feed source), S (proximity to the sea) and H

and O (latitude) as well as the half-lives of tissues that can be of use for meat authentication. Currently, this information is not available for livestock animals.

Recently, Phillips and Eldridge (2006) proposed a novel approach, the so-called "isotope clock", that potentially allows scientists to estimate the time of previous changes in the diet of animals. In order to use this isotope clock, it is necessary to analyse at least two tissues with distinctly different half-lives. For meat authentication, such tissues should be sourced from a single sample obtained from a single animal. Muscle and lipid were previously shown to fulfil this requirement in gerbils (Tieszen, Boutton, Tesdahl, & Slade, 1983). Furthermore, research on the half-lives of triacylglycerols in human skeletal muscle (Sacchetti, Saltin, Olsen, & van Hall, 2004) and polar lipids in rat brain tissue (Freysz, Bieth, & Mandel, 1969) revealed distinctively different half-lives for these two lipid fractions (29 h and 20–40 days, respectively). Thus, it may be possible to obtain more detailed pre-slaughter dietary information on meat animals by analysing different biochemical fractions of the same meat sample.

The objective of this research was to estimate half-lives of ovine muscle tissue and its associated lipids and lipid fractions by measuring multiple stable isotope ratios following a controlled diet-switch prior to slaughter. Furthermore, we estimated diet-tissue fractionation of various elements in several tissues. Finally, we focused on the possibility of extracting two tissues or tissue fractions with distinctive half-lives from meat that could be used as an isotope clock (Phillips & Eldridge, 2006).

3.2. Materials and methods

3.2.1. Animals and feeds

Between March and April 2006, 28 purebred Belclare lambs (14 males and 14 females) were born at the Teagasc Production Research Centre, Athenry, Co. Galway, Ireland and were taken from their mothers at pasture 2.5 ± 2.1 days (mean ± standard deviation (SD), $n = 28$) after birth and initially raised on artificial milk for 6 weeks. The lambs were slowly weaned from the milk substitute during this time and introduced to a commercial diet (control diet). The control diet (Thomas McDonagh & Sons Ltd., Dromod, Co. Leitrim, Ireland) consisted of a mixture of cooked and flaked C_3 and C_4 plant material including barley flakes, maize flakes, maize gluten, cane, molasses and oats. In June 2006, the animals were moved to the Teagasc

Grange Beef Research Centre, Dunsany, Co. Meath, Ireland and maintained on the control diet.

Prior to the diet-switch, all lambs were statistically blocked according to sex and within treatment groups assigned at random to either a high energy allowance (HEA) or low energy allowance (LEA) of the experimental diet for 0 (control), 14, 28, 56, 98, 154 and 231 days (treatments T0, T14, T28, T56, T98, T154 or T231, respectively). The lambs were penned individually, weighed periodically and the feed allowances adjusted, based on body weight, to ensure a target weight gain of 50 g d^{-1} and 150 g d^{-1} for animals receiving the LEA and HEA, respectively.

The experimental diet was formulated to have a similar metabolisable energy (Agricultural and Food Research Council, 1993) as the commercially available control diet. The control diet had a metabolisable energy of 11.4 ± 0.3 MJ kg^{-1} dry matter (DM) (mean $\pm$ SD, $n = 8$) while the pelleted maize concentrate and maize silage had metabolisable energies of 12.6 ± 0.1 MJ kg^{-1} DM (mean $\pm$ SD, $n = 9$) and 10.1 ± 0.3 MJ kg^{-1} DM (mean $\pm$ SD, $n = 9$), respectively (see Table 1). Thus, the experimental diet had a theoretical metabolisable energy of 12.4 MJ kg^{-1} DM after consideration of DM and DM digestibility of each diet component. The experimental diet consisted of 76% (wet weight basis) of pelleted maize concentrate containing seaweed (48 kg t^{-1} concentrate; Arramara Teoranta, Kilkieran, Co. Galway, Ireland), produced in a single batch at the Teagasc Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland, and 24% (wet weight basis) of maize silage. The feed was offered to the animals in a single meal each morning while the lambs had *ad libitum* access to water at all times.

This study was carried out under licence from the Irish Government Department of Health and Children and with the approval of Teagasc, the Irish Agricultural and Food Development Authority. All procedures used complied with national regulations concerning experimentation on farm animals.

Table 1. Metabolisable energy, δ^2H, δ^{13}C, δ^{18}O and δ^{34}S of all feed stuffs (dried) and δ^2H and δ^{18}O of drinking water at both research stations (means ± SD).

Feed component	Energy [MJ kg^{-1} DM]	δ^2H [‰]	δ^{13}C [‰]	δ^{18}O [‰]	δ^{34}S [‰]
Control diet ($n = 8$)	11.4 ± 0.3	−53.6 ± 3.9	−22.6 ± 1.4	23.8 ± 1.2	4.1 ± 1.0
Experimental diet					
Pelleted concentrate ($n = 9$)	12.6 ± 0.1	−21.4 ± 2.6	−12.5 ± 0.5	25.9 ± 0.5	8.3 ± 0.8
Maize silage ($n = 9$)	10.1 ± 0.3	−45.9 ± 7.1	−12.1 ± 0.3	26.1 ± 1.3	5.1 ± 0.6
Composite (theoretical)	12.4	−23.6	−12.5	25.9	8.2
Water					
Water (Athenry; $n = 4$)		−30.7 ± 0.3		−5.0 ± 0.0	
Water (Dunsany; $n = 15$)		−44.6 ± 0.7		−6.7 ± 0.2	

3.2.2. Feed and water samples

Weekly feed samples were collected and stored at −20 °C until analysis. For SIRA, monthly samples of the three feed stuffs (control diet, pelleted maize concentrate and maize silage) were oven dried at 40 °C for 48 h. The dried samples were then first milled to a particle size of <1 mm using a commercial feed mill (Christy & Norris Ltd., Chelmsford, UK) before being powdered using a ball mill (Glen Creston Ltd., Stanmore, UK). For H and O analysis, feed was defatted according to Radin using a mixture of *n*-hexane and 2-propanol (3:2, v/v) (Radin, 1981). Powdered (C and S) or powdered, lipid-free (H and O) feed were finally weighed into tin capsules for SIRA.

Water samples were collected regularly throughout the experiment. In total, 19 samples were collected; four samples at the research station where the animals were born (Athenry, Co. Galway) and a further 15 samples at the research station where the diet-switch experiment was carried out (Dunsany, Co. Meath). All water samples were kept frozen at −20 °C until analysis.

3.2.3. Preparation of muscle and lipid samples

The muscle *Longissimus dorsi* (LD) was collected from all animals 24 h *post-mortem*, trimmed of superficial adipose tissue, vacuum packed and stored frozen at −20 °C until analysis. Furthermore, a sample from the subcutaneous adipose tissue (SAT) located close to

the LD was collected 24 h *post-mortem*. For SIRA, a sample from the geometrical centre of the muscle was collected and freeze dried (Edwards Pirani 501 Freeze Dryer, Edwards Ltd., Crawley, UK) for 168 h to ensure absolute dryness of the samples. Prior to analysis, 300 mg of muscle were sub-sampled from every collected sample and defatted according to Radin (1981) and the extracted lipid was collected. Dry, lipid-free muscle samples were stored in Eppendorf vials in a desiccator prior to weighing into tin capsules for SIRA.

The intra-muscular lipid (IML) fraction obtained following de-fatting was retained and stored in $CHCl_3$. The amount of IML was estimated following evaporation of the solvent under a stream of N_2 and determined as % of muscle dry matter (% DM). The SAT collected close to the LD was also "de-fatted" according to Radin (1981). The lipid collected was transferred to a 4 ml brown screw cap vial, re-dissolved in $CHCl_3$ and stored frozen at $-20\,°C$ until analysis. The IML (10 mg) was further separated into neutral lipids (NL), free fatty acids (FFA) and polar lipids (PL) using solid phase extraction (SPE) according to Kaluzny, Duncan, Merritt, and Epps (1985). To estimate the amounts of NL, FFA and PL in IML, the solvents were evaporated under a stream of N_2. After weighing, the IML and its fractions were re-dissolved in $CHCl_3$ and stored frozen at $-20\,°C$ until weighing. Purity of the lipid fractions was determined using thin layer chromatography (TLC) according to the procedure described by Bateman and Jenkins (1997).

For SIRA, lipids dissolved in $CHCl_3$ were transferred into pre-weighed tin capsules using a Hamilton syringe. The solvent was then slowly evaporated by placing the tin capsules onto a glass plate on a hotplate set to $40\,°C$. Finally, the tin capsules were weighed and wrapped for analysis.

3.2.4. Stable isotope analysis

The natural abundance levels of carbon stable isotopes were measured in feed, lipid-free muscle, SAT, IML, NL and PL using a Costech ECS4010 elemental analyser (Costech, Milan, Italy) connected to a Thermo-Finnigan Delta Plus XP gas isotope ratio mass spectrometer *via* a Conflo III interface (Thermo-Finnigan GmbH, Bremen, Germany). The natural abundance levels of sulphur stable isotopes ($^{34}S/^{32}S$) were measured in six replications in feed and lipid-free muscle using a Vario EL Cube elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany) connected to an IsoPrime mass spectrometer

(GVI, Manchester, UK). Natural abundance levels of hydrogen and oxygen stable isotopes were measured in lipid-free feed, lipid-free muscle, IML and water through pyrolysis of the sample in a high-temperature conversion/elemental analyser (TC/EA) coupled to a Delta XL Plus Mass Spectrometer (Thermo-Finnigan GmbH, Bremen, Germany).

Stable isotope ratios are expressed in the δ-notation in parts per thousand (‰) according to the equation:

$$\delta‰ = \frac{R_{sample} - R_{reference}}{R_{reference}} x1000$$

where R is the ratio of the heavier to the lighter stable isotope (e.g., $^{13}C/^{12}C$) in the sample (R_{sample}) and the reference material ($R_{reference}$). Porcine collagen and olive oil (EU TRACE project FP6-2003-FOOD-2-a Inter-Comparison Material) were analysed at the beginning, in the middle and at the end of each run to correct for instrumental drift and to determine the inter-batch variability of $\delta^{13}C$ analyses when analysing feed and fat-free muscle (porcine collagen) and SAT, IML, NL and PL (olive oil). For H, O and S analysis, casein (EU TRACE project FP6-2003-FOOD-2-a Inter-Comparison Material) was used to correct for exchangeable hydrogen according to the "comparative equilibration technique" (Wassenaar & Hobson, 2003) as well as instrumental drift and inter-batch variability.

The analytical precision of $\delta^{13}C$ measurements for the standard materials (NIST 1577b "Bovine Liver", US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, USA; NSB 22 "Mineral oil", International Atomic Energy Agency, Vienna, Austria) measured along with the samples was better than 0.2‰ (mean = −21.4‰) for NIST 1577b and better than 0.1‰ (mean = −29.7‰) for NBS 22. Analytical precision of δ^2H and $\delta^{18}O$ measurements for the standard materials (NBS 22 and IAEA CH6 "Anu Sucrose", both International Atomic Energy Agency, Vienna, Austria) measured along with the samples was better than 3‰ (mean = −120‰) for NBS 22 and better than 0.5‰ (mean = 36.4‰) for IAEA CH6 for δ^2H and $\delta^{18}O$, respectively. The analytical precision of $\delta^{34}S$ measurements for standard materials (Chicken ICM, EU TRACE project FP6-2003-FOOD-2-a Inter-Comparison Material) measured along with the samples was better than 0.2‰ (mean = 5.1‰). Samples were referenced to Vienna Pee-Dee Belemnite for C, to Standard Mean Ocean Water for H and O and to Cañon Diablo Troilite for S.

The following mass balance model (Scrimgeour & Robinson, 2004) was used to calculate the theoretical composite δ value of the experimental diet: xxxx

$$\delta_{ED} = \frac{P\delta_P + S\delta_S}{P + S}$$

In this equation, δ_{ED} is the theoretical δ value of the composite, experimental diet, δ_P and δ_S are the δ values of the two dietary components pelleted concentrate (*P*) and maize silage (*S*), respectively, and *P* and *S* are the product of the amount of the dietary component fed to the animals, the DM and the DMD (Harrison et al., 2010) of the dietary components *P* and *S*, respectively.

3.2.5. Statistical analysis and modelling of muscle and lipid turnover

A paired *t*-test was used to determine differences in C stable isotope ratios measured in LD and lipid types and fractions collected from animals receiving the LEA and HEA. Analysis of co-variance (ANCOVA) was used to determine whether sex, EA or the duration the animals received the experimental diet influenced the yields of IML, NL and PL using carcass weight as a covariate to correct for the differences in carcass weight. General linear models were fitted to H and O stable isotope ratios from LD to determine differences in slope factors for lambs receiving the LEA and HEA (SAS 9.1, SAS Institute Inc. Cary, NC, USA).

The software package SPSS v.12 (SPSS Inc., Chicago, IL, USA) was used to model the muscle C and S turnover. The model used was based on the one-pool model previously described by Hesslein, Hallard, and Ramlal (1993):

$$C(t) = C_E + (C_0 - C_E) \times e^{-a_* t}.$$

Here, $C(t)$ is the isotopic signature at any time *t* while C_0 and C_E are the isotopic signatures at the beginning and at isotopic equilibrium, respectively. Finally, the turnover constant *a*, the sum of the growth rate and the metabolic tissue replacement, can be used to calculate the half-lives, $t_{1/2}$ of the muscle:

$$t_{\frac{1}{2}} = \frac{\ln 2}{a}$$

3.3 Results and discussion

3.3.1. Feed and water samples

A summary of the SIRA results for feed and water samples is presented in Table 1. After consideration of the DM and DMD (Harrison et al., 2010), the composite experimental diet had theoretical δ^2H, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{34}S$ values of -23.6‰, -12.5‰, 25.9‰ and 8.2‰, respectively. Thus, the δ^2H, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{34}S$ values of the composite, experimental diet were elevated by 30.0‰, 10.1‰, 2.1‰ and 4.1‰ for H, C, O and S, respectively compared to the control diet.

Since the two research stations where the animals were born (Athenry, Co. Galway) and maintained during the experiment (Dunsany, Co. Meath) are located in the west and in the east of Ireland, respectively, the drinking water was depleted by -13.9‰ and -1.7‰ for H and O, respectively with the animals being exposed to lower δ^2H and $\delta^{18}O$ water values with the switch in drinking water when they were moved to the east in June 2006. In September 2006, when the experimental diet was introduced, the animals experienced a second switch in δ^2H and $\delta^{18}O$ values, but this time towards less negative δ^2H and more positive $\delta^{18}O$ values in the diet (see Table 1). It is known that drinking water affects the δ^2H and $\delta^{18}O$ values of human hair (Ehleringer et al., 2008 and Sharp et al., 2003) and of bacteria (Kreuzer-Martin, Lott, Dorigan, & Ehleringer, 2003). As the switch in drinking water preceded the switch in diet by three months, the animals may already have been partly equilibrated to the new δ^2H and $\delta^{18}O$ values in drinking water when the diet switch occurred.

3.3.2. Yields of lipids and lipid fractions

The average yield of lipid extracted from lean, freeze-dried ovine LD was 105.3 ± 37.7 g kg^{-1} DM (mean $\pm$ SD, $n = 28$). ANCOVA revealed that the yield of IML decreased with increasing duration of consumption of the experimental diet when carcass weight was used as a covariate ($p = 0.049$; $n = 28$), but did not depend on the sex or EA.

The average yields of NL, PL and FFA from the IML following SPE were estimated to be $83.0 \pm 4.8\%$, $15.0 \pm 4.4\%$ and $2.0 \pm 1.6\%$, respectively. TLC confirmed the purity of the three lipid fractions. The yields of NL and PL depended significantly on the length the animals were on the experimental diet when the carcass weight was used as a covariate ($p = 0.0094$ and $p = 0.0142$, respectively; $n = 28$) resulting in higher yields of NL and lower yields of PL

for animals slaughtered after 231 days as compared to animals slaughtered prior to the diet-switch (control).

3.3.3. Stable isotope ratios in muscle

The results of the SIRA of muscle H, C, O and S are presented in Fig. 1. The $\delta^{13}C$ values of LD (Fig. 1a) had been measured previously (Harrison et al., 2010) but were measured again to permit direct comparisons of muscle and lipid stable isotope ratios from the same samples. Fig. 1a shows clearly that the muscle sampled from animals on the HEA recorded higher $\delta^{13}C$ values than muscle from animals on the LEA ($p = 0.0003$). The application of the exponential one-pool model, described by Hesslein et al. (1993), determined the $\delta^{13}C$ half-lives in ovine LD to be 75.7 and 91.6 days for muscle collected from lambs receiving the HEA and LEA, respectively. The calculated fractionation between the muscle and the control diet at the beginning of the experiment was +1.9‰. This estimate was lower than previously determined diet-muscle fractionations of +3.0‰ in beef (Bahar et al., 2009), but slightly higher than estimates of approximately +1‰ in rodents (Arneson and MacAvoy, 2005 and MacAvoy et al., 2005).
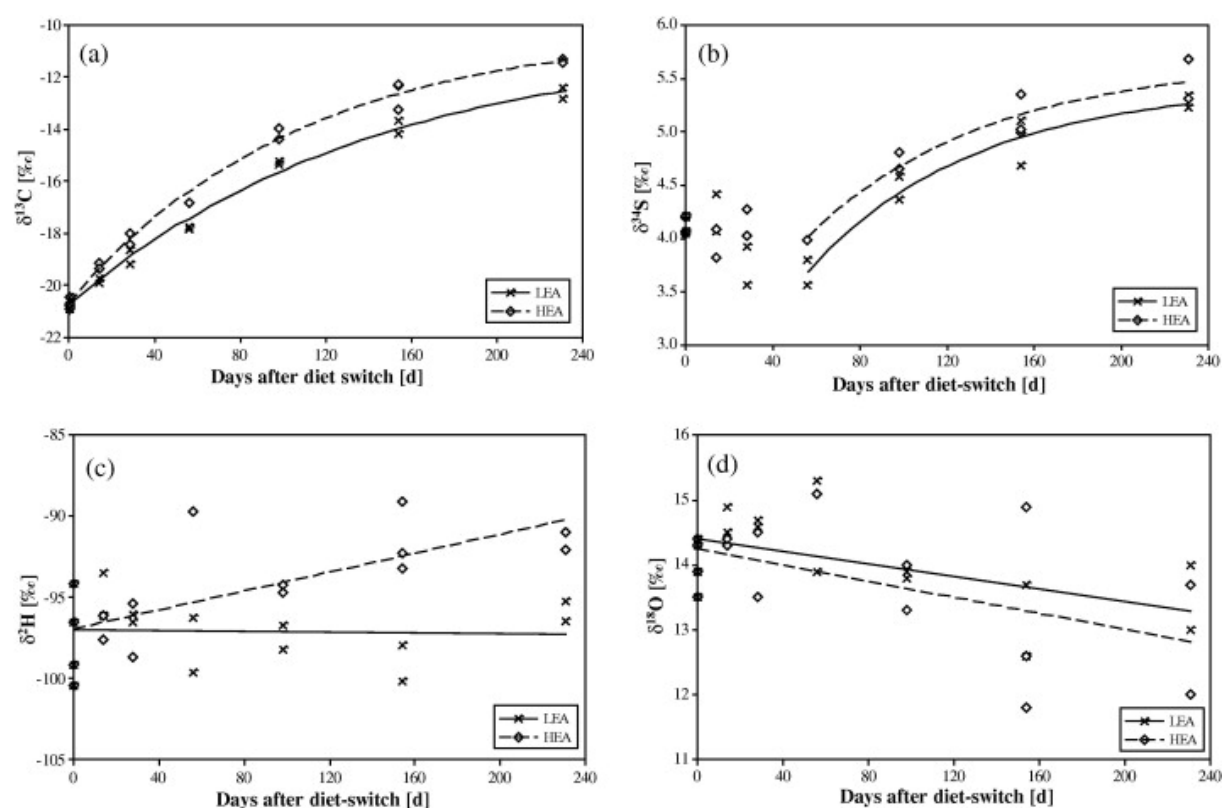
Fig. 1. The stable isotope ratios of (a) carbon, (b) sulphur, (c) hydrogen and (d) oxygen LD for lambs raised on a low (LEA) and high (HEA) energy allowance for up to 231 days.

The results of the S SIRA are presented in Fig. 1b. No diet-tissue fractionation between the defatted muscle and the control diet was found, which is in good agreement with previous observations on mice (Arneson & MacAvoy, 2005).

For N, it is known that $\delta^{15}N$ values can increase during nutritional stress (Fuller et al., 2005). Yet, to our knowledge, no such studies have been undertaken on $\delta^{34}S$ values. Our animals possibly experienced a nutritional stress after the diet switch, when they had to adapt to the new diet; this stress could explain the drop in $\delta^{34}S$ values. In a similar experiment, fish reared at two different temperatures on three different EAs of the same feed showed a similar drop in $\delta^{34}S$ values shortly after introduction to the new diet (Barnes & Jennings, 2007). However, in contrast to our experiment, only fish receiving the low EA in warm water and fish receiving the medium EA in cold water showed such a drop in $\delta^{34}S$ values. Referring to the same experiments, Barnes et al. stated that the EAs needed to be raised for fish receiving the medium and low EAs due to their low live weights (Barnes, Sweeting, Jennings, Barry, & Polunin, 2007). Therefore, we explain the drop in $\delta^{34}S$ values observed in ovine LD with the previously experienced nutritional stress after the diet switch. To determine whether this difference in $\delta^{34}S$ values for animals receiving different EAs is real, future diet-switch experiments need to give animals more time to equilibrate to the new diet.

As a result of this decrease in $\delta^{34}S$ right after the diet switch, we applied the one-pool model as described by Hesslein et al. (1993) only from day 56 in order to calculate half-lives for S. This analysis yielded half-lives of 53.3 days and 57.7 days for lambs receiving the LEA and HEA, respectively. As these half-lives are considerably shorter than those determined above for C, we also calculated the half-lives received from C SIRA from day 56 to make the S and C half-lives comparable. This analysis yielded similar half-lives for C (55.9 days and 55.0 days for animals receiving the LEA and HEA, respectively) as previously calculated for S. Therefore, we conclude that C and S half-lives are comparable in ovine LD. This is in contrast to findings by Bahar et al. (2009) who found a slower turnover of S (219 days) as

compared to C (151 days) in bovine LD and Hesslein et al. (1993) who detected a slower metabolism of S as compared to C and N in fish.

As pointed out previously, the animals experienced a change in drinking water towards more negative δ^2H and δ^{18}O values three months prior to the diet-switch. Fig. 1c and d shows the results of the H and O SIRA, respectively. Although the δ^2H values of LD showed a large spread shortly after the diet switch, linear models fitted to the data revealed a significant difference in the steepness of the slopes ($p = 0.0015$). While the linear model fitted to the data received from lambs on the LEA had a negative slope, this significant difference in slope factors suggests that dietary H is the main source contributing to muscle δ^2H values.

This finding is in good agreement with a study on the cells and spores of *Bacillus subtilis* (Kreuzer-Martin et al., 2003) which showed that only approximately 30% of the H in the bacterium was taken up from the water with the remainder having its origin in the solids of the growth medium. Experiments conducted by Sharp et al. (2003) and Ehleringer et al. (2008) who estimated that approximately 31% and 27%, respectively, of H in human hair keratin is derived from drinking water are in good agreement with the estimates made by Kreuzer-Martin et al. (2003). Furthermore, Hobson, Atwell, and Wassenaar (1999) estimated that approximately 20% of H in all metabolically active tissues such as muscle and lipid are derived from drinking water. Thus, we hypothesise that most of the H used to build ovine muscle tissue had its origin in the feed rather than in the drinking water. Therefore, the fractionation of δ^2H between the muscle and the control diet was estimated to be −44‰ at the beginning of the experiment.

In contrast to the H results, the δ^{18}O values in ovine muscle increased until day 56 and decreased thereafter until the end of the experiment 231 days after the diet-switch (Fig. 1d). The linear models fitted to the results showed negative slopes for δ^{18}O values from LD obtained from lambs receiving the LEA and HEA. However, statistical analysis showed no significant difference between slopes fitted to the results obtained ($p = 0.695$). Due to this lack of difference in δ^{18}O values between animals receiving the LEA and HEA we conclude that water was the main source of muscle O.

This finding, again, is in good agreement with findings on bacteria raised on growth media with various δ^2H and δ^{18}O values (Kreuzer-Martin et al., 2003). In their analysis of the bacterium *Bacillus subtilis*, an estimated 70% of the bacterium's O originated from the water

of the growth medium rather than from the growth medium itself. However, Ehleringer et al. (2008) estimated that only 35% of hair keratin O is derived from drinking water. These findings do not agree with the observations made in our research and we hypothesise that drinking water is the main source of muscle O. We did not, therefore, calculate any diet-tissue fractionations for O.

3.3.4. Stable isotope ratios in lipids and lipid fractions

The results of the C SIRA are presented in Fig. 2. Analysis of $\delta^{13}C$ values revealed less negative $\delta^{13}C$ for animals on the HEA than for those on the LEA for all lipid types and lipid fractions (SAT, IML, NL and PL; $p < 0.0047$). SAT, IML and NL all showed comparable diet-tissue fractionation at the beginning of the experiment (-1.3 ± 0.2‰). However, the PL fraction displayed a somewhat larger diet-tissue fractionation of -2.3‰. These fractionations were smaller than the previously observed -3‰ for lipid in gerbils (Tieszen et al., 1983).

Fig. 2. Carbon stable isotope composition determined in various lipid types and fractions (a) SAT, (b) IML, (c) NL and (d) PL from lambs raised on a low (LEA) and high (HEA) energy allowance for up to 231 days.
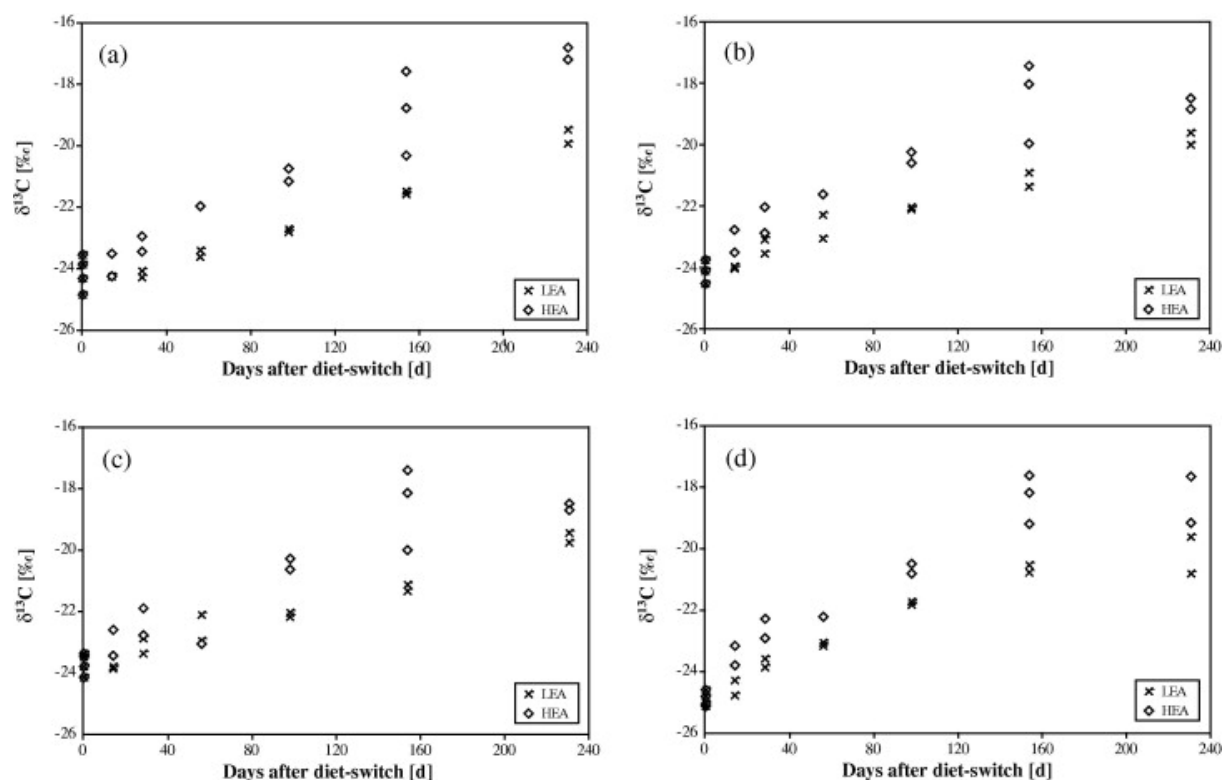
Fig. 2a shows the results of the C SIRA of SAT. Neither the δ^{13}C values of lipid samples from animals receiving the HEA nor of those receiving the LEA showed or approached a plateau. In fact, ovine SAT had only recorded a change in δ^{13}C values of 5.3‰ and 8.0‰ for animals receiving the LEA and HEA, respectively. This is much lower than the recorded changes in muscle (8.5‰ and 9.6‰ for animals fed the LEA and HEA, respectively).

Due to the linear increase in animal live weight (Harrison et al., 2010), we attributed the change in δ^{13}C values of SAT to newly produced lipid rather than turnover of existing SAT. Such a rapid increase in adipose cells in sheep starting at approximately five months of age was previously reported in lambs (Hood and Thornton, 1979 and Vernon, 1992). This increase in adipose cells followed a sigmoid curve until the sheep reached a constant adipose cell count at approximately 11 months of age. From this age on, no new adipose cells were produced. However, existing adipose cells still increased in diameter (Hood & Thornton, 1979).

Fig. 2b–d illustrates the results of C SIRA of IML extracted from ovine LD and its fractions NL and PL. Although all δ^{13}C profiles of animals receiving the HEA were approaching, or already showed, a plateau region, none of these profiles showed the expected change in δ^{13}C values of approximately +10.1‰ as a result of the diet-switch. In fact, the changes observed in δ^{13}C values for animals fed the HEA were only 7.1‰, 6.8‰ and 7.5‰ for IML, NL and PL, respectively. For animals receiving the LEA, the δ^{13}C profiles of IML and NL were still increasing, while the δ^{13}C profiles of PL appeared to approach a plateau. The observed changes in δ^{13}C values for animals fed the LEA were 4.9‰, 4.7‰ and 5.5‰ for IML, NL and PL, respectively. This slow response to new dietary C is in contrast to findings from Tieszen et al. (1983) who observed shorter half-lives in gerbil lipids than in muscle (15.6 and 27.6 days, respectively). Furthermore, the faster response of PL as compared to NL was not expected. This is in contrast to findings by Sacchetti et al. (2004) and Freysz et al. (1969) who reported half-lives of 29 h and 20–40 days for triacylglycerols in human muscle and phospholipids in rat brain tissue, respectively.

As a result of the almost linear increase of δ^{13}C values for animals on the LEA and the early showing of plateau regions in the δ^{13}C profiles of animals on the HEA, we were unable to calculate half-lives for any of the lipid fractions using the one-pool model. Furthermore, previous research also showed that even though lipids are regularly broken down and rebuilt,

products of this lipolysis are regularly re-esterfied or retained in the adipose cell (Vernon, 1980). Due to this possible re-esterfication of free fatty acids, we believe that ovine IML may never be completely turned over and will therefore always retain the signatures of past diets. Consequently, the isotope clock approach (Phillips & Eldridge, 2006) using muscle and lipid or different lipid types or fractions does not appear practicable for detecting short term dietary changes in lambs.

The results of H and O SIRA on IML are presented in Fig. 3. The δ^2H and $\delta^{18}O$ values of animals on both EAs increased from the beginning towards the end of the experiment, yet there was no significant difference between EAs. The observed increases in δ^2H and $\delta^{18}O$ values of IML indicate that the switch in diet had more influence on the δ^2H and $\delta^{18}O$ values than the switch in drinking water. These findings are in good agreement with findings by Hobson et al. (1999) who estimated that approximately 20% of lipid hydrogen are derived from drinking water. However, these observations are in contrast to the findings in muscle for which only muscle δ^2H values were strongly connected to feed δ^2H values, whereas the muscle $\delta^{18}O$ values were strongly connected to the $\delta^{18}O$ values of the drinking water (see previous section). Furthermore, the observed δ^2H and $\delta^{18}O$ values of ovine IML were in the same range as those of bovine IML reported by Heaton et al. (2008) and of quail abdominal lipids reported by Hobson et al. (1999). We estimated the fractionation between IML and the control diet to be −172.7‰ and −11.5‰ for H and O, respectively prior to the diet-switch which is significantly larger than the shift of 57–62‰ for H observed in quail lipids by Hobson et al. (1999).

Fig. 3. The changes in (a) δ^2H values and (b) $\delta^{18}O$ values in intra-muscular lipid for lambs raised on a low (LEA) and high (HEA) energy allowance for up to 231 days
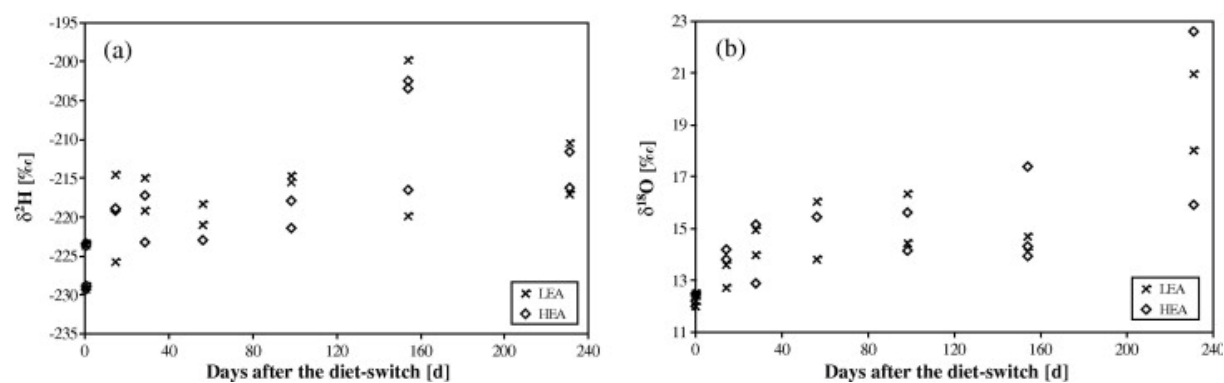
3.4. Conclusions

C SIRA of LD demonstrates the strong dependence of tissue turnover on the EA whereby tissues of animals receiving a HEA experience faster tissue turnover than animals receiving a LEA. Analysis of $\delta^{34}S$ in LD reveals no significant diet-tissue fractionation and comparable half-lives to those observed for C. Analysis of δ^2H and $\delta^{18}O$ values in LD suggests that most of the assimilated muscle H and O originates from the feed and water, respectively. Analysis of two lipid classes (IML, SAT) and two lipid fractions of the IML (NL, PL) suggests slow turnover of all analysed lipid classes and fractions, possibly due to re-esterfication of already accumulated fatty acids. Therefore, applying the isotope clock approach (Phillips & Eldridge, 2006), using muscle, muscle lipids and lipid fractions for estimating past diet-switches, appears not to be feasible in sheep. However, the diet-tissue fractionations for various elements and tissues reported here should be helpful in interpreting future food authenticity and traceability studies.

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CHAPTER IV: EFFECT OF ORIGIN, BREEDING AND PROCESSING CONDITIONS ON THE ISOTOPE RATIOS OF BIOELEMENTS IN DRY-CURED HAM

Abstract

The stable isotope ratios (SIR) of the bioelements ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) of the defatted dry matter and marbling and subcutaneous fat fractions, were assessed on 86 ham samples belonging to six different types, with the aim of ascertaining the effect of origin and production system on 11 isotopic ratios. The ham types were obtained from pigs reared in three regions, examining in every location one different production factor at two levels of expression: pig genotype (local breed *vs.* industrial hybrid) in Friuli (Italy), pig feeding regime (Bellota *vs.* Campo) in Extremadura (Spain) and ham seasoning time (mid *vs.* end) in Emilia (Italy). The isotopic composition of meteoric water and the dietary abundance of C_4 plants allowed to distinguish Italian PDO from Spanish hams. The contrasting treatments investigated within the regional batches generated promising differences in SIR for tracing the whole ham production system, including the processing procedure.

Keywords: IRMS, dry-cured ham, origin, husbandry system, pork processing

4.1. Introduction

The stable isotopes ratios (SIR) of bioelements, which depend on botanical, geographical, agronomic and climatic factors, transmitted from water and plants to animal products, have been widely proposed for meat authenticity and origin assessment. As recently reviewed by Schmidt, Rossmann, Rummel, and Tanz (2009), studies on pork are few in comparison with those on ruminant meat species. The pioneering experiments of DeNiro and Epstein (1978) presented the $^{13}C/^{12}C$ (expressed as $\delta^{13}C$ values) of the major biochemical fractions of pork, analyzed as an experimental food of flies. Mitchell, Steele, and Hare (1993) then changed the $^{13}C/^{12}C$ levels in pig tissues by switching the animals from two opposite C_3 or C_4 plant diets. Eventually the isotope ratio mass spectrometry (IRMS) was used around year 2000 to trace Iberian swine production system. González-Martin and colleagues from Salamanca University first differentiated the fresh pork from fattening Iberian pigs according to their feeding regime on the basis of $^{13}C/^{12}C$ in adipose tissue samples (González-Martin, González-Pérez, Hernández Méndez, Marqués-Marcia, & Sanz Poveda, 1999) and then, by joint analysis of

$^{13}C/^{12}C$ and $^{34}S/^{32}S$ of liver tissue, they discriminated both fattening diet and pig breed (González-Martin, González-Pérez, Hernández Méndez, & Sánchez González, 2001). Other studies on stable isotopic ratios were carried out on pigs to evaluate the mechanisms influencing the fractionation of body tissues (Nardoto et al., 2006, Tuross et al., 2008, Warinner and Tuross, 2009 and Warinner and Tuross, 2010) or individual molecular compounds (Hare et al., 1991, Howland et al., 2003 and Stott et al., 1997) and their implication for ecology and archeology. These researches make an important contribution to understanding the production factors (genetic type, age, growth rate, feeding composition, nutritive level, etc.) affecting the isotopic signatures of swine tissues and their relationship with geographical origin and breeding system. However, isotopic fractionation might occur even during pig meat processing and storage (Thiem, Lüpke, & Seifert, 2004). As claimed by Schmidt et al. (2009), who concluded their review stating that IRMS method "has so far not been applied to meat products", further experiments would be necessary in order to fully understand the influence of biochemical pathways on isotopic shifts during the manufacturing processes of meat.

Among meat products, dry-cured ham is a valuable traditional one which originated in southern European countries, where it is often guaranteed by a protected designation of origin (PDO) and represents an important part of the agro-food economy. As an example, PDO dry-cured ham (*prosciutto*) is the main product of the Italian pig industry and more than 80% of pig production is destined for the PDO traditional Italian ham market (Renaville et al., 2010). To obtain unique quality traits, the production of PDO dry-cured hams is subjected to rules established by several consortia concerning the characteristics of the raw meat (geographical origin, breed of pigs, feeding regime and rearing system) and the processing conditions (salting, curing and ripening; Toldrá, 2002).

Consequently the aim of this paper is twofold: (i) ascertain the effect of various production factors concerning both pig husbandry and thigh processing procedures on stable isotope ratio variability of the most important bioelements and (ii) confirm the efficacy of IRMS as a tool for tracing dry-cured ham origin and authenticity, by using simultaneously 11 SIR data from three ham fractions.

4.2. Material and methods

4.2.1. Experimental design

The hams were made from pigs reared in three geographical areas [Friuli, Emilia (IT), Extremadura (ES)], where different production factors were studied (Table 1). The effect of pig genetic type was examined on 36 hams from heavy pigs of two genotypes (black local breed and Goland industrial white hybrid) kept on the same diet and breeding conditions in Friuli. The influence of feeding regime was analyzed by comparing the isotope ratios of 26 Dehesa de Extremadura PDO hams, from heavy Iberian pigs fattened outdoors on grazed feedstuffs without ("Bellota" ham) or with ("Campo" ham) concentrate supplements (Sánchez del Pulgar et al., 2011). The processing influence was examined on 24 hams from industrial white hybrid, heavy pigs (Italian Large White × Italian Landrace) reared on the same farm and diet in Emilia and seasoned in three plants for two different times.

Table 1. Main characteristics of the examined ham types.

Ham Type	Pig origin	Productive factor	Factor levels	No. of hams
1	Friuli	Pig genotype	Black local breed	24
2			White industrial hybrid	12
3	Extremadura	Feeding regime	Outdoor+ supplement (Campo)	20
4			Outdoor (Bellota)	6
5	Emilia	Seasoning time	Mid (240 days)	12
6			End (405 days)	12

4.2.2. Ham samples and tissue fractions

The ham samples were collected in their processing plants at the end of seasoning (types 1–4 and 6, Table 1) or at mid-seasoning (type 5). A section of *Biceps femoris* muscle (BF) with the surrounding subcutaneous fat (SCF) was taken. The two tissues were separated and the two samples were individually vacuum-packed and frozen at −18 °C until the time of analysis, when BF and SCF were cut into small pieces. The BF pieces were dried completely with the aid of a lyophilizer (freeze-drier) and 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 (DFDM), essentially protein. The SCF pieces were directly extracted with petroleum ether for 6 h in a Soxhlet apparatus to obtain the SCF fraction. Afterwards the DFDM, FAT and SCF fractions (after evaporating the solvent) were stored in an appropriate container in a vacuum desiccator until measurement.

4.2.3. Measurements by IRMS

Measurement of the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of ham fractions was carried out as described by Perini, Camin, Bontempo, Rossmann, and Piasentier (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. The $δ^{2}H_{DFDM}$ values were corrected according to the "comparative equilibration technique" (Wassenaar & Hobson, 2003).

For the measurement of the $^{34}S/^{32}S$ ratios we used an elemental analyser (EA Flash 1112 ThermoFinnigan, Bremen, Germany) connected to an isotope ratio mass spectrometer (Delta plus XP mass spectrometer, ThermoFinnigan). The DFDM 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), quartz wool (2 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 ($δ^{34}S = +0.5‰$) and NBS 127 ($δ^{34}S = +20.3‰$), through the creation of a linear equation.

The uncertainty (2σ) of measurements was <0.3‰ for the $δ^{13}C$ and $δ^{15}N$ analysis, and respectively <3‰, <0.6‰ and <0.8‰ for the $δ^{2}H$, $δ^{18}O$ and $δ^{34}S$ values.

4.2.4. Statistical analysis

The statistical analysis of data was performed using the SPSS Statistics version 17 for Windows (SPSS Inc., Chicago, IL, USA). The data for each stable isotope ratio were summarized as mean and standard deviation values. The effect of ham type on each stable isotope ratio was investigated using ANOVA, followed by the Sidak test for multiple comparison or, in the case of unequal variance (Levene's test) in the ham type samples, using Kruskal–Wallis's test, followed by Dunnett's T3 test for post hoc comparisons. The comparison between the ratios of the same isotope in different ham fractions (e.g. $δ^{13}C_{DFDM}vs.$

$\delta^{13}C_{FAT}$ *vs.* $\delta^{13}C_{SCF}$) was performed by the GLM Repeated Measures procedure, after having verified the variance–covariance matrix sphericity by Mauchly's test. The post hoc multiple comparison tests were then performed to evaluate the significance of the pair differences across the levels of the within-subjects factor (e.g. $\Delta C_{SCF-FAT} = \delta^{13}C_{SCF} - \delta^{13}C_{FAT}$). The associate variance between pairs of isotope ratios was evaluated using the Pearson correlation coefficient, *r*.

Principal component analysis (PCA) was performed to describe dimensionality and explain the variability of the multiple data set comprising all the isotope ratios analyzed in ham fractions, as described in detail by Perini et al. (2009).

4.3. Results and discussion

4.3.1 Stable isotope ratios variability

Overall, 11 SIR data were examined in three ham fractions: defatted dry matter (DFDM) and fat (FAT) of *Biceps femoris* and subcutaneous adipose tissue (SCF). Their descriptive statistics are presented in Table 2, divided into four sub-tables, from (a) to (d).

Table 2. Descriptive statistics of the stable isotope ratios and ham type effect on their variability.

Ham fraction[i]	DFDM			FAT			SCF		
Ham type	mean	P^{ii}	sd	mean	P^{ii}	sd	mean	P^{ii}	sd
a. Carbon, $\delta^{13}C$ [‰] V-PDB									
Friuli, local breed	-19.0	c	.10	-21.0	b	.28	-20.3	c	.21
Friuli, industrial hybrid	-19.3	b	.18	-20.9	b	.27	-20.6	b	.21
Extremadura, Campo	-22.3	a	.63	-24.4	a	.76	-24.0	a	.97
Extremadura, Bellota	-22.1	a	.08	-24.7	a	.27	-24.8	a	.19
Emilia, mid seasoning	-18.5	d	.23	-19.0	c	.48	-18.6	d	.48
Emilia, end seasoning	-18.6	d	.24	-19.0	c	.30	-18.7	d	.38
Mean[iii]	-19.8	C	.17	-21.4	A	.23	-21.0	B	.24
b. Hydrogen, δ^2H [‰] V-SMOW									
Friuli, local breed	-89	b	1.8	-240	cd	3.8	-233	b	2.7
Friuli, industrial hybrid	-90	ab	3.2	-240	bcd	6.9	-233	b	2.5
Extremadura, Campo	-74	c	5.4	-245	bc	6.0	-232	b	5.3
Extremadura, Bellota	-74	c	2.9	-233	d	3.7	-223	c	2.9
Emilia, mid seasoning	-89	b	2.3	-255	a	6.6	-240	a	3.2
Emilia, end seasoning	-94	a	3.7	-247	b	3.5	-241	a	4.8
Mean[iii]	-86	C	.89	-244	A	.82	-234	B	.65

c. Oxygen, $\delta^{18}O$ [‰] V-SMOW

Friuli, local breed	10.8 [b]	.39	19.7 [b]	1.06	17.3 [b]	.47
Friuli, industrial hybrid	11.0 [b]	.46	19.0 [b]	1.84	17.2 [b]	.39
Extremadura, Campo	14.4 [c]	.91	21.3 [c]	1.12	19.7 [c]	1.11
Extremadura, Bellota	15.9 [d]	.50	25.3 [c]	2.27	20.9 [c]	.68
Emilia, mid seasoning	10.0 [a]	.50	16.5 [a]	.83	15.6 [a]	.51
Emilia, end seasoning	10.7 [b]	.42	16.7 [a]	.52	16.3 [a]	.71
Mean[iii]	11.8 [A]	0.21	19.4 [C]	0.29	17.6 [B]	0.19

d. Nitrogen, $\delta^{15}N$ [‰] AIR, and Sulphur, $\delta^{34}S$ [‰] V-CDT, in DFDM fraction[i]

Ham type	mean P^{iv}	sd	mean P^{ii}	sd
Friuli, local breed	6.2 [e]	.23	4.1 [b]	.38
Friuli, industrial hybrid	5.4 [d]	.17	4.5 [c]	.21
Extremadura, Campo	5.1 [c]	.22	4.7 [c]	.11
Extremadura, Bellota	4.6 [b]	.21	5.2 [d]	.12
Emilia, mid seasoning	4.2 [a]	.19	4.3 [b]	.12
Emilia, end seasoning	4.3 [ab]	.32	3.2 [a]	.16
Mean	5.2	.76	4.3	.58

[i]: DFDM: *Biceps femoris* defatted dry matter; FAT: *Biceps femoris* crude fat; SFC: subcutaneous fat. [ii]: ham type means (on the column) with different superscript (a,b,c,d) differ at $P \leq 0.05$ (Dunnett T3 test). [iii]: ham fraction means (on the row) with different superscript (A,B,C) differ at $P \leq 0.01$ (repeated measure design). [iv]: ham type means (on the column) with different superscript (a,b,c,d,e) differ at $P \leq 0.05$ (Sidak test).

4.3.1.1. Carbon

The mean $\delta^{13}C$ values of the various ham fractions (Table 2a) are consistent with those reported by González-Martin et al., 1999 and González-Martin et al., 2001 and by Nardoto et al. (2006). As described in ruminant species (Piasentier, Valusso, Camin, & Versini, 2003), the $\delta^{13}C$ value in protein (–19.8‰, on average) was significantly more positive than in fats (FAT: –21.4‰; SCF: –21.0‰; $P < 0.01$) as a consequence of the depletion in ^{13}C during the oxidation of pyruvate to acetyl-CoA in the biosynthesis of lipids (DeNiro & Epstein, 1977) and the possible enrichment in ^{13}C occurring during amino acid cycling (Hare et al., 1991 and Howland et al., 2003). The difference between fat fractions (0.4‰; $P < 0.01$) may result from their diversity in fatty acid (FA) composition and in the ratio between lipid assimilation and *de novo* synthesis. Indeed, while the essential linoleic acid is more abundant in SCF than in FAT (12.6% *vs*. 8.0% total lipids, respectively; Piasentier, Favotto, Saccà, Sepulcri, & Vitale, 2009) and is directly incorporated from dietary FA into the tissue without fractionation, the oleic acid is less abundant in SCF than in FAT (41.4% *vs.* 44.7% total

lipids, respectively; Piasentier et al., 2009) and is routed but also biosynthesized, and thus depleted, from the dietary biomolecular carbon sources (Stott et al., 1997).

The $\delta^{13}C$ values of protein and fat fractions were highly correlated ($r_{DFDM/FAT} = 0.96$, $r_{DFDM/SCF} = 0.97$, $r_{FAT/SCF} = 0.98$, $P < 0.01$; Table 3). In all the fractions, the main source of variability was represented by swine geographical origin, related to the dietary abundance of the C_4 plants, that explains the less negative values of Italian hams (approx. 50% of maize) in comparison with the Spanish ones, which were obtained outdoors on grazed feedstuffs, dominated by C_3 plant materials (Smith & Epstein, 1971).

Table 3. Pearson correlation between stable isotope ratios.
Pearson correlation between stable isotope ratios.

	$\delta^{13}C$ [‰]DFDM	$\delta^{13}C$ [‰]FAT	$\delta^{13}C$ [‰]SCF	$\delta^{2}H$ [‰]DFDM	$\delta^{2}H$ [‰]FAT	$\delta^{2}H$ [‰]SCF	$\delta^{18}O$ [‰]DFDM	$\delta^{18}O$ [‰]FAT	$\delta^{18}O$ [‰]SCF	$\delta^{15}N$ [‰]DFDM
$\delta^{13}C_{[‰]FAT}$	.96**									
$\delta^{13}C_{[‰]SCF}$	.97**	.98**								
$\delta^{2}H_{[‰]DFDM}$	-.87**	-.84**	-.82**							
$\delta^{2}H_{[‰]FAT}$	-.19	-.38**	-.27*	.13						
$\delta^{2}H_{[‰]SCF}$	-.53**	-.66**	-.66**	.47**	.55**					
$\delta^{18}O_{[‰]DFDM}$	-.92**	-.89**	-.89**	.89**	.27*	.56**				
$\delta^{18}O_{[‰]FAT}$	-.72**	-.82**	-.81**	.62**	.55**	.70**	.74**			
$\delta^{18}O_{[‰]SCF}$	-.88**	-.89**	-.89**	.80**	.32**	.53**	.88**	.79**		
$\delta^{15}N_{[‰]DFDM}$	.06	-.17	-.09	-.07	.46**	.33**	-.09	.23*	.11	
$\delta^{34}S_{[‰]DFDM}$	-.66**	-.69**	-.68**	.68**	.19	.55**	.63**	.64**	.59**	0.10

**significative correlations : $P \leq 0.01$. *significative correlations : $P \leq 0.05$.

In Friuli, the local breed showed a slight tendency towards an ^{13}C enrichment, compared to the white hybrid, both in protein ($\delta^{13}C_{DFDM}$ values respectively equal to $-19.0‰$ *vs.* $-19.3‰$; $P < 0.05$) and subcutaneous ($\delta^{13}C_{SCF}$: $-20.3‰$ *vs.* $-20.6‰$; $P < 0.05$) fractions. The two types of pigs differed highly in their growth rate, the local breed being characterized by a lower daily weight increment (500 g/day *vs.* 820 g/day, respectively) than the late maturing, white

hybrid, fed an identical diet and raised in the same conditions. Warinner and Tuross (2010), in a controlled diet experiment of juvenile pigs, found evidence for substantial ^{13}C enrichment in apatitic and proteinaceous tissues in a reduced growth pig as compared to controls, suggesting that growth rate is a factor that may affect fractionation in multiple tissues. Moreover, the autochthonous breed presented a deeper lard layer than industrial hybrid (45 mm *vs.* 21 mm, respectively). As a consequence of the higher rate of subcutaneous fat deposition, the local genetic types are expected to have a more diluted phospholipids (PL) content and a higher proportion of neutral lipids (NL) in their subcutaneous fat in comparison with the industrial hybrid, and thus a less negative $\delta^{13}C_{SCF}$ value, in agreement with the findings that PL fraction displays a more negative diet–tissue fractionation than NL fraction (Harrison et al., 2011).

4.3.1.2. Hydrogen isotopes

The stable hydrogen isotope composition is summarized in Table 2b. Similar to findings by Tuross et al. (2008) in pigs raised on a controlled diet and with one water source, both the fat fractions were highly ^{2}H depleted in comparison with defatted muscle (mean values −86‰ *vs.* −244‰ *vs.* −234‰, respectively for δ^2H_{DFDM}, δ^2H_{FAT} and δ^2H_{SCF}; $P < 0.01$), confirming that the hydrogen stable isotopes in protein and lipids are incorporated according different biosynthetic pathways involving isotope fractionations (Hobson, Atwell, & Wassenaar, 1999). The δ^2H of muscle fractions (DFDM and FAT) were not correlated with each other ($r = 0.13$; $P > 0.05$; Table 3) but correlated with δ^2H_{SCF} ($r = 0.47$ and 0.55, respectively; $P < 0.01$). The Extremadura hams showed a higher δ^2H_{DFDM} mean value (−74‰) in comparison with Northern Italian hams (−90‰, on average). The values are correlated ($r = 0.92$) with the annual means of deuterium isotope composition of precipitation (δ^2H_{water}) at the locations of pig breeding, estimated from the average altitude, latitude and longitude, using the prediction model available at http://wateriso.eas.purdue.edu/waterisotopes/ (average δ^2H_{water} in Extremadura, −32‰, Emilia, −43‰ and Friuli, −47‰, breeding sites; Bowen & Revenaugh, 2003). Even if only 20–30% of hydrogen body protein derives from drinking water (Hobson et al., 1999), the δ^2H_{DFDM} of hams appear to have memorized the deuterium signature of the water consumed by pigs. The relationship among δ^2H values from the meteoric water estimates and the fat fractions, particularly the intramuscular one, was low, as demonstrated by the high and significant difference between "Bellota" and "Campo" hams, the values of the

latter Spanish product being comparable with those of Italian hams. Instead, the raw fat extract was characterized by a significant dietary effect. We obtained similar findings comparing the δ^2H values of DFDM and FAT of various lamb meat types (Perini et al., 2009). Deuterium values were also affected by the seasoning time of the ham, but without a clear tendency. Indeed, during ripening, the protein fraction of hams from Emilia pigs underwent a deuterium depletion (δ^2H_{DFDM}: −89‰ *vs.* −94‰, respectively in mid and end seasoning time; $P < 0.05$; Table 2b), while fat fraction underwent an enrichment (δ^2H_{FAT}: −255‰ *vs.* −247‰; $P < 0.05$).

4.3.1.3. Oxygen isotopes

In agreement with the results of Tuross et al. (2008), the defatted muscle of ham was on average significantly depleted in ^{18}O in comparison with both types of fat (mean values: $\delta^{18}O_{DFDM} = 11.8‰$ *vs.* $\delta^{18}O_{FAT} = 19.4‰$ *vs.* $\delta^{18}O_{SCF} = 17.6‰$; $P < 0.01$; Table 2c). The oxygen isotope ratios of animal body products are correlated to that of "cell water", which by itself is a product of drinking water, oxidation water and organically bound oxygen in the diet. The shift of the $\delta^{18}O$ values of individual body compounds to that of "cell water" depends on characteristic isotope effects of the involved functional groups. The predominant functional group for oxygen in proteins is the carbonamide group, enriched in 18O by ∼22‰ versus cell water. The corresponding functional group of triglycerides is the ester group, that are enriched in 18O of ∼28‰ versus cell water (Schmidt, Werner, & Rossmann, 2001). This explain the higher δ18O values in fat. Differences in between the two types of fats may be due to different contents of phospholipids.

In all the examined ham fractions, characterized by good bivariate correlation between $\delta^{18}O$ (0.74–0.88%; $P < 0.01$; Table 3), Extremadura samples showed higher $\delta^{18}O$ values than Northern Italian ones. These differences reflect the related differences in the isotopic composition of meteoric water ($\delta^{18}O_{water}$), estimated on the basis of the geographical data, using the already cited prediction model (average $\delta^{18}O_{water}$ in Extremadura, −5.5‰, Emilia, −6.7‰ and Friuli, −7.1‰, breeding sites; Bowen & Revenaugh, 2003). As with deuterium, ^{18}O content of hams showed a geographic and climatic gradient caused by systematic global

variations in the isotope composition of precipitated water, transferred to some extent to the isotopic values of pork fractions. In fact, at least for muscle, oxygen derives mainly from that of the ingested water (Harrison et al., 2011), which for pig is mainly the local water sources, because the feedstuffs that might be imported by the modern husbandry systems into their regions are dry concentrates that contain almost no water.

However, significant variations of $\delta^{18}O$ values of protein were observed within the same region. In Extremadura, ham types from pigs supplemented with concentrates showed a lower stable isotopic oxygen ratio (Campo ham: $\delta^{18}O_{DFDM} = 14.4‰$; Table 2c) in comparison with pigs fed in the field (Bellota: $\delta^{18}O_{DFDM} = 15.9‰$; $P < 0.05$). The variation may be at least partially accounted for by a different intake of tap water, which is depleted in ^{18}O in comparison with plant water from the same region due to the evapotranspiration processes occurring in plants (Camin et al., 2008 and Kornexl et al., 1997). Indeed, pigs fed concentrates were expected to drink more depleted tap water than pigs kept on the range because field feeds contain higher amounts of plant water than concentrates (Soto et al., 2008).

Ham types from Emilia pigs presented a significant, even if close to the measurement uncertainty, effect of seasoning time, responsible for a ^{18}O enrichment of defatted muscle fraction (10.0‰ *vs.* 10.7‰, in mid and end cured hams, respectively, $P < 0.05$), probably due to biochemical or enzymatic reactions taking place during the seasoning time, such as oxidation or proteolysis and lipolysis, which can generate isotopic fractionation (e.g. Schmidt et al., 2001).

4.3.1.4. Nitrogen isotopes

The mean $\delta^{15}N$ of DFDM (Table 2d) ranged between 4.2‰ and 6.2‰, an interval basically consistent with the literature $\delta^{15}N$ values for muscles and proteinaceous materials in pigs (Hare et al., 1991, Nardoto et al., 2006 and Warinner and Tuross, 2010). The variability between ham types was accounted for by differences in the geographical origin of the pigs and, within a production region, by differences in both genetic type, in Friuli, and feeding regime, in Extremadura.

Geographical patterns in $\delta^{15}N$ values have already been found for meat (e.g. Perini et al., 2009), milk (Camin et al., 2008) and milk products (Bontempo et al., 2011 and Camin et al.,

2004). These have been attributed to the different ^{15}N content of local feeds supplied to animals which, in turn, was a consequence of climate and soil conditions and agronomic procedures in different geographical areas. However, the use of δ^{15}N values in the pork industry for tracing the origin of products requires special attention. Indeed, while ruminants are generally provided with local forages and feed sources, in modern pig production systems variable amounts of feedstuffs have a non-local origin, which might distort the inferred geographical patterns.

The Friuli local, slow growing pigs were significantly enriched in ^{15}N compared to white, fast growing hybrids (δ^{15}N$_{DFDM}$ 6.2‰ *vs.* 5.4‰; $P < 0.05$; Table 2d). In the already cited experiment on juvenile pigs, the proteinaceous tissues, particularly bone collagen and hair, of the reduced growth subject were isotopically enriched in nitrogen compared to the rest of the cohort (Warinner & Tuross, 2010), in agreement with previous studies demonstrating that depressed growth rate in a juvenile mammal can result in elevated tissue δ^{15}N levels (see literature cited by Warinner & Tuross, 2010). The difference in δ^{15}N between genetic types was probably linked to differences in protein metabolism, since increased growth rates involve an intensive production of new tissues with high rates of protein syntheses and reduced levels of protein turnover and degradation. The primary sources of nitrogen isotopic fractionation are amino acid deamination and transamination. Deamination enzymes preferentially remove amine groups with ^{14}N and generate waste nitrogen (i.e. ammonia, uric acid, or urea) which is isotopically lighter than body protein (Martínez delRio, Wolf, Carleton, & Gannes, 2009). Thus, higher δ^{15}N values in local breeds than in industrial hybrids might indicate relatively more nitrogen excretion, due to a higher ratio between protein degradation and protein synthesis. In contrast, lower δ^{15}N values in industrial hybrids suggest a lower ratio of protein degradation to protein synthesis, and relatively less nitrogen excretion. Even the higher level of δ^{15}N$_{DFDM}$ in "Campo" compared to "Bellota" hams (5.1‰ *vs.* 4.6‰; $P < 0.05$) might be at least partially linked to different nitrogen excretion caused by feeding regime (Martínez delRio et al., 2009). Pigs producing the fresh thighs for "Campo" ham probably had preferential ^{15}N retention, provided feed supplementation allowed them to ingest higher amounts of protein than pigs submitted to an extensive feeding regime to produce "Bellota" ham (Soto et al., 2008). However the difference might also be directly due to different isotopic values of the diets.

4.3.1.5 Sulfur isotopes

The range of $^{34}S/^{32}S$ ratio (Table 2d) was quite narrow, from 3.2‰ to 5.2‰, an interval comparable with that described for the liver tissue from the groups of Iberian and white swine fed on two diets (3.1‰ to 5.8‰; González-Martin et al., 2001).

Sulfur is an essential element for animal organisms that must be obtained from diet. It is predominantly found in the amino acids cysteine, methionine, and taurine as well as in various vitamins and cofactors such as thiamine, vitamin B, biotin, and coenzyme A (Richards, Fuller, Sponheimer, Robinson, & Ayliffe, 2003). As with nitrogen, in plants and animals the largest part of organic sulfur is concentrated in protein (Tanz & Schmidt, 2010). Sulfur amino acids accounted for $84 \pm 20\%$ of the sulfur in corn, wheat, alfalfa, soybean meal, fish meal, chicken meal, pork meat and bone meal (Florin, Felicetti, & Robbins, 2011). $\delta^{34}S$ was not correlated with $\delta^{15}N$ ($r = 0.11$; $P > 0.05$, Table 3). As suggested by Florin et al. (2011) the two variables are not directly and fully related, because while $\delta^{15}N$ reflects the metabolism of all amino acids, $\delta^{34}S$ reflects the metabolism of only sulfur amino acids.

The $\delta^{34}S$ values of plant organic matter are influenced by those of the soils on which it grows and reflected in those of the animals fed on it. Thus $^{34}S/^{32}S$ are generally regarded in terms of providing product signatures on a small geographic scale (Perini et al., 2009), because this element is controlled by the local bedrock (igneous or sedimentary, acidic or basic) and atmospheric deposition and is also influenced by microbial processes in the soil.

However, in our data set, unlike the other light elements, sulfur showed a relatively low variability accounted for by pig geographical origin in comparison with that due to the different procedures of pig production and ham processing followed in the various regions. In particular, $\delta^{34}S$ values were significantly lower in hams produced in Friuli from a local breed compared to white industrial hybrids ($\delta^{34}S_{DFDM}$: 4.1‰ *vs.* 4.5‰; $P < 0.05$; Table 2d) and in "Campo" compared to "Bellota" hams (4.7‰ *vs.* 5.2‰; $P < 0.05$), even if the differences are comparable with the measurement uncertainty. These findings are consistent with those highlighted by González-Martin et al. (2001), who showed lower $\delta^{34}S$ levels in liver samples from the local Iberian pigs in comparison with the white pigs fed the same diet (3.1‰ *vs.* 5.6‰) and from Iberian pigs fed commercial feed compared to those receiving acorn (3.1‰ *vs.* 5.7‰). Moreover, end-seasoned Italian hams showed slightly but significantly lower $\delta^{34}S$

values than mid-seasoned ones (3.2‰ *vs.* 4.3‰; $P < 0.05$). It is not clear if this small difference indicates a sulfur isotope discrimination occurred during biochemical processes of animal metabolism and thigh processing. A deeper evaluation of the phenomenon is thus suitable. However, the Strecker degradation of sulfur amino acids taking place during seasoning and generating dimethyl disulfide and other similar volatile compounds (Flores, Grimm, Toldrá, & Spanier, 1997) can determine isotopic fractionation (Fry, Gest, & Hayes 1988).

4.3.2. Multivariate analysis of the stable isotope ratio data set of ham

The multivariate approach allows the simultaneous analysis of the isotopic signatures from multiple fractions, the individual variability of which has been examined separately in previous paragraphs. It is useful for understanding the interrelationship between SIR of various bioelements and the level of independent information they provide, following different fractionation patterns in the different tissues and biochemical fractions.

The isotope data set was investigated using PCA, which provided a geometric representation of the data structure. The 91.7% original data variance was summarized by four uncorrelated PCs. Each of them accounted for a significant piece of information (eigenvalues 1). The scores were computed for each ham sample and plotted, together with the isotope-ratio loadings, on two bi-plots (i.e. scores + loadings plot), one for the first two PCs (Fig. 1) and the other for the last two PCs (Fig. 2). The first principal component (PC1) accounted for 52.9% of total variability and was loaded (we are only referring to the SIR data with loading values > 0.67) positively with $\delta^{13}C$ of the various ham fractions, and negatively with water elements: $\delta^{2}H_{DFDM}$ and $\delta^{18}O$ of the various fractions. Along PC1 the ham types were separated on the basis of their origin: all the Italian ham samples had positive scores while all the Spanish ones had negative scores. The second principal component (PC2) explained 17.1% of the total variance and was positively correlated with $\delta^{2}H$ of fat fractions. PC3 and PC4 (Fig. 2) accounted for a smaller and comparable proportion of total variance (11.6% and 10.1%, respectively) and were positively and highly correlated with the isotope ratio of only one bioelement each: sulfur and nitrogen, respectively. Each of the last three PCs provided a separation of the ham samples produced following an alternative procedure in the same region. Along PC2 "Bellota" hams were segregated from "Campo" ones produced in

Extremadura; on PC3 the scores of mid seasoned samples were clearly differentiated from those of the end seasoned hams, both from pigs reared in Emilia; along PC4 the hams produced in Friuli from the local breed were separated from those provided by the industrial hybrid.

Fig. 1. Principal component analysis of stable isotope ratios in dry-cured hams. Bi-plot of sample scores, grouped by ham types, and isotope ratio loadings with the first two principal components (PC); in parentheses, the percentage of the total variance explained by each PC.
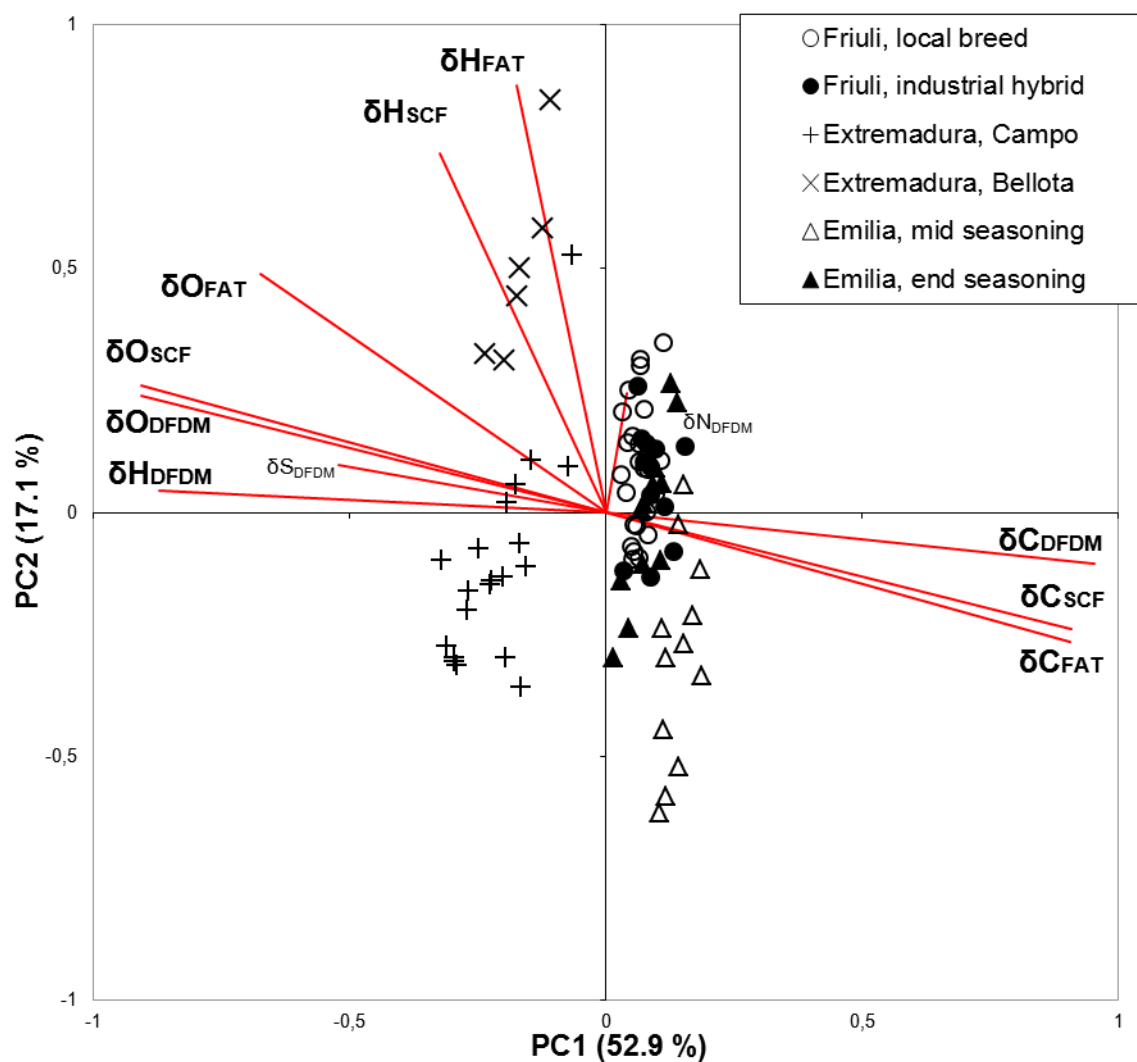
Fig. 2. Principal component analysis of stable isotope ratios in dry-cured hams. Bi-plot of sample scores, grouped by ham types, and isotope ratio loadings with the last two PCs; in parentheses, the percentage of the total variance explained by each PC
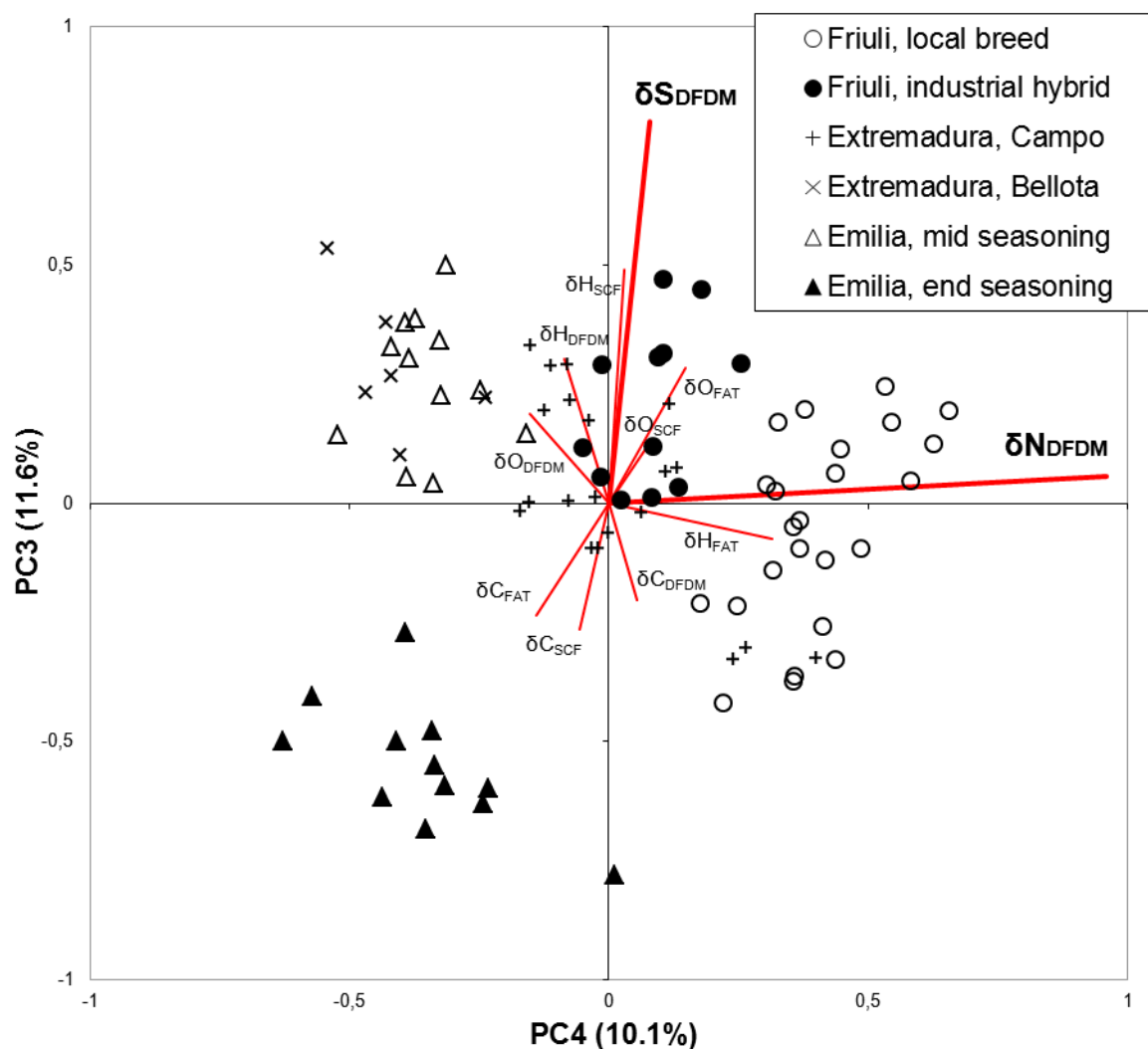
These findings are in line with those previously obtained for other meat types (Osorio et al., 2011, Perini et al., 2009 and Schmidt et al., 2009); however they were achieved with only three discrete batches of hams characterized by contrasting treatments that were associated with interesting, but generally small differences in the isotope ratios (Bahar et al., 2008). Thus, the evaluation and interpretation of the causes of isotope ratio variability has to be regarded as an initial step in confirming the potential use of SIR as a tool for tracing the whole ham production system, including the processing procedure. A greater dataset

comprising samples from a wider and representative range of geographical, seasonal, biological and technological conditions must be considered before applying multi-element SIR to the authentication of hams.

4.4. Conclusions

The variability of the stable isotopic ratios of bioelements of dry-cured ham is affected by pig origin. The production conditions and, among these, the length of the ripening–drying phase, when the ham develops its characteristic flavor and texture while drying and undergoing enzymatic hydrolysis, oxidation and chemical changes, can generate different isotope ratios. In that period the stable heavier isotopes of the protein fraction underwent an enrichment for oxygen, while the isotope trend for sulfur and hydrogen was less clear, with a small depletion of sulfur, a depletion of deuterium in protein and an enrichment in marbling fat. Further insights are needed to confirm and better understand the phenomenon, that may have considerable impact on authentication of this luxurious pork product.

The dimensionality in the structure of the isotope ratio data confirmed the usefulness of considering the main bioelements simultaneously, because either they or their linear combinations provided essential information about independent sources of ham type discrimination.

The results highlight that it is possible to use SIR of bioelements to distinguish Italian PDO hams from hams produced in other areas. A following work with a representative number of PDO hams and imitators is necessary to develop a robust traceability model usable to verify the authenticity of commercial ham and eventual fraudulent mislabeling.

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CHAPTER V: USING BIOLEMENT ISOTOPE RATIOS TO DEDUCE BEEF ORIGIN AND ZEBU FEEDING REGIME IN CAMEROON

Abstract

The aim of this study was to deal with a lack of knowledge regarding the stable isotope composition of beef from zebu cattle reared in tropical Africa. Sixty beef carcasses belonging to the most popular zebu breeds were thus selected and sampled at the slaughter house of Yaounde. The stable isotope ratios of five bio-elements - H, O, C, N and S - were analysed in protein and fat muscle fractions. Zebu beef from Cameroon has a particular isotope profile, characterised by lower $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ values than those reported in other areas of the world, as a consequence of the almost exclusive use of tropical C4 pasture grasses for cattle feeding and of the geographic and climatic gradient in the isotope composition of precipitatation water. Within Cameroon, multi-element analysis give promising results for tracing the regional origin of beef and some aspects of the cattle breeding system, such as the animal's nutritional status.

5.1 Introduction

The stable isotope ratio (SIR) of bio-elements - such as H, O, C, N and S - in muscle fractions has been extensively used for assessing beef origin and cattle production systems in many areas of the world (e.g. Boner and Förstel, 2004; Horacek and Min, 2010; Yanagi et al., 2012). However, for zebu cattle reared in tropical Africa, there is no information about isotope signatures and their variability due to geographical distribution and animal feeding systems. 70% of the population in Cameroon is economically dependent on agriculture, essentially represented by livestock production. There are around six million cattle in Cameroon, mostly belonging to the *Zebu* breed.

In order to improve meat and milk productivity for the *Gudali zebu* breed in Cameroon, international cooperation between Italy and Cameroon has recently been started up.

The aim of this study was to deal with this lack of knowledge and to describe the possible use of SIR in beef fractions to trace feeding histories in zebu cattle production systems in tropical Africa.

5.2 Materials and methods

5.2.1 Animals, carcass characteristics and sample collection

Sixty beef carcasses belonging to the most popular zebu breeds (Goudali, White Fulani and Red Mbororo) were selected and sampled at the slaughter house of Yaounde, to represent the variability of young bulls (aged between 3 and 5) slaughtered at the abattoir in the capital of Cameroon. The subcutaneous fat colour of the carcass was visually evaluated on the lateral face of the left side, using a three-level qualitative scale (white, cream or yellow; Figure 1). After chilling at 4 °C for 24 hours, a sample of *Longissimus lumborum* muscle (LM) was taken from the left side of the carcasses, by cutting a three-centimetre thick chop from the section between the 12th and 13th rib, but over the LM. The samples were divided into two sub samples, which were vacuum-packed, rapidly frozen and stored at -20 °C until the time of preparation for isotopic and FA assay.

Figure 1. The three-level scale used for visual assessment of subcutaneous fat colour.

The samples were divided according to the origin and the fat colour (linked to the type fo diet).

5.2.2 Cattle origin

Before slaughtering, the geographical and ecological origin of the cattle, was carefully recorded, along with other characteristics. The Republic of Cameroon covers an area of 475,000 square km, lying between longitudes 8° and 16° East of the Greenwich Meridian, and latitudes 2° and 13° North of the equator. There is an estimated cattle population of 6.5 million in the area, reared in five ecological zones and ten administrative regions (Figure 2).

Figure 2. Map of Cameroon showing the five agro-ecological zones and the ten administrative regions
Experimental cattle origin is summarised in Table 1.

Table 1. Origin of the cattle (see Figure 2 for geographical explanation)

Ecological zone	Region	Sampled Cattle
Guinean high savannah	Adamawa	40
Guinean high savannah	East Region	10
Western high plateaus	Northwest	10
TOTAL NUMBER		60

5.2.3 Sample preparation and analysis

The first subsample was subjected to extraction of total lipids according to Folch et al. (1957). In particular, after mincing, 1.5 g of meat were taken and added to nonadecanoic acid (C19:0), then homogenised in 30 ml of chloroform-methanol mixture (2:1 v/v) using a Ultra-Turrax T 25 basic (Ika-Werke); the tissue was then filtered by vacuum filtration using Whatmann filter paper. The extract was washed with 8.5 ml of 0.88% (w/v) KCl, mixed vigorously for 1 min, and then left overnight. The organic phase was separated and the solvents were evaporated under vacuum at 40°C. FA methyl esters were prepared using HCl methanolic (Christie, 1993). Lipid sample was mixed with 2 ml of hexane and 3 ml of HCl metanolic in 20 ml glass tubes with Teflon lined caps. The mixture was heated at 70°C for 2 hours in a metal block and cooled to room temperature; then, methyl esters were extracted in 2 ml of hexane after addition of 5 ml K_2CO_3 of 6% (w/v) and Na_2SO_4. Samples stood for 30 min and were centrifuged. The upper hexane layer was removed, concentrated under nitrogen, then diluted in hexane and stored until measurement.

Methyl ester analysis was performed using Carlo Erba gas chromatography (HRGC 5300 mega-series) fitted with an automatic sampler (Model A200S) and FID detector; 1 μl of sample was injected into the gas chromatography in split mode (split ratio 1:30). The conditions used were the following: Omegawax fused silica capillary column (30 m x 0.32 mm i.d., film thickness 0.25 μm) (Supelco Inc., Bellafonte, PA), programmed temperature from 160°C to 240°C at 4°C/min and from 200°C to 240°C at 10°C/min, and then held for 5min. Helium at 1.2 ml/min was the carrier gas used. FA methyl esters were identified using external standards and quantified using C19:0 as internal standard and expressed as a percentage of the total lipids identified.

The second subsample was 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 (DFDM), essentially protein. Afterwards the DFDM and FAT fractions were stored in an appropriate container until measurement. Measurement of the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^2H/^1H$ and $^{18}O/^{16}O$ ratios of DFDM 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. The δ^2H_{DFDM} values were corrected according to the "comparative equilibration technique" (Wassenaar & Hobson, 2003). For the measurement of the $^{34}S/^{32}S$ ratios we used an elemental analyser (EA Flash 1112 ThermoFinnigan, Bremen, Germany) connected to an isotope ratio mass spectrometer (Delta plus XP mass spectrometer, ThermoFinnigan). The DFDM sample ($\sim$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), quartz wool (2 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 the $\delta^{13}C$ and $\delta^{15}N$ analysis, and respectively <3‰, <0.6‰ and <0.8‰ for the δ2H, $\delta^{18}O$ and $\delta^{34}S$ values.

5.2.4. Statistical analysis

Statistical analysis of data was performed using the SPSS Statistics version 17 for Windows (SPSS Inc., Chicago, IL, USA). The data for each stable isotope ratio were summarised as mean and standard deviation values. The effect of beef origin and fat colour on each stable isotope ratio and fatty acid profile was investigated using ANOVA. The associate variance between isotope ratios and fatty acid profile was evaluated using the Pearson correlation coefficient, r.

Canonical discriminant analysis was carried out to evaluate whether multivariate separation for classifying beef origin could be based on the stable isotopic signatures of bio-elements and

to verify which isotope ratios contribute towards enabling this classification. The most discriminant ratios were selected by a stepwise procedure and the significance of each discriminant function was evaluated on the basis of Wilks' Lambda statistics. The success of discrimination was measured by the proportion of observations incorrectly allocated to groups, using '10-fold' cross-validation.

5.3. Results and discussion

The $\delta^{13}C_{DFDM}$ values found in Cameroonian beef (-11.8‰ ± 1.22‰; table 2) fall in the highest part of the range reported in literature. For example, similar values were recorded by Schmidt et al. (2005) in beef samples from USA (-12.3‰ ± 0.1‰) and Brazil (-10.0‰ ± 0.6‰); by Nakashita et al. (2008) and Horacek and Min (2010) also for beef produced in the USA; by Guo et al. (2010) and Yanagi et al. (2012) in cattle tissues from some provinces in China and farms in Japan. In all cases, the authors suggested that these very low $\delta^{13}C$ values may have resulted from the high content of C4 plants in cattle diet. Indeed, mean $\delta^{13}C$ values of terrestrial C3 and C4 plants are -27‰ (range -35‰ to -21‰) and -13‰ (range -14‰ to -10‰), respectively (Kelly, 2000). In our Cameroonian zebu beef samples, the low negative $\delta^{13}C$ values also reflect the almost exclusive use of tropical C4 pasture grasses as cattle feed (Pamo, 2008).

As already described for other kinds of meat (Piasentier et al., 2003; Perini et al., 2012), the $\delta^{13}C$ values of protein and fat fractions were correlated ($r_{DFDM/FAT}$=0.81; P<0.01; data not reported), even if the $\delta^{13}C$ mean value in protein (-11.8‰) was significantly more positive than that in fat (-17.8‰; P<0.01), as a consequence of the depletion in ^{13}C during the oxidation of pyruvate to acetyl-CoA in the biosynthesis of lipids and the possible enrichment in ^{13}C occurring during amino acid cycling. In the fat fraction, both the examined sources of variability – beef origin and subcutaneous fat colour – reached the threshold of significance in their effect on $\delta^{13}C$ values.

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Table 2. Stable isotope ratios (observed mean and standard deviation).

Fat colour	Region	No.	$\delta^{13}C_{[‰]DFDM}$[i] Mean	SD	$\delta^{13}C_{[‰]FAT}$[i] Mean	SD	$\delta^2H_{[‰]DFDM}$ Mean	SD	$\delta^2H_{[‰]FAT}$ Mean	SD	$\delta^{18}O_{[‰]DFDM}$ Mean	SD	$\delta^{18}O_{[‰]FAT}$ Mean	SD	$\delta^{15}N_{[‰]DFDM}$ Mean	SD	$\delta^{34}S_{[‰]DFDM}$ Mean	SD
White	Adamawa	7	-12.2	1.44	-18.7	1.87	-60.5	4.99	-174.1	6.30	17.4	0.90	23.7	1.26	4.70	0.58	8.09	0.84
	Northwest	6	-13.1	2.55	-20.9	2.53	-60.9	7.36	-172.4	7.70	17.3	0.97	22.2	0.96	5.80	0.73	8.98	1.27
	East	3	-11.3	0.70	-17.0	0.36	-66.8	5.67	-176.6	6.33	17.3	0.44	24.6	0.14	4.70	0.89	7.39	0.50
	Total	**16**	**-12.4**	**1.89**	**-19.2** ^A	**2.39**	**-61.8**	**6.19**	**-173.9** ^B	**6.58**	**17.4**	**0.82**	**23.3**	**1.35**	**5.11**	**0.85**	**8.30**	**1.11**
Cream	Adamawa	15	-11.5	0.78	-18.1	1.32	-63.2	4.23	-177.9	7.03	17.4	0.52	23.8	1.31	4.53	0.80	7.86	0.88
	Northwest	2	-11.7	1.10	-17.7	0.99	-61.1	0.81	-178.8	10.16	17.3	0.85	22.2	0.30	5.25	0.06	8.32	0.49
	East	5	-12.3	0.90	-17.6	0.98	-65.4	0.94	-178.8	6.57	16.9	0.75	24.2	1.49	4.81	0.48	7.16	0.38
	Total	**22**	**-11.7**	**0.85**	**-18.0** ^{AB}	**1.21**	**-63.5**	**3.69**	**-178.2** ^{AB}	**6.80**	**17.3**	**0.61**	**23.7**	**1.36**	**4.66**	**0.72**	**7.74**	**0.82**
Yellow	Adamawa	18	-11.3	0.67	-16.6	1.11	-62.8	5.71	-185.2	7.73	17.4	0.92	23.5	1.14	4.31	0.62	7.73	0.57
	Northwest	2	-12.0	0.30	-17.7	0.19	-64.2	3.17	-186.0	2.84	16.3	0.49	21.7	0.29	5.16	0.33	8.48	0.21
	East	2	-11.6	1.15	-15.8	1.34	-60.5	2.35	-181.2	1.94	17.9	0.35	26.9	0.18	4.33	1.12	7.79	0.87
	Total	**22**	**-11.4**	**0.70**	**-16.6** ^B	**1.12**	**-62.7**	**5.28**	**-184.9** ^A	**7.10**	**17.3**	**0.92**	**23.6**	**1.56**	**4.39**	**0.66**	**7.80**	**0.59**
Total	**Adamawa**	**40**	**-11.5**	**0.92**	**-17.6** ^{AB}	**1.58**	**-62.5**	**5.04**	**-180.5**	**8.37**	**17.4**	**0.77**	**23.6** ^B	**1.20**	**4.46** ^b	**0.69**	**7.84** ^b	**0.74**
	Northwest	**10**	**-12.6**	**2.06**	**-19.6** ^B	**2.52**	**-61.6**	**5.76**	**-176.4**	**8.80**	**17.1**	**0.90**	**22.1** ^C	**0.76**	**5.56** ^a	**0.64**	**8.75** ^a	**1.01**
	East	**10**	**-11.9**	**0.93**	**-17.1** ^A	**1.09**	**-64.8**	**3.71**	**-178.6**	**5.60**	**17.2**	**0.68**	**24.8** ^A	**1.48**	**4.68** ^b	**0.68**	**7.35** ^b	**0.52**
	Total	60	-11.8	1.22	-17.8	1.86	-62.8	4.99	-179.5	8.08	17.3	0.78	23.6	1.42	4.68	0.78	7.91	0.86

[i]: DFDM: *Longissimus lumborum* defatted dry matter; FAT: *Longissimus lumborum* crude fat.
[a,b,c]: means of the levels of a factor (on the column) with different superscript differ at $P \leq 0.05$.
[A,B,C]: means of the levels of a factor (on the column) with different superscript differ at $P \leq 0.01$.

The $\delta^2 H_{DFDM}$ values of Cameroonian zebu (-62.8‰ ± 4.99‰; table 2) are higher than those observed by Horacek and Min (2010) in beef produced in Korea, USA, Mexico, Australia, or New Zealand. These enriched hydrogen isotope ratios could be a result of both the local climate and free-range pasture feeding. Indeed, because 20–30% of hydrogen body protein derives from drinking water (Hobson et al., 1999), the hydrogen isotopic composition of beef protein is likely to have memorised the deuterium signature of the water that the zebu has consumed, which is in its turn affected by regional fractionation due to the global hydrological cycle.

Furthermore, the hydrogen present in the carbohydrates and protein of feed also contributes to the total metabolic pool of hydrogen in the animal's body. So, if cattle is fed with fresh feed , such as herbage at pasture, a significant fraction of the daily water demand is ingested with the plant material. As plant water is enriched in ^2H as compared to groundwater, due to evapotranspiration processes, the $\delta^2 H$ ratio of beef is also enriched.

As already observed in other meats, the fat fraction was highly ^2H depleted in comparison to defatted muscle (mean values -62.8‰ *vs.* -179.5‰ respectively for $\delta^2 H_{DFDM}$ and $\delta^2 H_{FAT}$; $P < 0.01$); however, the two muscle fractions were correlated with each other ($r = 0.36$; $P < 0.01$; data not reported), and the regional average values of both muscle fractions were correlated with the annual means of deuterium isotope composition of precipitation ($\delta^2 H_{water}$) in the main cities of the regions (Ngaundéré in Adamawa, Bamenda in the North-West and Bertuoa in the West) estimated from the average altitude, latitude and longitude, using the prediction model available at http://wateriso.eas.purdue.edu/waterisotopes/ (average $\delta^2 H_{water}$ in Ngaundéré, -29‰, Bamenda, -38‰ and Bertuoa, -24‰; Bowen and Revenaugh, 2003).

As with deuterium, the $\delta^{18}O_{DFDM}$ average value of zebu beef (17.3‰ ± 0.78‰; table 2) is higher than that reported for beef from other areas of the world (Nakashita et al., 2008), probably as a consequence of the geographic and climatic gradient caused by systematic global variations in the isotope composition of precipitation water, transferred to some extent to the isotopic values of beef (Harrison et al., 2011). The oxygen isotope ratios of the different muscle fractions were different (mean values 17.3‰ *vs.* 23.6‰, respectively for $\delta^{18}O_{DFDM}$ and $\delta^{18}O_{FAT}$; $P < 0.01$), probably because of the isotope effects of their predominant oxygen functional groups (i.e. the carbonamide group for oxygen in proteins, and the ester group for

oxygen in triglycerides, respectively enriched in ^{18}O by ~22‰ and ~28‰ as compared to cell water; Schmidt et al., 2001). The oxygen isotope ratios of the different muscle fractions were correlated each other (r=0.49%; P<0.01) in relation to the isotopic composition of meteoric water ($\delta^{18}O_{water}$), estimated on the basis of the geographical data, using the previously cited prediction model (average $\delta^{18}O_{water}$ in Ngaundéré, -4.9‰, Bamenda, -6.2‰ and Bertuoa, -4.4‰; Bowen & Revenaugh, 2003).

The regional $\delta^{15}N_{DFDM}$ means ranged between 4.51‰ (Adamawa) and 5.4‰ (Northwest, P<0.05; Table 2). Geographical patterns in $\delta^{15}N$ values have already been found for meat (e.g. Perini et al., 2009). They are probably caused by the different ^{15}N content of local grass on which the zebu grazes, which in turn, could be a consequence of climate and soil conditions in the different geographical areas.

The variability of $^{34}S/^{32}S$ ratio was also influenced by the regional origin of beef (8.59‰ *vs.* 7.45‰ respectively, in the north-western and eastern regions P<0.05; Table 2). The $\delta^{34}S$ values are generally regarded in terms of providing product signatures on a small geographic scale (Perini et al., 2009), because this element is controlled by the local bedrock (igneous or sedimentary, acidic or basic) and atmospheric deposition and is also influenced by microbial processes in the soil.

After having separately examined the individual variability of the isotopic signatures from multiple fractions, a multivariate approach was considered. Stepwise discriminant analysis was performed to trace beef origin on the basis of the stable isotopic signatures of the product and to verify which isotope ratios contribute towards the suitable classification of the different ham types. The results are given in Table 3, as the number and percentage of correctly classified observations. Four stable isotope ratios were selected, due to their significant contribution in the discrimination of beef origin, sorted by entering them in the following order: $\delta^{15}N_{DFDM}$; $\delta^{34}S_{DFDM}$; $\delta^{18}O_{FAT}$; $\delta^{2}H_{DFDM}$. The selected model allowed corrected empirical allocation of 81.7% of beef samples and corrected cross-validation of 75% of individual samples. These findings are in line with those previously obtained for other meat types (Perini et al., 2009; Schmidt et al., 2009; Osorio et al., 2011), even if slightly lower. This result, less clear than previous ones, is likely linked to the fact that some of the zebus sent by train from Ngaundéré (and thus recorded as of Adamawa origin) to Yaoundé for slaughtering could have travelled on foot from their actual original area in the East.

Table 3. Results of the best reclassification of beef samples from zebu of different origin on the basis of the linear discriminant functions calculated using stable isotope data

		Region	Predicted beef origin			Total
			Adamawa	Northwest	East	
Original i	Count	Adamawa	30	5	5	40
		Northwest	0	10	0	10
		East	0	1	9	10
	%	Adamawa	75.0	12.5	12.5	100.0
		Northwest	.0	100.0	.0	100.0
		East	.0	10.0	90.0	100.0
Cross-validated ii	Count	Adamawa	26	7	7	40
		Northwest	0	10	0	10
		East	0	1	9	10
	%	Adamawa	65.0	17.5	17.5	100.0
		Northwest	.0	100.0	.0	100.0
		East	.0	10.0	90.0	100.0

i : 81.7% of original grouped cases correctly classified.

ii : 75% of cross-validated grouped cases correctly classified.

At slaughter, in African environmental conditions, it is easy to identify the subcutaneous fat colour of the carcasses. It is widely known that the fat colour of beef can influence the reasons why consumers appreciate and purchase the product. As reviewed by Dunne et al. (2009) changes in fat yellowness were often attributed to animal diet and to the proportion of forage to concentrate in particular, due to the β-carotene content of the forage. Feeding grass to cattle can increase the unsaturated fatty acid of their beef (Nuernberg et al., 2005).

In our conditions all the zebu were fed exclusively on natural pasture. The colour of subcutaneous fat recorded during slaughtering was significantly related to fat content and composition (Table 4). Independently of the origin, the carcasses with yellow subcutaneous fat showed the fattest meat. The higher fat content of meat, and of carcasses, could be due to

higher herbage intake by animals, consequently leading to the yellower colour of the subcutaneous fat of carcasses. Moreover, beef from carcasses with yellow fat had a fatty acid profile characterised by the highest content of total SFA and MUFA (Table 4) and the lowest percentage of total n-3 and n-6 PUFA.

Table 4. Relationship of *Longissimus lumborum* fat content (Total lipids, TL, % dry matter) and fatty acid profile (%TL) with subcutaneous fat colour and the isotope composition of *Longissimus lumborum* fat fraction

| Subcutaneous fat colour:	Fatty acid profile				Correlation	
	White	Cream	Yellow	SE	δ^2H[‰]$_{FAT}$	δ^{13}C[‰]$_{FAT}$
no. of samples	16	22	22			
Total lipids	3.3^b	4.1^b	7.1^a	2.61	-.478**	.413**
SFA	47.1b	49.5ab	52.2a	5.12	-.514**	.335**
MUFA	33.1b	35.3b	38.3a	4.21	-.315 *	.273 *
PUFA-n3	6.4a	5.0b	3.2c	1.57	.655**	-.466**
PUFA-n6	13.4a	10.2b	6.2c	3.50	.675**	-.506**
PUFA	19.8a	15.2b	9.4c	4.95	.665**	-.483**

a,b,c : means on the row with different superscript differ at $P{\leq}0.05$.

*, **: correlation significance $P{\leq}0.05$ and $P{\leq}0.01$, respectively.

The isotopic composition of the fat fraction of muscle, and in particular the δ^2H[‰]$_{FAT}$ and δ^{13}C[‰]$_{FAT}$ values, was significantly affected by the subcutaneous fat colour (Table 4). As shown in Figure 3, zebu with white subcutaneous fat ("white type") showed a clear tendency to be more enriched in ^{2}H isotopes and more depleted in ^{13}C isotopes than the "yellow type" , while the "cream type" represented an intermediate condition. These trends were correlated with fat composition. Indeed ^{2}H enrichment and ^{13}C depletion are significantly correlated with a high PUFA content (Table 4), while ^{2}H depletion and ^{13}C enrichment are correlated with a high SFA content. These differences among groups could be due to the different fat content of carcasses. A lower phospholipid/neutral lipid ratio could be hypothesised for the yellow fat group, which also had the highest fat content in meat. Indeed, this group had the

highest content of total SFA, mainly present in the neutral fraction, and lower content of long chain PUFA, which are mainly present in the phospholipid fraction. Phospholipids, which are polar lipids, are mainly located in cell membranes, whereas neutral lipids consisting mainly of triacylglycerols, the major constituents of reserve fat, and free fatty acids are mainly stored in adipocytes (De Smet et al., 2004). In other words, as a consequence of better nutritional status and consequently a higher rate of subcutaneous fat deposition, the "yellow types" are expected to have a more diluted phospholipid (PL) content and a higher proportion of neutral lipids (NL) in their subcutaneous and marble fat in comparison with the "white types", and thus a less negative $\delta^{13}C_{FAT}$ value, in agreement with the findings that PL fraction displays a more negative diet-tissue fractionation than NL fraction (Harrison et al., 2011).

Figure 3. Distribution of beef samples from zebu carcasses with different subcutaneous fat colour, in relation to stable hydrogen and carbon isotope ratios in *Longissimus lumborum* fat fraction
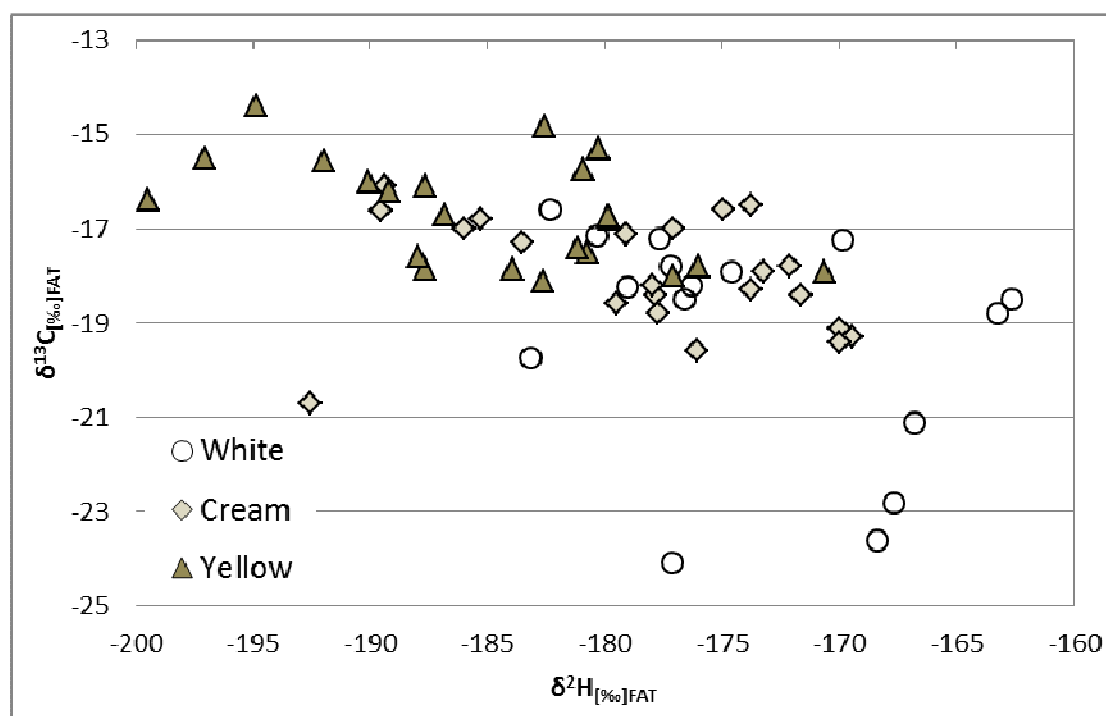
5.4. Conclusions

Zebu beef from Cameroon showed a specific isotope profile, characterised by lower $\delta^{13}C$, δ^2H and $\delta^{18}O$ values than those reported in other areas of the world, as a consequence of the almost exclusive use of tropical C4 pasture grasses for cattle feed and the geographic and climatic gradient in the isotope composition of precipitation water.

Within the country, multi-element analysis gave promising results for tracing the regional origin of beef and some aspects of the cattle breeding system, such as the animal's nutritional status.

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CHAPTER VI: FINAL CONCLUSIONS

In this thesis the effect of different factors on the stable isotope ratio (SIR) variability of the most important bioelements (^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{18}O/^{16}O, ^{34}S/^{32}S) was evaluated in different types of tissues, including pig and ovine muscle, muscle lipids and lipid fractions.

In the first experiment we demonstrated that a diet-switch (switch to two isotopically distinct diets with two different energy allowances (EAs)) caused a change in the δ^{13}C and δ^{34}S isotopic values of muscle *Longissimus dorsi* (LD) during tissue turnover (240 days). The variation was greater with a HEA experimental diet (high energy allowances).

The results showthat the δ^{18}O values in ovine muscle were largely influenced by the water ingested and their isotopic trend was independent of the type of diet (HEA or LEA) while most of the H used to build ovine muscle tissue depended on feed rather than the drinking water.

The determination of multiple stable isotope ratios (δ^2H, δ^{13}C, δ^{18}O and δ^{34}S) in ovine muscle indicated diet-muscle fractionations to be -44.0‰ +1.9‰ and 0‰ and diet-intra muscular lipid fractionations to be -172.7, -1.3‰ and -11.5‰ for H, C and O respectively, prior to the diet-switch. Analysis of the δ^{34}S in LD revealed no significant diet-tissue fractionation and comparable half-lives to those observed for C.

Applying the isotope clock approach (Phillips & Eldridge, 2006), using muscle, muscle lipids and lipid fractions to estimate past diet-switches, does not appear to be feasible in sheep. The estimated half-lives of lipids were too long to be used for tracking short term dietary changes in lambs and probably also in other meat animals. However, the diet-tissue fractionations for various elements and tissues reported here should be helpful in interpreting future food authenticity and traceability studies.

On the basis of this, it is important to consider the tissues turnover and the diet change effect in every isotopic study, to prevent possible mistake during the results evaluation.

The origin of a food product, in our case dry-cured ham and beef, as demonstrated in this thesis, can affect the variability of the stable isotopic ratios of bioelements. Moreover, in the

case of ham, the production conditions and the length of the maturing–drying phase can generate different isotope ratios. In this period the stable heavier isotopes of the protein fraction underwent oxygen enrichment, while the isotope trend for sulphur and hydrogen was less clear, with a small depletion of sulphur, depletion of deuterium in protein and an enrichment in marbling fat. Further insight is needed to confirm and better understand the phenomenon, which may have a considerable impact on authentication of this prestigious pork product.

The dimensionality in the structure of the isotope ratio data confirmed the usefulness of considering the main bioelements simultaneously, because either they or their linear combinations provided essential information about independent sources of ham type discrimination.

The isotopic effect produced by ham technical process isn't able to mask the effect of diet and geographical origin.

The results highlight that it is possible to use the SIR of bioelements to distinguish Italian PDO hams from hams produced in other areas. Subsequent studies with a representative number of PDO hams and imitators will be necessary to develop an effective traceability model which can be used to verify the authenticity of commercial ham and eventual fraudulent mislabelling.

With the analysis of Zebu beef from Cameroon we confirm the important effect of geographical origin on the stable isotope ratio variability of the most important bioelements. These samples show a specific isotope profile, characterised by lower $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ values than those reported in other regions around the world, as a consequence of the almost exclusive use of tropical C4 pasture grasses for cattle feed and the geographic and climatic gradient in the isotope composition of precipitation water.

Within Cameroon, multi-element analysis gave promising results for tracing the regional origin of beef and some aspects of the cattle breeding system, such as animal nutritional status.

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