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"Stable Isotope Ratio Analysis in the study of animal matrixes"

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Abstract

The principle of the stable isotope ratio analysis (SIRA) lies in the different amounts of isotopes of the same element in different compounds and/or products.

Among the applications of this technique, one of the most important is the one for the traceability of food products. SIRA makes it possible to obtain information about the geographical origin of different foodstuffs and about the dietary regime of the animals used to obtain products such as milk and meat, by considering the isotopes of the major bioelements, i.e., carbon ($^{12}C/^{13}C$), nitrogen ($^{14}N/^{15}N$), sulphur ($^{32}S/^{34}S$), oxygen ($^{16}O/^{18}O$) and hydrogen ($^{1}H/^{2}H$). The aim of this thesis was to apply SIRA to the study of different animal products, considering the factors that influence the isotopic ratios to better interpret the obtained results.

First, the principles of SIRA and its application through elemental analysis and gas chromatography coupled to isotope ratio mass spectrometry (EA-IRMS and GC-IRMS, respectively) are described. Furthermore, the isotopic ratios of the major bioelements, as well as their factors of influence are also considered. Moreover, four scientific works on samples of animal origin (bovine fat, cheese, edible insects, different lamb matrices) are presented and discussed.

The first work considered two groups of multiparous cull cows fed according to two different dietary regimes, based on products deriving from plants characterized by either C3 or C4 photosynthetic cycle. The different paths C3 and C4 plants follow for CO_2 fixations result in discriminating carbon isotopic ratios ($\delta^{13}C$). Therefore, the ability to distinguish between animals directly comes from the isotopic differences in the feeding regimes. In this work, different cow compartments (rumen, liver and meat) lead to the diet-based discrimination of the animals. The presented results were obtained by analysing the $\delta^{13}C$ of both the bulk lipidic extract through EA-IRMS and the single fatty acids through GC-IRMS in each compartment. Furthermore, it is worth considering that several chemical reactions resulting in isotopic fractionation (i.e., the change in the relative abundance of two isotopes of the same element during a physical or chemical process) take place in the bovine organism. On this basis, the compound-specific analysis of the fatty acids in the different compartments of the C3 cows (taken as reference group) gave the opportunity to study the fractionation processes taking place in the bovine organism, from the diet to the meat.

In the second work, sulphur isotopic values of 725 casein samples were provided. The aim of the study was to discriminate between PDO (Protected Designation of Origin) Italian cheese, not PDO cheese and foreign competitors. As for the third work, the isotopic ratios of 44 samples of edible insects were discussed. The samples were either classified as farmed insects or insect-based food items. The aim of the study was to create a preliminary dataset for this *novel food* and to attempt to discriminate between the two mentioned categories, in order to prevent future frauds concerning these new products. Finally, the fourth study was a review that aimed to collect and process, under an innovative perspective, all scientific works concerning the isotopic ratios of different lamb matrices (i.e., meat, fat, wool, plasma, erythrocytes and faeces). This work made it possible to obtain a general overview of the subject and to deepen the analysis of the factors that influence isotopic ratios in animal matrices, focusing on their diet and their geographical origin.

In conclusion, in this work SIRA represents the common thread that connects four scientific works on stable isotopes of animal matrices. The obtained results are meaningful for the development of stable isotopes research, and they also have a practical use, opening to the possibility to discriminate between different typologies of animal products, and being thus useful for traceability purposes.

Riassunto

L'analisi dei rapporti degli isotopi stabili (SIRA), basata sulla differenza quantitativa tra isotopi dello stesso elemento in composti/sostanze, è stata utilizzata nel corso degli ultimi decenni per la tracciabilità degli alimenti. Tramite la SIRA è possibile ottenere informazioni sull'origine geografica dei prodotti e sul tipo di dieta somministrata agli animali da cui tali prodotti derivano.

Nella presente tesi vengono esposti i principi su cui si basa la SIRA e le tecniche tramite cui viene effettuata, in particolar modo l'analisi elementare e la gascromatografia accoppiate alla spettrometria di massa isotopica (EA-IRMS e GC-IRMS, rispettivamente). Vengono inoltre descritti i fattori che influenzano i principali bioelementi, quali carbonio ($^{12}C/^{13}C$), azoto ($^{14}N/^{15}N$), zolfo ($^{32}S/^{34}S$), ossigeno ($^{16}O/^{18}O$) ed idrogeno ($^{1}H/^{2}H$). Infine, vengono presentati quattro lavori scientifici in cui sono stati analizzati i rapporti isotopici di campioni di origine animale (grasso bovino, formaggio, insetti commestibili e diverse matrici di agnello).

Nel primo lavoro sono stati considerati due gruppi di vacche alimentate con piante a ciclo fotosintetico C3 e C4. La diversa modalità di fissazione della CO_2 durante la fotosintesi fa sì che le piante C3 e C4 abbiano valori isotopici del carbonio ($\delta^{13}C$) distinguibili tra loro. Tale differenza nella dieta si trasmette alle matrici dell'animale. I $\delta^{13}C$ dei diversi comparti della vacca (rumine, fegato e carne) hanno permesso di distinguere gli animali in base al tipo di dieta. Tale discriminazione è stata ottenuta sia grazie all'analisi degli estratti lipidici tramite EA-IRMS, sia grazie all'analisi composto-specifica tramite GC-IRMS, valutando i $\delta^{13}C$ dei singoli acidi grassi nei diversi compartimenti. Inoltre, è da considerarsi che all'interno dell'organismo bovino avvengono reazioni chimiche che comportano processi di frazionamento isotopico (ossia la predilezione, da parte di una particolare reazione, di un isotopo rispetto ad un altro). Pertanto, tramite analisi composto-specifica dei singoli acidi grassi nelle vacche del gruppo C3, prese come campione di riferimento, è stato possibile effettuare delle considerazioni sul percorso metabolico di tali composti.

Nel secondo studio sono stati presi in considerazioni i valori isotopici dello zolfo ($\delta^{34}S$) di 725 campioni di caseina per distinguere i formaggi appartenenti a DOP italiane da prodotti nazionali senza DOP o da formaggi a pasta dura di competitori esteri. Nel terzo lavoro sono stati invece misurati i rapporti isotopici di 44 campioni di insetti commestibili, divisi in insetti da allevamento e prodotti commerciali a base di insetti. L'analisi isotopica ha permesso di

creare un primo set di valori di riferimento per questo *novel food*. È stata inoltre valutata la possibilità di discriminare tra insetti da allevamento e prodotti commerciali, in modo da fornire un primo strumento contro eventuali frodi alimentari. Il quarto studio, infine, rappresenta un lavoro compilativo che raccoglie e ripropone tutto il materiale disponibile in letteratura riguardante l'analisi isotopica di diverse matrici di agnello (carne, grasso, lana, plasma, eritrociti e feci). Lo studio ha permesso di avere un quadro generale dell'argomento trattato e di approfondire l'analisi dei fattori che influenzano i valori isotopici delle matrici animali, focalizzando in particolar modo l'attenzione sulla dieta degli animali e sulla loro origine geografica.

Nella presente tesi, in conclusione, l'analisi degli isotopi stabili rappresenta il filo comune che unisce quattro lavori riguardanti campioni di origine animale. I risultati ottenuti sono non solo significativi per lo sviluppo della ricerca dell'analisi isotopica, ma anche validi da un punto di vista pratico, aprendo alla possibilità di discriminare campioni di tipologie diverse e di poter essere utilizzati, pertanto, nell'ambito della tracciabilità dei prodotti.

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Chapter 1

Stable Isotope Ratio Analysis

1.1 Principles and definitions

Carbon, nitrogen, sulphur, hydrogen and oxygen (C, N, S, O and H) are the main elemental constituents of organic matter and have different stable isotopes (^{13}C,^{12}C; ^{15}N, ^{14}N; ^{36}S, ^{34}S, ^{33}S, ^{32}S; ^{18}O, ^{17}O, ^{16}O; ^{1}H, ^{2}H). By 1931, it had long been suspected that there were differences in the chemical properties of the isotopes of an element, but it was the discovery of the rare heavy isotopes of the light elements ^{13}C, ^{15}N, ^{17}O and ^{18}O, in 1929, and particularly that of deuterium in 1931, that led to the detection of such differences (Krouse et al., 1991). Isotopes of the same element have the same number of protons but a different number of neutrons, entailing differences in their mass and in their nuclear spin. Despite having the same number of electrons and chemically behaving the same way, isotopes present slightly different physical properties, due to the different energy they need for physical changes, such as breaking and forming bonds (Wagner, 2005). Owing to the non-equivalence in the properties of isotopes, their content in natural substances varies. The atomic weight of the same carbon ranges from 12.01144 to 12.01002 and the isotopic composition from 0.010225 to 0.011574. Even if these variations are not large, they exceed the accuracy with which they can be measured (up to ± 0.000001) by several orders of magnitude (Galimov, 1985). This characteristic is the base principle of isotopic fractionation, defined as the relative partitioning of the heavier and the lighter isotopes between two coexisting phases in a natural system (Ramkumar, 2015). Isotopic fractionation is the consequence of the redistribution of isotopes between interacting compounds, which increases the relative concentration of an isotope in one compound while decreasing it in another (Galimov, 1985).

1.2 Isotope Ratio Mass Spectrometry (IRMS)

The stable isotope ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, $^{18}O/^{16}O$, and $^{2}H/^{1}H$ are analysed through isotope ratio mass spectrometry (IRMS). According to the International Union of Pure and Applied Chemistry (IUPAC) protocol, the values are denoted in delta in relation to the international V-PDB (Vienna-Pee Dee Belemnite, fossil) for $\delta^{13}C$, V-SMOW water (Vienna-Standard Mean Ocean Water, water) for $\delta^{2}H$ and $\delta^{18}O$, Air for $\delta^{15}N$ and V-CDT (Vienna-Canyon Diablo Troilite, mineral) for $\delta^{34}S$. As a scale of isotope-delta values must be

normalized (anchored) by a substance that can be measured by other laboratories, the mentioned delta values are normalized by international measurement standards (e.g. consider the VSMOW–SLAP scale for $\delta^{2}H$ and $\delta^{18}O$ and the VPDB–LSVEC scale for $\delta^{13}C$) (Coplen, 2011). The delta values are expressed according to the following general formula:

Equation 1. $$\delta i\, E = (iR_{SA} - iR_{REF}) / iR_{REF}$$

where i is the mass number of the heavier isotope of element E (for example, ^{13}C); R_{SA} is the isotope ratio of a sample (such as for C: number of ^{13}C atoms/number of ^{12}C atoms or, as approximation, $^{13}C/^{12}C$); R_{REF} is the isotope ratio of the internationally recognized reference material. As the resulting numbers are too small to be easily used, the delta values are multiplied by 1000 and expressed in units "per mil" (‰). Currently, delta values are more often expressed in 'urey' (symbol 'Ur') that can be used with the full range of prefixes permissible in the SI system, including milli-urey instead of ‰ (Brand & Coplen, 2012). Furthermore, for isotopic ratios calculation, a two-point linear correction, made by using appropriate reference materials whose isotopic ratios include those of the samples, is carried out (Coplen, 2011).

The IRMSs are specifically designed to measure small differences in the abundances of isotopes with a high degree of precision. The two most used instrument configurations are named dual-inlet IRMS (DI-IRMS) and continuous flow IRMS (CF-IRMS). In the first case, the gases are generated off-line, and pumped into the sample bellows of the system. A second bellow contains the reference gas, and sample and reference are alternatively admitted into the ion source. On the other hand, in continuous-flow instruments, the dual inlet system is replaced by a carrier gas flow. This type of design is particularly suitable for elemental analysers, gas chromatographs, or equilibrium devices. In both cases, to carry out the measure, the analytes must be converted into simple gases such as H_2, CO, CO_2, N_2, and SO_2 before entering the ion source of an IRMS.

Although IRMS coupled with elemental analysers (that will be further described in **Chapter 1.3**) have been successfully applied in several fields, one major drawback is that it is a technique for bulk items, meaning that the resulting data are representative of the entire sample, which can contain many hundreds or thousands of different chemical compounds. Therefore, it has been observed in several studies that stable isotope ratios measured in specific compounds or fractions derived from a product are much more informative than the same parameters determined in the original bulk sample. Compound Specific Isotope Analysis

(CSIA) can be carried out, for instance, by coupling IRMS with gas chromatography, as will be discussed in **Chapter 1.4**.

All IRMS instruments use electron-impact ionization sources, a single magnetic-sector analyser, and multiple Faraday detectors for analog measurement of ion currents. Ionization sources in IRMS instruments are typically "tight", providing higher gas pressure in the ionization volume and increasing ionization efficiency. As a result, gas exchanges in the ion source are relatively slow, negating the possibility of analysing chromatographic peaks less than a few seconds in width. The energy of the ionizing electrons is typically 50–150 eV, and is adjusted separately for each gas species, to maximize both the yield of molecular ions and the linearity of the isotope ratio, i.e., minimizing changes in isotope ratio at different ion beam currents. Ions are accelerated by either a repeller plate or extraction lens, or some combination of the two. Relatively large extraction potentials – up to 10 kV – are required to minimize ion–molecule reactions in the ion source (de Groot, 2004).

Ions are accelerated to typical energies of 2.5–10 keV and are separated in a homogeneous magnetic field. All modern instruments designed for GC-IRMS use electromagnets to provide a mass range that is typically 1– 80 amu at a 3 kV accelerating voltage. The design of these instruments requires a relatively large electromagnet, and magnet hysteresis is significant. While IRMS instruments are capable of scanning across mass ranges, the process is slow (typically a1 amu/s) and they are thus always operated at a constant magnetic field to monitor a single group of masses. Ion optics are chosen to maximize ion transmission and to limit fluctuations in ion beam intensity with small changes in the magnetic field (de Groot, 2004).

Stability, rather than sensitivity, is of paramount importance for the detectors, so Faraday cups connected to high-gain (typically 10^8–10^{12}) electrometers are used for analog measurement of ion currents. The use of multiple detectors to monitor two or three masses simultaneously effectively cancels fluctuations in ion beam intensity (McKinney et al., 1950) and obviates the need for peak jumping. While the design of the Faraday detectors themselves has changed little over the past 50 years, reducing noise and stray capacitance in the electrometers remains an area of active development, particularly for D/H analyses (Sessions, 2006).

1.3 Elemental Analyser and Thermal Conversion Elemental Analyser - Isotope Ratio Mass Spectrometry (EA-IRMS and TC/EA-IRMS)

1.3.1 Principles of the technique

The bulk isotopic composition of a certain type of sample can be determined by using an elemental analyser coupled with an IRMS (EA-IRMS). Depending on the specific set-up, it is possible to measure the isotopic ratios of H, C, N, O and S in a variety of solid and liquid matrices.

In combustion mode (EA-IRMS), tin capsules used for sample introduction combust in an exothermic reaction, reaching a temperature of up to 1800°C for a few seconds. When the considered instrument is set up for simultaneous analysis, it is possible to measure C/N/S at the same time. In this case, nitrous oxides which may form during combustion are reduced by Cu to N_2; after removal of water, the gases N_2, CO_2 and SO_2 are separated in an isothermal GC column and the $^{15}N/^{14}N$, $^{13}C/^{12}C$ and $^{34}S/^{32}S$ ratios are measured sequentially. Depending on the instrument configuration, the reactors, adsorption filters and analytical columns to be used are different. The reactors can be quartz tubes or special steel tubes. The filling materials used both for reactors and adsorption filters also vary according to the analytical determination required (www.thermoscientific.com).

On the other hand, the pyrolysis or high-temperature conversion mode (TC/EA-IRMS) is employed for the analysis of the isotope ratio of hydrogen and oxygen. Through this system, the oxygen and hydrogen present in the sample are converted to CO and H_2, respectively. The process is rapid and quantitative in a reducing environment at high temperatures, typically exceeding 1400 ˚C. The reactor consists of a glassy carbon tube with glassy carbon filling, ensuring that neither sample nor reaction gases can get into contact with oxygen-containing surfaces (e.g., Al_2O_3) while at high temperatures. The reaction gases are separated in an isothermal gas chromatograph, which is also part of the TC/EA. The gases are admitted to the IRMS via the universal continuous flow interface. The continuous flow interface makes automatic sample gas dilution and the generation of reference gas pulses possible, enabling the individual referencing of each sample gas peak (www.thermoscientific.com). Samples are weighted and loaded into silver capsules and dropped into the reactor (Bicchi, 2017).

As for the analysis of $^{2}H/^{1}H$, an additional expedient must be considered. It is well established that the deuterium measurements of organic materials are problematic compared to other

isotopes analyses, due to the uncontrolled hydrogen isotopic exchange between the exchangeable organic hydrogens in the samples and those of the laboratory moisture. Therefore, if left uncorrected, δ^2H of an identical sample in laboratories in different geographic locations will yield different results. Changes in the deuterium isotopic ratios could also be detected due to geographical and seasonal changes in the hydrogen isotopic composition of ambient moisture (Wassenaar & Hobson, 2000). Therefore, a comparative-equilibration method must be used. The equilibration can be carried out by leaving both the samples and the standards at laboratory air moisture for at least 96 hours and then placing both of them in a desiccator with P_2O_5 under in a nitrogen atmosphere before the analysis (Werner & Brand, 2001). As an alternative, an offline steam equilibration method, providing deuterium measurements not influenced by uncontrolled hydrogen isotope exchange with laboratory ambient moisture, can be used (Wassenaar & Hobson, 2000, 2003).

1.3.2 EA-IRMS and TC/EA-IRMS in the present thesis

As for the EA-IRMS analyses carried out and described in this thesis, the following procedure was applied. As for $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$, the samples were weighed using a microbalance (XM1000P Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany), placed into tin capsules and loaded into an IRMS (IsoPrime, Isoprime Limited, Germany) after total combustion in an EA (VARIO CUBE, Isoprime Limited, Germany). The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ isotopic values were calculated against international reference materials or in-house working standards (ST), through the creation of a linear equation. In-house standards ST1 ($\delta^{13}C$=-23.51‰, $\delta^{15}N$=6.56‰), ST2 ($\delta^{13}C$=-21.98‰, $\delta^{15}N$=7.38‰), ST3 ($\delta^{34}S$=0.41‰) and ST4 ($\delta^{34}S$=-24.96‰) have been used, being calibrated against international reference materials, namely, for $^{13}C/^{12}C$: fuel oil NBS-22 ($\delta^{13}C$=-30.03‰), sucrose IAEA-CH-6 ($\delta^{13}C$=-10.45‰) (IAEA-International Atomic Energy Agency, Vienna, Austria), and L-glutamic acid USGS 40 ($\delta^{13}C$=-26.39‰ and $\delta^{15}N$=-4.52‰ (U.S. Geological Survey, Reston, VA, USA); for $^{15}N/^{14}N$: L-glutamic acid USGS 40 and potassium nitrate IAEA-NO3 ($\delta^{15}N$=+4.7‰); for $^{34}S/^{32}S$: Barium sulphates IAEA-SO-5 ($\delta^{34}S$=+0.5‰) and NBS 127 ($\delta^{34}S$=+20.3‰). The maximum standard deviations of repeatability accepted were 0.3‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$.

On the other hand, as for the TC/EA-IRMS analyses performed to measure the $^2H/^1H$ and $^{18}O/^{16}O$ ratios, the samples were weighed into silver capsules and measured simultaneously. This was done by using an IRMS (Finnigan DELTA XP, Thermo Scientific) coupled with a pyrolyzer (Finningan DELTA TC/EA, high temperature conversion elemental analyser,

Thermo Scientific). Each sample was weighted and analysed in duplicate. The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotopic values were calculated against two international reference materials, namely Keratins CBS (Caribou Hoof Standard $\delta^{2}H$=-157 ± 2‰ and $\delta^{18}O$=+3.8 ± 0.1‰) and KHS (Kudu Horn Standard, $\delta^{2}H$=-35 ± 1‰ and $\delta^{18}O$=+20.3 ± 0.2‰) from U.S. Geological Survey, used to normalise$^{18}O/^{16}O$ and $^{2}H/^{1}H$ values. The maximum standard deviations of repeatability accepted were 0.5‰ for $\delta^{18}O$ and of 4‰ for $\delta^{2}H$.

1.4 Gas Chromatography - Isotope Ratio Mass Spectrometry (GC-IRMS)

1.4.1 Principles of the technique

The premise for the use of a gas chromatography – isotope ratio mass spectrometry (GC-IRMS) system is that the compounds constituting the sample mixture are suitable for GC analysis, that is, they are volatile and thermally stable. Polar compounds such as fatty acids (FAs) are not volatile enough to be analysed directly through GC-IRMS. Therefore, they require a derivatization process, that is, a chemical reaction that converts the fatty acids in the corresponding esters and makes them more volatile and thus more suitable for the GC analysis. As the described reaction entails the addition of one or more carbons to the starting fatty acid, depending on the derivatization agent used, the carbon isotopic ratio of the just mentioned reactant must also be determined and a correction to the carbon isotopic ratio of the analysed fatty acid must be considered (Bicchi, 2017). The correction procedure will be further described in **Chapter 1.4.2**.

Analytical systems for stable isotope detection of gaseous analytes are commercially available and are commonly sold as three discrete units: the GC, the IRMS and the interface between them. A generic system is shown in **Figure 1**. Interfaces are sold either as combustion or pyrolysis devices, with both flavors commonly incorporated into a single apparatus. Most reports today use acronyms describing the nature of the GC-to-IRMS interface, including GC-C-IRMS for combustion or GC-P-IRMS (or GC-py-IRMS) for pyrolysis (Sessions, 2006). In the GC-IRMS instrument, the compounds eluted from the chromatography column are carried by the helium flow, combusted in the interface, and carried into the source of the IRMS. At the very beginning, only ^{13}C could be identified (W.A. Brand, K. Habfast, M. Ricci, 1989), but later modifications were made that made it possible to measure the N, O, and H isotope ratios (Brand et al., 1994; Hilkert et al., 1999). Carbon and nitrogen compounds eluting from the chromatographic column are then directed towards a combustion reactor (an alumina tube

containing Cu, Ni and, Pt wires maintained at 940°C) where they are oxidatively combusted, passing through it. This is followed by a reduction reactor (an alumina tube containing three Cu wires maintained at 600°C) to reduce any nitrogen oxides to nitrogen (Merritt et al., 1995). On the other hand, for hydrogen and oxygen, a high-temperature thermal conversion reactor is required (Begley & Scrimgeour, 1997). After GC separation, the hydrogen bound in organic compounds must be quantitatively converted into gaseous H_2 before being analysed in the IRMS. Quantitative conversion is achieved by high-temperature conversion (T > 1400 °C) (Hilkert et al., 1999).

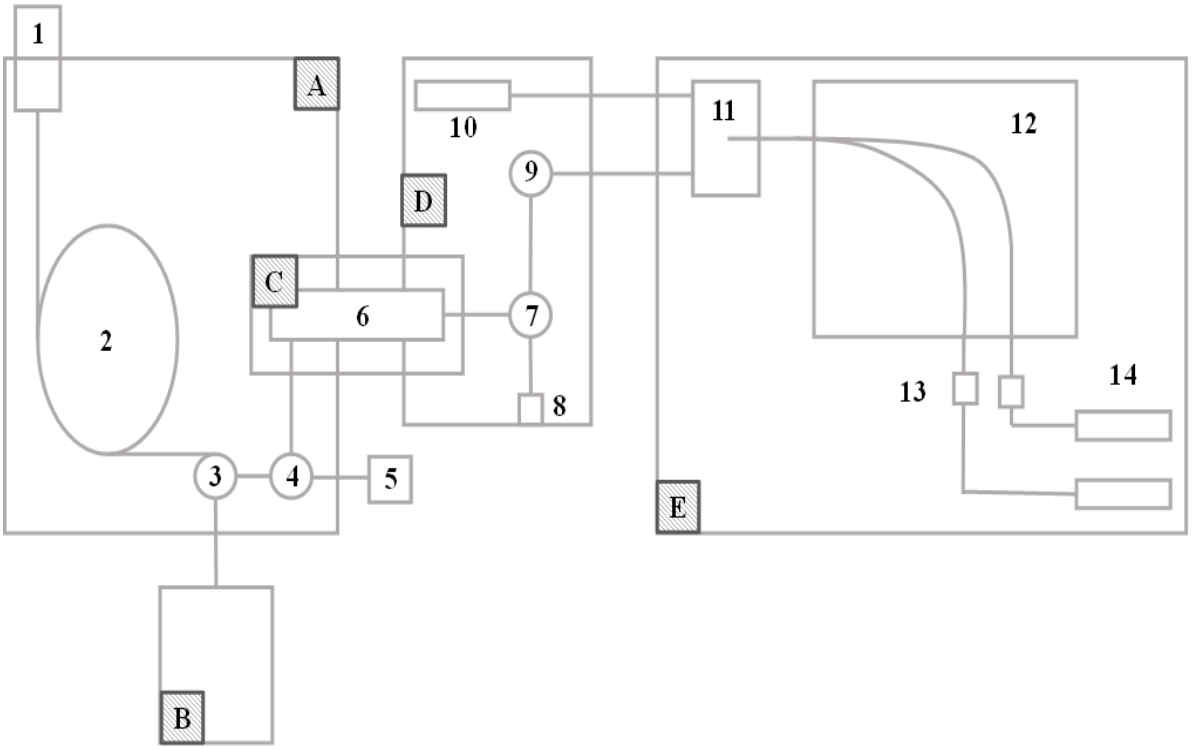


Figure 1. Schematic of a typical GC-IRMS system. The major components are **A**. Gas chromatograph, **B**. Single-quadrupole mass spectrometer, **C**. Gas Chromatograph Conversion Unit, **D**. Communication Unit, **E**. Isotope Ratio Mass Spectrometer. Other components are **1**. injector, **2**. analytical column, **3**. first splitter, **4**. second splitter, **5**. flow measuring point, **6**. combustion/pyrolysis reactor, **7**. unions, **8**. backflush valve, **9**. open split, **10**. reference gas injector, **11**. electron impact ionization source, **12**. magnetic-sector mass analyser, **13**. Faraday detectors, **14**. analog electrometers.

1.4.2 GC-IRMS analyses in the present thesis

In this thesis, this gas chromatographic technique in its combustion configuration (GC-C-IRMS) has been used for the CSIA of fatty acids of bovine fat (Pianezze, Corazzin, Perini, Camin, et al., 2021), as it will be described in **Chapter 3**. In particular, the instrument set-up is described as follows. The GC (Trace GC Ultra, Thermo Scientific, Bremen) was equipped

with an autosampler (Triplus, Thermo Scientific, Bremen) and with a BPX-70 polar fused silica capillary column (60 m length, 0.32 mm internal diameter, 0.25 μm film thickness) supplied by Trajan Scientific and Medical (Victoria, Australia). The BPX-70 column made it possible to obtain a good chromatographic resolution. The GC injector temperature was set at 250 °C. The GC oven program started at 50 °C for 4 min; then, the temperature was ramped up increasing by 30 °C/min to 170 °C (hold time 0 min), 2 °C/min to 200 °C (hold time 0 min) and finally 1 °C/min to 210 °C (hold time 8 min). Helium (5.0) was used as carrier gas with a constant flow rate of 2.0 mL/min. The GC column was thus connected to a first splitter, dividing the flow in two parts and sending 1/10 of it to a conventional MS, while the remaining 9/10 of the flow were sent to the IRMS.

The MS was a single quadrupole (ISQ Thermo Scientific, Bremen) and was used to identify the compounds exiting the GC column. The sample was carried from the GC to the MS through a transfer line at a temperature of 250°C. The temperature value of the MS ion source was set to 250°C too. The GC-MS analyses were performed in full scan mode (m/z 35-600). The different compounds were identified by comparing the mass spectra acquired at 70 eV with the NIST library data (NIST Standard Reference Database 1A NIST/ EPA/NIH Mass Spectral Library (NIST 08) and NIST Mass Spectral Search Program (Version 2.0f)).

As for IRMS analysis, it is worth highlighting that the single compounds exiting the GC column must be converted into CO_2 to obtain their carbon isotopic ratio. Thus, the compounds were first combusted into CO_2 and H_2O in a combustion furnace reactor (GC IsoLink II Conversion Unit), set at 1030 °C and consisting of a non-porous alumina tube (320 mm long) containing three wires (Ni/Cu/Pt, 0.125 mm diameter, all 240 mm long) braided and centred end-to-end within the tube. Water was subsequently removed through a Nafion dryer (integrated in the IsoLink) before the analyte gases were transferred to the IRMS via ConFlo IV universal continuous flow interface (Thermo Scientific, Bremen), which makes it possible to use reference gases for standardization instead of reference bulk material.

As previously described in **Chapter 1.4**, the FAs must be converted in the corresponding esters, which are more volatile compounds, in order to be analysed through GC-C-IRMS. In this thesis, the FAs were converted to fatty acid methyl esters (FAMEs) by using methanol as the derivatization agent, as it will be further described in **Chapter 3.2.2**. Therefore, beside the correction of the instrumental drift and the two-point linear one, a further correction must be taken into account, in order to consider the contribution of the derivatization agent on the $\delta^{13}C$

of the analyte. Indeed, the $\delta^{13}C$ values of the FAMEs relate to the FAs carbon content and to the contribution of the methanol used for the derivatization. Thus, the $\delta^{13}C$ values were calculated first against two international reference materials injected separately before and after each analytical run: Eicosanoic Acid Methyl Esters USGS70 ($\delta^{13}C$=-30.53‰) and USGS71 ($\delta^{13}C$=-10.5‰). The values were denoted as described in **Equation 1** of **Chapter 1.2**. Second, the correction due to the reagents used in the derivatization process was considered. An empirical correction was applied to determine the actual $\delta^{13}C$ values of FAs ($\delta^{13}C_{FA}$, in **Equation 2**) by applying the following equation:

Equation 2. $$(C_n + 1)\,\delta^{13}C_{FAME} = C_n\delta^{13}C_{FA} + \delta^{13}C_{Me}$$

where $\delta^{13}C_{FAME}$, $\delta^{13}C_{FA}$ and $\delta^{13}C_{Me}$ are the carbon isotopic values of the FAME, FA and methyl group of methanol (Me), respectively. The C_n value is the number of atoms of carbon in the FA, and the $\delta^{13}C$ of Me (-35.8‰ ± 0.1‰) was determined using EA-IRMS against two international reference materials, as reported in **Chapter 1.3.2**.

Chapter 2

Factors of Influence on the isotopic ratios of the major elements of animal matrices

2.1 Carbon isotopic Ratio

The carbon stable isotope ratio ($\delta^{13}C$) of the different animal matrices (e.g., meat or milk) is influenced by the photosynthetic pathway of the plants in the animal meal (Galimov, 1985). According to the way the plants carry out CO_2 fixation, they can be categorized in three groups: C3 (which is the most represented), C4 and CAM plants. C3 plants (such as rice, beet, wheat, barley) carry out the photorespiration only through the Calvin cycle. The first step of this pathway is the fixation of CO_2 by the carbon-fixing enzyme RuBisCO, which produces the three-carbon compound (3-PGA), that gives the C3 plants their name (O'Leary, 1988).

On the other hand, C4 (such as maize, cane and sorghum) and CAM (such as pineapple and agave) plants found an alternative to the previously described photosynthetic pathway, in order to minimize photorespiration. The C4 photosynthesis first produces a four-carbon intermediate (oxaloacetate) through PEP carboxylase, a non-RuBisCO enzyme. The compound thus formed is converted into a three-carbon intermediate (malate) which will be therefore involved in the Calvin cycle, exactly as for C3 plants. Finally, CAM plants use the crassulacean acid metabolism (CAM) pathway to minimize photorespiration, collecting sunlight during the day and fixing carbon dioxide at night (O'Leary, 1988).

It is known that C4 plants have $\delta^{13}C$ values between -14 and -12‰, while C3 plants values range between -30 and -23‰ (Knobbe et al., 2006; Molkentin & Giesemann, 2007, 2010). Forages and concentrates included in animal diets may derive from both C3 and C4 plants. In temperate areas, forages, both directly grazed or consumed in stable, are usually constituted by C3 plants, while in subtropical and tropical areas C4 plants are the most common (**Figure 2**). This phenomenon provides a basis for discriminating meat from different geographical origins, considering that forages are grazed or consumed where they grow. However, the concentrate feedstuffs supplementing the forage-based diets complicate the discrimination, because they are commercialized and used out of their harvesting area and are based on both C3 and C4 plants (Camin et al., 2007).

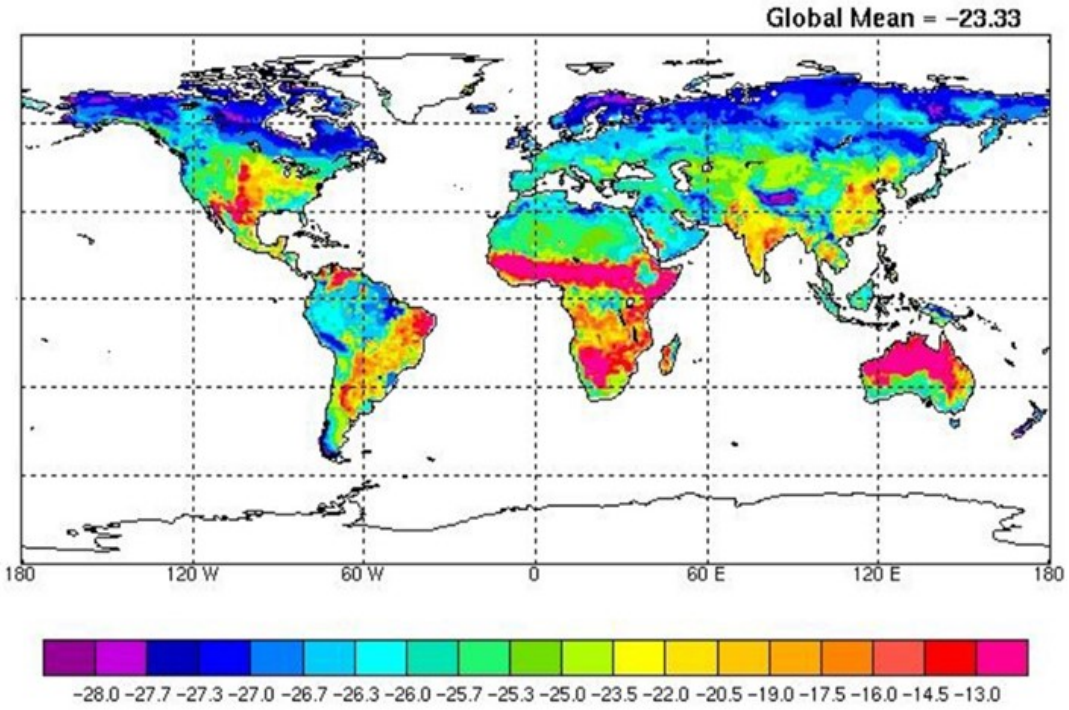


Figure 2. Model estimates of plant organic $\delta^{13}C$ (Suits et al., 2005)

Several environmental factors (Sun et al., 2012), as well as the climate of the regions the vegetable basis of the diet grew on, may also affect the carbon isotopic ratio of animal components. In particular, as high humidity results in high C isotope fractionation during plant biosynthesis, the $\delta^{13}C$ of humid regions has been reported to be more negative than that of arid ones (Camin et al., 2007). Indeed, plants normally exhibit higher $\delta^{13}C$ values when high temperatures, low air humidity and a high ground-water deficit lead to narrower stomatal apertures in the leaves of plants (Barbour et al., 2000).

2.2 Nitrogen Isotopic Ratio

The nitrogen stable isotope ratio ($\delta^{15}N$) in animal matrices depends on several parameters. The trophic level leads to an increase of $\delta^{15}N$ with each passage from the simplest to the most complex form, an example of which is a lamb suckling from the sheep (O'Brien, 2015). Also, the environmental conditions influence the nitrogen isotopic ratio not only of plants but also of the animals that feed on them, high aridity being associated with higher $\delta^{15}N$. Indeed, it was reported that warm and arid regions can result in food commodities with relatively high nitrogen isotopic ratios (Camin et al., 2004). On the other hand, the inclusion of leguminous in animal diet can lead to lower $\delta^{15}N$, as they are nitrogen-fixing plants (Perini et al., 2009). Furthermore, the nitrogen isotopic ratio depends on the soil fertilizers. In particular, synthetic

fertilizers, produced from atmospheric nitrogen via Haber process, have $\delta^{15}N$ values between -4 and +4 ‰, while organic fertilizers are characterised by values between +0.6 and +36.7‰ (Bateman et al., 2007; Otero et al., 2004). Again, this influences the plants that are fertilized and therefore on the animals that feed on them. Finally, seaweed or other products from the marine ecosystem are characterised by relatively high ^{15}N contents (Rossmann et al., 2000), therefore their inclusion into animal diet may contribute to enhance the nitrogen isotopic ratios of their matrices. A summary diagram of all the factors influencing the nitrogen isotopic ratio in plants and soil is provided in **Figure 3**.

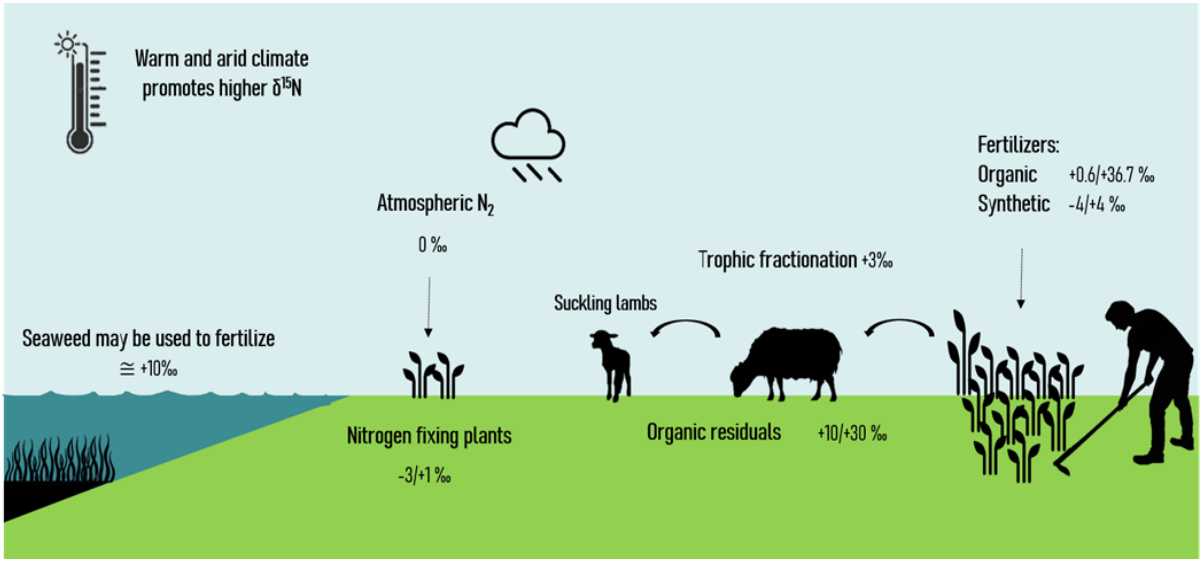


Figure 3. Parameters influencing the $\delta^{15}N$ in plants and soil

2.3 Sulphur Isotopic Ratio

The sulphur isotopic ratios ($\delta^{34}S$) of animals tend to reflect the values of the plants they fed on. Indeed, the sulphur trophic shift between animals and their diet was estimated in literature between 0‰ and 1‰ (Harrison et al., 2011; McCutchan et al., 2003; (Tanz) et al., 2015). In turn, the $\delta^{34}S$ values of the plants are influenced by many factors, such as the active microbial processes taking place into the soil, the aerobic or anaerobic growing conditions (Rubenstein & Hobson, 2004) of the plant itself and also the anthropogenic impact, regarding the sulphur released from industries and other human made sources (Böttcher, 2001; Strauch et al., 2001). Furthermore, chemical fertilizers that contain abundant sulphur can modify the $\delta^{34}S$ values of the native soils. Thus, available sulphates in the soils under intensive agricultural use come from both native and anthropogenic sources (Mizota & Sasaki, 1996).

Moreover, the geology of the soil the plant grew on can influence their isotopic ratios. The original $\delta^{34}S$ of meteoric sulphur is assumed to be 0‰, while environmental processes involving the oxidation or reduction of sulphuric compounds alter this value resulting in large fractionation effects. The majority of the measured sulphur isotopic values range between −20 and +30‰, nevertheless extreme values of −50‰ as well as + 40‰ have been observed (Fritz & Fontes, 1980), resulting from the reduction of sulphates or oxidation of sulphides with fractionation exceeding a $\Delta^{34}S$ of 25‰ (Böttcher, 2001).

Another factor influencing the $\delta^{34}S$ of plants is the closeness to the sea (sea-spray effect). Sulphur in aquatic environments is mainly present as sulphate (SO_4^{2-}) that, in modern oceans, is characterised by a $\delta^{34}S$ of +20.3‰ (Ault &Kulp, 1959; Rees, 1978; Shima&Thode, 1961). Furthermore, it is well known that one of the main natural sources of sulphate is sea salt (Wadleigh et al., 1994), that can be spread into the atmosphere by the oceans both by the mechanical disintegration of water into small, quickly evaporating drops (Liss&Slinn, 1983; Maclntyre, 1970) and by gaseous diffusion through the air/water interface (Zafiriou&Morari, 1985). In this way, natural sulphates coming from the sea can influence the $\delta^{34}S$ of plants growing in coastal areas and therefore the values in the animals fed on these plants.

2.4 Hydrogen and Oxygen Isotopic Ratios

The isotopic ratios of hydrogen and oxygen ($\delta^{2}H$ and $\delta^{18}O$) in animal matrices reflect those of drinking water and food in different proportions. As reported in literature, the $\delta^{2}H$ and $\delta^{18}O$ of water are strictly related parameters (Araguas et al., 1996) that depend on variables such as latitude, altitude, closeness to the sea (Bowen et al., 2005). It is, indeed, established that the heavy isotope content of precipitated water and snow varies noticeably and systematically across the globe, providing a label that is transferred into animal tissues through their diet and drinking water (Araguas et al., 1996) (**Figure 4**).

The feed turned out to be the main source of animal muscle H (Hobson et al., 1999; Sharp et al., 2003). Nevertheless, Perini et al. proved that even though only around 30% of body protein hydrogen derives from drinking water (Araguas et al., 1996), the H isotopic composition of defatted dry mass memorize the deuterium signature of meteoric water (Perini et al., 2009).

Drinking water showed to be the main source of muscle oxygen in animals (Biondi et al., 2013; Mekki et al., 2016; Perini et al., 2009). Furthermore, in addition to the geo-climatic aspects that derive from this correlation, the $\delta^{18}O$ values of defatted dry mass are reported to be related to

the animals' feeding regime. The $\delta^{18}O$ also depends on oxygen contained in the air, even though this has little effect on $\delta^{18}O$ due to the fairly constant isotopic values of atmospheric oxygen (Hobson & Koehler, 2015).

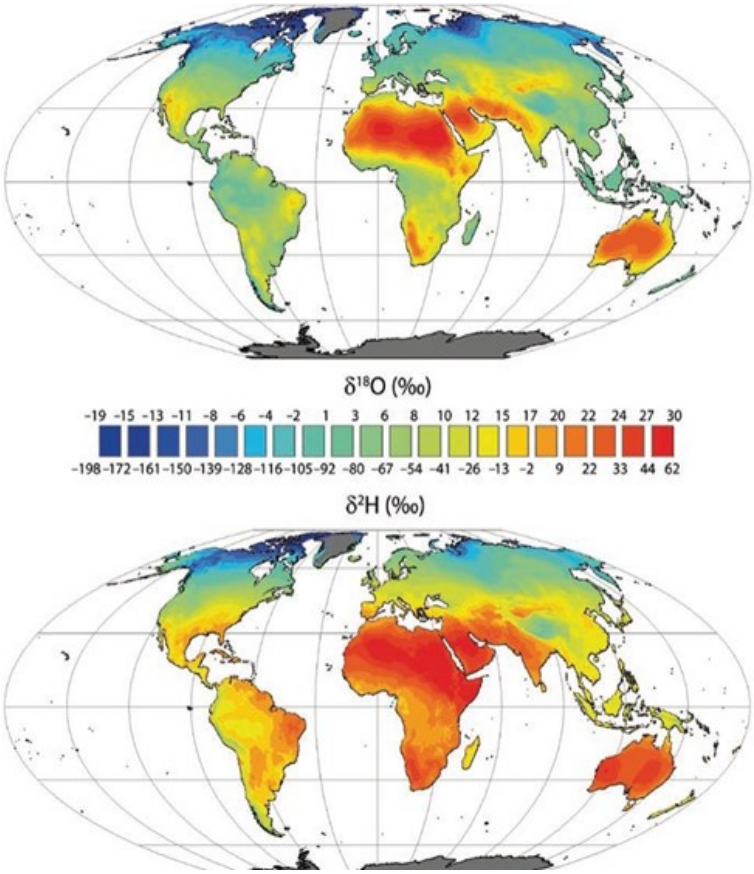


Figure 4. Global mean annual average leaf water $\delta^{18}O$ and $\delta^{2}H$ isoscapes for the sites of evaporation within leaves(West et al., 2009)

Chapter 3

Stable isotope ratio analysis to detect differences in four matrices of Simmental cows fed on C3 and C4 diets and to study the fractionation processes in the bovine organism

3.1 Background

Fatty acids, carboxylic acids with a long aliphatic chain present in both adipose tissue and muscle of animals, strongly contribute to various aspects of meat quality and are central to its nutritional value (Wood et al., 2008). In particular, the intramuscular FAs content was shown to affect the flavour, juiciness, tenderness and visual characteristics of meat (Miller, 2002). The FAs composition determines the firmness/oiliness (because the different FAs have different melting points) of adipose tissue and the oxidative stability of muscle, which in turn affects flavour and muscle colour (Wood et al., 2008).

As for the biosynthetic pathway the FAs follow in the cow organism, we have to consider that they may derive either from the diet only, as is the case with essential linoleic and linolenic FA, or from *de novo* endogenous synthesis, or both (Galli & Risé, 2006). Before depositing into the tissues, dietary FAs undergo substantial transformations into the digestive tract. First, the hydrolyzation of complex lipids deriving from the diet, carried out by bacteria and protozoa in the rumen, produces long chain fatty acids (LCFAs) and other organic compounds (Buccioni et al., 2012). Then, the free FAs released during hydrolysis are converted to saturated ones, primarily stearic and secondarily palmitic acids through biohydrogenation (Christie & Han, 2012). On exiting the rumen, the FAs flow into the duodenum, where the absorption takes place. Furthermore, the FAs reach the liver carried by the blood, whose flow, together with the FAs concentration, influences their supply to this organ (Hocquette&Bauchart, 1999).

The presented project was divided into two parts: the first one aimed to evaluate if each step of the previously described path may involve changes in the carbon isotopic ratio ($\delta^{13}C$), given a specific dietary regime (in this case, a C3-based one). The aim was also to figure out if the analysis of stable isotopes could represent a new tool to follow the FAs life cycle to the end. A compound-specific stable isotope approach was considered as the most suitable method to investigate the FAs $\delta^{13}C$ throughout the bovine organism.

The second aim of this project was to discriminate between cows fed on different feeding regimes, based on C3 or C4 plants. In particular, there is a growing interest in the capability of

SIRA to discriminate between pasture and stall-based feeding systems. Indeed, meat from animals raised on pasture is considered to be healthier with respect to that obtained by stall-fed animals, due to the higher content in omega-3 fatty acids, conjugated linoleic acid and oleic acid (Simopoulos, 2002). During this step, both bulk and compound-specific analyses were carried out. Although bulk analysis represents a fast and reliable method, it has been demonstrated that compound-specific analyses can provide more detailed information (van Leeuwen et al., 2014). In this sense, FAs analysis may provide a deeper understanding of the processes taking place in the animal organisms and may figure as a more precise dietary regime information supplier.

As for the first part of the project, which will be discussed in **Chapter 3.3.1**, a group of six cull cows fed exclusively on C3 plants was involved. The $\delta^{13}C$ of single FAs was measured in the diet and in four different animal matrices, i.e., rumen, duodenal content, liver and meat, through GC-C-IRMS. A set of six FAs was considered, including myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2n-6) and linolenic acid (C18:3n-3). As for the second part, which will be described in **Chapter 3.3.2**, the same set of matrices (excluding the duodenum) and FAs (excluding C14:0) was studied, but, unlike in the first step, two groups of cows (n=13 animals in total) fed on different dietary regimes (either C3- or C4- based) were involved. In this way, a comparison between the two groups could be carried out, based both on the $\delta^{13}C$ of single FAs and on the bulk $\delta^{13}C$.

3.2 Materials and Methods

The study was carried out according to EU Directive 2010/63/EU. All the procedures were routine and non-invasive. Even though formal approval was not required, the ethical committee of University of Udine was consulted and approved the trial (Prot. No.8/2018).

3.2.1 Description of the samples

Thirteen Italian Simmental multiparous cull cows were assigned, on the basis of their body weight (601±21.4 kg; P>0.05), age (83±9.8 months; P>0.05) and body condition score (3.1±0.14 points; P>0.05, (Edmonson, 1989), to two dietary treatments, either C3 (C3 group; n=6) or C4 (C4 group, n=6 for the bulk analysis and n=7 for the CSIA analysis). The C3 group only will be considered in **Chapter 3.3.1**, while a comparison between C3 and C4 group will be carried out in **Chapter 3.3.2**. The groups differed in the metabolism of the plants the diet was based on, as previously mentioned. The C3 group was fed on hay offered *ad libitum* and

received 6.9 kg dry matter (DM) of a concentrate composed of barley meal, wheat meal, wheat bran, soybean meal, hempseed cake, mineral and vitamins. The hay was composed of 7.1% DM crude protein (CP), 70.1% DM neutral detergent fiber (NDF) and had net energy for lactation (NEL) of 4.3 MJ/kg of DM, while the concentrate was composed of 13.9% DM CP and 7.8 MJ NEL / kg DM. The C4 group was fed on corn silage offered *ad libitum* and received 3.2 kg DM of a concentrate composed for the 84% of corn meal and corn gluten (C4 plants), while C3 plants (soybean meal and hempseed cake) represented the remaining part. The corn silage was composed of 7.8% DM CP, 42.8% DM NDF and 5.9 MJ/kg of DM NEL, while the concentrate had 23.4% DM CP and 7.7 MJ NEL / kg DM. The diets were formulated hypothesizing a target daily weight gain of 1.1 kg/d and a forage intake of 14 kg DM for all the animals according to the Institut National de la Recherche Agronomique (INRA) standards (Agabriel & Institut national de la recherche agronomique (France)., 2007). The concentrates were individually offered in twice-daily meals and were completely consumed by the animals. The intake of corn silage was 13.2 ± 1.32 kg of DM. The experimental period lasted 4 months. During the entire experimental period, the animals stayed healthy, as verified by periodical clinical examinations by the veterinary.

In order to ensure the individual daily forage (hay and silage) intake, cull cows were equipped with a noseband pressure sensor (RumiWatch system, ITIN-HOCH GmbH, Liestal, CH) to evaluate the feeding behaviours, including eating time as well as chews and number of boluses (Corazzin et al., 2020; Romanzin et al., 2018)**.** In particular, the relationship between feeding behaviour and forage intake had been studied in a preliminary period that involved the same animals and hay and silage batches similar to those used during the experimental period (Pahl et al., 2016). During the trial, the mean DM intake of hay and silage were 9.3 ± 1.0 kg of DM and 13.0 ± 1.2 kg of DM, respectively. The DM weight of C3 and C4 diet was 25.3 and 38.5 g/kg DM, respectively. The dietary plants in the two groups were classified as 100% C3 (C3 group) and as over 95% C4 (C4 group). Samples of hay, silage and concentrates were collected every two weeks, dried at 65°C in a forced draft oven for 48h and used for stable isotopes and FAs analyses. The individual intake of hay, silage and concentrates was considered to calculate the average $\delta^{13}C$ and FAs amount of the diets.

The day before slaughter, the animals accessed only their morning meal. The cull cows were slaughtered at a live weight similar between experimental groups (703 ± 23.2 kg; P>0.05), in an EU-licensed abattoir 40 km far from the farm, and within 20 min from their arrival. At

slaughter, individual samples of rumen (approx. 100 g of representative content), duodenum (approx. 50 g of representative content, collected at about 1m from pylorus), liver (approx. 100 g) and meat (approx. 100 g of m. longissimus thoracis at 6-7 rib level) were immediately collected and refrigerated. Samples were freeze-dried (Lyo Quest, Telstar, Legnago, IT) and grinded by a laboratory blender for stable isotope and FAs analyses. The pH of rumen fluid was measured using a pH-meter (Hanna, HI 8424, Padova, IT) equipped with a glass electrode (Crison, 5232, Barcelona, E). The average pH of the rumen was 6.5 ± 0.05, being similar between experimental groups ($P>0.05$) and all the animals showed a value within the normal range (6.2 - 7.2) (Constable et al., 2016), meaning that cows had normal ruminal fermentation balance.

3.2.2 Preparative of the samples

Lipid extraction was performed using the Accelerated Solvent Extraction (ASE 350 Dionex, Thermo Fisher Scientific, Ro, Italy) with chloroform:methanol (2:1, v/v) (Folch, 1975). Samples were placed in 22 mL extraction cells in variable amounts (from 1.0 to 2.5 g), according to the expected fat content. A weighted amount of heneicosanoic acid (C21:0), or nonadecanoic acid (C19:0) for forage sample, was added as a standard for lipid determination. The sample loading was completed by filling with Diatomee hydromatrix (Agilent Technologies, USA) and sealing-off each extraction cell with a cellulose filter. After extraction, the solvent was evaporated with Univapo 100 ECH (Vacuum concentrator centrifuge, Elettrofor, scientific instrument, Ro, Italy) and then with a nitrogen stream, until a constant weight was achieved. The extract weight (fat) was obtained by subtracting the vial tare from the total weight. The samples thus obtained were finally stored in hexane and divided into two aliquots, in order to carry out both the bulk analyses through EA-IRMS (prior hexane evaporation, as described in **Chapter 1.3.2**) and the derivatization.

The derivatization procedure permits obtaining FAMEs prior transesterification of triglycerides, by following the method described by Perini et al., 2019. An amount of 2 mL of hexane extract was put in a reactor, the solvent was evaporated under a stream of N_2 and 1.5 mL of a sulphuric acid/methanol (1:16 v/v) mixture was added. The sample was then heated at 100 °C for 2 h and shaken 4 times during the heating. An amount of 5 mL of a saturated NaCl−water solution and 3 mL of hexane were added to the cooled solution, shaken vigorously. The mixture was allowed to stratify until the upper organic layer became clear. The organic

phase was thus picked up with a micropipette and injected for GC-MS and GC-C-IRMS analyses.

The analytical procedure carried out for the GC-C-IRMS analysis is described in **Chapter 1.4.2**. As for the GC-MS analysis that allowed the quantification of the single FAs, it is briefly described as follows. The GC-MS analyses were performed in EI mode (70 eV) with a 5977E MSD system, a single-quadrupole with hyperbolic rod sensors (Agilent Technologies, USA), with 7820A GC system and equipped with a 7693A autosampler and automatic split/splitless injector. An amount of 1µL was injected in split mode (1:50) and separations were carried out on a HP-88 fused silica capillary column (100 m length, 0.25 mm internal diameter coated with 0.20 µm film thickness, 88% - Cyanopropy-aryl-polysiloxane) (Supelco, Bellefonte, PA). The GC oven program started for 5 min at 50°C; then, the temperature was ramped up (8°C/min) to 160°C (hold time 4 min), then the temperature was increased again (0.6°C/min) to 200°C (hold time 1 min) and finally (15°C/min) to 240°C (hold time 5 min). The total run was 98 min long and in post run the temperature was set to 245°C for 5 min in order to clean the column. The equilibration time was set to 2 min. Helium (6.0) was used as carrier gas with a constant flow rate of 1.2 mL/min. The temperature values of the ion source and the quadrupole were set to 240 °C and 150 °C, respectively; the transfer line was set to 230°C. The GC-MS analyses in the full scan mode (m/z 50-600) were performed after a solvent delay of 15 min, with 3 microscan/s.

3.2.3 Data analysis

Results obtained through CSIA analyses were corrected as reported in **Chapter 1.4.2**. Statistical analysis was performed using R software (vers. 3.4.0 (2017) and 4.0.4 (2021)). The normality of the data distribution was tested using the Shapiro-Wilk test and skewness and kurtosis were estimated using model residuals. When appropriate, data were transformed for parametric testing and, if the model assumptions were not yet satisfied, generalized estimating equations applied on the same model were considered. For multiple comparisons, Bonferroni adjustments were used. P-value<0.05 and <0.01 indicates significant statistical differences.

As for the first part of the project (**Chapter 3.3.1**), taking into account only C3 group and

focusing on the fractionation processes in the bovine organism, the effect of the body compartment (rumen, duodenum, liver and meat) on the variables was assessed with a mixed model for repeated measures, considering body compartment as a within-subject factor (Wang

and LA Goonewardene, 2004). Individual animals were treated as random factors. Tukey's test was considered for multiple comparisons. In the text, values are reported as mean ± SE.

As for the second part of the project (**Chapter 3.3.2**), considering the comparison between C3 and C4 groups both in bulk and in CSIA analyses, variables were analysed with a mixed model for repeated measures that considered body compartment (feed, rumen, liver and meat) and diet (C3, C4) as within and between subject factor, respectively. The interaction compartment x diet was also considered and the analyses were carried out as suggested by (Wang and LA Goonewardene, 2004).

3.3 Results and Discussion

3.3.1 Part 1 - Stable isotope ratio analysis to study the fractionation processes in bovine organism (Pianezze, Corazzin, Perini, Camin, et al., 2021)

As previously described, in this first part of **Chapter 3.3**, six cull cows fed exclusively on C3 plants have been considered. The $\delta^{13}C$ of single FAs has been measured in the diet and in four different animal matrices, i.e., rumen, duodenal content, liver and meat (loin) through GC-C-IRMS. In particular, a set of six FAs both non-essential (C14:0, C16:0, C18:0, C18:1n-9) and essential (C18:2n-6, C18:3n-3) has been considered.

The FAs content and profile are reported in **Table 1**, while the results for carbon isotopic analysis carried out on the single FAMEs through GC-C-IRMS are discussed throughout the main text. The statistic that has been carried out is described in **Chapter 3.2.3**.

The metabolic path starts with the rumen. All chemical reactions pertaining to the FAs can entail isotopic fractionation processes, and therefore a change in their $\delta^{13}C$. Dietary fats are subject to chemical processes such as lipolysis and biohydrogenation carried out by ruminal microorganisms (Enjalbert et al., 2017). Moreover, ruminal bacteria can carry out a *de novo* synthesis of both straight-chain and branched-chain FAs (Craninx et al., 2006). Although important from a qualitative point of view, the amount of *de novo* FA synthesis by ruminal bacteria is low (M. B. Jenkins & Lion, 1993). It can however explain the similarity between the $\delta^{13}C$ of the FAs in the diet and the rumen, which had already been reported in other studies (DeNiro & Epstein, 1978; Stott et al., 1997).

Item	Diet	Rumen	Duodenum	Liver	Loin	RMSE	P-value
Content, mg/g DM							
C14:0	0.08^{d}	0.30^{bc}	0.16^{cd}	0.44^{b}	2.92^{a}	0.041	<0.01
C16:0	3.50^{C}	3.26^{C}	4.51^{C}	9.93^{B}	32.84^{A}	0.090	<0.01
C18:0	0.49^{C}	7.63^{B}	7.77^{B}	23.75^{A}	25.23^{A}	1.987	<0.01
C18:1n-9	2.34^{C}	0.43^{D}	2.05^{C}	5.39^{B}	41.13^{A}	0.085	<0.01
C18:2n-6	6.98^{A}	0.17^{C}	3.67^{B}	6.05^{A}	2.49^{B}	0.734	<0.01
C18:3n-3	1.86^{a}	0.06^{c}	0.27^{b}	0.38^{b}	0.38^{b}	0.207	<0.01
Fat	25.3^{C}	27.2^{C}	36.1^{C}	71.5^{B}	122.5^{A}	10.72	<0.01
Profile, %FA							
C14:0	0.52^{B}	2.05^{A}	0.84^{B}	0.66^{B}	2.49^{A}	0.374	<0.01
C16:0	22.22^{B}	21.87^{B}	20.81^{B}	14.81^{C}	27.89^{A}	2.319	<0.01
C18:0	3.06^{D}	51.63^{A}	35.56^{B}	35.73^{B}	21.80^{C}	3.745	<0.01
C18:1n-9	14.72^{B}	2.91^{D}	8.68^{C}	8.02^{C}	35.01^{A}	1.897	<0.01
C18:2n-6	43.59^{A}	1.20^{E}	17.20^{B}	9.07^{C}	2.16^{D}	1.657	<0.01
C18:3n-3	11.62^{A}	0.41^{CD}	1.27^{B}	0.57^{C}	0.33^{D}	0.740	<0.01

$^{A,B,C,D,E:}$ P<0.01 within row; $^{a,b,c,d,e:}$ P<0.05 within row; RMSE: root mean square error

Table 1. Estimated marginal means of fat and FAs in diet, rumen, duodenum, liver and meat (loin) of cows, indicating the root mean error square (RMSE) and the P value.

The myristic acid (C14:0) content increases in the rumen with respect to the diet (P<0.05; **Table 1**), as reported by other authors (Abuelfatah et al., 2016; Vargas et al., 2020). Palmitic (C16:0) and stearic acids (C18:0) are the most abundant FAs in rumen (**Table 1**). The results of the present study agree with the observation, made by Loften et al., 2014, that the amount of palmitic acid in the diet and in the rumen remains similar. As with concentration, the $\delta^{13}C$ of the palmitic acid in the rumen ($\delta^{13}C_{C16:0\_RUMEN}$ = -36.02±0.31‰) and in the diet ($\delta^{13}C_{C16:0\_DIET}$ = -36.28±2.85‰) were similar (P>0.05). On the other hand, the concentration of stearic acid greatly increased in the rumen with respect to the diet (**Table 1**; P<0.05), while the $\delta^{13}C$ remained similar ($\delta^{13}C_{C18:0\_DIET}$ = -34.78±1.90‰; $\delta^{13}C_{C18:0\_RUMEN}$ = -35.12±0.22‰; P>0.05). As already mentioned, stearic acid is the end product of the biohydrogenation of linoleic and linolenic FAs in rumen (Elgersma et al., 2006), hence, its concentration depends on the sum of the dietary fraction and the endogenous synthetized one.

As already mentioned, oleic acid is subject to intense biohydrogenation occurring into the rumen. This is why its percentage, as for linoleic and linolenic acids, decreases from the diet to the rumen (P<0.05, **Table 1**). Different bacteria have different capacities to biohydrogenate polyunsaturated FAs in this matrix. They are usually divided into a group A and a group B. The bacteria of group A can hydrogenate linolenic and linoleic acid to (mostly) trans-11-octadecenoic acid, while the bacteria of group B can hydrogenate a wide range of octadecenoic

acids, including cis-9-oleic and linoleic acid, with stearic acid (C18:0) as end product. As reported in other studies, regardless of the type of animal, level of intake and type of diet, linoleic acid seems to be biohydrogenated to a smaller extent than linolenic acid (Scollan et al., 2003). These three FAs, subjected to a partial ruminal biohydrogenation, showed different evolution of their dietary $\delta^{13}C$. Indeed, the ratio diminished for oleic acid ($\delta^{13}C_{C18:1n-9\_RUMEN}$ = -35.97±1.13‰), remained stable for linoleic acid ($\delta^{13}C_{C18:2n-6\_RUMEN}$ = -35.06±1.36‰) and increased for linolenic acid ($\delta^{13}C_{C18:3n-3\_RUMEN}$ = -32.27±1.18‰).

The digestive process of the LCFAs starting in the rumen, ends in the small intestine, where they are absorbed by epithelial cells and esterified (Bauchart, 1993). The duodenum content is thus representative of the final part of the absorption process. The duodenal digestibility of the FAs considered is high and varies from 73-79% for palmitic acid to 85-89% for oleic acid (Moate et al., 2004).

Differently from all the other FAs, C14:0 in the duodenal content did not represent a percentage of the total FAs high enough to permit the measure of its $\delta^{13}C$ (**Table 1**). Myristic acid is indeed broken down by digestive metabolism more efficiently than the other FAs, due to its shorter chain (Leighton et al., 1984; Piot et al., 1999).

Palmitic and stearic acids are the most abundant FAs in the duodenum (**Table 1**). The concentration of palmitic acid in the duodenum was similar to that observed in the diet and in rumen ($P>0.05$), while the amount of stearic acid in the duodenum was comparable to that observed in rumen ($P>0.05$), but higher than that in the diet ($P<0.01$). Previous studies showed that changes in the concentration of palmitic acid from the diet to the duodenal flow were much less than those for the stearic acid and that the amount of stearic acid flowing into the duodenum was generally higher than that of any other (Loor et al., 2004). The quantification carried out in the present work supports this assumption. However, the $\delta^{13}C$ gets less negative going from the rumen to the duodenum for both the FAs ($\delta^{13}C_{C16:0\_DUOD}$ = -32.40±1.39‰; $\delta^{13}C_{C18:0\_DUOD}$ = -33.26±0.96‰; $P<0.01$).

As for the oleic acid, its flow from the rumen into the duodenum is less intense than that of stearic acid, as reported also in other studies (Smith et al., 2006). Nevertheless, its concentration increases going from the rumen to the duodenum ($P<0.01$, **Table 1**) and at the same time its $\delta^{13}C$ becomes less negative than the same value in rumen ($\delta^{13}C_{C18:1n-9\_DUOD}$ = -31.05±0.89‰, $P<0.01$). The higher content of oleic acid in the duodenum with respect to the rumen could be

due to the incomplete biohydrogenation of linolenic and linoleic acids, which can result in C18:1n-9 as a sub product (Hocquette & Bauchart, 1999).

As for the linoleic and linolenic acids, their content in the duodenum is higher than the one found in rumen ($P<0.05$, **Table 1**) but lower than the same value in the diet ($P<0.05$, **Table 1**). Linoleic $\delta^{13}C$ changes less appreciably going from the rumen to the duodenal content ($\delta^{13}C_{C18:2n\text{-}6\_DUOD}$ = -34.10±0.31‰) than for the other FAs. As for linolenic acid, the $\delta^{13}C$ of the duodenal content ($\delta^{13}C_{C18:3n\text{-}3\_DUOD}$ = -35.48±1.09‰) is comparable to that of the diet but lower than that of the rumen.

The liver is the major site of FAs metabolism. The FAs are transported from the digestive tract to the liver through blood, in an amount proportional to their concentration (Bruss, 1993). Once in there, FAs undergo several chemical processes, like *de novo* synthesis, and they can also be stored (Hocquette & Bauchart, 1999), which contributes to explain the differences in FAs content in this matrix with respect to the duodenum and the rumen (**Table 1**). On the other hand, the $\delta^{13}C$ of all the FAs turned out to be not significantly different from those found in the duodenum ($\delta^{13}C_{C16:0\_LIVER}$ = -31.62±0.49‰; $\delta^{13}C_{C18:0\_LIVER}$ = -33.16±0.43‰; $\delta^{13}C_{C18:1n\text{-}9\_LIVER}$ = -30.74±2.07‰; $\delta^{13}C_{C18:2n\text{-}6\_LIVER}$ = -33.72±0.41‰; $P>0.05$). From this point of view, the results of the present study may indicate that the main activity of the liver is to store FAs rather than to synthesize or to oxidase them. Indeed, as reported in literature the *de novo* FA synthesis in ruminants' liver is very limited (Hocquette & Bauchart, 1999).

It was not possible to collect the $\delta^{13}C$ of either myristic or linolenic acids, due to their low concentration with respect to the other FAs considered (**Table 1**). As reported in the literature, myristic acid does not accumulate within the liver (Bauchart et al., 1998). This may be explained by a modification of the oxidation process: myristic acid, due to its shorter chain, is more efficiently broken down than the other LCFAs (Christensen et al., 1989; Leighton et al., 1984; Piot et al., 1999).

The absorption of the FAs from the duodenum finally leads to their deposition in the adipose tissues. In this way, meat can be considered as the last step of the FAs path. The content of myristic, stearic, palmitic and oleic acids in meat is the highest of all the matrices considered. On the other hand, the content of both linoleic and linolenic was lower than the same value in the diet, but higher than that in the rumen (**Table 1**).

It was possible to detect myristic acid in meat in amounts allowing the $\delta^{13}C$ assessment ($\delta^{13}C_{C14:0\_MEAT}$ = -28.58±1.95‰), likely because of *de novo* synthesis occurring in the tissue.

Oleic acid is the most abundant FA in meat (**Table 1**), probably because of the activity of delta-9 desaturase, which involves the conversion of the stearic acid into the oleic acid in adipose cells (Smith et al., 2006). Consequently, the concentration of stearic acid in meat decreased to a lower value than that of oleic acid (**Table 1**). Most of the stearic acid in adipose tissue, in turn, likely comes from the elongation of palmitic acid (Sampath & Ntambi, 2005), which is the second most abundant FA in meat (**Table 1**). Van Leeuwen et al., 2017 found out that C16:0 is the major product of FA synthase, therefore its presence in muscle neutral lipids derives from *de novo* synthesis in typical ruminant diets. Palmitic, stearic and oleic acids showed a significant enrichment of ^{13}C in the duodenal content, where the absorption takes place ($P<0.01$), which is also associated with *de novo* FA synthesis (Richter, Spangenberg, Willems, et al., 2012).

The amount of linoleic and linolenic acid found in meat is comparable to that found in the duodenum, showing an intense absorption of these FAs and the dietary origin of them both. The $\delta^{13}C$ value of the linoleic acid ($\delta^{13}C_{C18:2n-6\_MEAT}$ = -26.96±1.98‰), as well as those of oleic ($\delta^{13}C_{C18:1n-9\_MEAT}$ = -27.61±1.63‰), palmitic ($\delta^{13}C_{C16:0\_MEAT}$ = -28.54±2.04‰) and stearic ($\delta^{13}C_{C18:0\_MEAT}$ = -29.46±1.54‰) acids are comparable to the isotopic values measured in other studies (van Leeuwen et al., 2017). Furthermore, the results of the present study are in agreement with the findings of Richter et al., 2012 who found more negative $\delta^{13}C$ of palmitic, stearic, oleic and linoleic acids in the diet than in the adipose tissues of lamb. As for linolenic acid, the amount of this FA was not high enough, compared to the other FAs, to enable the collection of its $\delta^{13}C$ (**Table 1**).

3.3.2 Part 2 - GC-C-IRMS on single FAs and EA-IRMS on bulk lipid to detect differences in four matrices of Simmental cows fed on C3 and C4 diets

As previously described, in this second part of **Chapter 3.3**, a set of four compartments (feed, rumen, liver and meat) and five FAs (C16:0, C18:0, C18:1n-9, C18:2n-6, C18:3n-3) has been considered, but, unlike in the first step, two groups of cows (n=13 animals in total) fed on different dietary regimes (either C3- or C4- based) were considered. In this way, a comparison between the two groups could be carried out, based both on the bulk analysis of $\delta^{13}C$ (**Chapter 3.3.2.1**) and on the CSIA carried out on the single FAs (**Chapter 3.3.2.2**).

3.3.2.1 EA-IRMS on bulk lipid (Pianezze, Corazzin, Perini, Sepulcri, et al., 2021)

The results of the statistical calculations carried out on the isotopic values and the relative amount of lipids are displayed in **Table 2** and described in **Chapter 3.2.3**. The columns named as C3 and C4 represent the diet effect and show therefore the mean values for all the data of groups C3 and C4 (including diet, rumen, liver and meat), respectively.

	Diet, plants (P)		**Compartment (C)**					**P value**		
	C3	**C4**	**DIET**	**RUMEN**	**LIVER**	**MEAT**	**SEM**	**P**	**C**	**P x C**
Fat (ee), % DM	5.36	7.30	2.35 **A**	1.09 **B**	7.63 **C**	15.51 **D**	0.353	<0.01	<0.01	<0.01
Fat ($\delta^{13}C$), ‰	-30.10	-21.57	-25.64 **A**	-29.92 **B**	-24.98 **C**	-22.80 **D**	0.183	<0.01	<0.01	<0.01

Table 2. Statistical calculations carried out on isotopic values and relative amount of lipids, A, B, C, D Within the same row and factor with ulike letters differ significantly at P<0.01

The main effects of plant type in the diet of the animals and in the compartment were significant (P<0.01), as the interaction compartment × plant type was significant (P<0.01) for both the considered variables. It means that the effects of the main factors cannot be interpreted separately.

From the perspective of the plant type, i.e., by considering the significant differences between group C3 and C4 in the same compartment, statistical differences have been found. Indeed, the $\delta^{13}C$ values of groups C3 and C4 were found to be statistically different in the diet (-32.55‰ vs. -18.74‰; P<0.01), rumen (-32.10‰ vs. -27.73‰; P<0.01), liver (-29.50‰ vs. -20.47‰; P<0.01) and meat (-26.25‰ vs. -19.35‰; P<0.01). In particular, C3 values turned out to be lower than C4 ones for all the compartments. The relative amounts of lipids in groups C3 and C4, were found to be statistically different in the diet (1.81% vs. 2.90% DM; P<0.01), liver (6.50% vs. 8.77% DM) and meat (11.95% vs 19.07%; P<0.01). On the other hand, the relative amount of lipids in the rumen of group C3 and C4 did not statistically differ (1.20%

vs. 0.98%; P>0.05). The consistent $\Delta\delta_{DIET}$ between the values of groups C3 and C4 ($\delta^{13}C_{C4\text{-}DIET}$ - $\delta^{13}C_{C3\text{-}DIET}$ = 13.81‰) results in differences in the other compartments ($\Delta\delta_{RUMEN}$ = 4.37, $\Delta\delta_{LIVER}$ = 9.03 and $\Delta\delta_{MEAT}$ = 6.90). The compartment characterised by the lower Δδ is the rumen. Due to the significant $\delta^{13}C$ between groups C3 and C4 in the liver and in the meat, the two compartments turned out to be a reliable tool to discriminate animals fed on different feeding regimes.

From the perspective of the compartment, the fat content for both groups decreased from diet to rumen, and increased from rumen to liver, showing the highest value in meat (P<0.05). This result agrees with the fact that fatty acids are mainly stored in the liver and the muscles, where they also undergo *de novo* synthesis (Nafikov & Beitz, 2007). On the other hand, the variation of the $\delta^{13}C$ among the different compartments was not the same for group C3 and group C4. Indeed, $\delta^{13}C$ of group C3 showed the trend $\delta^{13}C_{DIET} < \delta^{13}C_{RUMEN} < \delta^{13}C_{LIVER} < \delta^{13}C_{MEAT}$, that agrees with the results reported by (Pianezze, Corazzin, Perini, Camin, et al., 2021), while group C4 showed the trend $\delta^{13}C_{RUMEN} < \delta^{13}C_{LIVER} < \delta^{13}C_{DIET}$ with differences between diet and liver, and also between liver and meat that were not statistically significant (**Figure 5**).

Figure 5. Variation and uncertainty of the measurement of the carbon isotopic ratio in groups C3 and C4 in the different compartments. ^{A,B,C}: P<0.01; ^{a,b,c,d}: P<0.05 within C3 and C4 groups.

One possible explanation for this unexpectedly low value of the rumen in the C4 group may lie in the reactions occurring during rumination. The anaerobic degradation of carbohydrates in the rumen produces volatile fatty acids, CO_2 and reducing equivalents. Some of the CO_2 is used as a hydrogen acceptor in the production of methane as in **Equation 3**.

Equation 3. $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$

This process entails a consistent isotopic discrimination, as hydrogenation to CH_4 involves preferentially light $^{12}CO_2$. The methane will be consequently depleted in ^{13}C with respect to the CO_2 it derives from (resulting in more negative $\delta^{13}C$ of CH_4). On the contrary, the remaining CO_2 will be enriched in ^{13}C, resulting in less negative values with respect to the feeding it derives from (Metges et al., 1990). Klevenhusen et al., 2009 reported values of CH_4 and CO_2 of -65.5‰ and -10.9‰, respectively, for a C4 diet (-15.2‰) in an *in vitro* experiment using rumen simulation technique. Starting from these assumptions, CH_4 and CO_2 (both deriving from ruminal activity and the atmospheric one) may somehow influence the $\delta^{13}C$ of the lipids they are in close contact with during the rumen digestion. In the same study the authors found short chain fatty acids (as n- and iso-butyrate and n- and iso-valerate) of rumen to be enriched in ^{13}C, resulting in more negative values with respect to the diet, in agreement with the results reported in the present work. An experimental project focusing on these hypotheses should be carried out to clarify the possible influence of $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ on $\delta^{13}C_{FAT}$.

3.3.2.2 GC-C-IRMS on single FAs

As the interaction compartment × diet resulted statistically significant for all the variables considered ($P<0.05$; data not reported), the main effects of compartment and diet could not be discussed separately. For this reason, the results of the interactions are reported in **Figures 6**, **7** and **8**.

First, the diet effect, given a fixed compartment, was considered by taking into account both the quantification and the carbon isotopic ratios. From an overall point of view, it is worth noting that both the quantification of the FAs and their $\delta^{13}C$ permitted to discriminate between the two different diets provided to the animals.

In C4 group feed, the concentration of all the FAs considered was higher than in C3 one ($P<0.05$), with the only exception of C18:3n-3 ($P<0.05$; **Figure 6**). The differences observed

in the FAs content of feed resulted in those observed in meat (P<0.05). Being essential, C18:2n-6 and C18:3n-3 are not synthesized in ruminants and their concentration in the adipose tissues is thus closely related to their dietary amount and to the proportion of that escaping the rumen (Chilliard et al., 2007).

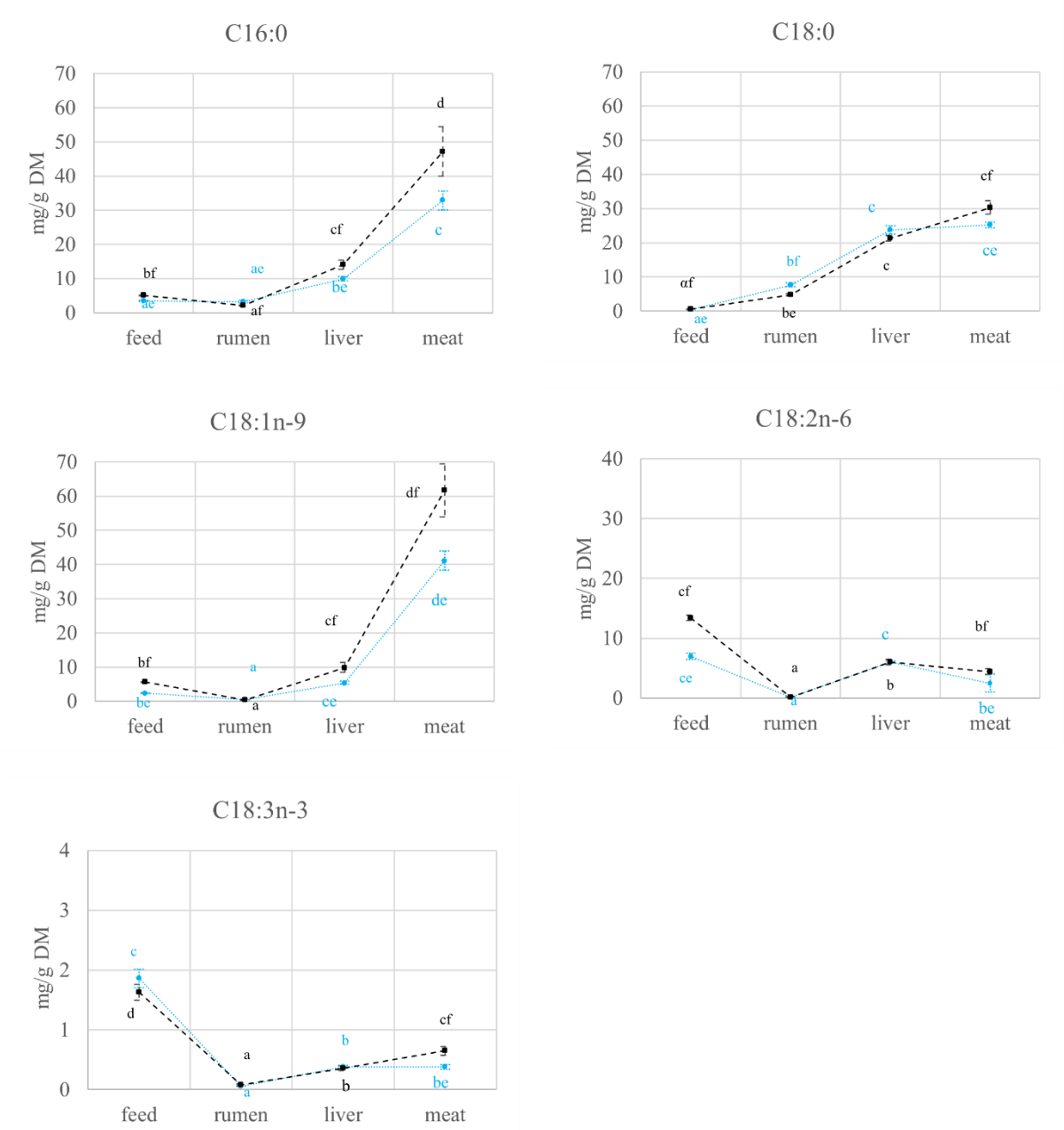


Figure 6. Variations (mean ± se) of FAs expressed as mg/gDM in compartments (feed, rumen, liver, meat) of cull cows fed with diets based on C3 (C3 group, blue) and C4 (C4 group, black) plants. [a,b,c,d]: *P*<0.05 within experimental group; [e,f]: *P*<0.05 compartment

The C4 group had a higher C18:2n-6 intake (feed compartment), but a lower C18:3n-3 with respect to the C3 group; however, focusing on the meat compartment, the C4 group had a higher content of both C18:2n-6 and C18:3n-3. Therefore, it could be hypothesized that the

fraction of C18:3n-3 escaping the rumen was higher in the C4 group, probably because of a lower retention time and/or a different rumen biohydrogenation with respect to the C3 one.

By focusing on **Figure 7**, where the FAs content is expressed as a percentage (g/100 g of total FAs), the C4 group feed had a higher content of C18:1n-9 and C18:2n-6, but a lower amount of C16:0, C18:0 and C18:3n-3 ($P<0.05$; **Figure 7**) with respect to the C3 one.

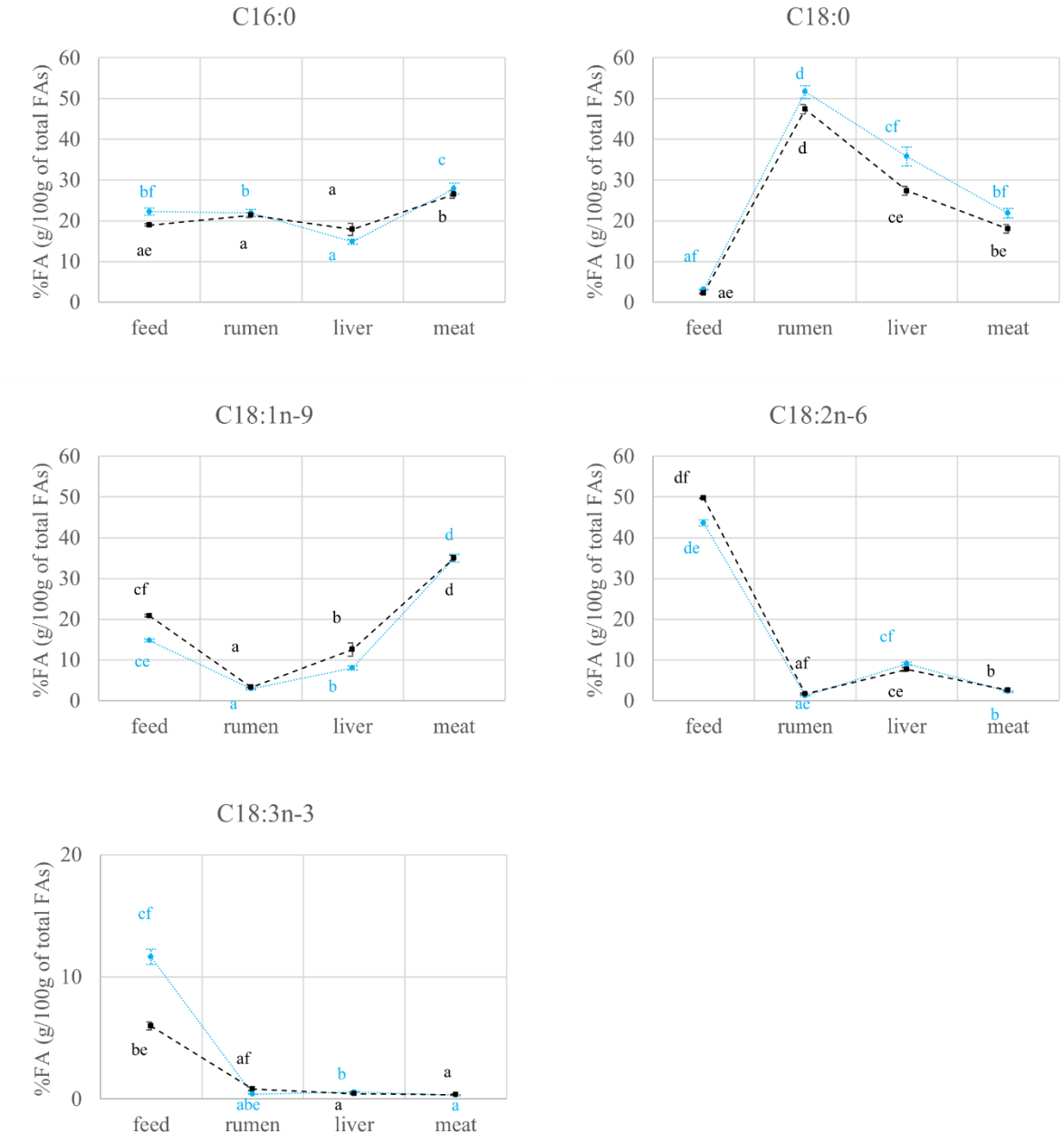


Figure 7. Variations (mean ± se) of FAs expressed as % (g/100 g of total FAs) in compartments (feed, rumen, liver, meat) of cull cows fed with diets based on C3 (C3 group, blue) and C4 (C4 group, black) plants. [a,b,c,d]: $P<0.05$ within experimental group; [e,f]: $P<0.05$ compartment.

This agrees with the high content of C18:1n-9 and C18:2n-6 in corn (Glasser et al., 2013), which represented the major component of the C4 group diet. The relative amount of C18:2n-6 ($P<0.05$) and C18:3n-3 ($P<0.05$) in rumen was higher for C4 than for C3 group. The differences between the FAs percentages in C3 and C4 rumen mostly mirrored those observed in the meat (**Figure 7**).

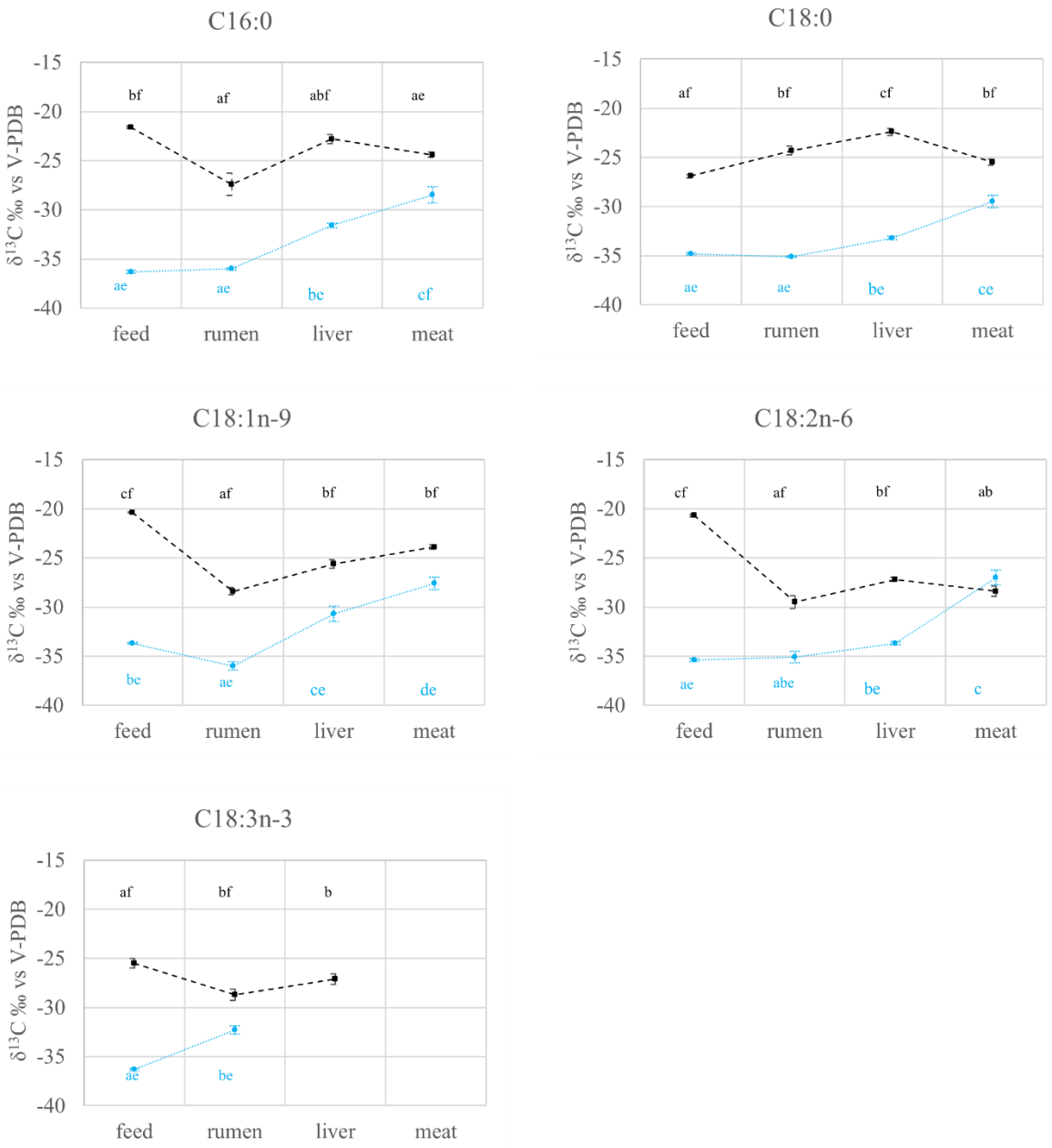


Figure 8. Variations (mean ± se) of $\delta^{13}C$ (‰) of fatty acids in different compartments (feed, rumen, liver, meat) of cull cows fed with diets based on C3 (C3 group, blue) and C4 (C4 group, black) plants. [a,b,c]: $P<0.05$ within experimental group; [e,f]: $P<0.05$ compartment.

By focusing on C18:3n-3, its percentage was statistically higher in the C4 group than in C3 one as for the rumen compartment, and numerically higher as for the meat compartment (**Figure 7**). These results agree with (Boerman et al., 2015), reporting that the FAs amount available for absorption in the intestine was similar to that leaving the rumen.

The $\delta^{13}C_{FEED}$ values were different between the two experimental groups for all FAs, and this probably led to the differences observed in all the other compartments, being C4 group values higher than those of the C3 one ($P<0.05$, **Figure 8**). These results agree with many other studies (Knobbe et al., 2006; Molkentin & Giesemann, 2007, 2010). The only exception, excluding those related to the lack of data for one or both diets, is represented by $\delta^{13}C_{C18:2n-6\_MEAT}$, being not statistically different in C3 and C4 diet ($P>0.05$; **Figure 8**). Therefore, except for $\delta^{13}C_{C18:2n-6\_MEAT,}$ the $\delta^{13}C$ of all FAs gave the possibility to discriminate, as for all compartments, between the cows belonging to C3 and C4 groups (**Figure 8**). Finally, the low concentration of C18:3n-3 in meat did not allow it to measure its $\delta^{13}C$.

After describing the dietary effect, given a fixed compartment and by considering both the quantification and the carbon isotopic ratios, the compartment effect, given the same diet, was therefore discussed.

As previously described, the FAs metabolic path starts with rumen. In here, the unsaturated FAs are mainly converted into C18:0, while a low percentage escape the biohydrogenation processes (Chilliard et al., 2007). In particular, the disappearance of C18:3n-3 and C18:2n-6 in the rumen averages 93% and 85%, respectively (Doreau & Ferlay, 1994). These results agree with the data we obtained, both for the C18:2n-6 (loss of 97.6% in C3 group and 98.7%in C4 group) and for the C18:3n-3 (loss of 96.8% in C3 group and 95.1% in C4 group). Being the end product of the majority of the biohydrogenation processes, C18:0 has the highest amount of all FAs in the rumen (**Figure 6**), which results 16- and 8-fold higher in the rumen than in the diet, for C3 and C4 diets, respectively.

In both groups the content of C18:0 increased ($P<0.05$), while that of C18:1n-9, C18:2n-2 and C18:3n-3 decreased ($P<0.05$), by passing from the diet to the rumen. Conversely, as for C16:0, a different behaviour was observed in the two groups, being its content in the C4 group lower in the rumen than in the diet, while vice versa in the C3 group ($P<0.05$; **Figure 6**). Considering C3 group, rumen had lower $\delta^{13}C_{C18:1n-9}$ ($P<0.05$) and higher $\delta^{13}C_{C18:3n-3}$ ($P<0.05$) than feed, being the $\delta^{13}C$ of the other FAs not statistically different in the two compartments. Conversely,

rumen of C4 group had lower $\delta^{13}C_{C16:0}$ ($P<0.05$), $\delta^{13}C_{C18:1n-9}$ ($P<0.05$), $\delta^{13}C_{C18:2n-6}$ ($P<0.05$), $\delta^{13}C_{C18:3n-3}$ ($P<0.05$), but higher $\delta^{13}C_{C18:0}$ ($P<0.05$) than feed (**Figure 8**).

Around 20% of lipids leaving the rumen are of microbial origin, mainly bacteria and protozoa (T. C. Jenkins, 1993). Some types of the former use carbon sources for growth with very specific ^{13}C signals or have a metabolism that results in characteristic isotopic ratios (Boschker & Middelburg, 2002). As the mean $\delta^{13}C$ for all FAs in rumen ranges from -36.0‰ to -27.4‰ regardless of the diet, the possibility that ruminal microbiota metabolism may result in products having this specific $\delta^{13}C$ range of values is not to be excluded. The only exception that has been ruled out from the mentioned range is C18:0 in the C4 group ($\delta^{13}C_{C18:0\_RUMEN\_C4}$ = -24.3±0.45‰). As for this specific FA, it has to be considered that its $\delta^{13}C$ can be influenced by a wider range of factors, being C18:0 the end product of the majority of the processes occurring in the rumen. As previously said, its fate is linked to that of C18:1n-9, C18:2n-6 and C18:3n-3 through desaturation processes (Chilliard et al., 2007; T. C. Jenkins, 1993). As these FAs are characterised by particularly high $\delta^{13}C$ values in the C4 diet (**Figure 8**), their conversion in C18:0 may lead to a relatively high $\delta^{13}C$ of this FA.

The framework gets more complicated by considering that diets rich in fat (T. C. Jenkins, 1993) have a depressant effect on the biohydrogenation processes taking place in rumen. In particular, a 10% of fat added into the diet leads to a reduction of fermentation of 50%, especially if unsaturated FAs are supplemented (T. C. Jenkins, 1993). This may have to be considered, to some extent, in the case of the C4 diet, which is 1.3 higher in fat content with respect to the C3 one (**Figure 6**).

Furthermore, it is well known that the corn silage has a much smaller particle size than hay and this could lead to lower retention time of the feed into the rumen (Leite et al., 2019). This factor may also significantly affect lipid metabolism by causing differences in the digestive processes and promoting the selective growth of some types of bacteria compared to others (Bickhart & Weimer, 2018). This could contribute in widening the different compartments effect between C3 and C4 groups as for the FAs profile and their $\delta^{13}C$.

Liver can be considered as a further step following the rumen in FAs metabolic path. The content of all FAs increased significantly ($P<0.05$) in this compartment with respect to the rumen, for both groups (**Figure 6**). Following the digestive process, the FAs reach the liver carried by blood. Its flow and the FAs concentration influence their supply to this organ, which

can be therefore seen as a following step to rumen in the FAs metabolic path (Bruss, 1993). Here, the FAs can be stored, in agreement with our results, or also esterified and oxidised. On the contrary, the *de novo* synthesis in the ruminant liver is very limited (Hocquette & Bauchart, 1999).

The $\delta^{13}C$ of all FAs increases (mostly being statistically different) by passing from the rumen to the liver for both diets (**Figure 8**). This may be due to the fact that the path in between the two compartments also entails chemical reactions, as previously mentioned, leading to fractionation processes. Indeed, depending on their origin (whether *de novo* synthesized or exogenous), the FAs have different fates in the liver: the former are likely channeled to triglycerides synthesis for very low density lipoproteins formation, while the latter are preferentially stored as triglycerides in lipid droplets (Wurie et al., 2012). Moreover, the diet type (with particular regard to fat content and FAs composition) and hormones regulate the partitioning of fats between the various hepatic metabolic pathways (Hocquette & Bauchart, 1999).

Finally, the FAs deposition in the adipose tissues may be seen as a final step in FAs metabolic path. The amount of C16:0, C18:0 and C18:1n-9 increased significantly ($P<0.05$) in the meat with respect to the rumen, regardless of the experimental group ($P<0.05$; **Figure 6**). As previously mentioned, the adipose tissue can be figured as one of the final steps of FAs metabolism. In this compartment, C16:0 is subjected to *de novo* synthesis and figures as a precursor for the production of C18:0 through elongation (Sampath & Ntambi, 2005; Smith et al., 2006). Moreover, C18:0 is partially converted into C18:1n-9 through the activity of delta-9 desaturase (Smith et al., 2006), which results evident by considering the FAs percentages in **Figure 7**. Indeed, C18:0 percentage in meat is lower than the value observed in rumen ($P<0.05$), while the highest value of C18:1n-9 was found in the meat ($P<0.05$; **Figure 7**). As expected, for both C3 and C4 groups, the contents of C18:2n-6 and C18:3n-3 are lower ($P<0.05$) in meat than in the diet, having these FAs a dietary origin and being partially subjected to rumen biohydrogenation (**Figure 6**).

As shown in **Figure 8**, the highest $\delta^{13}C$ of all FAs of the C3 group was found in meat ($P<0.05$). On the other hand, in the C4 group meat compartment, only $\delta^{13}C_{C18:1n-9}$ resulted to be higher than the same value observed in rumen ($P<0.05$). Furthermore, all the other FAs of the C4 group had similar $\delta^{13}C$ in meat and rumen ($P>0.05$). Considering that the enrichment of $\delta^{13}C$ is also related to the *de novo* synthesis (Richter, Spangenberg, Klevenhusen, et al., 2012), it is

possible to hypothesize that the portion of FAs of exogenous origin and the portion of FAs deriving from the endogenous synthesis in meat could be different in C3 and C4 group.

3.4 Conclusions

In the study presented in **Chapter 3**, 13 Italian Simmental cull cows, divided in two groups according to the diet they fed on (either C3- or C4-based), were considered.

In Part 1 (**Chapter 3.3.1**), the first group only (cows fed on a C3-based diet) was considered. In this part, by using GC-C-IRMS, the $\delta^{13}C$ values of both nonessential (myristic, palmitic, stearic, oleic) and essential (linoleic and linolenic) FAs were measured in the diet and in four animal matrices (rumen, duodenal content, liver and loin). It was found that the $\delta^{13}C$ of all the FAs became less negative going from the diet to muscle. Considering rumen and loin as the starting and the final step of the metabolic path, the difference between the average $\delta^{13}C$ of the six FAs was $\Delta\delta = (\delta^{13}C_{(RUMEN)} - \delta^{13}C_{(LOIN)}) = 7.05 \pm 1.32$. A relative partitioning of heavier and lighter carbon isotopes occurred during fat digestion and metabolism. However, the isotope fractionation affected the individual FAs with a different intensity within the various bovine matrices, according to the chemical and physiological processes they underwent. Therefore, stable isotope analysis turned out to be a promising tool in the challenging aim that is the comprehension of the metabolic path of FAs in bovine organisms.

As for Part 2, a comparison between the two groups of cows was considered, thanks to the bulk lipid analysis through EA-IRMS (**Chapter 3.3.2.1**) and to the CSIA of five FAs through GC-C-IRMS (**Chapter 3.3.2.2**). In this part, all matrices of Part 1, except the duodenal content, were considered.

The bulk analysis (**Chapter 3.3.2.1**) made it possible to detect a different trend in the variation of the $\delta^{13}C$ from one compartment to the other as for C3 and C4 groups, which provides information on the fractionation processes taking place into the cow organism. Moreover, the $\delta^{13}C$ values of liver and meat lipids were statistically different between groups C3 and C4, having a significant carbon isotopic ratio shift ($\Delta\delta_{LIVER} = 9.03$, $\Delta\delta_{MEAT} = 6.90$). This leads to the possibility to use these parameters to discriminate efficiently between cows fed on different feeding regimes based on C3 and C4 products.

On the other hand, the CSIA supported by the quantification of the FAs (**Chapter 3.3.2.2**) made it possible to discriminate between different diets in all the compartments, with the only exception of C18:2n-6 in the meat. The mentioned analyses also helped in shedding light on the processes the FAs undergo in the bovine organism and on how their metabolic path changes

depending on the diet. Nevertheless, this topic is far from being completely understood, thus more studies, especially on the various chemical reactions taking place into the rumen, should be carried out.

Chapter 4

$\delta^{34}S$ for tracing the origin of cheese and detecting its authenticity (Pianezze et al., 2020)

4.1 Background

Stable isotope ratios of cheese have been studied in several papers, both for the authentication of premium products and for the attribution of geographical origin. Focusing on studies that were carried out on Italian samples, Brescia et al., 2005 managed to characterise the geographical origin of 14 mozzarella cheese samples coming from two different zones in southern Italy using carbon and nitrogen isotopic ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, expressed as $\delta^{13}C$ and $\delta^{15}N$) and ^{1}H-NMR analysis of aqueous mozzarella extracts. Manca et al., 2001 achieved a good differentiation in samples of Pecorino coming from three different regions in southern Italy (Apulia, Sardinia and Sicily) using $^{13}C/^{12}C$ and $^{15}N/^{14}N$ values in cheese casein coupled with amino acid ratios such as Thr/Pro and Ile/Pro. Faberi et al., 2018 analysed and obtained precise identification of the area of 118 samples of cheese belonging to five Italian PDO categories (Taleggio PDO, Asiago PDO, Pecorino Toscano PDO and Provolone Valpadana PDO) using five isotopic parameters: $\delta^{13}C$ and $\delta^{15}N$ in casein fraction and in bulk cheese and $\delta^{13}C$ in the fat fraction.

As regards foreign studies, Magdas et al., 2019 managed to characterise 66 samples of cheese coming from three different regions of Transylvania in Romania. They used $^{13}C/^{12}C$ of bulk cheese and extracted casein corroborated with the elemental profile. Silva et al., 2014 provided an initial database for the comparison of dairy products from the Marajo Island in the Amazon River basin with other dairy products in Brazil and around the world. They used $\delta^{18}O$ and $\delta^{2}H$ in sample water together with $\delta^{13}C$ and $\delta^{15}N$ in bulk cheese.

A few studies were also carried out using sulphur stable isotope ratio ($^{34}S/^{32}S$, expressed as $\delta^{34}S$) in cheese casein to characterise the geographical origin of cheese samples, coupled with isotopic ratios of other elements and in different components (Bontempo et al., 2012). Camin et al. used $\delta^{34}S$, $\delta^{13}C$ and $\delta^{15}N$ of casein, as well as $\delta^{18}O$ and $\delta^{13}C$ in glycerol, to distinguish between cheese samples coming from France, Spain and Italy. They found that $\delta^{34}S$ is strongly influenced by factors such as climatic conditions and proximity to the sea. Moreover, they pointed out the correlation between $\delta^{34}S$ and $\delta^{15}N$, which was relevant, as the two parameters seemed to depend on similar factors (fertilization procedure, mineralization and demineralization reactions, leaching, climatic conditions, and proximity to the sea) (Camin et

al., 2004; Luo et al., 2016). Bontempo et al., 2011 attempted to distinguish between seven kinds of milk bovine cheese produced in the alpine and pre-alpine Italian areas using the isotopic ratio of C, N, S, O and H and the content of 49 mineral elements. Nevertheless, $\delta^{34}S$ in casein was not considered in the statistical analysis as it had been measured only in a subset of samples. Camin et al., 2012 were able to both discriminate between Parmigiano Reggiano PDO and 11 imitators and to characterise seven types of European hard cheese. They measured the isotopic values of S, N, H and C in casein together with the content of 55 elements in 260 samples of cheese, obtaining excellent classification success rates (>98%).

Thus, to date, $\delta^{34}S$ has been exploited less than other stable isotope ratios and has not yet been thoroughly investigated. Moreover, $\delta^{34}S$ has always been considered in association with other parameters. In this work, we aim to focus primarily on this isotopic ratio considering $\delta^{34}S$ casein of 725 samples of cheese, which is, to the best of our knowledge, the widest range of samples ever considered in works on the isotopic values of cheese. Differences in the sulphur isotopic ratio between two types of Italian PDO cheese, that is, Grana Padano PDO and Parmigiano Reggiano PDO, and not PDO cheese samples, are to be taken into consideration. Furthermore, the focus is on both the Grana Padano PDO and the Parmigiano Reggiano PDO zones, to evaluate the differences in $\delta^{34}S$ of the samples coming from the different regions and provinces of Italy. Subsequently, we are going to check whether $\delta^{34}S$ allows for distinguishing between Italian cheese samples (considering Parmigiano Reggiano PDO and Grana Padano PDO and not PDO samples as a whole) and the cheese samples of 9 foreign competitors. Finally, we are going to point out the potentialities of sulphur isotopic ratio with respect to other isotopic parameters linked to geographical origin: $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$ (Bontempo et al., 2012). Thus, the aim of this work was to show the potential of $\delta^{34}S$ in characterizing the type of cheese, as well as in determining its geographical origin, analysing a huge number of samples coming from all over the world.

4.2. Materials and methods

All samples were provided by the Grana Padano and the Parmigiano Reggiano consortia. The group of 725 samples is divided into two subgroups: Italian samples and foreign samples (**Figure 9A-B**). The former group is made up of 304 samples of Grana Padano PDO, 107 samples of Parmigiano Reggiano PDO and 52 samples of Italian not PDO hard cheeses, whereas the latter group is made up of 262 samples of foreign cheeses. From among the foreign samples, only the countries represented by a number of samples larger than 10 were considered

in the statistical analysis. The sample classification and the geographical localization of both Grana Padano and Parmigiano Reggiano PDO products are shown in **Figure 9A-B**. The years of sampling range from 2008 to 2018, but most of the samples are dated 2010.

A

B

Italian samples (463)			**Foreign samples** (262)	
Product	**Region**	**Province(s)**	**N of samples**	**Country**
Grana Padano (304)	Piedmont	Cuneo	**>10**	Czech Republic (35)
	Lombardy	Bergamo, Pavia, Lodi, Mantua, Cremona, Brescia		Austria (14)
	Emilia Romagna	Piacenza		Germany (39)
	Veneto	Padua, Verona, Rovigo, Vicenza		Poland (12)
	Trentino	Trento		France (17)
Parmigiano Reggiano (107)	Lombardy	Mantua		USA (22)
	Emilia Romagna	Reggio Emilia, Bologna, Modena, Parma		Latvia (30)
Not PDO (52)				Russia (12)
				Lithuania (34)
			<10	Holland (8)
				China (8)
				Belgium (6)
				Spain (4)
				Japan (2)
				Switzerland (4)
				Luxemburg (3)
				Australia (1)
				New Zealand (2)
				Hungary (3)
				Estonia (1)
				Ukraine (1)

Figure 9. **A** Geographical localization of the sampling points of the PDO Italian samples; **B** Classification of Italian and foreign samples

The $\delta^{34}S$, $\delta^{2}H$, $\delta^{15}N$ and $\delta^{18}O$ was determined in casein fraction obtained after defatting cheese. The details of the analysis are described in **Chapter 1.3.2**. In particular, the stable isotope ratios of H, N and O were analysed according to the procedures described in Camin et al., 2015 and in the UNI official standard 11692 2017 (Camin et al., 2015).

As for the statistics, data were evaluated using Statistica For Windows v 13.1 (StatSoft Inc., Tulsa, OK, USA). Statistically significant differences were found using a Kruskal-Wallis test. Differences were considered to be statistically significant at $p<0.05$. The remainder of the analyses was performed in R (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/) relying on tidyverse (Hadley Wickham (2017). tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse) and ggplot (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.) for data manipulation and visualization.

4.3 Results and discussion

Grana Padano PDO and Parmigiano Reggiano PDO, as already mentioned, are two types of Italian hard cheeses that are produced in specific areas of northern Italy. The two PDO zones partially overlap in the regions of Lombardy and Emilia Romagna. As they are premium products, well known all over the world, it is fundamental to improve the tools that allow for distinguishing between these products of excellence and those of Italian and foreign competitors. The Kruskal-Wallis test did not reveal a significant difference between samples due to the year of production. The same test showed that $\delta^{34}S$ makes it possible to statistically distinguish between Parmigiano Reggiano and the other two groups at $p<0.001$, as shown in **Figure 10A**. Mean values and standard deviations for the different types of cheese and for the different regions and provinces of each PDO zone are shown in **Table 3**.

Focusing on Grana Padano PDO, its zone covers several provinces in the Italian regions of Piedmont, Lombardy, Emilia Romagna, Veneto and Trentino, according to production rules (https://www.granapadano.it/it-it/disciplinare-grana-padano-dop.aspx). A Kruskal-Wallis test was performed to verify whether it was possible to characterise cheese samples coming from different regions within a PDO zone.

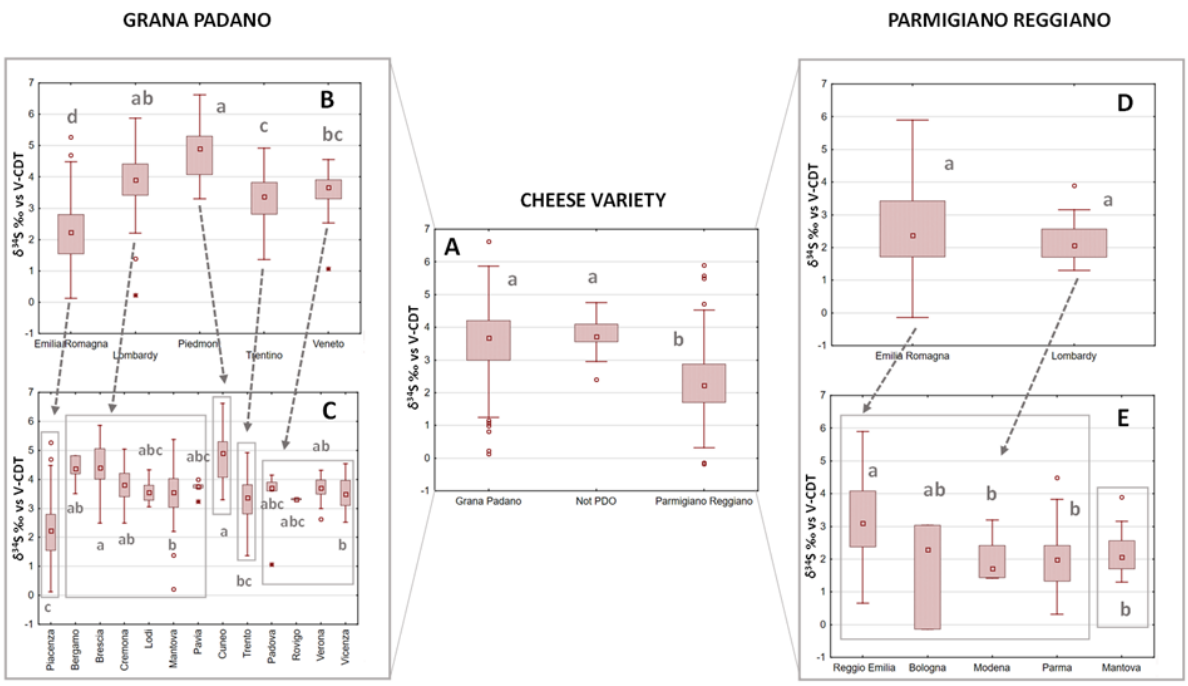


Figure 10. A $\delta^{34}S$ values of Grana Padano PDO, Parmigiano Reggiano PDO and not PDO samples; **B** $\delta^{34}S$ values of Grana Padano PDO cheese samples produced in different regions; **C** $\delta^{34}S$ values of Grana Padano PDO cheese samples produced in different provinces; **D** $\delta^{34}S$ values of Parmigiano Reggiano PDO cheese samples produced in different regions; **E** $\delta^{34}S$ values of Parmigiano Reggiano PDO cheese samples produced in different provinces

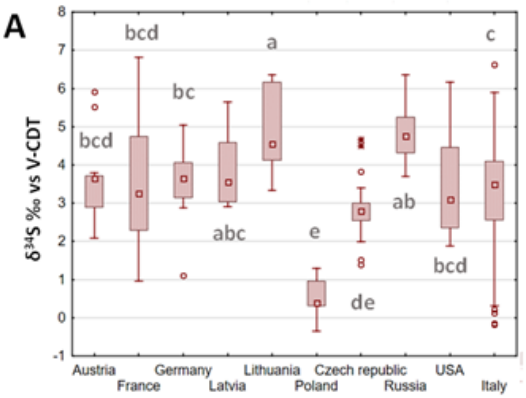
As we can see in **Figure 10B**, statistical discrimination ($p<0.05$) was achieved between Emilia Romagna samples, characterised by lower $\delta^{34}S$ values, and the rest of the Grana Padano PDO group. A possible reason for lower $\delta^{34}S$ in this region could be the presence of sulphur-bearing limestones from the ancient times of mines, resulting in post-depositional bacterial sulphate reduction (Dessau et al., 1962) which decreases the $\delta^{34}S$ of soil (Detmers et al., 2001). Piedmont resulted in having higher values ($p<0.05$) than Veneto and Trentino. If we focus on Veneto and Lombardy (**Figure 10C**), the two Grana Padano PDO regions for which we collected samples from more than one province, we can see that sulphur isotopic values do not change appreciatively within the same region. Significant differences ($p<0.05$) were found on performing a Kruskal-Wallis test (**Figure 10C**). The significant difference between the provinces of Cuneo and Piacenza reflects the already mentioned distinction between the regions of Piedmont and Emilia Romagna. Focusing on Parmigiano Reggiano PDO, the box plots in **Figure 10D-E** do not show a significant difference between the two regions of Emilia Romagna (with different sampling provinces to Grana Padano PDO) and Lombardy or between the provinces of Reggio Emilia and Bologna on one hand, and Bologna, Modena, Parma and Manua on the other hand.

Type of cheese	δ^{34}S ‰vs V-CDT Mean value	St. Dev.	Region	δ34S ‰vs V-CDT Mean value	St. Dev.	Province	δ^{34}S ‰vs V-CDT Mean value	St. Dev.
Grana Padano (304)	**3,6**	**1,0**	*Emilia Romagna (49)*	**2,3**	**1,0**	*Piacenza (49)*	**2,3**	**1,0**
			Lombardy (158)	**3,9**	**0,9**	*Bergamo (6)*	**4,3**	**0,5**
						Brescia (55)	**4,4**	**0,8**
						Cremona (22)	**3,7**	**0,7**
						Lodi (8)	**3,6**	**0,4**
						Mantova (62)	**3,5**	**0,9**
						Pavia (5)	**3,7**	**0,3**
			Piedmont (15)	**4,7**	**1,0**	*Cuneo (15)*	**4,7**	**1,0**
			Trentino (24)	**3,3**	**0,8**	*Trento (24)*	**3,3**	**0,8**
			Veneto (58)	**3,5**	**0,6**	*Padova (7)*	**3,4**	**1,1**
						Rovigo (2)	**3,3**	**0,0**
						Verona (20)	**3,7**	**0,4**
						Vicenza (29)	**3,5**	**0,5**
Parmigiano Reggiano (107)	**2,4**	**1,2**	*Emilia Romagna (67)*	**2,6**	**1,3**	*Reggio Emilia (31)*	**3,3**	**1,3**
						Bologna (3)	**1,9**	**1,0**
						Modena (8)	**2,0**	**0,7**
						Parma (25)	**1,9**	**1,0**
			Lombardy (15)	**2,2**	**0,7**	*Mantova (15)*	**1,9**	**1,0**
			Not assigned (25)	**1,9**	**0,9**			
Not PDO (52)	**3,8**	**0,5**						

Table 3. Mean and Standard Deviation of δ^{34}S of Grana Padano, Parmigiano Reggiano and not PDO cheese and the regions and the provinces of Grana Padano PDO and Parmigiano Reggiano PDO zones

Italian cheese is one of the most appreciated and valued in the world, therefore, its authenticity must be protected against competitive products from other countries. In this work, we compared 262 samples of foreign competitors with 463 samples of Italian cheeses (Grana Padano PDO, Parmigiano Reggiano PDO and those of Italian competitors). A Kruskal-Wallis test was performed to check the differences between the various groups. The statistical analysis only involved those countries represented by a number of samples higher than 10. Results are reported in **Figure 11A**. The δ^{34}S mean values as well as the standard deviation of the considered countries are shown in **Figure 11B**.

A



B

Country of origin	Mean value δ^{34}S ‰ vs V-CDT	St. Dev.
Austria	**3,5**	**1,0**
France	**3,5**	**1,6**
Germany	**3,7**	**0,7**
Latvia	**3,9**	**0,9**
Lithuania	**4,9**	**1,0**
Poland	**0,6**	**0,5**
Czech republic	**2,8**	**0,6**
Russia	**4,9**	**0,8**
USA	**3,5**	**1,4**
Italy	**3,3**	**1,2**

Figure 11. A Box Plot of δ^{34}S of cheese samples from different countries; **B** Mean Values and standard deviation of δ^{34}S of the different countries

As shown in the box plot (**Figure 11A**), cheese samples produced in Lithuania have the highest $\delta^{34}S$ values, with a mean value of 4.9 ± 1.0. Although Lithuanian mean value is fairly different to others measured, its values overlap with those of the closest countries, Latvia and Russia. On the other hand, Poland's values only overlap with those of the Czech Republic, and this country shows the lowest mean value in the set (0.6 ± 0.5). Moreover, Schellenberg et al. found particularly low $\delta^{34}S$ values in honey samples produced in Poland, in comparison with other countries considered in the study (Germany, Portugal, Spain, France, Greece, United Kingdom, Iceland, Ireland, Denmark, Austria and Italy) (Schellenberg et al., 2010).

The stable isotope ratios conventionally used for food geographical traceability are $\delta^{2}H$ and $\delta^{18}O$, as they are strictly related to the geographical and climatic characteristics of the production area (Camin et al., 2016). In the case of cheese, $\delta^{15}N$ has also shown, to some extent, a link with the geographical origin. In particular, Bontempo et al. found that lower $\delta^{15}N$ casein values were associated with cheeses produced at higher altitudes (Bontempo et al., 2012). On the other hand, $\delta^{13}C$ determined in cheeses is a major indicator of the type of diet fed to the animal, in particular, the content of C3 and C4 plants (Valenti et al., 2017).

In order to verify the role of stable isotope ratio of S compared to other geographical isotopic parameters, $\delta^{34}S$ values were plotted against $\delta^{15}N$, $\delta^{18}O$ and $\delta^{2}H$ values (**Figure 12)**, also considering the plot between the other variables. $\delta^{13}C$ was not considered because it is more related to the composition of animal diet than to the geographical origin. As expected, a positive correlation was observed between $\delta^{18}O$ and $\delta^{2}H$, which are well known parameters related to the geographical and climatic characteristics of the area, as they derive from the isotopic values of the local drinking water (Bontempo et al., 2012; Camin et al., 2008). $\delta^{34}S$ does not correlate with any of the other three isotopic ratios, nor with $\delta^{15}N$, which is different to what was observed for European cheeses (Camin et al., 2004). This indicates that $\delta^{34}S$ is affected by other different factors and that it can provide further geographical differentiation.

This is also evident from **Figure 13**, which shows the estimated density distribution for $\delta^{2}H$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$ in the set of Italian samples (Grana Padano, Parmigiano Reggiano and not PDO). $\delta^{2}H$, $\delta^{18}O$ and $\delta^{15}N$ show a quite complete overlap of their values in the three sample classes due to the geographically similar origins of the samples. At odds, $\delta^{34}S$ shows a clearly different distribution for Parmigiano Reggiano samples compared to the Grana Padano group and the not PDO cheeses (**Figures 13** and **Figure10A**).

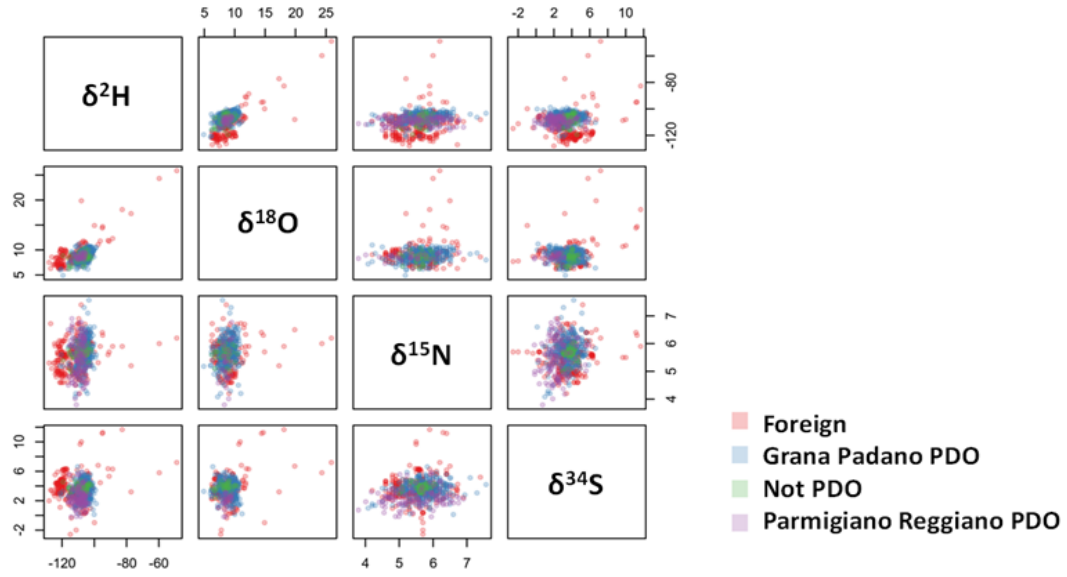


Figure 12. Correlation plots among $\delta^{34}S$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{2}H$

The reason is linked to the fact that $\delta^{34}S$ is not influenced by geographical coordinates such as $\delta^{2}H$ and $\delta^{18}O$, but mainly by the geological features of the soil (Schmidt et al., 2005) which can also change on a small scale. In this study, $\delta^{34}S$ appears to be less related to soil pedology and fertilization practice, being unrelated to $\delta^{15}N$.

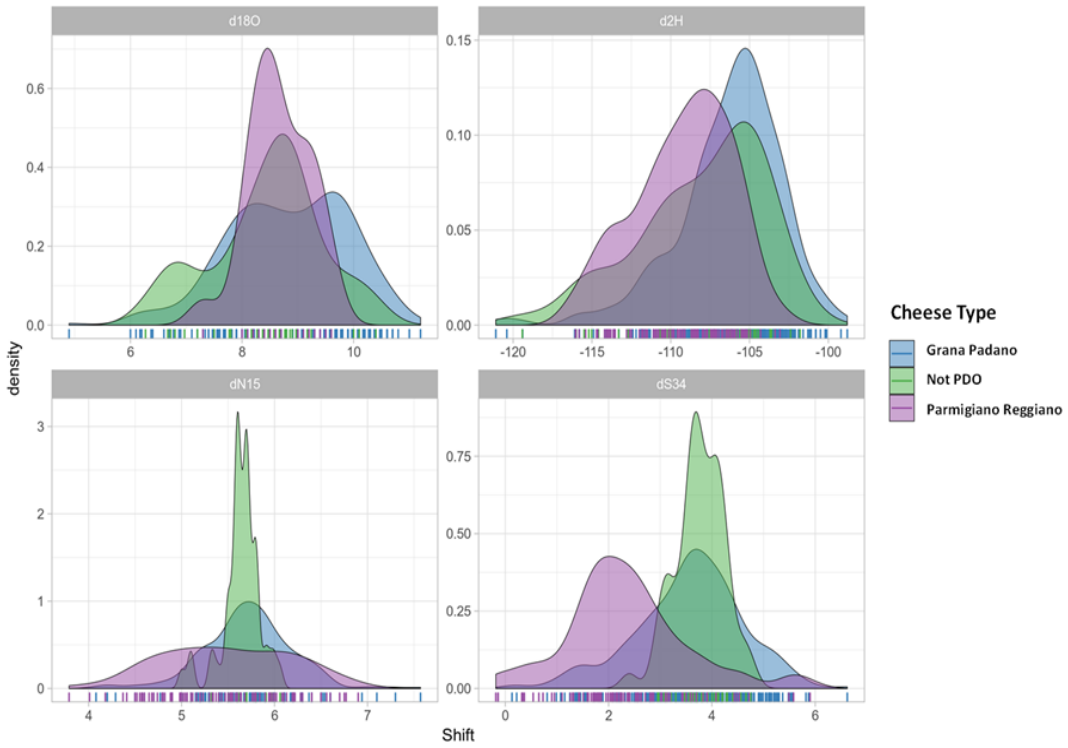


Figure 13. Density distribution for $\delta^{2}H$, $\delta^{18}O$, $\delta^{15}N$ and $\delta^{34}S$ in the set of Italian samples

This means that to differentiate foods produced in narrow areas with similar climate and geographical coordinates, stable isotope ratios such as $\delta^{2}H$ and $\delta^{18}O$ are sometimes not useful. This is also true of $\delta^{15}N$ since it is linked more to soil pedology and fertilization practice. In this case, $\delta^{34}S$, being influenced by other factors such as soil geology, can be the most suitable stable isotope ratio for discrimination.

4.4 Conclusions

A huge $\delta^{34}S$ value dataset of cheese was analysed. The $\delta^{34}S$ made it possible to characterise Grana Padano PDO, Parmigiano Reggiano PDO and the not PDO cheese samples, together with the different PDO product regions and provinces.

When considering cheeses produced in narrow areas, $\delta^{34}S$ becomes the most important stable isotope ratio for discrimination. The reason is that $\delta^{34}S$ is not linked to the climate and geographical coordinates of the production area, but it depends mainly on the geological features of the soil, which can also change on a small scale.

Chapter 5

Stable isotope ratio analysis for the characterisation of edible insects (Pianezze, Perini, et al., 2021)

5.1 Background

Entomophagy, or the act of eating insects, has been part of the culinary tradition of many countries since ancient times, but started to increase in popularity only recently, gaining momentum especially in Western countries. Nowadays, more than 100 countries and about two billion people in the world practice entomophagy, mostly in some parts of Asia, Africa and Latin America (Barennes et al., 2015). There are over 2000 species of edible insects in the world (Roos, 2018), but the most commonly consumed belong predominantly to the orders Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Odonata, Isoptera, and Diptera (van Huis, 2013). Edible insects can be consumed at different life stages as larvae, pupae and adult insects (Stamer, 2015).

Despite the common prejudices that make people reject entomophagy, they are a valuable source of protein, fibers, fats, vitamins (i.e., ascorbic and folic acid, thiamine, riboflavin, niacin) and minerals (i.e., calcium, potassium, magnesium, phosphor, sodium, and iron). Most species of insects generally contain high levels of proteins (50-70% on dry basis), resulting in good digestibility (Sosa & Fogliano, 2017). As for fats, their content ranges from 10 to 50% on dry basis (Xiaoming et al., 2010). The percentage can differ according to the life stage of the insect (the amount is usually higher in the larvae compared to the adults), while their composition in terms of fatty acids depends on factors such as the species, sex, stage of life, diet and environmental temperature (Oonincx et al., 2015). The fats of the insects, which are generally liquid at room temperature, can derive from their diet or from endogenous synthesis. The essential fatty acids contained in their fat are linoleic (18:2n-6) and linolenic (18:3n-3) acids, which are more abundant in insects than in other animal and vegetal sources (Lucas et al., 2020).

Besides the nutritional value of edible insects, entomophagy also provides environmental advantages (Govorushko, 2019). As sustainability is one of the emerging themes of our times, the inclusion of insects in our diet can help reduce the amount of water and land used for livestock. As reported in other works, indeed, the land area and the water needed to rear insects is less consistent than those needed for the production of milk, chicken, pork, and beef (Nadeau

et al., 2015; Oonincx et al., 2012). Rearing insects also entails a very high conversion of food into protein, giving solution to the growing demand by humanity for food and nutrition, and especially for animal protein (Govorushko, 2019). Furthermore, the inclusion of insects into our diet would contribute to the decrease of greenhouse gasses and ammonia emissions deriving from this practice (Nadeau et al., 2015; Oonincx et al., 2010).

From a regulatory point of view, in Europe insects are considered as a "novel food" by Regulation 2015/2283EC. Meanwhile, only a limited number of studies have been carried out to investigate the chemical and microbiological safety of edible insects (Baiano, 2020; Poma et al., 2017, 2019). The European Food Safety Authority (EFSA) mentioned *Musca domestica*, *Zophobas atratus*, *Galleria mellonella*, *Bombyx mori*, *Achroia grisella*, *Locusta migratoria migratorioides*, *Schistocerca Americana*, *Tenebrio molitor*, *Hermetia illucens*, *Acheta domesticus*, *Gryllodes sigillatus* and *Alphitobius diaperinus* as the species having the greatest potential to be used as food and feed in Europe (EFSA Journal, 2015).

Stable isotope ratio analysis of light elements (C, N, S, O and H) has been widely used to investigate food authenticity. Examples are reported for wine (Dordevic et al., 2013), vinegar (Camin et al., 2013; Perini et al., 2014), orange juice (Simpkins et al., 1999), coffee (Santato et al., 2012), olive oil (Camin et al., 2010), cheese (Camin et al., 2012; Pianezze et al., 2020), saffron (Perini et al., 2020), tomatoes (Bontempo, Camin, et al., 2011; Bontempo et al., 2020), honey (Schellenberg et al., 2010), cereals (Asfaha et al., 2011) and also food supplements (Perini et al., 2017). It has also been recognised since the 90s, by several standardisation organizations (CEN, AOAC, OIV, EU Reg.), as an official method to detect adulterations in wine, honey, fruit juice, vinegar, cheese and other food matrices (Rossmann, 2001). An authenticity or adulteration assessment encompasses the analysis of genuine samples to create reference datasets (Camin et al., 2017; Donarski et al., 2019) which are then used for comparing the data of samples on the market. Isotopic values are therefore available for several categories of food, but to the best of our knowledge, no isotopic values for edible insects are yet reported in literature.

The $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$ of 40 defatted farmed insect samples and 4 insect-based food items, as well as the $\delta^{13}C$ of their fats, have been measured using IRMS. The aim was to provide the first background reference stable isotope ratios that could be useful for future investigations on their authenticity. This first dataset, once confirmed by analysing a larger set of samples, can be used to characterise the different species of edible insects and then, based

on data comparison, to verify their authenticity, i.e., the correspondence to what is reported on the label of insect-based food products, in terms of insect composition and origin.

5.2 Materials and Methods

The current dataset includes 44 samples, 40 of which were edible insects authorized for human consumption and 4 were insect-based food items, purchased between November 2015 and September 2018 from different European and Asian shops, e-shops, and supermarkets. A detailed description of the collected samples is reported elsewhere (Poma et al., 2017, 2019) and summarized in **Table 4**.

SAMPLE	ORDER	PRODUCT	LIFE STAGE	ORIGIN CONTINENT	$\delta^{13}C‰$ defatted (vs V-PDB)	$\delta^{15}N‰$ defatted (vs Air)	$\delta^{34}S‰$ defatted (vs V-CDT)	$\delta^{2}H‰$ defatted (vs V-SMOW)	$\delta^{18}O$ defatted (vs V-SMOW)	$\delta^{13}C$ fat vs (V-PDB)
O1	Orthoptera	Natural	Adult	Europe	-23.5	3.3	4.2	-87.2	18.1	-27.3
O2	Orthoptera	Natural	Adult	Europe	-23.5	3.5	5.2	-83.3	17.1	-27.9
O3	Orthoptera	Seasoned	Adult	Asia	-19.9	4.3	4.5	-95.9	16.2	-23.8
O4	Orthoptera	Natural	Adult	Europe	-23.5	3.2	5.7	-84.9	18.1	-27.5
O5	Orthoptera	Natural	Adult	Europe	-21.3	4.1	6.4	-73.6	20.1	-24.2
O6	Orthoptera	Natural	Adult	Europe	-28.7	4.8	4.4	-88.5	19.8	-32.7
O7	Orthoptera	Natural	Adult	Europe	-28.4	6.1	4.7	-89.6	19.5	-32.5
O8	Orthoptera	Natural	Adult	Asia	-22.6	3.9	2.1	-90.9	19.6	-25.5
O9	Orthoptera	Natural	Adult	Europe	-28.9	4.1	5.7	-92.2	18.6	-33.0
O10	Orthoptera	Natural	Adult	Europe	-28.6	6.0	4.5	-86.3	18.2	-32.1
O11	Orthoptera	Natural	Adult	Asia	-20.3	2.0	2.9	-89.4	14.6	-22.1
O12	Orthoptera	Natural	Adult	Asia	-18.7	3.1	1.6	-85.4	17.0	-25.6
O13	Orthoptera	Seasoned	Adult	Asia	-17.9	4.7	2.3	-49.2	26.7	nd
O14	Orthoptera	Natural	Adult	Asia	-26.3	5.2	1.2	-101.1	17.7	nd
O15	Orthoptera	Natural	Adult	Asia	-12.2	9.9	-0.1	-67.5	22.3	-21.1
O16	Orthoptera	Natural	Adult	Asia	-18.9	2.5	-2.6	-98.7	15.7	-19.8
O17	Orthoptera	Natural	Adult	Asia	-25.2	10.5	7.8	-76.6	19.2	-31.2
O18	Orthoptera	Natural	Adult	Europe	-28.4	5.5	2.8	-83.1	19.4	-32.5
O19	Orthoptera	Natural	Adult	Europe	-27.9	4.7	1.7	-90.1	20.4	-32.5
C1	Coleoptera	Natural	Larva	Europe	-24.3	4.7	5.2	-75.6	19.9	-28.4
C2	Coleoptera	Natural	Larva	Europe	-23.9	5.5	4.6	-77.2	19.6	-28.3
C3	Coleoptera	Natural	Larva	Asia	-20.2	4.4	0.7	-77.5	18.9	-23.0
C4	Coleoptera	Natural	Larva	Europe	-24.5	5.4	5.2	-70.9	19.9	-28.6
C5	Coleoptera	Natural	Larva	Europe	-24.3	4.9	2.8	-71.2	19.9	-28.6
C6	Coleoptera	Natural	Larva	Asia	-26.9	1.8	7.7	-77.1	17.8	-32.3
C7	Coleoptera	Natural	Larva	Asia	-24.8	5.9	1.6	-59.7	20.3	-31.3
C8	Coleoptera	Natural	Larva	Asia	-24.5	11.5	0.8	-58.5	19.0	-29.8
C9	Coleoptera	Natural	Larva	Asia	-24.2	5.4	5.6	-64.4	22.6	-28.6
C10	Coleoptera	Natural	Larva	Asia	-24.5	6.0	2.9	-71.5	19.6	-29.1
C11	Coleoptera	Natural	Larva	Asia	-17.9	3.2	3.0	-100.7	15.0	-19.4
C12	Coleoptera	Natural	Larva	Asia	-27.3	2.3	4.4	-80.5	20.5	-32.2
C13	Coleoptera	Natural	Larva	Europe	-23.9	5	1.9	-77.8	19.9	-28.5
C14	Coleoptera	Natural	Larva	Europe	-24.1	6.5	3.2	-71.6	19.9	-29.2
L1	Lepidoptera	Natural	Larva	Asia	-28.9	6.4	-2.4	-99.6	13.5	-35.7
L2	Lepidoptera	Seasoned	Larva	Asia	-21.4	6.7	2.0	-55.5	26.0	-34
L3	Lepidoptera	Seasoned	Larva	Asia	-27.6	5.6	2.4	-87.6	18.2	-35.4
L4	Lepidoptera	Natural	Larva	Europe	-23.8	6.6	-2.6	-70.3	16.4	-27.9
He1	Hemiptera	Natural	Larva	Asia	-27.8	2.1	3.2	-82.6	14.9	-31.1
Od1	Odonata	Natural	Larva	Asia	-31.3	7.6	-0.6	-83.7	17.5	-37.6
Hy1	Hymenoptera	Seasoned	Larva	Asia	-19.6	1.9	2.8	-59.3	28.9	-30.0
IBFI1	Commercial	IBFI	-	-	-26.2	3.9	2.9	-40.9	27.4	nd
IBFI2	Commercial	IBFI	-	-	-25.1	4.3	4.1	-43.7	29.9	nd
IBFI3	Commercial	IBFI	-	-	-26	4.1	2.4	-62.9	24.0	-29.9
IBFI4	Commercial	IBFI	-	-	-23.7	2.1	3.8	-46.6	27.6	nd

Table 4. Dataset of $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$ of the defatted samples and $\delta^{13}C$ of the fats

Briefly, the selected species of edible insects belonged to six different Orders: Orthoptera (n=19), Coleoptera (n=14), Lepidoptera (n=4), Hemiptera (n=1), Odonata (n=1), Hymenoptera (n=1) and were purchased in their natural state (n=35, i.e., without addition of any ingredients) or seasoned (n=5, i.e., with added flavours and dressings, such as sugar, soy sauce and syrup). Insect-based food was plant-based food containing small percentages of insects (5-10% in weight) and were buffalo worm-based "bugballs", cricket croquettes, and buffalo worm-based "bugburgers". Multiple insects composing a sample were pooled for analysis, freeze-dried, homogenized, and stored in aluminium foil at −20°C pending analysis. The fat content of each sample was determined gravimetrically (Poma et al., 2017, 2019).

To separate the fats from the defatted fraction, all samples were washed with three 10 mL aliquots of ethyl ether: petroleum ether (1:2, *v/v*). The washed samples were then dried, while the etheric aliquots were collected and evaporated to get the fatty fraction. The procedure was performed according to (Camin et al., 2004). Finally, to exclude the presence of carbonates, all samples were treated with 10 μL of HCl (0.1 N) and dried in an oven at 60 °C overnight. The defatted samples were thus weighed and analysed through EA-IRMS ($^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$) and TC/EA ($^{2}H/^{1}H$ and $^{18}O/^{16}O$) as described in **Chapter 1.3.2**.

Statistical analysis was carried out using RStudio version 1.2.5033 (2019). Non-parametric tests were applied due to the low number of samples. The Kolmogorov-Smirnov test was used to compare two independent groups and the Kruskal-Wallis test was used to compare more than two independent groups. Moreover, a dendrogram was performed in RStudio to display potential sample groupings in clusters formed according to similar features.

5.3 Results and Discussion

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ dataset, grouped based on the order of each edible insect sample, is represented in **Figure 14**. Values of $\delta^{13}C$ ranged from -12.2 to -31.3‰ (**Table 4**). The carbon stable isotope ratio is related to the diet of the insect. In particular, it is known that C4 plants have $\delta^{13}C$ values between -14 and -12‰, while C3 plants range between -30 and -23‰ (Knobbe et al., 2006). A few insect samples showed $\delta^{13}C$ higher than -23‰, up to -12.2‰ for an Asian grasshopper, likely attributable to ingestion of C4 plants. Anyway, most samples had $\delta^{13}C$ values fitting the range of a C3 plant, indicating this is a major component of insects' diet. Kruskal-Wallis test has been carried out, yielding no significant differences among orders (**Figure 14A**).

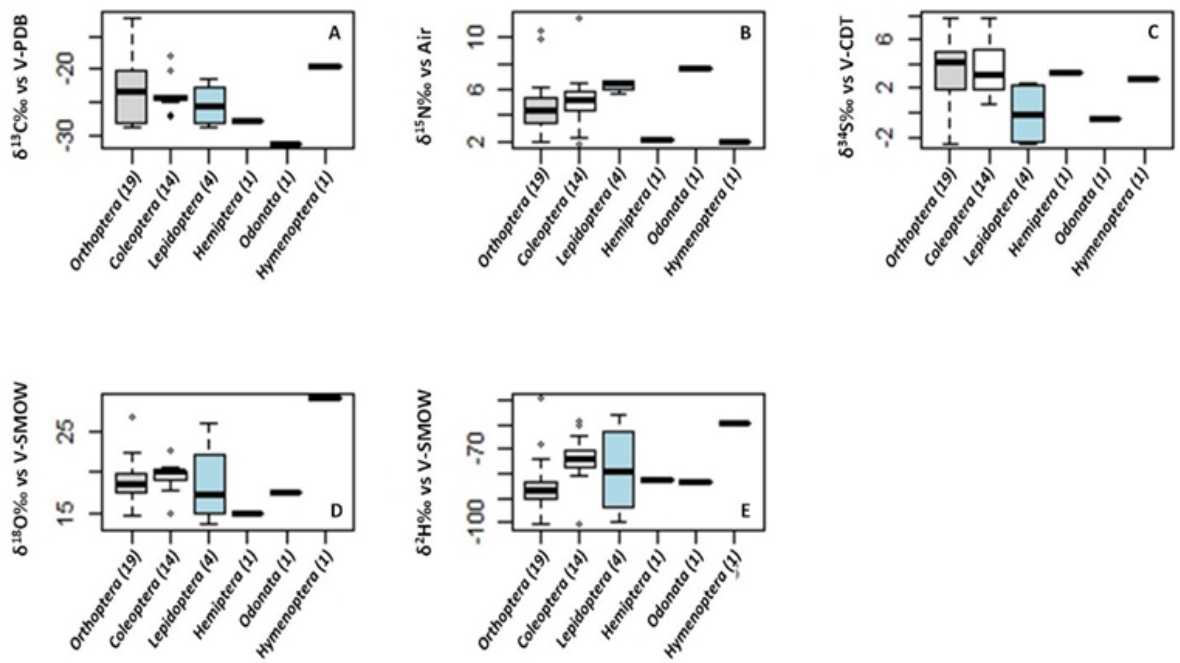


Figure 14. A, $\delta^{13}C$, **B**, $\delta^{15}N$, **C**, $\delta^{34}S$, **D**, $\delta^{18}O$ and **E**, $\delta^{2}H$ of the defatted samples grouped according to the order of the insects (number of samples belonging to each order is given in brackets)

Values of $\delta^{15}N$ in insects ranged from 1.8 to 11.5 ‰ (**Table 4**). The Kruskal-Wallis test was carried out, but did not show any significant differences among the orders (**Figure 14B**). The nitrogen stable isotope ratio of the animals is closely related to their trophic level (O'Brien, 2015). Herbivorous species are expected to have a lower $\delta^{15}N$ than carnivorous ones, as ^{15}N accumulates along the trophic chain (Camin et al., 2016). The range of values found here for insects indicates an omnivorous diet, consisting of both plants and animals. The $\delta^{15}N$ and $\delta^{13}C$ values reported in this work resemble the isotopic values described in an article on Asian longhorn beetles (Heinrich & Collins, 2017).

Finally, the values of $\delta^{34}S$ ranged from -2.6 to 7.8‰ (**Table 4**). As for $\delta^{13}C$, the $\delta^{34}S$ values of animals tend to reflect the values of the plants they feed on. Indeed, the sulphur trophic shift between animals and their diet was estimated in literature between 0‰ and 1‰ (Harrison et al., 2011; (Tanz) et al., 2015). In turn, the $\delta^{34}S$ values of the plants are influenced by factors like the geology of the soil the plant grew on (e.g., presence of sulphides in the soil, type of underlying local bedrocks), aerobic or anaerobic growing conditions (Rubenstein & Hobson, 2004) of the plant itself, active microbial process into the soil, closeness to the sea (sea-spray effect), and the fertilization practices (Krouse et al., 2000; Rubenstein & Hobson, 2004). To

test the differences among the orders (**Figure 14C**), the Kruskal-Wallis test was used, giving no significant differences. Since the sulphur isotopic ratio is also influenced by the geology of the soil in which the insect dietary plants grow, the samples were divided in 2 groups: from Asia (n=23) and from Europe (n=17). The $\delta^{34}S$ of the European samples ($\delta^{34}S$ 3.9±3.1‰) showed statistically higher values that of the Asian ones ($\delta^{34}S$ 2.3±2.6‰) ($p<0.05$). For $\delta^{13}C$ and $\delta^{15}N$, we did not find significant differences between Asian and European insects.

As reported in the literature, $\delta^{2}H$ and $\delta^{18}O$ of water are strictly related parameters (Araguas et al., 1996) that depend on variables such as latitude, altitude, closeness to the sea (Bowen et al., 2007). For plants, the correlation between $\delta^{2}H$ and $\delta^{18}O$ is still valid, but not that strict, as the only source of hydrogen is the water absorbed through the roots, while oxygen derives from both O_2 and CO_2 absorbed through the stomata (Barbour, 2007). As the sources of oxygen and hydrogen of the insects are both the water they drink and the plants they eat, their $\delta^{2}H$ and $\delta^{18}O$ is influenced by such factors. The correlation between the oxygen and hydrogen isotopic ratios ($\delta^{2}H = 3.41‰\ \delta^{18}O - 144‰$; $r^2 = 0.84$), is represented in **Figure 15**. A similar correlation has already been reported in literature concerning isotopic ratios of oxygen and hydrogen in human scalp ($\delta^{2}H = 5.73‰\ \delta^{18}O - 166‰$; $r^2 = 0.873$) (Ehleringer et al., 2008). Furthermore, the slope of the correlation found in this work is similar to that described in another one concerning crickets and spiders, in which the slope was reported to range between 3.8 and 5.8 (McCluney & Sabo, 2010).

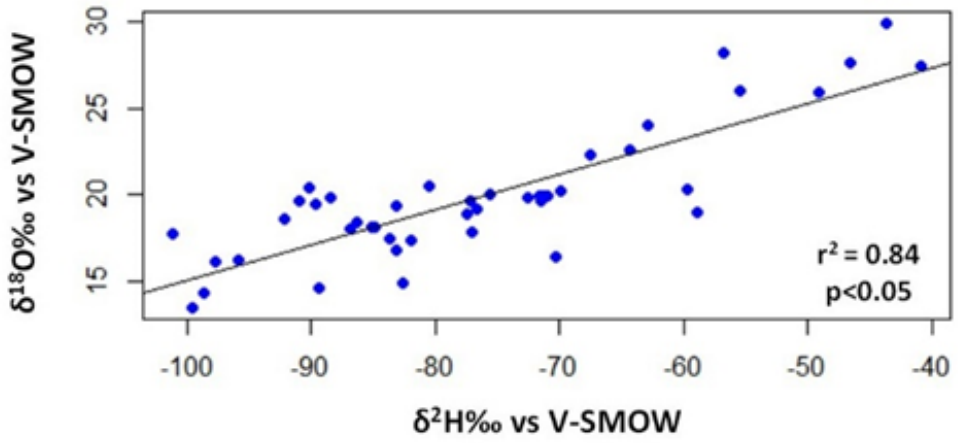


Figure 15. Linear correlation between $\delta^{18}O$ and $\delta^{2}H$

Our results ranged from -40.9 to -101.1‰ for $\delta^{2}H$ and from 13.5 to 29.9‰ for $\delta^{18}O$ (**Table 4**) and as for the other isotopic data, they were not significantly different from one insect order to another (Kruskal-Wallis test) (**Figure 14D, E**). These ranges of values resemble the isotopic

ratios described in other studies on insects (Heinrich & Collins, 2017; Myers et al., 2012). Our hydrogen and oxygen isotopic ratios seem to be higher in insects compared to animal muscle, probably because of the presence of chitin with higher δ^2H values compared to collagen (Soto et al., 2017). As for $\delta^{13}C$, $\delta^{15}N$, δ^2H and $\delta^{18}O$, we did not find significant differences between Asian and European insects. The only significant difference was between larvae (-74.9±12.1‰) and adults (-84.9±11.9‰), with significantly higher δ^2H values in larvae, probably due to the different tissue composition. Moreover, we found higher δ^2H and especially $\delta^{18}O$ values, even though they were not statistically significant, in seasoned samples (mean values -77.1±18.4‰ and 23.2±5.6‰, respectively) in comparison to natural samples (-76.6±14.9‰ and 18.6±2.1‰, respectively). A possible reason thereof can be the presence of sugar in the seasoning samples, as sugar shows high $\delta^{18}O$ values of around 30‰ (Houerou et al., 1999; Spangenberg, 2006).

The $\delta^{13}C$ of fats ranged from -19.4 to -37.6 ‰. $\delta^{13}C_{FAT}$ and $\delta^{13}C_{DEFATTED}$ are expected to be related, as the only source of carbon is the diet. The linear correlation resulting from our dataset is represented in **Figure 16** ($\delta^{13}C_{FAT}$ = 0.955 $\delta^{13}C_{DEFATTED}$ - 5.89, r^2 = 0.73). An average depletion of 4.9‰ in fat compared to the defatted samples was calculated, in line with the findings of other authors in other animal species (Camin et al., 2018; Kiljunen et al., 2006; Smet et al., 2004). Due to the depletion in the lighter isotope (fractionation) during the synthesis of fats, the $\delta^{13}C_{DEFATTED}$ (which is actually the carbon isotopic ratio of the insect proteins) is higher than $\delta^{13}C_{FAT}$ (Deniro & Epstein, 1978; Piasentier et al., 2003).

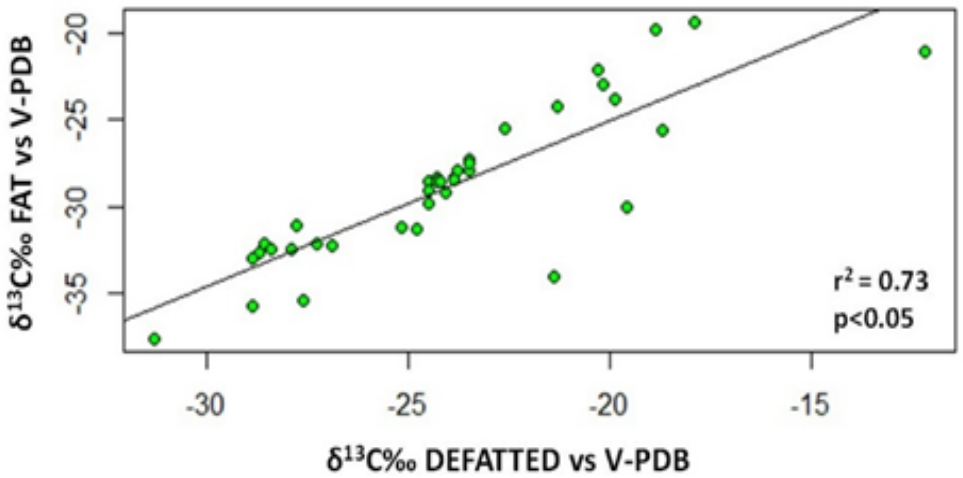


Figure 16. Linear correlation between $\delta^{13}C$ in defatted samples and $\delta^{13}C$ in fats

The isotopic values of the 4 insect-based food items are reported in **Table 4**. The insect-based food items IBFI1, IBFI2, IBFI3 and IBFI4 contained 7.4%, 15.8%, 8.9% and 10.0% of insects, respectively (Poma et al., 2017). Their mean isotopic values ($\delta^{13}C_{DEFATTED}$ = -25.3±1.1‰, $\delta^{15}N_{DEFATTED}$ = 3.6±1.0‰, $\delta^{34}S_{DEFATTED}$ = 3.3±0.8‰) are comparable (not statistically different, as shown by the Kolmogorov-Smirnov test) to the defatted insect. As for $\delta^{13}C_{FAT}$, it was possible to measure only one value among the 4 food items (-29.9‰) due to the low fat concentration of the samples. As for $\delta^{2}H_{DEFATTED}$ (-48.5±9.9‰) and $\delta^{18}O_{DEFATTED}$ (27.2±2.4‰), the insect-based food items present significantly higher values compared to the insects. If we divide the farmed insects into natural and seasoned ones, the insect-based products show values of $\delta^{2}H_{DEFATTED}$ and $\delta^{18}O_{DEFATTED}$ similar to those of the seasoned insects. (**Figure 17**).

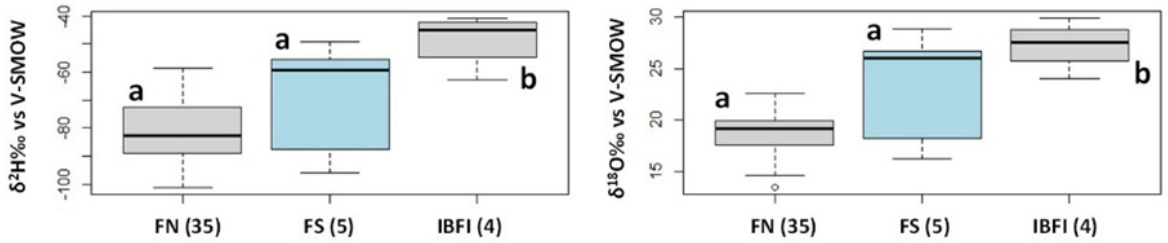


Figure 17. $\delta^{2}H$ and $\delta^{18}O$ values vs type of product considered, whether farmed natural (FN) or seasoned (FN) insects or insect-based food items (IBFI). Significantly different mean values are identified by different letters ($p<0.05$)

This variation seems therefore imputable to the seasoning added to the insects. The results are represented in **Figure 17**, where F are the farmed insect samples (natural and seasoned) and IBFI are the insect-based food items. The Kolmogorov-Smirnov test revealed significant differences between the IBFI and F samples in terms of both $\delta^{2}H$ ($p<0.05$) and $\delta^{18}O$ ($p<0.05$). Sugar, soy sauce, syrup, amino acids, vegetal oils, and flavours are just a few of the ingredients that can be added to insects and these additives can change their isotopic values. Higher $\delta^{18}O$ values may be explained with the addition of sugars to the insects. Indeed, as reported in several works, sugar usually has an oxygen isotopic ratio of around 30‰ (Houerou et al., 1999; Spangenberg, 2006).

To check whether the samples of the dataset can be grouped in clusters according to similar features, a dendrogram was created (**Figure 18**). This representation makes it possible to display potential likenesses between groups of samples, to highlight a clustering feature, i.e. a specific characteristic which appears in different and apparently non connected samples. The

number on the x axis corresponds to the code shown in **Table 4**, while the hierarchical level of aggregation, which represents the distance or dissimilarity between clusters, is shown on the y axis.

By considering a hierarchical level of 40, three groups can be distinguished: Group 1 (20 samples) labelled in blue, Group 2 (6 samples) in yellow and Group 3 (18 samples) in red, (**Figure 18**). Group 1 and Group 3 are mainly composed of natural samples (90% and 94%, respectively), while Group 2 includes 3 insect-based food items and 3 seasoned insects. This confirms the ability of isotopic analysis, in particular $\delta^2H_{defatted}$ and $\delta^{18}O_{defatted}$, to differentiate seasoned from natural insects, as previously discussed. Focusing on the differences between Group 1 and Group 3, most (75%) of the first one is represented by natural adult insects, while the second is mostly represented by natural insects in their larval stage (78%). Thus, the insect life stage seems to be the clustering feature that makes it possible to discriminate between the two groups, as discussed for hydrogen isotopic ratio.

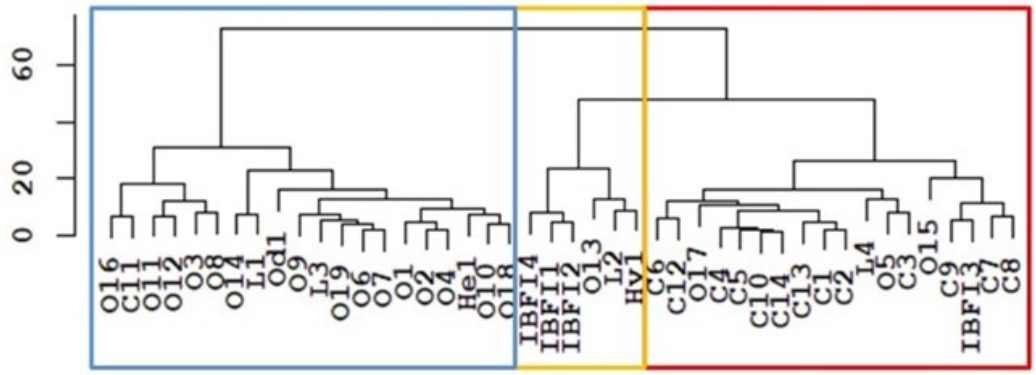


Figure 18. Dendrogram of the dataset. Group 1 (n=20, blue); Group 2 (n=6, yellow); Group 3 (n=18, red). On the x axis is reported the same code as in **Table 4**, on the y axis the dissimilarity between clusters.

5.4 Conclusions

In the work presented in **Chapter 5**, the $\delta^{13}C$, $\delta^{34}S$, $\delta^{15}N$, δ^2H and $\delta^{18}O$ of the defatted samples and the $\delta^{13}C$ of the fat have been provided for different edible insect species and some insect-based food items. As edible insects had never been investigated with stable isotope ratio analysis before, these data can be used to give a first isotopic characterisation of this product and, once confirmed by the analysis of a larger dataset, can be used as reference of authenticity

in order to verify the compliance of what is reported on the label of insect-based food products on the market. Parameters like $\delta^{2}H$ and $\delta^{18}O$ appear to allow the differentiation between natural and seasoned insects and insect-based food items. This is due to the addition of ingredients and flavours to the latter, which makes their isotopic values shift as against the values of natural insects. Furthermore, other parameters seem to be promising in the characterisation of the insect diet ($\delta^{13}C$), geographical origin ($\delta^{34}S$) and the trophic level ($\delta^{2}H$) of edible insects.

Chapter 6

Tracing lamb meat with stable isotope ratio analysis (Pianezze, Camin, et al., 2021)

6.1 Background

Even though in the Mediterranean area lamb has been widely appreciated by consumers for a long time (Gürsoy, 2006), in recent years it gained more popularity due to its higher protein and lower fat content, and to its reduced contamination risk as against other animal meat (Sun et al., 2012).

In an overall context of growing interest in animal products traceability and in their geographical origin identification, the European Commission decided to assign PDO and PGI marks (Sacco et al., 2005), such as Agnello di Sardegna (EC Reg. n. 138 of 24.01.01) or Abbacchio Romano (EC Reg. n. 507 of 15.06.09), with the aim to protect typical lamb products and increase consumer confidence. The animal products belonging to the categories mentioned above are, in most cases, associated with a specific area (Camin et al., 2007) and defined by the feeding regime, due to its effect on animal tissue composition and thus on meat quality (Geay et al., 2001). In particular, the interest in the possibility of discriminating between pasture and stall-based feeding systems lies in its implication in organic and sustainable production systems (Biondi et al., 2013; Mekki et al., 2016; Perini et al., 2009). Moreover, meat from animals raised on pasture is considered to be healthier than that obtained from stall-fed animals, due to the higher content of omega-3 fatty acids, conjugated linoleic acid and oleic acid (Simopoulos, 2002).

An effective defence for typical products requires the development of innovative analytical techniques, such as stable isotope analysis of the light elements (carbon, nitrogen, sulphur, hydrogen and oxygen), that has been used for more than 20 years in food authenticity controls and food traceability (Rossmann, 2001). In particular, it has been widely used to characterise animal products such as milk (Bontempo et al., 2012; Knobbe et al., 2006; Molkentin & Giesemann, 2007), honey (Bontempo et al., 2017), cheese (Camin et al., 2004) and meat (Bahar et al., 2005; Camin et al., 2016). Indeed, as isotopic features are transferred from feeds to animal products, the isotopic values of animal matrices are expected to be related to their feeding regime and to their geographical origin. Therefore, stable isotope ratio analysis figures as one of the most suitable techniques for authentication purposes.

In order to normalise the results and anchor delta values to the relevant conventional delta scale (e.g., as for $\delta^{18}O$, a scale in which $\delta^{18}O$ of VSMOW = 0 ‰ and $\delta^{18}O$ of SLAP = −55.5 ‰), at least two reference materials matching the matrix and bracketing the values of the samples are needed. In this way it is possible to compensate for differences in response of analytical instrumentation, which commonly compress isotope−delta scales and minimize matrix effects according to the "principle of identical treatment of sample and reference material" (Dunn et al., 2020; Meier-Augenstein & Schimmelmann, 2019). So far, certified secondary reference materials for lamb meat are not available. There are some secondary standards available today as IAEA and USGS reference materials with certified values for $\delta^{13}C$ and $\delta^{15}N$ in organic and proteinaceous compounds, and a few for $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$ (e.g., USGS Caribou Hoof Standard (CBS) and Kudu Horn Standard (KHS) or IAEA Benzoic Acid (601 and 602)). As already mentioned in **Chapter 1.3.1**, the issue is particularly important for $\delta^{2}H$, because in compounds with exchangeable H, exchanges occur with the air humidity, altering therefore the original $\delta^{2}H$ values. This makes it sometimes difficult to compare the data obtained (Meier-Augenstein & Schimmelmann, 2019), which must therefore be normalised. A possible solution is reported by (Wassenaar & Hobson, 2003), who stated that it is firstly necessary to balance the sample and the reference material, in particular for deuterium, to allow the exchange of exchangeable hydrogens with the same atmospheric air, and then to normalise the data. The method is called "comparative equilibration technique". The different protein matrices exchange differently, and it would therefore be useful to have a reference material with the same composition of the samples being analysed. Another option is to calculate the fraction of exchangeable H atoms experimentally, by equilibrating the samples using water vapours with different isotopic compositions. The difference between the total $\delta^{2}H$ values of a sample, equilibrated with two distinct water vapours, could then be used to calculate the contribution of exchangeable H atoms (Wassenaar & Hobson, 2000).

The aim of this review was to provide an alternative and innovative overview on geographical identification and traceability of lambs, by describing in detail five different light elements (carbon, nitrogen, sulphur, hydrogen and oxygen), their ranges of variability and how they are investigated in the case of lamb matrices (namely defatted meat, fat, wool, plasma, erythrocytes and faeces).

6.2 Carbon Stable Isotope Ratio of defatted lamb meat and other matrices

A description of the factors influencing the carbon isotopic ratio of animal matrices are described in **Chapter 2.1**. The $\delta^{13}C$ of the defatted meat (or defatted dry mass, DDM) is representative of the diet of the animal, as this parameter is mainly affected by the amount of C3 (pasture or C3 concentrates) and C4 (generally deriving from concentrate containing C4 plants such as maize) feedings in the diet (Bahar et al., 2005) and is thus capable of providing information about the feeding regime of European lambs (Camin et al., 2007; Mekki et al., 2016; Moreno-Rojas et al., 2008; Perini et al., 2009; Piasentier et al., 2003; Qie et al., 2021; Sacco et al., 2005; Sun et al., 2012, 2016). To visualize how the carbon isotopic ratio in the DDM manages to differentiate between lambs fed on different diets, the plot of the $\delta^{13}C_{DDM}$ vs $\delta^{15}N_{DDM}$ (as these parameters are often analysed as a fixed couple) values, taken from the literature, is displayed (**Figure 19**).

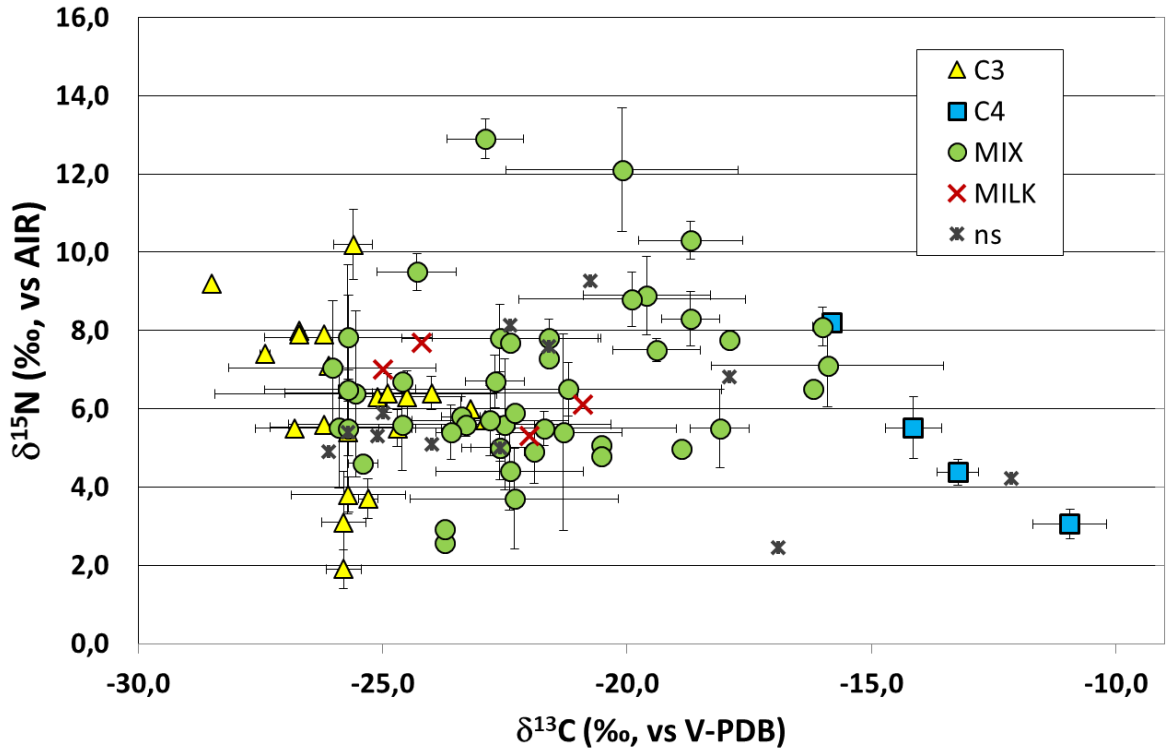


Figure 19. Carbon isotopic ratio vs nitrogen isotopic ratio for DDM samples from lambs fed with diets based on milk (MILK), C3, C4, or both C3 and C3 (MIXED) plants of the cited works (Biondi et al., 2013; Camin et al., 2007; Erasmus et al., 2016, 2018; Liu et al., 2020; Mekki et al., 2016; Perini et al., 2009; Piasentier et al., 2003; Qie et al., 2021; Sacco et al., 2005; Sun et al., 2012, 2016).

Nevertheless, as for lambs grazed in extra-European territories, it is worth noting that, as the pasture may also include several C4 plants, such as in the case of African pastures, it is not

always possible to associate a high carbon isotopic ratio with the use of C4 concentrates (Erasmus et al., 2016). Furthermore, it has been demonstrated that $\delta^{13}C$ is also a powerful tool to detect a diet switch from a C3-pasture to a C3-concentrate, thanks to slight differences in isotopic composition, as it makes it possible to discriminate between pasture-fed lambs and other lambs (stall-fed, grazed and switched from pasture to stalls for 37 or 14 days before slaughter). In the same work, the $\delta^{13}C_{DDM}$ proved to be correlated to both $\delta^{13}C_{PLASMA}$ and $\delta^{13}C_{ERYTHROCYTES}$ (Biondi et al., 2013).

As for traceability, the carbon isotopic ratio of DDM, often considered together with nitrogen rather than with other light elements, has been successfully used in the authentication of lambs coming from both different European countries (Camin et al., 2007; Piasentier et al., 2003) and different sites of Italy (Perini et al., 2009), China (Liu et al., 2020; Sun et al., 2012, 2016) and Africa (Erasmus et al., 2016). The differences found in meat of different origins seem to be related not only to the type of diet but also to the differences in the isotopic composition of the plants that the animals eat. Plants generally show higher $\delta^{13}C$ values when high temperatures, low air humidity and a high ground-water deficit led to narrower stomatal apertures in leaves (Barbour et al., 2000). This evidence was already collected in other studies on saffron (Perini et al., 2020) and goji berries (Bertoldi et al., 2019).

Besides DDM, the second most investigated matrix for stable isotope analysis is the lamb fat. Several works reported the $\delta^{13}C$ values of defatted lamb meat as less negative than the same values in the fat (Biondi et al., 2013; Liu et al., 2020; Mekki et al., 2016; Moreno-Rojas et al., 2008; Perini et al., 2009; Piasentier et al., 2003), as a consequence of the depletion in ^{13}C during the synthesis of lipids (DeNiro & Epstein, 1977; Tieszen et al., 1983). Nevertheless, the two parameters showed a mutual correlation (Liu et al., 2020; Perini et al., 2009; Piasentier et al., 2003). Similar values are also reported by van Leeuwen et al., 2017 in a study on the $\delta^{13}C$ of neutral and polar intramuscular fat of lambs fed on four different diets and by Richter, Spangenberg, Willems, et al., 2012 in a study on fatty acids of lambs fed exclusively on C3 plants and grazed in sites at different elevations. In the last cited work, $\delta^{13}C_{FAT}$ alone was sufficient to discriminate ($p<0.05$) between Engadin lambs grazing mountain or lowland pastures, thus turning out to be a suitable tool for traceability purposes. At increasing altitudes, indeed, the partial pressure of atmospheric CO_2 declines, leading to a decrease in the CO_2 uptake (Jensen & Bahr, 1977; Woodrow & Berry, 1988). This results in less discrimination against the heavier C isotope in the plants during photosynthesis (Körner et al., 1988) and may

explain the differences in the $\delta^{13}C$ values found between mountain and lowland pasture grass, and therefore in the lamb fat samples.

Furthermore, $\delta^{13}C$ of lamb plasma and erythrocytes has been investigated. The $\delta^{13}C$ of lamb plasma seems to be a more reliable tool than that of erythrocytes to detect dietary changes, giving significant differences between lambs fed on C3 pasture only and those provided with C3 concentrates in different experimental periods. In particular, $\delta^{13}C$ of plasma is reported to become less negative going from lambs fed on pasture to those fed on concentrates, according to the differences (but still ranging in the C3 values interval) in the isotopic values of the relevant feed. Furthermore, all works agree that lower $\delta^{13}C$ values were found in plasma than in erythrocytes in pasture-fed lambs, while in concentrate-fed lambs the values were always higher in plasma than in erythrocytes (Biondi et al., 2013; Bontempo et al., 2016).

As for wool, a significant correlation between $\delta^{13}C$ in defatted lamb meat and wool is reported in literature, both parameters being strictly related to the carbon isotopic ratios of the feed (Moreno-Rojas et al., 2008; Sun et al., 2016). Moreno-Rojas et al., 2008 found no overlap between the carbon isotopic ratios of wool and DDM coming from lambs fed on pasture, C3-based and C4-based concentrates. This finding suggests that $\delta^{13}C$ of wool can provide useful information for traceability purposes even without slaughtering the animals.

Matrices such as faeces and teeth have also been considered a matter of study in the stable isotope analysis of lambs. Neto et al., 2019 reported different $\delta^{13}C_{FECES}$ of lambs depending on the different proportions of C4 and C3 plants included in their diet. The carbon isotopic ratios of lamb teeth, on the other hand, are reported by Zazzo et al., 2010. The study showed how $\delta^{13}C$ changes along the tooth crown and depends also on the sampled tooth. Even though the study had archaeological purposes, not related to traceability, the 28 lambs grew in Ireland and were fed on different diets for different periods of time, and this provides an overview of the isotopic ratio of this unexplored matrix, that may be useful for traceability purposes (Zazzo et al., 2010).

6.3 Nitrogen Stable Isotope Ratio of defatted lamb meat and other matrices

A description of the factors influencing the nitrogen isotopic ratio of animal matrices is provided in **Chapter 2.2**. In most cases, the nitrogen isotopic ratio of meat is used to obtain information about the dietary regime. In particular, for traceability purposes, it has been used to distinguish lambs fed on pasture from those fed on concentrate, whether C3 (Biondi et al.,

2013), C4 or both (Biondi et al., 2013; Moreno-Rojas et al., 2008; Sun et al., 2016) and from those fed mainly on leguminous plants such as alfalfa hay (Devincenzi et al., 2014; Prache et al., 2009) or lucerne (Macari et al., 2017). As leguminous plants are characterised by relatively low nitrogen values, the $\delta^{15}N_{DDM}$ of the lambs provided with this dietary component resulted, in all cited studies, to be statistically lower than that of lambs fed on grass. Furthermore, the value turned out to be linearly related to the proportion of leguminous plants included in the diet (Devincenzi et al., 2014). From this point of view, once the lack of synthetic fertilizers in the agricultural practices has been ascertained, $\delta^{15}N$ may become a suitable tool to detect the addition of leguminous plants in the lamb diet and also to predict the amount of this addition.

On the other hand, further studies have been conducted to detect the geographical origin of lamb meat by using $\delta^{15}N_{DDM}$. Although geographical traceability was the principal aim of these works, in most cases the discriminating information depended on the different diets the lambs were fed on, and not from parameters related to the geo climatic conditions of the land they grew on. Indeed, peculiar plant $\delta^{15}N$ values (Erasmus et al., 2016, 2018) or the presence of leguminosae (Erasmus et al., 2016, 2018; Mekki et al., 2016; Qie et al., 2021) in the grazed grassland were shown to influence the nitrogen isotopic ratios of defatted meat, thus becoming the discriminant element for differentiating between lambs grazing different areas. Furthermore, an analogous discriminating power may be attributed to the exclusive presence of milk in the lamb diet: indeed, as previously mentioned, the trophic effect derived from suckling leads to higher $\delta^{15}N_{DDM}$ (Mekki et al., 2016; Perini et al., 2009).

Nevertheless, studies on isotopic ratios of lambs grazed in different European countries (Piasentier et al., 2003) or different regions of the same country (Perini et al., 2009), reported significant differences between lamb types that did not depend on the feeding regime, but on environmental parameters. Indeed, lamb types fed on similar diets but in different countries provided defatted meat with different nitrogen isotopic ratios. In agreement with this statement, further studies showed how arid climatic conditions can lead to higher $\delta^{15}N_{DDM}$, whereas humidity and a cold climate contribute to lower values (Camin et al., 2007; Erasmus et al., 2016, 2018; Mekki et al., 2016; Perini et al., 2009; Piasentier et al., 2003; Sun et al., 2016).

Finally, fertilization practices are known to contribute to the variation in the nitrogen isotopic

ratio of the plants, which is transferred to lamb $\delta^{15}N_{DDM}$. In particular, relatively high lamb $\delta^{15}N_{DDM}$ values may reflect the use of organic fertilizers in pasture, while relatively low

$\delta^{15}N_{DDM}$ values may be associated with the use of synthetic fertilizers (Mekki et al., 2016; Perini et al., 2009; Sun et al., 2012, 2016). On the other hand, high $\delta^{15}N_{DDM}$ values in lambs grazed in coastal regions have been attributed to the inclusion of seaweed, or other products from the marine ecosystem, in the fertilization practices (Camin et al., 2007).

Besides DDM, wool was also considered to discriminate between different lamb feeding regimes (Moreno-Rojas et al., 2008; Sun et al., 2016). Sun et al., 2016 reported both $\delta^{15}N_{DDM}$ and $\delta^{15}N_{WOOL}$ to be powerful tools to distinguish between the agricultural and pastoral regions of China ($p<0.05$). Moreno-Rojas et al., 2008 showed how the $\delta^{15}N$ of the wool turned out to be suitable to discriminate between lambs fed on herbage and those fed on concentrate, thus making it possible to distinguish between the two groups without slaughtering the animals. Moreover, a higher trophic shift (defined as the difference between the $\delta^{15}N$ of the diet and the tissue under consideration) was noticed for wool with respect to defatted meat.

Biondi et al., 2013 and 2009 and Bontempo et al., 2016 argued that the $\delta^{15}N$ of plasma and erythrocytes of lambs is an unreliable indicator of diet change from pasture to concentrate.

6.4 Sulphur Stable Isotope Ratio of defatted lamb meat and other matrices

A description of the main factors influencing sulphur isotopic ratio is provided in **Chapter 2.3**. The sulphur isotopic ratio of lamb matrices has not been investigated as deeply as the nitrogen and carbon isotopic ratios mentioned above. To the best of our knowledge, two works reported $\delta^{34}S$ to be a powerful tool to discriminate between lamb types, whereas it was not feed dependent (Mekki et al., 2016; Perini et al., 2009). The latter finding is in contrast with a work by Biondi et al., 2013 which states that the diet affected the sulphur isotopic ratio of the defatted meat of lambs subjected to four different dietary treatments during an 89-day experimental period. As for the environment the lambs grew in, the $\delta^{34}S$ of their defatted meat is reported to depend on the closeness to the sea. Higher values of $\delta^{34}S$ for sites close to the sea have been found in Italian (Camin et al., 2007; Mekki et al., 2016; Perini et al., 2009), Greek (Camin et al., 2007) and Tunisian (Mekki et al., 2016) territories and are due to the fallout of oceanic sulphates with high $\delta^{34}S$ values (+20‰) along the coasts (so named "sea spray effect"). Nevertheless, as $\delta^{34}S$ is capable to provide signatures on a small geographic scale, due to the local geological sulphate variability (Perini et al., 2009), there may be sites quite close to the sea, which are characterised by relatively high sulphur isotopic ratios. This is the case of the Italian regions of Tuscany and Sicily, which, despite their nearness to the coast, revealed to

have low $\delta^{34}S$ due to the high amount of volcanic sulphur, characterised by relatively low $\delta^{34}S$ values (Camin et al., 2007; Perini et al., 2009). Finally, low sulphur isotopic ratios can be related to low ^{34}S content in reduced sulphur (sulphides) in the soil, as it has been hypothesized to explain the low $\delta^{34}S$ of lamb meat produced in Germany (Camin et al., 2007).

To the best of our knowledge, only the paper by Bontempo et al., 2016 concerned lambs and reported sulphur isotopic ratios in matrices other than defatted meat. In this study, sulphur isotopic values in the plasma and erythrocytes of lambs differed after 14 days of switching from pasture to concentrate-based diets. This means that the $\delta^{34}S$ of plasma can be a suitable tool for traceability purposes, as it can detect the changes in lamb diet.

6.5 Hydrogen and Oxygen Stable Isotope Ratio of defatted lamb meat

A description of the main factors influencing hydrogen and oxygen isotopic ratio is provided in **Chapter 2.4**. All the publications in the relevant literature used exchangeable reference materials for hydrogen and oxygen isotope analysis (Biondi et al., 2013; Camin et al., 2007; Mekki et al., 2016; Perini et al., 2009; Piasentier et al., 2003; Qie et al., 2021; Shumin et al., 2011; Sun et al., 2016) even if it is not always clear in those papers if the 'comparative equilibration technique' has been used (Wassenaar & Hobson, 2003). The $^{2}H/H$ values of DDM were corrected against IHR casein reference material with an assigned value of $\delta^{2}H$ (-113‰), ICM collagen (-65.9‰) or USGS keratins CBS (-197‰) and KHS (-51.4‰) while the IAEA benzoic acid 601 (23.3‰) or keratins CBS (3.8‰) and KHS (20.3‰) were used for $^{18}O/^{16}O$ analysis of DDM. Anyway, the use of the single reference material does not meet the data normalisation requirement against at least two reference materials bracketing the values of the samples, even if for casein its value matches those of the samples.

To the best of our knowledge, the values of $\delta^{18}O_{DDM}$ reported in the literature concern lambs from Tunisia (Mekki et al., 2016), Italy (Biondi et al., 2013; Perini et al., 2009), China and New Zealand (Qie et al., 2021). Mekki et al., 2016 provided oxygen isotopic values for the meat of lambs grazed in four different sites of Northwest Tunisia. The mean $\delta^{18}O_{DDM}$ of the areas ranged from 15.0‰ to 16.4‰. The mean values seemed to vary according to the closeness of the sampling point to the coast, thus higher values were associated with sites relatively near to the sea. An exception was represented by AinDraham (Lat.North 36°46'36.35, Long. 8°40'49.75) which had an intermediate $\delta^{18}O_{DDM}$ even though it was the site closest to the coast. The altitude of this sampling point may explain the fact: indeed, even though it is just 18.5 km

away from the sea, this area stands at 756m asl. Therefore, altitude and coast effect may contribute in different ways to the oxygen isotopic ratio. In this study, PLS-DA carried out by considering a set of isotopic ratios ($\delta^{18}O_{DDM}$, $\delta^{15}N_{DDM}$, $\delta^{34}S_{DDM}$ and $\delta^{13}C_{FAT}$) gave an average accuracy of 92% for four different Tunisian regions, providing a suitable tool for Tunisian lamb traceability.

Perini et al., 2009 provided $\delta^{18}O$ values for the meat of lambs grazing in different regions of Italy and subject to different feeding regimes. In this study, the mean $\delta^{18}O$ values ranged from the 11.6‰ of Friuli Venezia Giulia, in the northern part of Italy, to the 17.0‰ of Sicily, in the South. Although the geographic influence can explain most results, it is worth noting that lambs supplemented with concentrates containing maize, a C4 plant, showed lower $\delta^{18}O$ than those fed mainly with forage or milk. This was explained by considering that the $\delta^{18}O$ of the cellulose of C3 plants can be higher than that of C4 plants, as the latter keep on synthesizing even in dry conditions, being heat- and light-adapted. Moreover, the inclusion of concentrate, low in water content, to the diet, forces the animals to drink higher amounts of 18O-depleted tap water, which leads to the decrease of the isotopic value in the meat. Further confirmation of this behaviour comes from the work Biondi et al., 2013 which, considering lambs reared in the same area in South Italy, showed statistically higher $\delta^{18}O$ DDM in the meat of animals fed exclusively on pasture than those fed with concentrates (15.5‰ and 13.5‰).

Qie et al., 2021 reported lamb samples from New Zealand to have relatively low $\delta^{18}O$ and $\delta^{2}H$ (10.81±0.75‰ and -125.34±4.44‰, respectively) than those collected in different regions of China (from 11.93±1.73‰ to 14.27±0.85‰, and from -97.90±7.74‰ to -120.44±6.48‰, respectively) probably due to the latitude effect. In this study, PCA-LDA carried out by considering $\delta^{18}O$, $\delta^{2}H$, $\delta^{15}N$ and $\delta^{13}C$ of DDM gave a discrimination accuracy for the different regions and countries of 96.67%.

On the other hand, $\delta^{2}H_{DDM}$ does not seem to be suitable to discriminate between the feeding regimes of different lambs (Biondi et al., 2013; Mekki et al., 2016; Perini et al., 2009). On the contrary, in terms of geographical discrimination, $\delta^{2}H$ turns out to be a powerful tool in lamb meat origin discrimination. Perini et al., 2009 gave $\delta^{2}H$ values for lambs reared in different regions of Italy, ranging from the -82.7‰ of a site in Sicily, at sea level and 5km away from the coast to the -97.0‰ of Trentino, an Alpine Mountain area. In this work, the hydrogen isotopic ratio showed a significant relationship with the mean annual deuterium isotope composition of precipitation ($r=0.66$, $p<0.04$). In agreement with this range of values Biondi

et al., 2013 reported δ^2H_{DDM} values for lambs reared in South Italy ranging from -84‰ to -90‰, similar to those reported by Camin et al., 2007 for lambs grazed in four different Italian regions. Moreover, the last cited work provided δ^2H_{DDM} values for lambs reared in other six countries besides Italy, reporting relatively high values for countries characterised by an arid climate (Greece) or for sampling sites near the sea and located at sea level (UK, South Italy) and relatively low values for areas with high continental and latitude effects and a cold and humid climate (Germany, North Italy). Finally, a high discrimination power for δ^2H was also reported in the attempt of discriminating between lambs coming from different regions of China. Indeed, both the combination of δ^2H_{DDM}, $\delta^{13}C_{DDM}$ and $\delta^{15}N_{DDM}$ (Shumin et al., 2011) and the combination of the just mentioned parameters together with δ^2H_{WOOL}, $\delta^{13}C_{WOOL}$ and $\delta^{15}N_{WOOL}$ (Sun et al., 2016), lead to a total correct classification of 88.9%. Once again, the isotopic analysis confirms its power in discriminating lambs coming from different regions within a country and between countries.

Besides defatted meat, also lamb fat has been investigated, in order to obtain hydrogen and oxygen isotopic ratios. As previously mentioned, the $\delta^{18}O$ and δ^2H values presented by Perini et al., 2009 and Mekki et al., 2016 resulted from the use of the same standards and can be therefore compared. Perini et al., 2009 reported values of $\delta^{18}O_{FAT}$ ranging from 14.5±1.65‰ to 23.6±0.26‰ and values of δ^2H_{FAT} ranging from -184.1±5.35‰ to -203.5±1.96‰, for lambs grazed in seven regions of Italy and subject to different feeding regimes. In this work, $\delta^{18}O_{FAT}$ and δ^2H_{FAT} showed a statistical correlation to the respective values in the defatted meat (r^2=0.449, $p<0.01$ for oxygen and r^2=0.577, $p<0.01$ for hydrogen). Similar values were reported in the study by Mekki et al., 2016, in which samples of fat from lambs grazed in different sites of Tunisia had $\delta^{18}O_{FAT}$ values ranging from 18.6‰ to 19.3‰ and δ^2H_{FAT} values ranging from -200.3‰ to -187.9‰. Conflicting results were obtained for the correlation between the $\delta^{18}O_{FAT}$ and δ^2H_{FAT} parameters and the precipitation, in turn related to the geographical origin of the samples. Indeed, while Perini et al., 2009 found a significant relationship between $\delta^{18}O_{FAT}$ and the meteoric water (r^2=0.81, $p<0.05$) and a non-significant relationship between δ^2H_{FAT} and precipitation (r^2=0.46, p=0.18), Mekki et al., 2016 showed opposite results (all the isotopic ratios, except $\delta^{18}O_{FAT}$ resulted to be statistically influenced by the geographical origin). Moreover, both works reported the comparative depletion in deuterium of the lamb fat as against the DDM, being the level of this depletion significantly lower in animals fed on forage than in other diets. The difference between δ^2H_{FAT} and δ^2H_{DDM} confirms that hydrogen stable isotopes are incorporated and fractionated differently into animal tissues (Hobson et al., 1999;

Sessions et al., 1999). On the other hand, lamb fat is reported to be enriched in ^{18}O than defatted meat. Indeed, muscle collagen contains high amounts of hydroxyproline, derived from the hydroxylation of proline with molecular oxygen, which leads to a remarkable depletion of ^{18}O (Schmidt et al., 2001). Perini et al., 2009 found that the increased amounts of ^{18}O of the fat were not constant across different lamb rearing systems, the difference between the fat and the DDM being more consistent for lambs fed only milk.

Lamb plasma has also been considered for stable isotope analysis. In literature, the $^{18}O/^{16}O$ in plasma was determined in 2 ml of defrosted samples after equilibration with CO_2 using an online preparation system that allowed CO_2/H_2O equilibration, interfaced with an IRMSr. Biondi et al., 2013 and Bontempo et al., 2016 described how $\delta^{18}O$ resulted statistically different at the starting and at the ending point (0.9‰ and -2.5‰, $p<0.001$, respectively) of an experimental period in which an abrupt switch from pasture to concentrate was applied to the diet of ten male Italian Merino lambs. Similarly, Biondi et al., 2013 showed that the $\delta^{18}O_{PLASMA}$ values of lambs fed on pasture or with concentrates resulted to be significantly different (-0.21‰ and -3.49‰, $p<0.001$, respectively). In this work, the multivariate analysis carried out by considering $\delta^{18}O_{PLASMA}$, together with $\delta^{13}C_{PLASMA}$, $\delta^{13}C_{DDM}$, $\delta^{2}H_{DDM}$ and $\delta^{34}S_{DDM}$, lead to a 100% level of accuracy in the classifications of the four lambs groups considered (stall-fed, grazed and switched from pasture to stalls for 37 or 14 days before slaughter). In both studies, the decrease in the oxygen isotopic ratio was attributed to the substitution of fresh herbage, ^{18}O-riched, with the concentrate in the diet of the lambs, which led to consequential higher ingestion of ^{18}O- depleted tap water (Bontempo et al., 2012). Finally, it must be considered that body fluids (such as milk, plasma, urine and blood) are richer in ^{18}O than drinking water, as isotope-light vapour is lost in respiration (Bryant et al., 1995; Kohn et al., 1996).

Finally, lamb wool was also considered in the study. (Sun et al., 2016) found that $\delta^{2}H_{WOOL}$ was significantly higher, but correlated to $\delta^{2}H_{DDM}$. This suggests that wool can give similar information as defatted muscle. In the previously cited study, lambs coming from pastoral and agricultural regions of China have no statistically different hydrogen isotopic ratios (-87.04 ± 9.45‰ and -85.35 ± 5.69‰, respectively).

6.6 Conclusions

Consumers around the world want to know and to be informed on what they are eating and where it comes from. As demonstrated in the review presented in **Chapter 6**, stable isotope

techniques can give an answer to this need, providing information about the geographical origin of the lamb and about the animals' diet. These features are, indeed, the elements that characterise valuable food products as PGI and PDO products. In this work, the isotopic ratios of the five major bioelements have been considered in different lamb matrices, in order to show the potential of stable isotope ratio analysis to characterise lamb meat. The statistical models adopted to validate the traceability systems are multidimensional and consider the different isotopes at the same time, allowing an overall validation. However, the various multivariate components systematically identify the tendency of isotope ratio of carbon to discriminate different animal diets, while the isotopic ratios of deuterium and oxygen tend to discriminate geographic origin. Based on the previously cited literatures, these models can reach and overcome 90% of total accuracy and this result can be increased by considering other additional variables such as fatty acids and trace mineral elements.

Once the technique has been shown valid for traceability purposes, a further step could be the definition of the analytical method and the creation of databases recording isotopic ratios of specific PGI and PDO lamb products, with the aim of creating reference systems to compare the data of actual samples, in order to be able to detect frauds or mislabels, as is already the case for some types of Italian hard cheese (described by the UNI 11692:2017 Regulation). To achieve this goal, it is necessary for the laboratories involved to use the same procedure and the same reference materials to provide comparable isotopic results, especially for $^{1}H/^{2}H$. It would be also interesting to create a lamb-based reference material, which can match the matrix of the analysed samples.

Chapter 7

General considerations and conclusions

In this thesis, stable isotope ratio analysis (SIRA) was used to study different animal matrices. Compounds such as fats, fatty acids (FAs), proteins and caseins were analysed in various animal products. Several preparative procedures were used to obtain the samples and techniques such as elemental analysis and gas chromatography, both coupled with isotope ratio mass spectrometry, were used to analyse them. The isotopic ratios of the major bioelements ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$) were used as a tool set to obtain information about the geographical origin of the samples, the diets that the animals under study were provided and, in general, to discriminate between different samples typologies.

In **Chapter 3**, various compartments (diet, rumen, duodenal content, liver and loin) of cows fed on C3- and a C4-based diets (C3 and C4 group) were considered. The $\delta^{13}C$ of the bulk lipids and of the single FAs made it possible to distinguish the two groups and to study the fractionation processes taking place into the bovine organism. In particular, the $\delta^{13}C_{\_BULK\_LIPID}$ of groups C3 and C4 were found to be statistically different in the diet (-32.55‰ vs. -18.74‰; $P<0.01$), rumen (-32.10‰ vs. -27.73‰; $P<0.01$), liver (-29.50‰ vs. -20.47‰; $P<0.01$) and meat (-26.25‰ vs. -19.35‰; $P<0.01$). On the other hand, as for the compound-specific analysis, the SIRA, together with the quantification results, made it possible to detect differences in the trend of the FAs $\delta^{13}C$ values (starting from the diet and going, through rumen and liver, to the meat) in group C3 and C4. Therefore, in order to better understand the changes that the FAs undergo in the bovine organism, further studies should be carried out, focusing on the biohydrogenation processes occurring in the rumen.

In **Chapter 4**, sulphur isotopic ratio made it possible to discriminate between Italian products such as Grana Padano PDO, Parmigiano Reggiano PDO and not PDO cheese. Moreover, foreign cheeses coming from both European and extra-European countries were characterised. In comparison with other stable isotopes ($\delta^{15}N$, $\delta^{18}O$ and $\delta^{2}H$), $\delta^{34}S$ showed a higher efficiency in discriminating products coming from close Italian areas, opening to the possibility to be used for Italian PDO cheese traceability.

In **Chapter 5**, edible insects were characterised by collecting the isotopic ratios of the major bioelements ($\delta^{13}C$ of the fat and $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$ of the defatted samples) using EA-IRMS. Both farmed insects (whether natural or seasoned) and insect-based food items

(IBFI) were included into the dataset. The SIRA made it possible to detect statistical differences into the dataset. Farmed edible insects could be discriminated depending on the geographical origin (Europe or Asia) by analysing the $\delta^{34}S$ and on the insect life-stage (adult or larva) by analysing the $\delta^{2}H$. Moreover, the $\delta^{18}O$ and $\delta^{2}H$ of the farmed insects were statistically different from those of the IBFI. Based on the obtained results, it may be supposed that, once widened the database, the SIRA may be useful for the authentication and the traceability of this *novel food*.

In **Chapter 6**, a review about the SIRA on lambs was proposed. All scientific works available in the literature were collected and data were gathered in order to provide an innovative point of view in the characterisation of this product. As for all works cited, information such as data, matrices under study, results obtained, methods and techniques used, were collected.

Stable isotope ratio analysis is an extremely valid tool, even though its use in food characterisation can still be further improved. The information that this technique provides is useful in the agricultural field, as well as in many others, such as food traceability, forensic science and the study of animal physiology.

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