Measurement: Sensors 18 (2021) 100088

Contents lists available at ScienceDirect

Measurement: Sensors

journal homepage: www.sciencedirect.com/journal/measurement-sensors

# Carbon isotopic ratio of lipid fraction to trace fractionation processes in cull cows organism and to discriminate between different feeding regimes

ARTICLE INFO

*Keywords*
Simmental cows
Carbon isotopic ratio
Dietary regime
Fractionation
Elemental analyser isotope ratio mass spectrometry

ABSTRACT

In the present work, the quantification of the lipids and the carbon isotopic ratio ($\delta^{13}C$) of diet, rumen, liver and meat lipids of cows fed on different diets (based on either C3 or C4 plants) are provided. The $\delta^{13}C$ of the four compartments had different trends in the two diets, which could give information about the fractionation processes and the factors influencing the lipids metabolic path in cows fed on different regimes. The $\delta^{13}C$ of liver and meat lipids resulted to be statistically different for cows of groups C3 and C4, and can therefore be a suitable tool to discriminate between the two groups.

## 1. Introduction

The lipid fraction of the meat is of fundamental importance, as it contributes to its flavour, juiciness, tenderness and visual characteristics [1]. The lipids ingested by cows through the diet are mostly in form of esters of glycerol. After ingestion, dietary esterified lipids are hydrolysed to free fatty acids (FAs) and glycerol [2]. Thus, FAs undergo biohydrogenation in the rumen, consisting in the reduction of the number of double bonds on their carbon chain [3]. Therefore, absorption takes place into the digestive tract. The FAs are then carried by blood to reach the liver. The haematic flow and the FAs concentration influence their supply to this organ [4]. The deposition and the lipogenesis into the tissues could be considered as the final step of the lipids metabolic path. Considering the complexity of the processes in which lipids are involved, a tool to investigate their fate in the bovine organism is required.

In most cases, marks certifying animal products quality, such as Protected Geographical Indication (PGI) and Protected Designation of Origin (PDO), correspond to a specific feeding regime, due to its effect on animal tissue composition and thus on meat quality [5]. Therefore, there is a growing interest in the discrimination between different feeding systems.

In recent years, stable isotope ratio analysis (SIRA) has been used to obtain information about the dietary regime of the animals. Several works have been carried out with the aim to discriminate between bovines [6–8], lambs [9] and sheep [10] fed on different feeding regimes by using SIRA. Carbon isotopes ($^{13}C$ and $^{12}C$) are the bioelement isotopes that were considered the most suitable to give information about the dietary regime of animals. Indeed, the photosynthetic pathway of the plants included in the animal diet is the main factor influencing the carbon isotopic ratio [11]. Plants that undergo the Calvin-Benson cycle for carbon dioxide fixation by the RuBisCo enzyme and obtain a three-carbon compound are called C3 plants [12]. On the other hand, C4 plants follow an alternative pathway to fix the $CO_2$, separating the photosynthetic functions between mesophyll and bundle sheath leaf cells, in order to avoid photorespiration (that is, the conversion of sugar phosphates back to carbon dioxide) [13]. The different pathway C3 and C4 plants follow in the fixation of $CO_2$ results in different carbon isotopic ratios of the plant itself. It is a well-known fact that C4 plants have $\delta^{13}C$ values between −14 and −12‰, while C3 plants values range between −30 and −23‰ [14] and that both may be included into forages and concentrates of animal diets.

In this work, the carbon isotopic ratios of feed, rumen, liver and meat lipid fractions of multiparous Simmental cows fed on a C3- (6 animals) and C4- (6 animals) based diet were collected. The aim of the study was to improve our understanding of the fractionation processes taking place in the cow organism, by tracing them thanks to $\delta^{13}C$, and to check whether the $\delta^{13}C$ of four different animal matrices could become a tool to discriminate between cows fed on C3 and C4 products.

## 2. Materials and methods

### 2.1. Description of the samples

The EU Directive 2010/63/EU on the protection of animals used for scientific purposes was applied in carrying out this present study. The procedure was routine and non-invasive. The ethical committee of the University of Udine approved the trial (Prot. No.8/2018). Regular clinical examinations by the veterinary assessed the good health of the cows during the experimental period.

### 2.2. Diet

Twelve Italian Simmental multiparous cull cows were randomly assigned to two dietary treatments differing in the metabolism of the main component plant of the diet, C3 (C3 group) or C4 (C4 group). The C3 group was fed on hay offered *ad libitum* and received 6.9 kg dry matter (DM) of a concentrate mainly composed by wheat meal, barley meal, and wheat bran. In this group, C3 products represented 100% of the diet ingredients. The C4 group was fed on corn silage offered *ad libitum* and received 3.3 kg DM of a concentrate composed by corn meal,

https://doi.org/10.1016/j.measen.2021.100088

Available online 5 October 2021

and corn gluten meal for around the 96%, while C3 plants (soybean meal and hempseed cake) represented the remaining part. The concentrates were offered in twice-daily meals. The experimental period lasted 4 months, and the animals were loose-housed with sawdust bedding. The individual DM intake of forages and concentrate were assessed as reported in Pianezze et al. (2021) [15]. The intake of hay and corn silage was 9.3 ± 1.01 kg and 13.2 ± 1.32 kg of DM, respectively. The day before slaughter, the animals accessed only their morning meal. At slaughter, individual samples of rumen, liver and meat (*m. longissimus thoracis* at 6th -7th rib level) were collected and freeze-dried (Heto freeze dryer, Analytical Control De Mori, Milan, Italy). After fine grinding (with a windmill blade at 4000 rpm × 10 s), the samples were stored until stable isotope and fatty acids analyses could be performed. On the other hand, samples of forage and concentrate were collected every two weeks for stable isotopes and lipids analysis. The samples included into the diet were dried at 65 °C in a forced draft oven for 48h in order to be analysed through SIRA.

### 2.3. Lipid extraction

Bulk fat samples were extracted using petroleum ether at 40–60° in a SER 148 extraction apparatus (Model Velp, Italy; Soxhlet Extraction, AOAC 2014). After removal of the solvent, the fat residue was suspended in 4 ml of hexane and transferred into vials with screw cap (bulk fat fraction). The amount of the fat fraction is mentioned as Fat (ee), % DM in Table 1.

### 2.4. Stable isotope ratio analysis

The $^{13}C/^{12}C$ was measured using an isotope ratio mass spectrometer (IsoPrime, Isoprime Limited, Germany) after total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited, Germany). According to the IUPAC protocol, the isotopic values are expressed as *delta* in relation to the international standard V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, as defined in (1).

$$\delta\ iE = (i\ R_{SA} - i\ R_{REF}) / i\ R_{REF} \quad (1)$$

where i is the mass number of the heavier isotope of element E, $R_{SA}$ is the respective isotope ratio of the sample and $R_{REF}$ is the relevant internationally recognised reference material [11]. The delta values are multiplied by 1000 and expressed in units "per mil" (‰). Two in-house working standards were used. They were calibrated against international reference materials: 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 ($\delta^{13}C = -26.39$‰) (U.S. Geological Survey, Reston, VA, USA). The uncertainty of the measurements (calculated as two standard deviations) was <0.3‰ for $\delta^{13}C$ values.

### 2.5. Statistical analysis

Statistical analysis was performed using R software, vers. 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. The effect of compartment (diet, rumen, liver and meat) and plant metabolism type (C3, C4) on the variables was assessed with a mixed model for repeated measures, as suggested by Wang and Goonewardene (2004) [16], considering compartment and plant metabolism type as within- and between-subject factor, respectively. The interaction of compartment × plant metabolism type was also considered. For multiple comparisons, the Bonferroni adjustments were considered.

**Table 1**
Statistical calculations carried out on isotopic values and relative amount of lipids.

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

A, B, C, D Within the same row and factor with unlike letters differ significantly at P<0.01

## 3. Results and discussion

The results of the statistical calculations carried out on the isotopic values and the relative amount of lipids are displayed in Table 1. 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.

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 characterized 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 is in agreement with the fact that fatty acids are mainly stored in the liver and the muscles, where they also undergo *de novo* synthesis [17].

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 et al. [15], while group C4 showed the trend $\delta^{13}C_{RUMEN} < \delta^{13}C_{LIVER} < \delta^{13}C_{MEAT} < \delta^{13}C_{DIET}$, being the meat not statistically different from the diet and the liver (Fig. 1).

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 defined in (2).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

This process entails a consistent isotopic discrimination, as

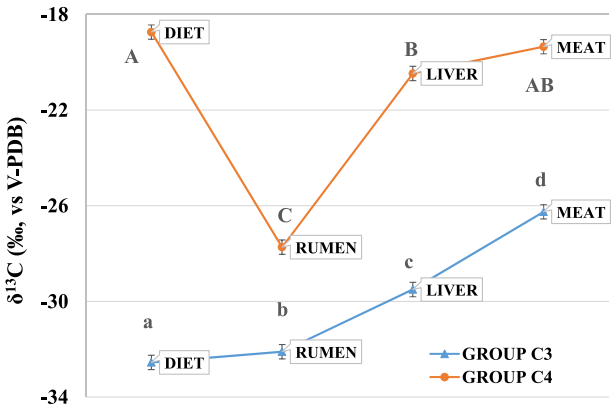


**Fig. 1.** Variation and uncertainty of the measurement of the carbon isotopic ratio in the different compartments of groups C3 and C4. $^{A,B,C}$: $P < 0.01$; $^{a,b,c,d}$: $P < 0.05$ within C3 and C4 groups.

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 [18]. Klevenhusen et al. 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 from the atmosphere) 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 [19]. 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 $\delta^{13}C_{FAT}$.

## 4. Conclusions

In the present work, the quantification of the lipids in the diet, rumen, liver and meat of 12 multiparous cows fed on different feeding regimes was carried out. The animals were divided into two groups and fed on a C3- and C4-based diet, respectively. The carbon isotopic ratio of lipids in the four compartments was also calculated. The variation of the $\delta^{13}C$ from one compartment to the other was different in C3 and C4 groups, which provides information on the fractionation processes taking place into the cow organism. Moreover, the $\delta^{13}C$ 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.

## Acknowledgments

Start-up 2018, project, Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, funded this research.

## References

[1] R.K. Miller, Factors affecting the quality of raw meat, Meat Process. (2002) 27–63, https://doi.org/10.1533/9781855736665.1.27.
[2] T.C. Jenkins, Lipid metabolism in the rumen, J. Dairy Sci. 76 (1993) 3851–3863.
[3] A. Buccioni, M. Decandia, S. Minieri, G. Molle, A. Cabiddu, Lipid metabolism in the rumen: new insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors, Anim. Feed Sci. Technol. 174 (2012) 1–25, https://doi.org/10.1016/j.anifeedsci.2012.02.009.
[4] J.F. Hocquette, D. Bauchart, Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals, Reprod. Nutr. Dev. 39 (1999) 27–48.
[5] Y. Geay, D. Bauchart, J.F. Hocquette, J. Culioli, Effect of nutritional factors on biochemical, structural and metabolic characteristics of muscles in ruminants, consequences on dietetic value and sensorial qualities of meat, Reprod. Nutr. Dev. 41 (2001) 1–26.
[6] M.T. Osorio, A.P. Moloney, O. Schmidt, F.J. Monahan, Beef authentication and retrospective dietary verification using stable isotope ratio analysis of bovine muscle and tail hair, J. Agric. Food Chem. 59 (2011) 3295–3305.
[7] B. Bahar, F.J. Monahan, A.P. Moloney, P. O'Kiely, C.M. Scrimgeour, O. Schmidt, Alteration of the carbon and nitrogen stable isotope composition of beef by substitution of grass silage with maize silage, Rapid Commun. Mass Spectrom. 19 (2005) 1937–1942.
[8] S. De Smet, A. Balcaen, E. Claeys, P. Boeckx, O. Van Cleemput, Stable carbon isotope analysis of different tissues of beef animals in relation to their diet, Rapid Commun. Mass Spectrom. 18 (2004) 1227–1232.
[9] J.M. Moreno-Rojas, V. Vasta, A. Lanza, G. Luciano, V. Ladroue, C. Guillou, A. Priolo, Stable isotopes to discriminate lambs fed herbage or concentrate both obtained from C(3) plants, Rapid Commun. Mass Spectrom. 22 (2008) 3701–3705.
[10] S. Prache, Diet authentication in sheep from the composition of animal tissues and products, Rev. Bras. Zootec. 38 (2009) 362–370.
[11] E.M. Galimov, Causes of fractionation of isotopes, in: E.M. Galimov (Ed.), The Biological Fractionation of Isotopes, Academic Press, Cambridge, 1985, pp. 1–15, https://doi.org/10.1016/b978-0-12-273970-5.50006-2.
[12] J.B. Reece, L.A. Urry, M.L. Cain, S.A. Wasserman, P.V. Minorsky, R.B. Jackson, Alternative mechanisms of carbon fixation have evolved in hot, arid climates, in: Campbell Biology (10th ed.), CA: Pearson, San Francisco, 2011, p. 201.
[13] L.J. Guralnick, A. Cline, M. Smith, R.F. Sage, Evolutionary physiology: the extent of C4 and CAM photosynthesis in the genera Anacampseros and Grahamia of the Portulacaceae, J. Exp. Bot. 59 (2008) 1735–1742, https://doi.org/10.1093/jxb/ern081.
[14] N. Knobbe, J. Vogl, W. Pritzkow, U. Panne, H. Fry, H.M. Lochotzke, A. Preiss-Weigert, C and N stable isotope variation in urine and milk of cattle depending on the diet, Anal. Bioanal. Chem. 386 (2006) 104–108.
[15] S. Pianezze, M. Corazzin, M. Perini, F. Camin, M. Paolini, A. Sepulcri, E. Saccà, C. Fabro, E. Piasentier, Fatty acids stable carbon isotope fractionation in the bovine organism. A compound-specific isotope analysis through gas chromatography combustion isotope ratio mass spectrometry, J. Chromatogr. A 1641 (2021) 461966.
[16] Z. Wang, L.A. Goonewardene, The use of MIXED models in the analysis of animal experiments with repeated measures data, Can. J. Anim. Sci. 84 (2004) 1–11.
[17] R.A. Nafikov, D.C. Beitz, Carbohydrate and lipid metabolism in farm animals, J. Nutr. 137 (2007) 702–705, https://doi.org/10.1093/jn/137.3.702. PMID: 17311965. doi: 10.1016/j.chroma.2021.461966.
[18] C. Metges, K. Kempe, H.-L. Schmidt, Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the $\delta^{13}C$ value of food in dairy cows, Br. J. Nutr. 63 (1990) 187–196.
[19] F. Klevenhusen, S.M. Bernasconi, T.B. Hofsetter, J. Bolotin, C. Kunz, C.R. Soliva, Efficiency of monolaurin in mitigating ruminal methanogenesis and modifying C-isotope fractionation when incubating diets composed of either C3 or C4 plants in a rumen rimulation technique (Rusitec) system, Br. J. Nutr. 102 (2009) 1308–1317.

Silvia Pianezze
*Department of Agricultural, Food, Environmental and Animal Sciences DI4A, University of Udine, 33100 Udine, Italy*
*Fondazione Edmund Mach, 38098 San Michele all'Adige, Trento, Italy*
*E-mail address:* silvia.pianezze@fmach.it.

Mirco Corazzin
*Department of Agricultural, Food, Environmental and Animal Sciences DI4A, University of Udine, 33100 Udine, Italy*
*E-mail address:* mirco.corazzin@uniud.it.

Matteo Perini*, Angela Sepulcri, Elena Saccà
*Department of Agricultural, Food, Environmental and Animal Sciences DI4A, University of Udine, 33100 Udine, Italy*
*Fondazione Edmund Mach, 38098 San Michele all'Adige, Trento, Italy*

Edi Piasentier
*Department of Agricultural, Food, Environmental and Animal Sciences DI4A, University of Udine, 33100 Udine, Italy*
*E-mail address:* edi.piasentier@uniud.it.

* Corresponding author.
*E-mail addresses:* matteo.perini@fmach.it (M. Perini), angela.sepulcri@uniud.it (A. Sepulcri), elena.sacca@uniud.it (E. Saccà).