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Beef Authentication and Retrospective Dietary Verification Using Stable Isotope Ratio Analysis of Bovine Muscle and Tail Hair

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ABSTRACT: Stable isotope ratio analysis (SIRA) was used as an analytical tool to verify the preslaughter diet of beef cattle. Muscle and tail hair samples were collected from animals fed either pasture (P), a barley-based concentrate (C), silage followed by pasture (SiP), or silage followed by pasture with concentrate (SiPC) for 1 year (n = 25 animals per treatment). The $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$, and $^{34}S/^{32}S$ isotope ratios in muscle clearly reflected those of the diets consumed by the animals. By applying a stepwise canonical discriminant analysis, a good discrimination of bovine meat according to dietary regimen was obtained. On the basis of the classification success rate, the $^{13}C/^{12}C$ and $^{34}S/^{32}S$ ratios in muscle were the best indicators for authentication of beef from animals consuming the different diets. Analysis of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ in tail hair sections provided an archival record of changes to the diet of the cattle for periods of over 1 year preslaughter.

KEYWORDS: authentication, beef, stable isotope ratio analysis, diet, muscle, tail hair

INTRODUCTION

Consumers are increasingly concerned about the origin and authenticity of the food, including meat, they purchase.[1] This concern has arisen for a number of reasons, including consumers seeking assurance about the safety of the food they consume. In addition, certain meats have specialty status because of particular production characteristics, for example, locally bred, pasture-fed, or organically raised, that merit protection through appropriate traceability and labeling systems.[2] An example of such a product is pasture-fed beef, often marketed as superior nutritionally as a result of increased levels of ω-3 fatty acids and conjugated linoleic acid arising from the consumption of grass.[3−7] Similarly, products with Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) status in the European Union require robust traceability systems to protect them from fraud.[8]

In these contexts, there is a need for reliable analytical methodologies to authenticate the dietary history of animals and the food derived from them. Multielement stable isotope ratio analysis (SIRA) has been shown to be particularly useful as it can provide information on the dietary background[9−15] and geographical origin[11,13,16−20] of meat. SIRA involves the measurement of ratios of stable isotopes of bioelements, mainly carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), hydrogen (D/H or $^{2}H/^{1}H$), oxygen ($^{18}O/^{16}O$), and sulfur ($^{34}S/^{32}S$). Because it is known that the stable isotope composition of these bioelements in animal tissues is influenced by the composition of the diet,[21,22] SIRA signatures provide information about the preslaughter diet consumed by animals and in some cases, by inference, the geographical origin of the animals.[11,13]

Although SIRA of muscle and adipose tissues, so-called integrating tissues, is useful for obtaining information about the preslaughter origin (dietary and geographical) of meat, the stable isotope ratios measured represent an "integrated" signature of the dietary inputs over a certain period preslaughter and, therefore, short-term changes to the diet during that period may go undetected.[23,24] If, for example, pasture-fed animals received nonpasture feed inputs, for example, a cereal concentrate, for a period preslaughter, this may go undetected either because the tissue turnover rate was insufficient to elicit a response in the tissue or because the stable isotope signature of the cereal concentrate was insufficiently different from that of the pasture.[24,25]

A powerful approach to reconstructing changes in diet over an animal's lifetime is the use of incremental tissues such as hair, hoof, or wool, which contain a record of changes to diet over time.[23,26−31] Incremental tissues such as these are metabolically inert tissues that are progressively laid down and remain unchanged thereafter, so that the isotope ratios are preserved during their growth and, thus, information about the diet assimilated at the time of tissue growth.[23,32] For example, shifts from a barley concentrate (C_3)-based diet to a maize (C_4)-based diet were clearly evident from stable isotope analysis of hair[30] and hoof[23,29] in cattle and of wool in sheep.[31] Interestingly, from a forensic perspective, an unplanned change in the diet of cattle on a maize-based diet was detected using stable isotope analysis of tail hair from cattle.[30]

Our hypotheses were first that measurement of the stable isotopes of light bioelements (C, N, H, and S) in Irish beef can be used to discriminate between beef produced under different pasture and concentrate-based production systems and second that C and N isotope analysis of bovine tail hair could provide evidence of temporal changes to the animals' preslaughter diets, that is, the animals' diet history, even when animals were fed only C_3-based diets.

MATERIALS AND METHODS

Animals, Diets, and Sampling. A detailed description of the animals and diets used in this experiment was published previously.[33] Briefly, 100 heifers at Teagasc, Animal and Grassland Innovation Centre

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(Grange, Dunsany, Co. Meath, Ireland) (53° 30′ N, 6′ 40′ W, 92 m above sea level) were assigned at random to one of four dietary treatments (25 heifers per treatment) between November 2006 and October/November 2007. All animals were grazed together on pasture (mainly *Lolium perenne* L. and *Poa* spp.) before being housed and assigned to the experimental diets. The four dietary treatments were grazed pasture outdoors (mainly *L. perenne* L., *Poa* spp., and *Trifolium repens* L.) from November 2006 to October/November 2007 (hereafter treatment P); grass silage offered ad libitum indoors from November 2006 to April 2007 and then grazed pasture from April to October/November 2007 (treatment SiP); grass silage offered ad libitum indoors from November 2006 to April 2007 and then grazed pasture plus 0.5 of the dietary dry matter (DM) as supplementary concentrate from April to October/November 2007 (treatment SiPC); concentrate and straw indoors (mean daily DM intake of 2.1 kg of concentrate and 1.4 kg of straw) from November 2006 to October/November 2007 (treatment C). The composition of the concentrate was 430 g kg^{-1} pelleted beet pulp, 430 g kg^{-1} rolled barley, 80 g kg^{-1} soybean meal, 35 g kg^{-1} molasses, 20 g kg^{-1} mineral/vitamin premix, and 5 g kg^{-1} lime. To assess temporal variability, grass and grass silage were sampled weekly and concentrate and straw were sampled monthly over the experimental period. All samples were frozen at −20 °C until processing for SIRA.

Animals were slaughtered according to European regulations at Meadow Meats Ltd., Rathdowney, Co. Laois, Ireland. Tails from eight animals (two animals per dietary treatment) were removed at slaughter and stored in plastic bags at −20 °C prior to processing. At 24 h post-mortem, the right *Longissimus dorsi* (LD) muscle was excised from each carcass. LD samples were vacuum packaged and transferred to Teagasc, Ashtown Food Research Centre, Dublin 15, Ireland, stored overnight at 4 °C, after which a 2.5 cm thick subsample (LD between the 9th and 10th ribs) was taken for analysis, vacuum packaged, and stored at −20 °C until processing for SIRA.

Preparation of Samples for Stable Isotope Ratio Analysis. *Feed Samples.* Samples of feed were selected to represent the 12 months of the experiment (22 grass, 6 silage, 7 concentrate, and 4 straw samples) and oven-dried at 40 °C for 48 h. These samples were then powdered using a ball mill (type MM2, Glen Creston Ltd., Stanmore, U.K.), and afterward subsamples of 3.5−4.5 mg of grass, silage, straw, and concentrate were weighed and packed into tin capsules for C and N isotope analysis and samples of 1.4−1.6 and 8−10 mg plus 10 mg of vanadium pentoxide were weighed and packed for H and S isotope analyses, respectively. For the concentrate ration, the pelleted beet pulp was isolated, dried, milled, and analyzed separately from the rest of the concentrate constituents.

Muscle Samples. Frozen LD muscle samples were cut into 1 cm cubes using a ceramic knife and then freeze-dried (Edwards Pirani 501 freeze-dryer, Edwards Ltd., Crawley, U.K.) for 4 days. After freeze-drying, samples were stored at −20 °C in plastic bags until lipid extraction. Total lipid from 3 g of the freeze-dried material was extracted using 2-isopropanol/hexane (2:3, v/v) according to the method of Radin.[34] The defatted muscle was separated from the solvent mixture by vacuum filtration and air-dried overnight in a container covered with aluminum foil to protect samples from the light. The lipid-free dry samples were stored in Eppendorf vials in a desiccator at room temperature prior to weighing for SIRA. An amount of 0.9−1.1 mg of the lipid-free dry muscle was weighed and placed into tin capsules for C, N, and H isotope analyses. For S isotope analysis, 1.9−2.1 mg of lipid-free dry beef muscle was weighed and 4 mg of vanadium pentoxide was added to the ultraclean tin capsules. Replicates were used to test the reliability of the IRMS (every fourth sample was measured in duplicate for C, N, and S isotopes, whereas every sample was measured in duplicate for H isotope analyses).

Tail Hair Samples. One long (>300 mm) and thick tail hair was chosen per animal and plucked from the tail skin with the follicle attached. The preparation procedure for isotopic analysis was as described by O'Connell et al.[35] Each hair was cleaned using soapy water and then defatted by two immersions of 30 min each in a solution of methanol and chloroform (2:1 v/v) using an ultrasonic bath. The samples were then rinsed twice in distilled water and oven-dried overnight at 60 °C. The individual hairs were serially cut into sections (30−60 sections each hair) using a scalpel and weighed on a precision balance until sufficient mass for isotope analysis was obtained. The length of each section (from 3.5 to 12.5 mm) was measured with either a ruler or digital callipers, and then sections were loaded into ultralight tin capsules for dual C and N isotope analysis. To maximize the resolution of the analysis of changes in ^{15}N and ^{13}C over the length of the hair, the weight of individual samples ranged from 0.15 to 0.25 mg, bearing in mind that the analytical limit of mass spectrometers is about 50 μg of carbon or nitrogen. Sequential hair samples obtained from the SiPC group (50−60 samples each hair) were analyzed, whereas every second sample section obtained from the P, SiP, and C groups was analyzed. The reason for the latter approach was that, apart from reducing the cost, maximum resolution was considered to be less important in the case of animals that did not switch diets (P and C groups) over the course of the experiment or of animals that switched between diets that were likely to be isotopically similar (SiP group).

To convert hair length to measurements in time, the position on the hair (in the case of the SiP and SiPC groups) where the diet switch occurred was identified from the stable isotope data. This point was identified as the first hair segment that showed a marked change in the isotopic values and was assigned a date of April 18, 2007, the date when animals switched diets. Using April 18 and the slaughter date for each animal, growth rates were estimated and assumed to be linear for the duration of the experiment. Individual growth rates were then taken into account for converting hair length units to temporal record units (calendar dates). In initial plots of isotopic values versus length or calendar dates, we observed an offset in the data collected when hair length was measured by ruler or by caliper. This offset was attributed to the lower accuracy of the ruler versus the caliper measurements of length. A conversion factor was therefore applied to samples measured using the ruler to correct the offset. The hair lengths analyzed ranged from 300 to 389 mm and, based on estimated growth rates, covered the period from July 25, 2006, to November 21, 2007.

Stable Isotope Ratio Analysis. The isotopic ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$, and $^{34}S/^{32}S$ of the freeze-dried muscle samples and feedstuffs and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ of defatted hair samples were determined using an Elemental Analyzer − Isotope Ratio Mass Spectrometer (EA-IRMS) Europa Scientific 20−20 (Sercon Ltd., Crewe, U.K.), equipped with a preparation module for solid and liquid samples (ANCA-SL). Stable isotope ratios were expressed using conventional δ notation in units of per mil (‰) relative to a suitable standard and defined as

$$\delta\ (‰) = [(R_{sample} - R_{reference}) - 1] \times 1000$$

where R_{sample} is the isotope ratio in the sample ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$, $^{34}S/^{32}S$) and $R_{reference}$ is the isotope ratio of the reference material. Results are referenced to Vienna Pee Dee Belemnite (V-PDB) for carbon, atmospheric N_2 for nitrogen, Vienna Canyon Diablo Troilite (V-CDT) for sulfur, and Vienna Standard Mean Ocean Water (V-SMOW) for hydrogen.

The isotopic values were calculated against in-house standards (powdered bovine liver, beet sugar, cane sugar, wheat flour, ammonium sulfate, mineral oil, polyethylene foil, whale baleen, egg shell membrane standard, barium sulfate, and silver sulfide), calibrated and traceable against international isotope reference standards: sucrose IAEA-CH-6 (International Atomic Energy Agency (IAEA), Vienna, Austria) for $^{13}C/^{12}C$, ammonium sulfate IAEA-N-1 (IAEA) for $^{15}N/^{14}N$, mineral oil NBS-22 (IAEA) for $^{2}H/^{1}H$, barium sulfate NBS-127, barium sulfate IAEA-SO-5 (IAEA) and silver sulfide IAEA-S-1 (IAEA) for $^{34}S/^{32}S$ measurements.

Table 1. Chemical Composition of the Dietary Components (Mean ± SD)

dietary component	no. of samples (n)	crude protein (g/kg DM)	crude ash (g/kg DM)	fat (g/kg DM)	digestibility (g/kg DM)
grass	12	215.4 ± 46.3	111.2 ± 8.2	38.1 ± 6.3	770.1
grass silage	6	167.7 ± 30.9	109.7 ± 4.2	39.9 ± 2.2	724.0
concentrate	12	134.0 ± 22.0	69.4 ± 14.6	19.2 ± 2.9	866.4
straw	12	48.0 ± 7.1	48.9 ± 8.5	15.6 ± 7.0	441.5

Table 2. Elemental and Isotopic Composition of the Dietary Components (Mean ± SD)

dietary component	no. of samples (n)	δ^{13}C (‰)	C (%)	δ^{15}N (‰)	N (%)	δ^{2}H (‰)	H (%)	δ^{34}S (‰)	S (%)
grass	22	−30.9 ± 0.8	42.7 ± 0.6	6.4 ± 1.8	3.0 ± 0.7	−128.8 ± 7.8	4.8 ± 0.5	4.9 ± 3.0	4.2 ± 0.9
grass silage	6	−29.2 ± 0.3	42.8 ± 0.5	5.0 ± 1.0	2.3 ± 0.4	−142.2 ± 7.6	5.2 ± 0.4	3.9 ± 0.5	0.3 ± 0.0
concentrate									
beet pulp pellets	7	−27.9 ± 0.9	42.0 ± 1.7	7.0 ± 0.6	1.4 ± 0.1	−122.4 ± 9.4	5.0 ± 0.2	4.5 ± 2.1	1.6 ± 1.6
other constituents[a]	7	−27.2 ± 1.3	43.4 ± 1.9	2.3 ± 0.5	2.8 ± 0.5	−98.6 ± 10.3	5.0 ± 0.2	8.0 ± 2.0	1.6 ± 1.7
composite value[b]		−27.5		3.6		−108.8		6.5	
straw	4	−29.2 ± 0.3	44.2 ± 0.6	1.4 ± 0.7	0.7 ± 0.1	−130.4 ± 3.0	5.5 ± 0.3	8.5 ± 0.8	0.1 ± 0.0

[a] Rolled barley, soybean meal, molasses, mineral/vitamin premix, and lime. [b] Theoretical composite values for the diet fed to the C group (concentrate plus straw) were calculated as −27.9, 3.4, −114.6, and 6.5‰ for δ^{13}C, δ^{15}N, δ^{2}H, and δ^{34}S, respectively.

The precision of measurements for freeze-dried muscle samples, as estimated by replicate analysis (n = 18) of powdered bovine liver standard (NBS-1577B, $\delta^{13}C_{V\text{-}PDB}$ = −21.60‰, $\delta^{15}N_{air}$ = 7.65‰) analyzed along with the samples was ±0.05 and ±0.10‰ (SD) for δ^{13}C and δ^{15}N, respectively. IA-R045 (ammonium sulfate, $\delta^{15}N_{air}$ = −4.71‰) (SD, n = 11, 0.12‰) and IA-R046 (ammonium sulfate, $\delta^{15}N_{air}$ = 22.04‰) (SD, n = 11, 0.06‰) were also run as quality control check samples for δ^{15}N, whereas beet sugar (IA-R005, $\delta^{13}C_{V\text{-}PDB}$ = −26.03‰) (SD, n = 11, 0.05‰) and cane sugar (IA-R006, $\delta^{13}C_{V\text{-}PDB}$ = −11.64‰) (SD, n = 11, 0.05‰) were run as controls for δ^{13}C. For S isotope analysis, IA-R036 (barium sulfate, $\delta^{34}S_{V\text{-}CDT}$ = 20.74‰) was used as a reference material during analysis of samples, and the analytical precision (SD, n = 20) was 0.2‰. Working standards IA-R036, IA-R025 (barium sulfate, $\delta^{34}S_{V\text{-}CDT}$ = 8.53‰), and IA-R026 (silver sulfide, $\delta^{34}S_{V\text{-}CDT}$ = 3.96‰) were used for calibration and correction of the oxygen-18 contribution to the sulfur isotope data. Replicate analysis of IAEA-SO-5 (barium sulfate, $\delta^{34}S_{V\text{-}CDT}$ = 0.5‰) and BWB II (whale baleen, $\delta^{34}S_{V\text{-}CDT}$ = 16.3‰) run concurrently with the samples gave mean $\delta^{34}S_{V\text{-}CDT}$ = 0.35‰ (n = 6) and 16.58‰ (n = 6), respectively. For H isotope analysis, the analytical precision (SD, n = 36) was 0.9‰ when IA-R002 (mineral oil, $\delta^{2}H_{V\text{-}SMOW}$ = −111.2‰) was analyzed along with the preweighed samples. Polyethylene foil (IAEA-CH-7, $\delta^{2}H_{V\text{-}SMOW}$ = 100.3‰) was also analyzed for δ^{2}H (SD, n = 54, 1.13‰). The sample capsules were comparatively equilibrated with capsules containing the keratin working standard BWB II (whale baleen, nonexchangeable $\delta^{2}H_{V\text{-}SMOW}$ = −108‰) and the egg shell membrane standard RSPB EGG (nonexchangeable $\delta^{2}H_{V\text{-}SMOW}$ = −93.8‰) for no less than 7 days prior to analysis to allow the exchangeable hydrogen in both samples and working standards to equilibrate fully with moisture in the laboratory air. Replicate analysis of BWB II and RSPB EGG run concurrently with the samples gave a mean $\delta^{2}H_{V\text{-}SMOW}$ = −106.9‰ (n = 19) and $\delta^{2}H_{V\text{-}SMOW}$ = −101.3‰ (n = 15), respectively. As the average $\delta^{2}H_{V\text{-}SMOW}$ data obtained for BWB II were within 1 SD of their known nonexchangeable δ^{2}H values, no correction for exchangeable hydrogen content was applied to the freeze-dried muscle samples. However, a simple correction for exchangeable hydrogen was applied to the $\delta^{2}H_{V\text{-}SMOW}$ data for the feed samples by using the measured $\delta^{2}H_{V\text{-}SMOW}$ value for BWB II measured within each batch of samples.

The quality control reference standards for ^{13}C and ^{15}N analysis of hair samples and the analytical precisions obtained were powdered bovine liver (NBS-1577B) (SD, n = 12, 0.13‰ for δ^{15}N and 0.09‰ for δ^{13}C), a mixture of ammonium sulfate and cane sugar (IA-R046/IA-R006) (SD, n = 4, 0.02‰ for δ^{15}N and 0.05‰ for δ^{13}C), a mixture of ammonium sulfate and sucrose (IAEA-N-1/IAEA-CH-6) (SD, n = 4, 0.04‰ for δ^{15}N and 0.05‰ for δ^{13}C), and bovine liver (IA-R042) (SD, n = 12, 0.24‰ for δ^{15}N and 0.12‰ for δ^{13}C) for δ^{13}C and δ^{15}N. Ammonium sulfate (IA-R046) was also analyzed along with the samples for δ^{15}N (SD, n = 3, 0.08‰), whereas beet sugar (IA-R005) (SD, n = 6, 0.29‰) and cane sugar (IA-R006) (SD, n = 5, 0.06‰) were run as controls for δ^{13}C.

Theoretical composite δ values of the feed rations were estimated using the mass balance model[36]

$$\delta_{RC}\ (‰) = (X\delta_X - Y\delta_Y)/(X + Y)$$

where δ_{RC} is the theoretical δ value of the ration composites, δ_X and δ_Y, are the δ values of the two dietary components X and Y, respectively, and X and Y are the product of the dry matter (DM), dry matter digestibility (DMD), and intake of each dietary component fed to the animals (Table 1) as well as the percentage of C, N, H, and S (Table 2) of the dietary components X and Y, respectively.

Statistical Analysis. An exploratory one-way analysis of variance (ANOVA) for each measured variable followed by a Tukey post hoc test was performed to assess the significance of the differences among groups of samples of different dietary origin using the SPSS 15.0 package for Windows (SPSS, Inc., Chicago, IL). The data were also subjected to multivariate statistical analysis to evaluate the possibility of differentiating bovine meat according to dietary regimen. Canonical discriminant analysis (CDA) was performed to evaluate whether separation for classifying animals on the basis of the feeding regimen could be based on the determined C, N, H, and S isotopes ratios and to verify which isotope ratios contribute toward classification. A stepwise method was used to select the most significant variables and to exclude the redundant ones from the model. The procedure generates a set of canonical discriminant functions based on the selected variables that provide the best discrimination between the dietary groups. Those functions can be applied to new samples that have measurements for the stable isotopic signatures of the determined bioelements but come from unknown dietary groups. The statistical significance of each discriminant function was evaluated on the basis of the Wilks' λ factor after the function was

Table 3. Mean ± SD $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{34}S$ Values of Bovine Muscle from Animals on Each of the Four Dietary Treatments[a]

	diet				
	P (n = 24)	SiP (n = 24)	SiPC (n = 25)	C (n = 25)	P value
$\delta^{13}C$ (‰)	−27.7 ± 0.2 d	−27.6 ± 0.1 c	−26.4 ± 0.2 b	−25.0 ± 0.1 a	<0.001
$\delta^{15}N$ (‰)	9.2 ± 0.4 a	8.9 ± 0.4 b	7.9 ± 0.2 c	6.3 ± 0.3 d	<0.001
$\delta^{2}H$ (‰)	−122.4 ± 1.9 c	−121.1 ± 3.3 c	−117.1 ± 1.9 b	−112.4 ± 4.7 a	<0.001
$\delta^{34}S$ (‰)	4.6 ± 0.7 b	4.7 ± 0.5 b	4.8 ± 0.5 b	6.0 ± 0.4 a	<0.001

[a] Different letters within a row indicate significant differences (P < 0.05) between groups. P, pasture; SiP, silage-pasture; SiPC, silage-pasture/concentrate; C, concentrate plus straw.

removed. To verify the stability of the model, a "leave-one-out" cross-validation was performed. The success of the discrimination was measured by the proportion of cases correctly classified using this cross-validation.

RESULTS AND DISCUSSION

Isotope Signatures of Feedstuffs. Grass had the most negative mean $\delta^{13}C$ value, whereas both grass silage and straw had slightly less negative values compared to grass (Table 2). The depletion in ^{13}C of the grass compared with the concentrate can be attributed to the high proportion of nonphotosynthetic material (grains) in the concentrate ration.[27,37] Differences between these C_3 feed constituents could also be due to variability in the water availability and the development level of the plant.[37] The $\delta^{13}C$ values are in agreement with those reported previously for C_3 grass,[12,27,31] barley-based concentrates,[29] C_3 grass silage,[12,27,38] and cereal straw.[12,24,29]

Among the feed components, straw had the lowest $\delta^{15}N$ value, whereas grass had the highest value (Table 2). The variability observed in plant N isotopic values even within a small area has been attributed to factors such as soil condition, N fertilization, intensity of crop practices, N availability, different pathways of N assimilation, N recycling within a plant, climate, and distance from the sea.[38−40] Plants that can fix N_2 from air have lower $^{15}N/^{14}N$ isotope ratios than those that only assimilate soil inorganic N, such as ammonium or nitrate.[22] The presence of soybean, which engages in N_2 fixation, in the concentrate ration could contribute to the lower $\delta^{15}N$ values found in the concentrate ration (Table 2). The low $\delta^{15}N$ values found are in agreement with those reported by Bahar et al.,[38] whereas the values for grass were considerably higher than those reported by Moreno-Rojas et al.,[41] likely due to different climatic, edaphic, and agronomic conditions.

The grass silage showed more negative $\delta^{2}H$ values than the grass and the other feedstuffs (Table 2). A similar ^{2}H isotopic pattern was reported by Camin et al.,[40] who observed more negative $\delta^{2}H$ values in grass (C_3 plants) than in barley-based concentrates. Hydrogen isotopes in the feedstuffs reflect the H isotopic ratio of the water available, which in turn is influenced by the average H isotopic ratio of the precipitation water.[8,17,42,43]

The concentrate and straw were enriched in ^{34}S compared to the other feed components (Table 2); there was also a small enrichment in ^{34}S in grass compared to grass silage. The $\delta^{34}S$ values in feedstuffs depend on the location (proximity to the sea) and season of production (atmospheric deposition),[14] as well as the use of organic fertilizers that may be enriched in ^{34}S.[13] Moreover, the local geological sulfate variability can influence the $\delta^{34}S$ values of soils and subsequently of the plants growing on these soils.[44]

Isotope Ratio in Bovine Muscle. The differences in $\delta^{13}C$ values of the dietary components (Table 2) were clearly reflected in those of the bovine muscle (Table 3). The mean $\delta^{13}C$ values for the P and SiP groups were more negative than that of the C group (P < 0.001), with the SiPC group being intermediate. The less negative mean $\delta^{13}C$ value of the muscle of the SiPC group compared to the muscle of the SiP group was clearly due to the introduction of the concentrate in the last 6 months of the experiment. The lower $\delta^{13}C$ values found in the P group than in the C group are in agreement with previous studies.[41,45] Bahar et al.[38] found more positive $\delta^{13}C$ values than those in the current study for muscle of animals fed grass silage, probably due to the different lengths of the experiments (5.5 months vs 12 months in the current study) and also the fact that all animals received 3 kg of concentrates daily in the study of Bahar et al.[38] Bahar et al.[24] demonstrated that isotopic equilibrium was not reached for C, N, or S in bovine muscle after 168 days on an experimental diet. Furthermore, Sponheimer et al.[46] demonstrated that large mammal muscle C requires a period of over a year to reflect 90% of a new diet signature. Thus, the turnover of C, N, and S in bovine skeletal muscle has been shown to be a slow process, and consequently the isotopic analysis of skeletal muscle alone is not adequate for the detection of short-term preslaughter dietary changes.[24] In agreement with previous studies,[16,21,47] enrichments in the $\delta^{13}C$ values were found in muscle compared to those of the diets. The isotopic values of light bioelements of animal tissues differ from those of their diets, and this difference is called the "diet−tissue shift".[41,48] The diet−muscle shifts for $\delta^{13}C$ in animals from the P and C groups were 3.2 and 2.9‰, respectively, which were in agreement with previous literature results.[21,38,41]

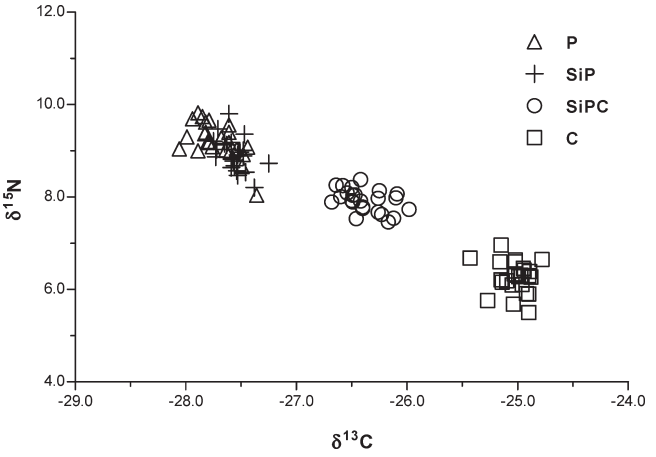

Figure 1. $\delta^{15}N$ versus $\delta^{13}C$ values of bovine muscle of animals on each of the four dietary treatments (P, pasture; SiP, silage-pasture; SiPC, silage-pasture/concentrate; C, concentrate plus straw).

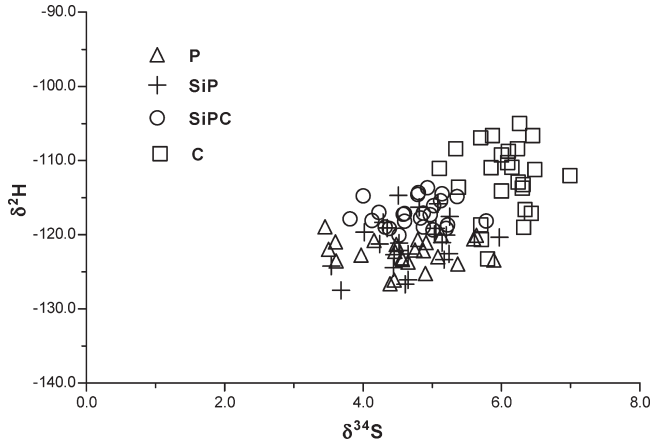

Figure 2. δ^2H versus δ^{34}S values of bovine muscle of animals on each of the four dietary treatments (P, pasture; SiP, silage-pasture; SiPC, silage-pasture/concentrate; C, concentrate plus straw).

The δ^{15}N values of the diets (Table 2) were also reflected in the δ^{15}N values of the muscle, and differences between the four groups were highly significant ($P < 0.001$) (Table 3). Muscle from the P group animals had a higher mean δ^{15}N value than that from the C group animals, with the mean δ^{15}N value for the SiPC group being intermediate between the C and P groups. The δ^{15}N values obtained for pasture-fed animals were considerably higher than those reported by Moreno-Rojas et al.,[41] probably due to differences in C_3 plant species consumed (fresh vetch, *Vicia sativa*, a legume, ad libitum). The diet−muscle shifts were 2.8 and 2.9‰ in animals from the P and C groups, respectively. Similar diet−muscle shifts were observed by other authors.[22,38]

Figure 1 is a plot of δ^{15}N versus δ^{13}C values of muscle samples from the four groups. Although the P and SiP groups overlapped, the isotopic signatures of these groups were quite distinct from those of the C and SiPC groups, thus giving a separation into three groups. Moreno-Rojas et al.[41] also reported a clear isotopic separation of lambs reared with grass or concentrate diets based on C_3 plants, when δ^{15}N versus δ^{13}C values in either lamb muscle or wool were plotted.

The mean δ^2H value of muscle of the C group was less negative ($P < 0.001$) than that of the P and SiP groups, which did not differ significantly (Table 3), showing that δ^2H values of the diets (Table 2) were reflected in the δ^2H values of the muscle. On the basis of changes in muscle δ^2H values in response to a switch in diet and drinking water, Harrison et al.[25] hypothesized that most of the H in ovine muscle originated in the feed rather than in the drinking water, and because all animals in the current study received the same drinking water (δ^2H value of -44.6 ± 0.7‰ measured previously[25]), the results of the current study with bovine muscle concur with those of Harrison et al.[25]

Muscle from the C group had higher δ^{34}S values ($P < 0.001$) than those from animals fed pasture-containing diets (P, SiP, SiPC) (Table 3), reflecting the high δ^{34}S values in the concentrate plus straw diet (Table 2). González-Martín et al.[10] revealed a correlation between δ^{34}S in the diet and δ^{34}S in Iberian swine tissues that permitted differentiation of Iberian swine on the basis of the diet received. The δ^{34}S value of natural compounds is influenced by several factors such as industrial sources, that is, fertilizers, or natural sources such as sea spray and volcanic emissions.[43]

Figure 2 is a plot of δ^2H versus δ^{34}S values of the muscle samples from the four groups. There was no spatial separation from samples belonging to different dietary groups. This finding makes it unlikely that δ^2H and δ^{34}S could be used alone as discriminatory variables to distinguish between feeding regimens.

Table 4. Results of Classification of Bovine Muscle Samples from Different Feeding Regimens on the Basis of the Discriminant Functions Calculated from the Stable Isotope Data[a]

		predicted diet membership				
	diet	P	SiP	SiPC	C	total
original count	P	17	7	0	0	24
	SiP	6	17	0	0	23
	SiPC	0	0	24	0	24
	C	0	0	0	25	25
original %	P	70.8	29.2	0.0	0.0	100.0
	SiP	26.1	73.9	0.0	0.0	100.0
	SiPC	0.0	0.0	100.0	0.0	100.0
	C	0.0	0.0	0.0	100.0	100.0
cross-validated count	P	17	7	0	0	24
	SiP	6	17	0	0	23
	SiPC	0	0	24	0	24
	C	0	0	0	25	25
cross-validated %	P	70.8	29.2	0.0	0.0	100.0
	SiP	26.1	73.9	0.0	0.0	100.0
	SiPC	0.0	0.0	100.0	0.0	100.0
	C	0.0	0.0	0.0	100.0	100.0

[a] 86.5% of original grouped cases correctly classified. 86.5% of cross-validated grouped cases correctly classified.

CDA was performed to classify the animals according to their feeding regimen on the basis of muscle stable isotopic signatures (C, N, H, S). Two stable isotope ratios were selected due to their significant contribution to the discrimination of beef samples: δ^{13}C and δ^{34}S. Meanwhile, δ^{15}N and δ^2H were excluded from the model because the information they provided was redundant. The results showed two discriminant functions. The first function was mainly correlated with the δ^{13}C values, which explained 99.6% of the variance and allowed the groups to be differentiated according to feeding regimen. On the other hand, δ^{34}S values proved to be the most discriminatory variable for the second discriminant function and accounted for 0.4% of the variation. The number and percentage of correctly classified samples are given in Table 4: 86.5% of the original grouped cases were correctly classified, and a similar correct classification was obtained when cross-validation analysis was performed. In the cross-validation all grouped C and SiPC cases were correctly classified, whereas of all samples belonging to the P and SiP groups, 29.2% (7 samples) and 26.1% (6 samples) were misclassified, respectively.

The separation between the four dietary regimens in the discriminant space was checked by plotting the first and second functions shown in Figure 3. The predictive capacity of the model decreased slightly when the four variables were considered together (without selecting the variables by the stepwise procedure), allowing a correct assignment of 85.4% of the original grouped cases and correct cross-validation of 83.3% of

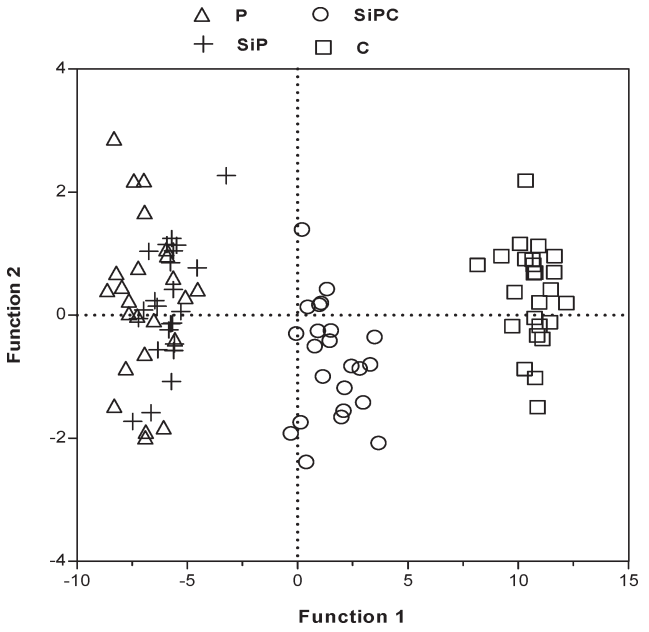

Figure 3. Discriminant analysis for authentication of beef samples based on different feeding regimens (P, pasture; SiP, silage-pasture; SiPC, silage-pasture/concentrate; C, concentrate plus straw).

individual beef samples (data not shown). However, when P and SiP groups were considered as belonging to one group (because the silage is conserved pasture), of animals fed a pasture-based diet, 100% of the original and cross-validated grouped cases were correctly classified using the signatures of both the four isotope ratios and the $\delta^{13}C$ and $\delta^{34}S$ isotope ratios (data not shown).

C and N Isotope Ratios in Tail Hair. The calculated rates of hair growth of animals in the C group (0.892 and 0.872 mm day^{-1}), the P group (0.894 and 0.997 mm day^{-1}), the SiP group (0.853 and 0.783 mm day^{-1}), and the SiPC group (0.835 and 0.930 mm day^{-1}) were in the range reported for tail hair by Schwertl et al.[49] and Zazzo et al.[30] Because the $\delta^{13}C$ and $\delta^{15}N$ values of the C and P groups increased from the start of the experimental diet to the slaughter dates, diet−hair shifts were estimated using the mean value of the last three isotopic values of the hair before slaughter.

The C isotopic signatures of consecutive hair segments from two different animals from each of the C, P, SiP, and SiPC groups are shown in Figure 4A. The $\delta^{13}C$ values were plotted versus time (calendar dates, dd/mm/yy) from the start of the experiment to the slaughter dates (approximately from November 2006 to November 2007). At the time of slaughter the $\delta^{13}C$ values of tail hair from the different groups reflected those of the diets (Table 2) and muscle (Table 3), with the P and SiP groups having the most negative $\delta^{13}C$ values, the C group having the least negative values, and the SiPC group having intermediate values (Figure 4A). The temporal isotopic patterns for tail hairs from animals in the same treatment groups were very similar, indicating good reproducibility of the method. A slight mismatch between hair samples from the same dietary treatment was not unexpected given that each hair was obtained from a different animal and that interindividual variability in hair growth rate was expected.[30]

The temporal C isotopic pattern of the hair and diet of two group C animals is shown in Figure 4B. Segmental $\delta^{13}C$ values of hair of those animals ranged between −24.1 and −27.1‰. A temporal variation was observed in the $\delta^{13}C$ values of the concentrate ration; that variation could be related to the fact that concentrate was made in different batches, although the formulation was the same for each batch. The lowest hair $\delta^{13}C$ value observed (estimated date October 1, 2006) corresponds to a time when animals were grazed on pasture before being assigned to the experimental diet (C). Once the animals were assigned to the concentrate ration (December 12, 2006), the $\delta^{13}C$ values quickly approached a plateau, following the temporal isotopic pattern of the dietary data and in agreement with the observations of Zazzo et al.[30] At slaughter, the two hairs were ∼3.1‰ enriched in ^{13}C relative to the diet, and this enrichment was similar to the 3.0‰ diet−hair shift found by Zazzo et al.[30]

The tail hair from two P group animals (Figure 4C) exhibited a rapid decrease in $\delta^{13}C$ values during the first 45 days after the start of the experimental diet to a minimum value of −29.1‰ in late December 2006, followed by an increase up to mid May 2007. The initial decrease and the increase thereafter may reflect the decrease in grass $\delta^{13}C$ up to February 2007 and the increase in $\delta^{13}C$ value of grass between February and June 2007. Temporal variation in the $\delta^{13}C$ values of the grass (diet P) was observed over the year with lower values in the (wetter) period from November to April and higher values in the (drier) period from April to November. At slaughter, the $\delta^{13}C$ of hair from the P group animals was enriched by 3.3‰ relative to the diet, and this enrichment was larger than those found in previous studies for cattle hair.[27,28,49] These differences in diet−hair shifts can be due to the large range of diets and environments used in the different studies. However, Cerling et al.[51] reported a similar mean $\delta^{13}C$ shift (3.1‰) between diet and keratin tissues in a range of wild ruminant mammals.

From the beginning of the experimental phase in November 2006 to April 2007, the tail hair from the two animals in the SiP group recorded gradual decreases in $\delta^{13}C$ values of 1.1 and 1.6‰, respectively (Figure 4D). This was followed by a slow increase to August 2007, coinciding with the diet switch from silage to grass and the seasonal increase in $\delta^{13}C$ of grass over that period, and a decrease thereafter as for the P group. Thus, the replacement of the silage with the pasture in the latter 6 months of the study is reflected in the $\delta^{13}C$ values of the tail hair. Data from one of the tail hairs (SiP1) related to the pre-experimental period 4 months (July 2006) prior to the start of the experimental feeding and indicated a $\delta^{13}C$ signature consistent with consumption of grass prior to the start of silage feeding.

Tail hairs from the SiPC group exhibited a similar $\delta^{13}C$ isotopic pattern to animals from the SiP group in the period early December 2006 to April 2007 (Figure 4A,E); this was expected as both groups received the same diet in this period. This was followed by an increase in $\delta^{13}C$ values from April 2007, when the animals were switched from silage to 50:50 DM pasture and concentrates, to mid-August 2007, followed by a slow decrease to mid-November 2007. The effect of the introduction of the concentrate is markedly reflected in the $\delta^{13}C$ values of the tail hair and indicates that the response of hair to dietary changes is rapid. Zazzo et al.[30] calculated that 25−32% of the total change in hair $\delta^{13}C$ values occurred within 1 day, with most carbon (90%) turned over in <3 months.

The nitrogen isotopic signatures of one tail hair from each of two different animals from the C, P, SiP, and SiPC groups are shown in Figure 5A. At the time of slaughter the $\delta^{15}N$ values of tail hair from the different groups reflected those of the diets (Table 2) and muscle (Table 3) with the values decreasing in the

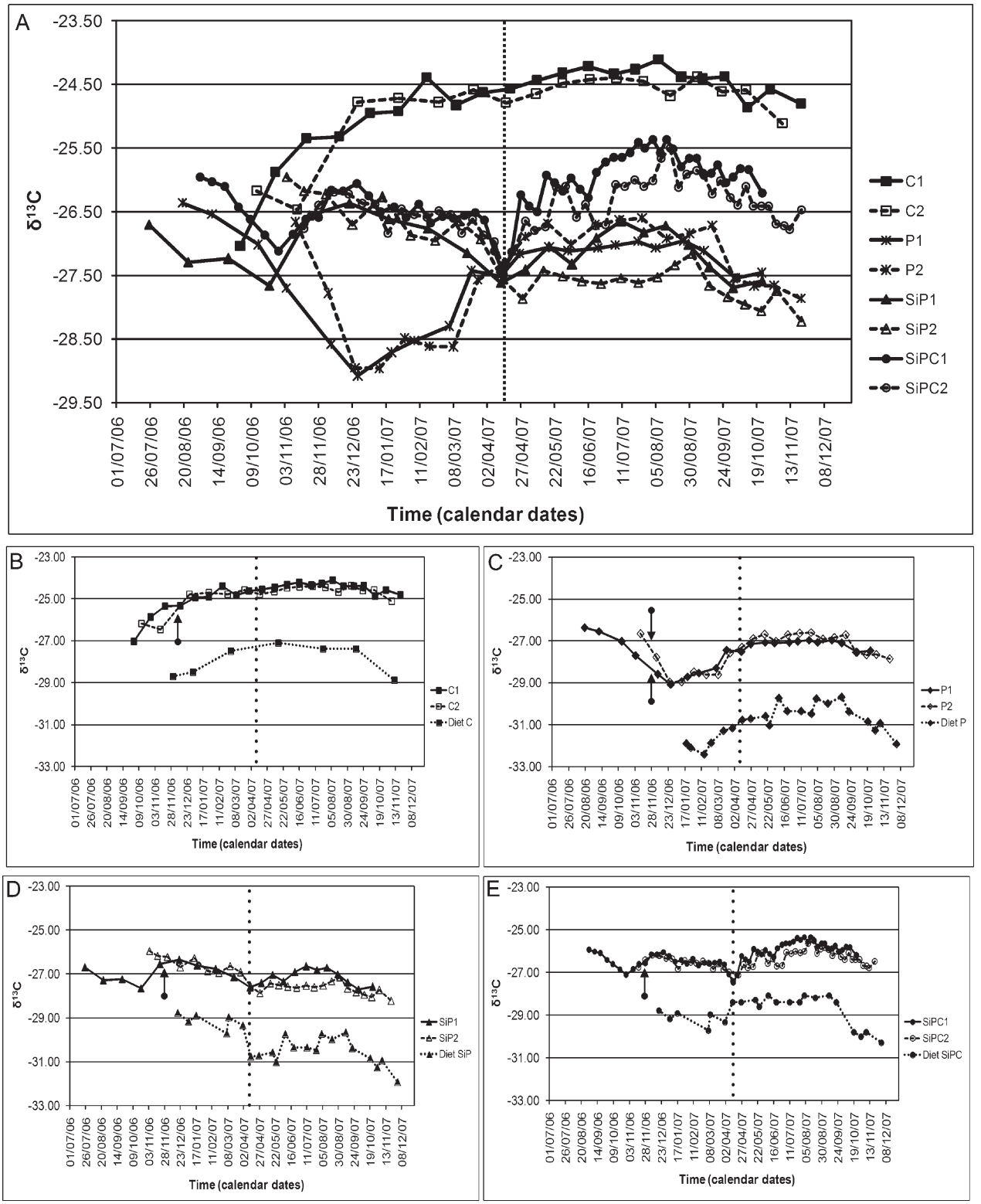

Figure 4. (A) Temporal changes in dietary and tail hair $\delta^{13}C$ of animals from the four dietary treatments. C1 and C2 represent tail hair from the concentrate-fed (C) group with experimental start date of December 12, 2006, and slaughter dates of November 21 and 7, 2007, respectively. P1 and P2 represent tail hair from the pasture-fed (P) group with experimental start date of November 28, 2006, and slaughter dates of October 23 and November 21, 2007, respectively. SiP1 and SiP2 represent tail hair from the silage-pasture-fed (SiP) group with experimental start date of November 28, 2006. and slaughter dates of October 23 and November 21, 2007, respectively. SiPC1 and SiPC2 represent tail hair from the silage-pasture/concentrate-fed (SiPC) group with experimental start date of November 28, 2006, and slaughter dates of October 23 and November 21, 2007, respectively. The dashed line indicates the diet switch (April 18, 2007). (B) Temporal changes in dietary (diet C) and tail hair $\delta^{13}C$ of animals from the concentrate-fed (C) group. (C) Temporal changes in dietary (diet P) and tail hair $\delta^{13}C$ of animals from the pasture-fed (P) group. (D) Temporal changes in dietary (diet SiP) and tail hair $\delta^{13}C$ of animals from the silage-pasture-fed (SiP) group. (E) Temporal changes in dietary (diet SiPC) and tail hair $\delta^{13}C$ of animals from the silage-pasture/concentrate-fed (SiPC) group. Arrows indicate the start date of the experimental period for the animals.

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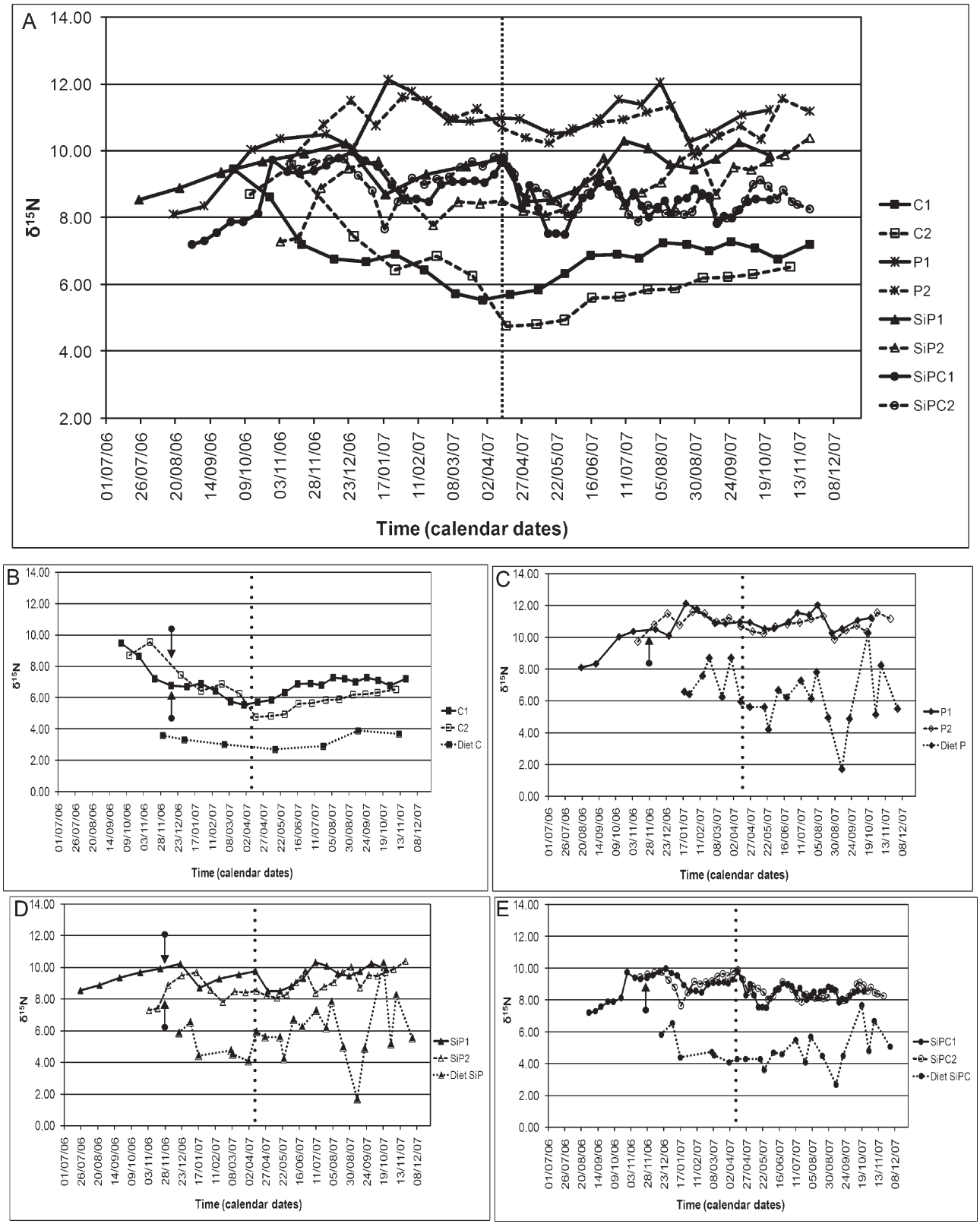

Figure 5. (A) Temporal changes in dietary and tail hair δ^{15}N of animals from the four dietary treatments (C1, C2, concentrate; P1, P2, pasture; SiP1, SiP2, silage-pasture: SiPC1, SiPC2, silage-pasture/concentrate). The dashed line indicates the diet switch (April 18, 2007). (B) Temporal changes in dietary (diet C) and tail hair δ^{15}N of animals from the concentrate-fed (C) group. (C) Temporal changes in dietary (diet P) and tail hair δ^{15}N of animals from the pasture-fed (P) group. (D) Temporal changes in dietary (diet SiP) and tail hair δ^{15}N of animals from the silage-pasture-fed (SiP) group. (E) Temporal changes in dietary (diet SiPC) and tail hair δ^{15}N of animals from the silage-pasture/concentrate-fed (SiPC) group. Arrows indicate the start date of the experimental period for the animals.

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order P > SiP > SiPC > C (Figure 5A). Segmental δ^{15}N values of tail hair from the C group ranged between 4.7 and 9.5‰ (Figure 5B). In general, the low δ^{15}N values found in the concentrate plus straw diet were reflected in the hair δ^{15}N signature. There was a decrease in δ^{15}N values during the first 6 months of the feeding trial, followed by a more modest increase (~1‰) from April to November 2007. The initial decrease (September 2006−early December 2006) corresponds to a period when the animals were receiving the pre-experimental diet, whereas the continued decrease to March/April 2007 followed by the increase to November 2007 reflects the trend in the δ^{15}N values of the C diet. The hair from cattle fed concentrate diets was consistently enriched in ^{15}N compared to the diet (3.3‰).

The δ^{15}N values of tail hairs from the P group showed considerable seasonal variability over the experimental period (Figure 5C). However, a substantial temporal variation over the year was observed in the δ^{15}N values of the grass and, for example, the decrease of δ^{15}N values in hair in the August/September 2007 period could reflect the marked decrease in δ^{15}N values observed in the pasture at that time. The mean δ^{15}N value of the tail hairs from the P group was 10.9‰ on the basis of the mean of data from November 2006 to November 2007. This high mean δ^{15}N value could reflect the high dietary protein level of the pasture (Table 1). Sponheimer et al.[39] investigated whether or not dietary protein levels affected herbivore δ^{15}N values and found that herbivores on high-protein diets (19% crude protein) had higher δ^{15}N diet−hair shifts than the same animals on low-protein diets (9% crude protein). The higher diet−hair shift (4.6‰) compared to that observed for the C group might be due to the different protein level of the diets (13.4% in C and 21.5% in P), but this requires further investigation.

When animals received grass silage from November 2006 to April 2007 (SiP group), δ^{15}N values were lower than those of the P group (Figure 5A), reflecting the lower δ^{15}N values of the silage compared to the pasture. From November 2006 to early February 2007 and from July to November 2007, both hairs from the SiP animals appeared to have different δ^{15}N isotopic patterns (Figure 5D). From February to April 2007, an increase in δ^{15}N values in both hairs was recorded. The diet switch from silage to pasture elicited an increase in δ^{15}N values from 8.0 to 10.4‰. Sponheimer et al.[39] established a period of 24 weeks to be sufficient for diet−hair nitrogen isotope equilibration in herbivores. Because the period for diet−hair N isotope equilibration is longer than that for C isotope equilibration,[30,39] the δ^{15}N values of the silage in the first 6 months of the experimental period had an effect on the δ^{15}N values of hair of SiP animals in the last 6 months; therefore, the P and SiP groups appeared separate for N in the last 6 month period (Figure 5A), whereas they overlapped for C (Figure 4A).

The δ^{15}N isotopic patterns of the two hairs from animals of the SiPC group from November 2006 to April 2007 (Figure 5E) were similar to that from the SiP group because they also received a diet based exclusively on grass silage. After the diet switch when animals received grazed pasture plus concentrate from April to October/November 2007, the δ^{15}N isotopic profile did not change greatly, showing the same pattern observed for the diet of the SiPC group. The reason for this could be the almost identical δ^{15}N values of the silage (5.0‰) and the pasture plus concentrate (4.9‰), which may be related to the similar dietary protein levels observed between the silage (16.8%) and a pasture plus concentrate diet (17.5%). Thus, the N isotopic values of cattle hair keratin reflected both the δ^{15}N value of the diet and the proportion of protein consumed in their diets.

Conclusions. SIRA of light elements (C, N, H, and S) in bovine muscle could be used to distinguish between beef from different feeding regimens with only minor isotopic differences, based not only on pasture or concentrates but also on feeding systems containing different proportions of these dietary components. Whereas C and N isotopic values in muscle did not allow discrimination between animals fed pasture and animals fed grass silage followed by pasture, isotopic analysis of short segments along cattle tail hair provided a distinctive isotopic archive for each group, allowing discrimination between them. Thus, for meat authentication, whereas SIRA analysis of a muscle sample gives data about animal production practice integrated over the animal's lifetime, natural ^{13}C and ^{15}N hair signatures provide a powerful tool to reconstruct changes in feed components offered to animals over periods of over a year and thus a tool to verify farm production practices.

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