End of Project Report

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CONFIRMATION OF THE DIETARY BACKGROUND OF BEEF FROM ITS STABLE ISOTOPE SIGNATURE

Authors

A.P. Moloney[a], B. Bahar[b,c], O. Schmidt[c], C.M. Scrimgeour[d], I.S. Begley[e], F.J. Monahan[b]

[a] *Teagasc, Grange Beef Research Centre, Dunsany, Co. Meath, Ireland*

[b] *UCD School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland*

[c] *UCD School of Biology and Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland*

[d] *Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK*

[e] *Iso-Analytical Ltd., Millbuck Way, Sandbach, Cheshire, CW11 3HT, UK*

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SUMMARY/CONCLUSIONS

Consumers are increasingly demanding information on the authenticity and source of the food they purchase. Molecular DNA-based technology allows animal identification, but without certification or a "paper-trail" but does not provide information about feed history or the production system under which the animal was reared. The stable isotopes of chemical elements (e.g. $^{13}C/^{12}C$, $^{15}N/^{14}N$) are naturally present in animal tissue and reflect the isotopic composition of the diet. The overall aim of this project was to determine the feasibility of using the stable isotopic composition as an intrinsic, biochemical marker to gain information about feed components used in the production of beef. Factors likely to affect the isotopic signature such as source of tissue, duration of feeding and production systems were examined. It is expected that this highly innovative and original technique will permit the identification of country of origin and dietary history of beef and so greatly assist efforts to market Irish beef, particularly in lucrative European markets. Sequential sampling and stable isotope analysis of bovine tail hair and hoof revealed that the two tissues can provide a detailed and continuous record of animal dietary history. Because hair can be sampled repeatedly and non-invasively, we anticipate that this approach will also prove useful for the investigation of short-term wildlife movements and changes in dietary preferences.

The conclusions from this project are:

- Multi-elemental stable isotope ratio analysis (SIRA) has the potential to assist in distinguishing organically from conventionally produced beef.

- Seasonal patterns in the isotopic composition of beef exist, probably reflecting seasonality in animal feeding practices modulated by tissue turnover rates.

- Consequently, there is a need to consider possible seasonal variation when applying multi-elemental SIRA to the authentication of beef in particular and to livestock-derived products in general.

- Maize silage used in the finishing diet of beef cattle could be detected and quantified by stable carbon isotope analysis of lipid-free beef muscle or lipid. Thus, $\delta^{13}C$ has potential as a marker for authenticating the origin of beef from production systems using different levels of maize and other C_4 plant products.

- The differences in the carbon and nitrogen stable isotope composition of a range of common feeds were reflected in the muscle of cattle thereby allowing SIRA to be used as a component, at least, of a scheme to authenticate the dietary history of beef from cattle consuming these feeds.

- While $\delta^{13}C$ showed little temporal variation in concentrate and silage feed materials, it was more variable in fresh grass over one growing season. Bulk muscle tissue reflected subtle isotopic differences between dietary components after about 100 days of consumption.

- The turnover of C and N was slow and similar for 2 muscles (half-lives of 151 and 157 days for striploin (*Longissimus dorsi*) and 134 and 145 days for fillet (*Psoas* major), respectively). For detection of short-term dietary changes, isotopic analysis of muscle alone is inadequate.

- Sequential sampling and stable isotope analysis of bovine tail hair and hoof revealed that the two tissues can provide a detailed and continuous record of animal dietary history. Because hair can be sampled repeatedly and non-invasively, we anticipate that this approach will prove useful for the investigation of short-term wildlife movements and changes in dietary preferences.

General Introduction

Food scares, in particular in the animal-derived food industry have lead to demands from consumers for greater knowledge of the food they consume. A critical component of any strategy to overcome beef-scares, regain competitive edge and ensure future competitiveness and sustainability will be an ability to guarantee quality, including authenticity, of beef. The need for methodologies to provide information about the origin and history of food has led to the development of molecular DNA-based methods that can trace meat cuts back to an individual animal and detect and identify meat species (Lockley and Bardsley, 2000). However, no methods are available to provide information on the geographical origin and feeding history of livestock independent of the producer or supplier of this livestock or when the source of meat is unknown. This project addressed this gap by assessing the usefulness of measurements of the isotopic composition of beef as an intrinsic, non-manipulative, biochemical marker of the origin and feeding history of beef cattle.

Stable isotope ratio analysis (SIRA) techniques measure ratios of naturally present stable isotopes of chemical elements (e.g. $^{13}C/^{12}C$, $^{15}N/^{14}N$). Dietary components have regionally typical isotopic "signatures" determined by climate (hydrogen, oxygen), vegetation composition (carbon), feed type (carbon, nitrogen), crop production practices (nitrogen), and proximity to the sea (sulphur). Use of SIRA in ecological studies (Schmidt et al., 1997, 1999) has demonstrated that the isotopic composition of animal tissue reflects the isotopic composition of the animals' diet. This relationship has been used successfully in both marine and terrestrial habitats to delineate geographical areas where (wild) animals are feeding and to infer the diet of animals from the isotopic composition of potential food sources (Hobson, 1999; Wada et al., 1993). Data from a preliminary study with beef indicated that the stable isotopic signature differs between grass-fed and concentrate-fed beef thus illustrating the potential of this technology as a tool to discriminate or authenticate beef (Schmidt et al., 2002). Little is known about factors that might influence the isotopic signature of beef, such as ration composition, duration of feeding and changes in body composition during growth, or on the variation in the isotopic signature between and within muscles, other tissues or due to production environment.

The overall aim of the project was to determine the feasibility of using novel (SIRA) techniques to gain information about the feed components used in the production of beef.

The specific objectives were:

- To determine whether differences exist in the stable isotope signatures of Irish beef produced under different production systems.
- To determine the effect of pre-slaughter diet on the stable isotope ratios of beef.
- To determine the kinetics of changes in stable isotope ratios of beef in response to changes in dietary regime.
- To determine the inter-muscle variation in stable isotope ratios within beef carcasses.
- To compare the isotopic signature of non-muscle bovine tissues

Carbon (^{13}C) and nitrogen (^{15}N) isotope analyses were carried out at the Stable Isotopes Unit, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, Scotland. Sulphur (S^{34}) isotope analysis was carried out by ISO-analytical Ltd., Unit 2, Zan Industrial Estate, Sandbach, Cheshire, United Kingdom.

Experiment 1: Stable carbon, nitrogen and sulphur isotopic differentiation of organically and conventionally produced Irish beef.

Introduction

Retail meat outlets now frequently offer consumers a choice of organically and conventionally produced beef. However, reliable scientific techniques for differentiating between the two types of beef are not available. Stable isotope analysis is a novel tool for the authentication of food products, including animal derived foods. The objective of this study was to ascertain whether differences exist in the isotopic composition of light elements (C, N and S) in organically and conventionally produced Irish beef.

Materials and Methods

Organically (n=15) and conventionally (n=17) produced beef samples were obtained from a variety of retail outlets in the Dublin area. Samples were freeze dried and powdered in a ball mill. The freeze dried samples (0.9–1.1 mg for C and N analysis and 7.5–8.5 mg for S analysis) were analysed by isotope ratio mass spectrometry. The values of the isotope ratios were calculated and expressed in delta-notation [δ per mille (‰)] according to the formula:

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

where, R is the ratio of the heavy to light stable isotope in a sample (R_{sample}) and a reference standard ($R_{reference}$). Results are referenced to an international standard (V-PDB). For each isotope, means were compared by t-test.

Results and Discussion

Type of production system had a significant effect on $\delta^{13}C$ ($P<0.001$), $\delta^{15}N$ ($P<0.001$) and $\delta^{34}S$ ($P<0.05$) values. The higher mean $\delta^{13}C$ value (–24.5‰) in conventional beef compared to that of organic beef (–26.0‰) suggests that the animals in the conventional production systems consumed some C_4 plant material, possibly maize, while those in the organic system consumed C_3 plant material only. Organic beef had a lower $\delta^{15}N$ value (6.6‰) than conventional beef (7.8‰). This may indicate that the organically reared animals consumed

a higher proportion of leguminous material such as clover. Legumes fix atmospheric N_2 (0 $\delta^{15}N$) and tend to have lower $\delta^{15}N$ values than non-legumes which depend on ^{15}N fertiliser-enriched soil N. Another possible explanation is that external N fertilizer inputs and consequent N losses are higher in conventional production systems, leading to a ^{15}N enrichment at the system level because ^{14}N is lost preferentially. The mean $\delta^{34}S$ value of organic beef (7.9‰) was higher than that of conventional beef (7.2‰) which may be due to the reliance of organic production systems on the use of organic manure for maintaining soil fertility. The additional S provided by the manure, together with the preferential loss of the lighter isotope (^{32}S) during the process of sulphur mineralization in soil may lead to ^{34}S enrichment in the surface soil. A combination of stable isotope ratios (Fig. 1) resulted in some segregation of samples into the two production systems.

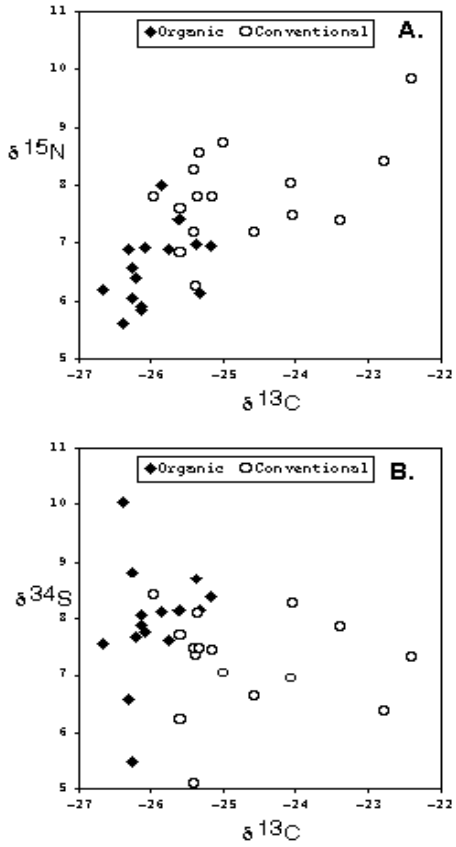


Figure 1: Scatter plots of $\delta^{13}C$ versus $\delta^{15}N$ (A) and $\delta^{13}C$ versus $\delta^{34}S$ (B) values

Conclusion

The preliminary data presented here suggest that multi-elemental SIRA has the potential to assist in distinguishing organically from conventionally produced beef.

Experiment 2: Seasonal variation in the stable carbon, nitrogen and sulphur isotope composition of retail organic and conventional Irish beef

Introduction

The potential of SIRA in authenticating organically produced beef was shown in Experiment 1. As the difference in isotopic compositions between indoor (mostly grain-based diets) and outdoor (mostly grass-based diets) dietary constituents are likely to result in altered isotopic compositions of animal tissues, seasonal variations in the isotopic composition of meat can be expected. The objectives of this study were to investigate the annual seasonal variation in the stable isotope composition of light elements (C, N and S) of organic and conventional retail Irish beef and to assess its implications for the isotopic authentication of organic beef.

Materials and methods

Organic beef samples were collected from two large supermarkets and two certified butcher shops dealing exclusively with organic beef. All sources were approved either by the Irish Organic Farmers' and Growers' Association (IOFGA) or the Organic Trust, Ireland, as certified organic beef retailers. Conventional beef samples were collected from the same supermarkets and three further butcher shops dealing only with conventional beef produced in Ireland. Of the total organic sample (n=127) collected, 96.5% were striploin; in instances where this was unavailable, round steak (2%) and sirloin (1.5%) were sampled. All conventional beef samples (n=115) were striploin.

Samples were collected once a week between July 2003 and June 2004. An uneven availability of organic beef throughout the year and the selling of beef from the same sources on consecutive weeks, especially by the small butchers, caused uneven numbers of beef samples collected each week. Collected samples were vacuum packaged and stored at –20°C until preparation for analysis using continuous flow isotope ratio mass spectrometry.

All samples were analysed for C and N. For S, only the samples supplied by one large supermarket were analysed (organic n=46, conventional n=54). Seasonal isotopic patterns were derived by time series analysis using MINITAB Release 14.13 (Minitab Inc., State College PA, USA). Randomness of the time series data was tested by

autocorrelation analysis of fortnightly means. Fortnightly mean $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values were used to calculate the moving average of three adjacent time points; centered moving averages are presented in the time series plots. The accuracy of the fitted time series was expressed in terms of MAPE (Mean Absolute Percent Error, expressed as a percentage), MAD (Mean Absolute Deviation, expressed in delta unit, ‰) and MSD (Mean Squared Deviation). For all three measures, smaller values indicate a better fitting model. Season-wise mean $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of organic and conventional beef were compared by t-tests.

Results and Discussion

Moving averages revealed a marked seasonality in $\delta^{13}C$ values for conventional beef which gradually became less negative from early December 2003 until early June 2004; the $\delta^{13}C$ of organic beef was only slightly less negative from early March to early May 2004 (Figure 2 i).

The observed elevated $\delta^{13}C$ values probably reflect indoor winter-feeding (November to March), when animals receive concentrates and conserved forages like hay and silages. Concentrate feeds mostly contain grains, which have relatively less negative $\delta^{13}C$ values than vegetative tissues including leaves and stems. Feeding of a high proportion of concentrates during winter resulted in delayed, less negative $\delta^{13}C$ values of meat in spring and early summer because of the slow C turnover in bovine skeletal muscle (cf. Experiment 6). On the other hand, the more negative and consistent $\delta^{13}C$ values in both types of beef in the present study from July to November likely reflect feeding of grass-clover pasture diets throughout the spring and summer.

The pronounced $\delta^{13}C$ seasonality in conventional beef probably reflects starker seasonal changes in the feeding regime used on conventional than organic farms. Unlike organic farms, some of the conventional animals (i.e. those with $\delta^{13}C$ less negative than –23‰) had possibly received considerable amounts of C_4 feedstuffs, most likely maize grain and/or maize silage during over-wintering (cf Experiment 4).

Grouping the data by season (Table 1) revealed that $\delta^{13}C$ values of conventional and organic beef differed significantly during winter (difference=0.7‰, $P<0.01$), spring (difference=1.4‰, $P<0.001$) and summer (difference=0.9‰, $P<0.05$) but not during

autumn (difference=0.2‰); the overall means were also significantly different SED=0.16, $P<0.001$).

Seasonal variation in N isotope composition

In conventional beef, $\delta^{15}N$ was remarkably invariant with an overall mean $\delta^{15}N$ value of 7.0‰ (95%CI=0.10) throughout the year; organic $\delta^{15}N$ was more variable, with slightly lower values during and autumn (Figure 2 ii).

Lower $\delta^{15}N$ values could be due to feeding of grass-clover during summer and legume concentrates during winter.

The $\delta^{15}N$ spacing between individual fortnightly means of organic and conventional beef revealed a maximum difference of 1.9‰ (SED=0.73, $P<0.05$) in the second fortnight of March (Figure 2 ii). In all but three fortnights organic beef had a somewhat lower $\delta^{15}N$ value than the conventional beef. Evidence from a number of studies suggests that elevated $\delta^{15}N$ values of the conventional beef reflect a system-wide enrichment of ^{15}N in conventional farms where the use of mineral fertilizers results in highly positive N input–output balances (Watzka, *et al*., 2006).

Grouping the data by season (Table 1) showed that the $\delta^{15}N$ value of organic and conventional beef differed during spring (difference=0.7‰, $P<0.01$), summer (difference=0.5‰, $P<0.05$) and autumn (difference=0.8‰, $P<0.001$) but not during winter (difference=0.3‰). In Experiment 1, the $\delta^{15}N$ values of organic and conventional Irish beef differed by 1.2‰ in samples collected from mid-February to mid-April 2002. Compared to that study, lower $\delta^{15}N$ in the same period could be due to a year-to-year variation in the feed $\delta^{15}N$ values.

3.3. Seasonal variation in S isotope composition

Compared to $\delta^{13}C$ and $\delta^{15}N$, the seasonal patterns in $\delta^{34}S$ were more complex (Figure 2iii). Between October to December 2003 and April to June 2004, the $\delta^{34}S$ of organic beef (n=46) was higher than that of conventional beef (n=54), whereas during rest of the year it was lower. The $\delta^{34}S$ value in both types of beef spaced maximally in the first fortnight of September (difference=3.7‰, P=0.28) and June (difference=2.7‰,

SED=0.09, $P<0.01$), but in the reverse order (Figure 2iii). Grouping the data by season (Table 1) failed to show any difference between mean $\delta^{34}S$ values of organic and conventional beef in any of the four seasons. During spring, the organic beef was somewhat enriched in ^{34}S compared to conventional beef.

The causes of this complex seasonality pattern of $\delta^{34}S$ in beef are unclear and explanatory background information (such as geographical origin of the samples) is lacking. Since there is little fractionation of $\delta^{34}S$ in normal animal metabolism (Peterson & Fry, 1987), the seasonal patterns in muscle $\delta^{34}S$ values likely reflect seasonality of $\delta^{34}S$ in feedstuffs. The latter can be diverse depending on, inter alia, the location (proximity to sea) and season of production (atmospheric deposition). Coastal effects are likely in an island the size of Ireland and the elevated $\delta^{34}S$ values of organic beef could be due to feeding of seaweed having much higher $\delta^{34}S$ values than terrestrial feedstuffs. Clearly, the dynamics of $\delta^{34}S$ are understood least and require more research.

Conclusions

These results show that seasonal patterns in the isotopic composition of beef exist, probably reflecting seasonality in animal feeding practices modulated by tissue turnover rates. The occurrence of seasonal variation in the isotopic composition of light elements reported here suggests strongly that there is a need to consider possible seasonal variation (and ultimately to understand their underlying causes including tissue turnover) when applying multi-elemental SIRA to the authentication of beef in particular and to livestock-derived products in general.

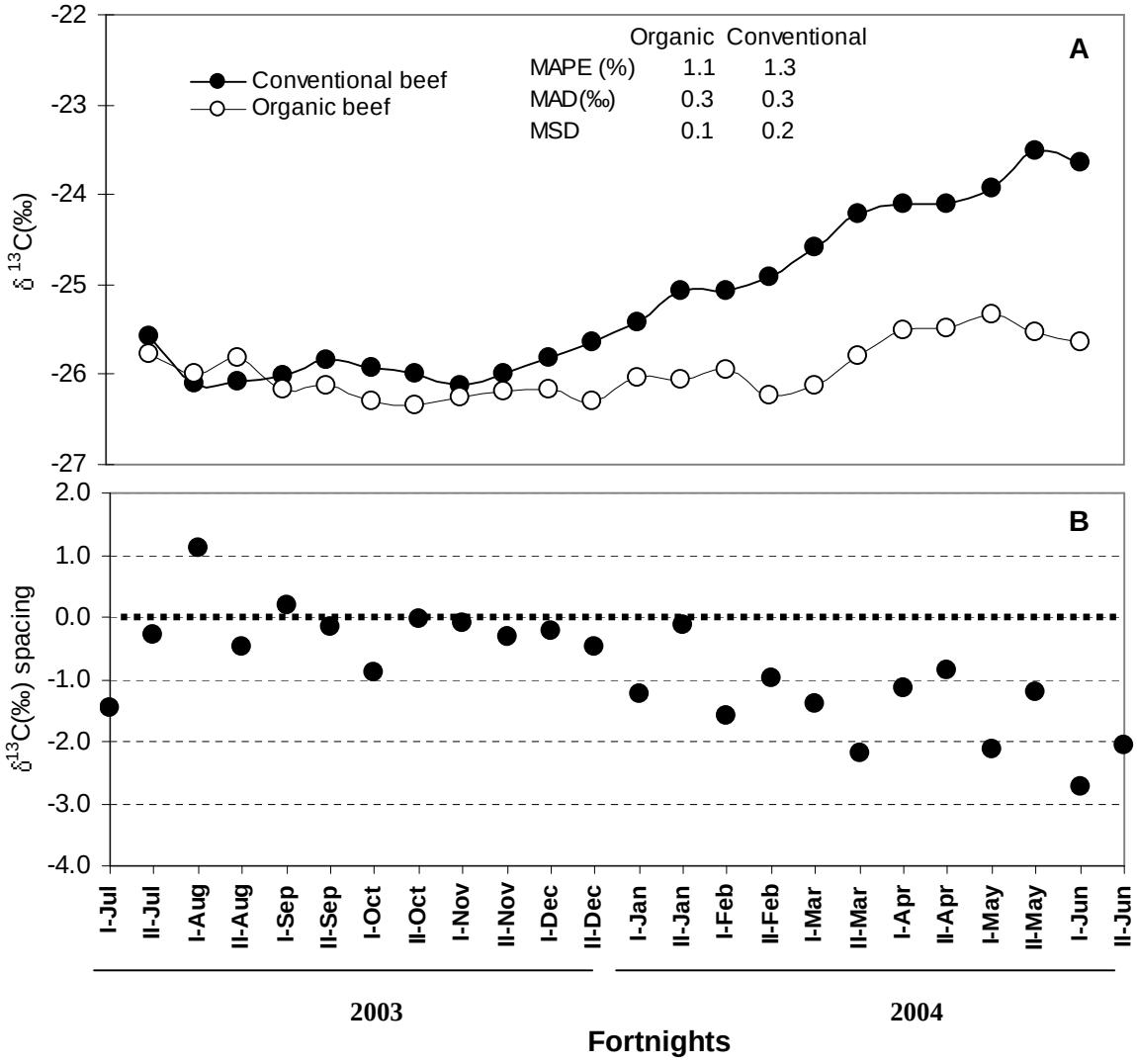


Figure 2 (i) Time series moving average plots of $\delta^{13}C$ (A), and the $\delta^{13}C$ spacing between fortnightly means of organic and conventional Irish beef (B). MAPE=Mean absolute percent error, MAD=Mean absolute deviation, MSD=Mean square deviation. First (I) and second (II) fortnights of the months and seasons (summer: June to August; autumn: September to November; winter: December to February; spring: March to May) are shown.

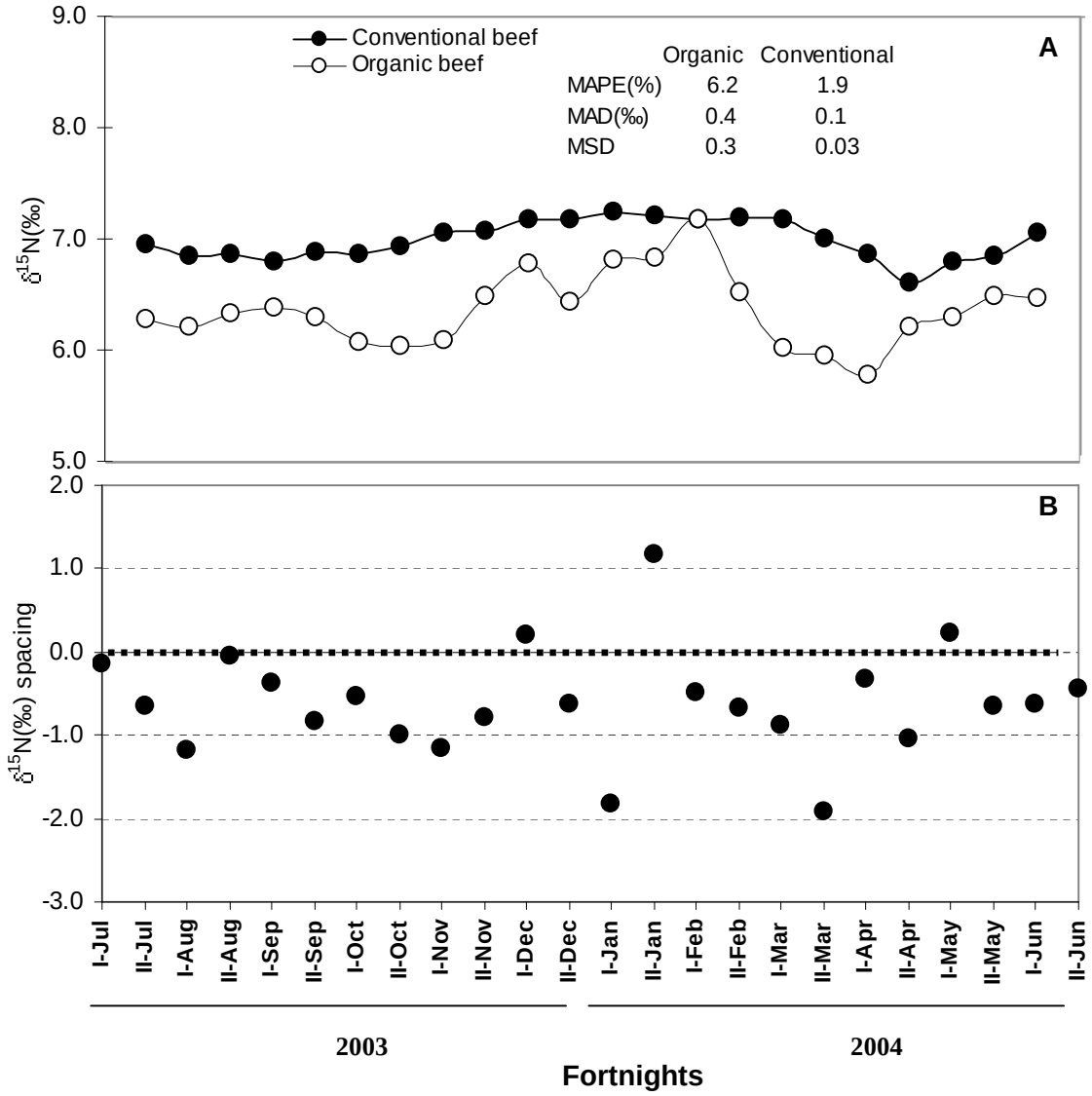


Figure 2 (ii) Time series moving average plots of $\delta^{15}N$ (A), and the $\delta\ ^{15}N$ spacing between fortnightly means of organic and conventional Irish beef (B). See Figure 2 (i) for other details.

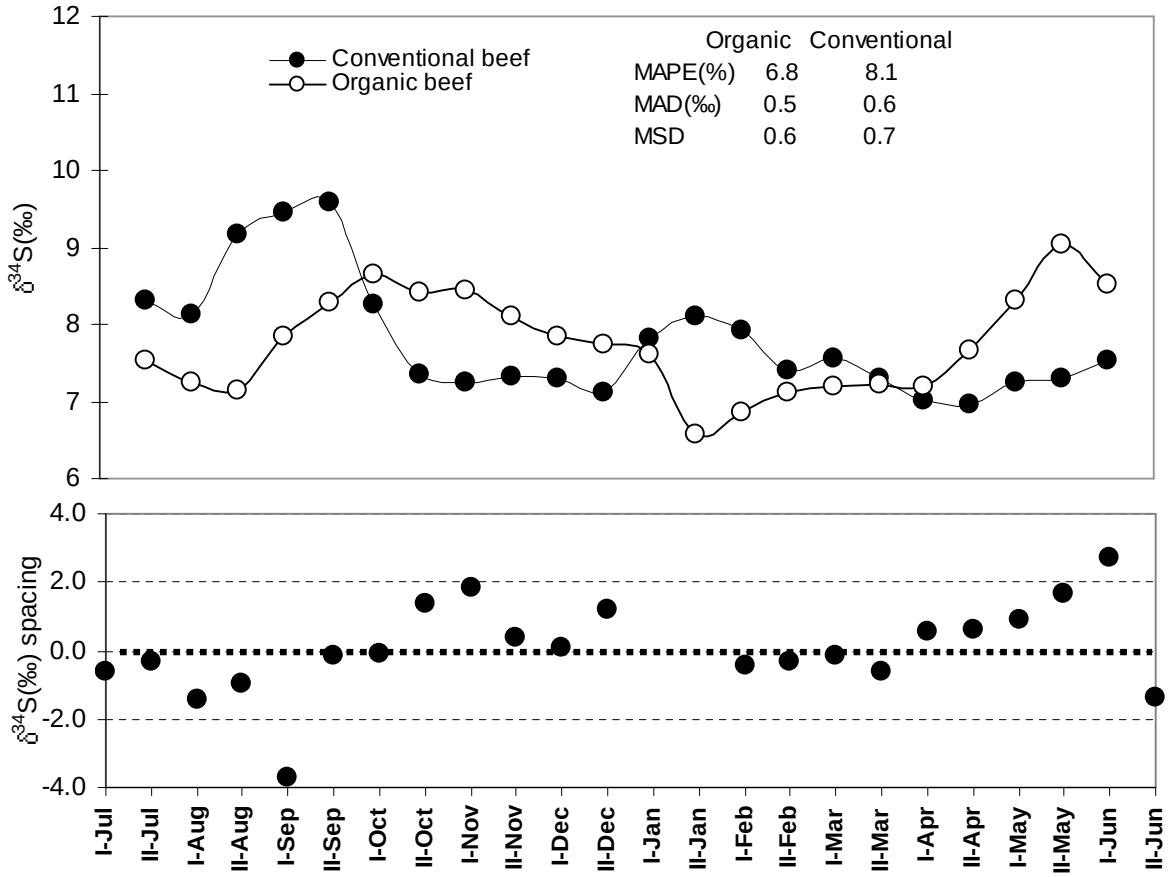


Figure 2 (iii) Time series moving average plots of δ ^{34}S (A), and the δ ^{34}S spacing between fortnightly means of organic and conventional Irish beef (B). Note that only samples from supermarket A were analysed for δ ^{34}S. See Figure 2 (i) for other details.

Table 1. Season-wise mean $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ of retail organic and conventional Irish beef

	$\delta^{13}C$			$\delta^{15}N$			$\delta^{34}S$		
	Organic	Conventional	SED	Organic	Conventional	*SED*	Organic	Conventional	*SED*
Dec–Feb (Winter)	–26.1 *(24)*	–25.4 *(23)*	*0.21***	6.9 *(24)*	7.2 *(23)*	*0.30*	7.4 *(6)*	7.7 *(9)*	*0.62*
Mar–May (Spring)	–25.5 *(31)*	–24.2 *(30)*	*0.32****	6.2 *(31)*	6.9 *(30)*	*0.25***	7.7 *(13)*	7.2 *(18)*	*0.31*
Jun–Aug (Summer)	–26.0 *(33)*	–25.2 *(28)*	*0.37**	6.4 *(33)*	6.9 *(28)*	*0.22**	7.6 *(14)*	8.0 *(14)*	*0.47*
Sep–Nov (Autumn)	–26.2 *(38)*	–26.0 *(34)*	*0.21*	6.2 *(38)*	7.0 *(34)*	*0.22****	8.4 *(13)*	8.3 *(13)*	*0.64*
Overall	–26.0 *(126)*	–25.2 *(115)*	*0.16****	6.4 *(126)*	7.0*(115)*	*0.12****	7.8 *(46)*	7.8 *(54)*	*0.26*

Number of samples (*n*) in parenthesis. Significance of difference: *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Experiment 3: Quantification of maize silage in a beef ration using carbon stable isotope ratio analysis of meat

Introduction

The potential of stable isotope ratio analysis to confirm the production origin of beef, i.e. whether the beef was organically or conventionally produced (was shown in Experiment 1 and Experiment 2). The carbon stable isotope composition (expressed as $\delta^{13}C$) of beef tissue primarily reflects the proportion of C_3 and C_4 photosynthetic plants consumed by cattle. The objective of this experiment was to establish a quantitative relationship between dietary C_4 plant intake and bovine muscle $\delta^{13}C$.

Materials and Methods

Forty-five continental crossbred heifers were fed with either grass silage (GS), an equal mixture (dry matter basis) of grass silage and maize silage (GMS), or maize silage (MS) *ad libitum* in a randomised block design (n=15). All animals also received 3 kg concentrates (composition per kg: 310 g citrus pulp, 460 g barley, 160 g soybean, 50 g molasses and 20 g mineral/vitamin mixture) daily. The *Longissimus thoracis et lumborum* muscles taken at 24 h post-mortem. Lipid free muscle (0.9-1.1 mg) and lipid samples (0.2-0.9 mg C) were analysed by continuous flow isotope ratio mass spectrometry. Data were subjected to analysis of variance (ANOVA) and regression analysis using SAS v.8.2 (SAS Institute).

Results and Discussion

The $\delta^{13}C$ of lipid free muscle from GS fed cattle (–25.1‰) was more negative than that from GMS (–22.1‰) and MS fed cattle (–18.1‰) (*SED*±0.1). This reflected the $\delta^{13}C$ values of dietary grass silage (–29.6±0.3‰), maize silage (–11.8±0.1‰) and the mixture of grass silage and maize silage (calculated $\delta^{13}C$, –21.0‰). A similar trend was evident in the lipid fraction, for which the $\delta^{13}C$ values were –29.2, –25.8 and –21.1‰ (*SED*±0.3) for GS, GMS and MS fed beef, respectively. Compared to lipid free muscle, the $\delta^{13}C$ of the lipid fraction was more negative. This depletion in ^{13}C likely reflected discrimination against ^{13}C during the process of lipid synthesis.

There was a linear relationship (P<0.001) between the proportion of maize carbon in the diets and $\delta^{13}C$ of lipid free muscle (r^2=0.98) and lipid (r^2=0.93) (Figure 3). The regression analysis indicated that, with 95% confidence, each 10% change in the dietary maize carbon corresponds to a 0.9 to 1.0‰ shift of $\delta^{13}C$ in lipid free muscle and a 1.0 to 1.2‰ shift of $\delta^{13}C$ in lipid. The regression lines for lipid free muscle ($\delta^{13}C$=0.0958 maize carbon % – 25.215) and lipid ($\delta^{13}C$=0.1117 maize carbon % – 29.338) had significantly different intercepts (differences 4.123‰, P<0.001) and slopes (differences 0.015, P<0.01), the latter possibly indicating an increasing tendency for maize carbon to be assimilated into beef intramuscular lipid during the fattening phase.

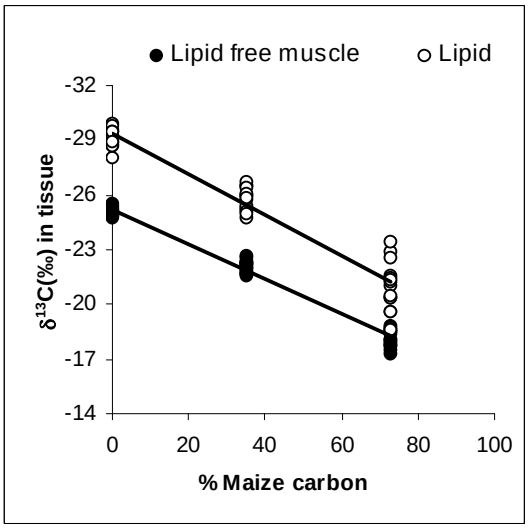


Figure. 3: Relationship between maize-derived carbon in the diet of beef cattle and carbon stable isotope composition ($\delta^{13}C$) of lipid free muscle and lipid.

Conclusions

Maize silage used in the finishing diet of beef cattle could be detected and quantified by stable carbon isotope analysis of lipid free beef muscle or lipid. Thus, $\delta^{13}C$ has potential as a marker for authenticating the origin of beef from production systems using different levels of maize and other C_4 plant products.

Experiment 4: Differentiation of beef according to the pre-slaughter diet of cattle using the stable isotope ratios of carbon and nitrogen

Stable isotope ratio analysis is one potentially useful technique for testing food authenticity. In Experiment 3, use of SIRA was shown to distinguish between beef from cattle fed grass silage or maize silage i.e. the $\delta^{13}C$ in livestock animal tissue primarily depended on the proportion of C_3 and C_4 photosynthetic plants consumed. The objective of this study was to determine the potential of SIRA to distinguish between beef from cattle that consumed, during the finishing phase, a range of feedstuffs commonly available in Ireland.

Materials and Methods

In an indoor study, continental cross-bred beef steers (n=14/feedstuff) were offered grass silage, maize silage (cv. Benecia), fermented whole-crop wheat (cv. Soissons), alkalage whole-crop wheat (cv. Soissons) and ad libitum concentrates (83% rolled barley). The alkalage was ensiled with 45 kg Home 'N' Dry (Volac International Ltd.)/t dry matter. Forages were offered ad libitum through individual Calan gates and supplemented with 3 kg concentrates/head/day. In a grazing study, continental cross-bred steers (n=14/feedstuff) were finished on either a conventionally managed sward i.e. the grass sward received approximately 200 kg N ha^{-1}, or an optimally managed grass-clover sward that received 50 kg N ha^{-1} in early spring. Animals were slaughtered after approximately 5 months of treatment. After cooling the carcasses for 24 h, longissimus muscle was sampled, vacuum packed and stored at -18^{o}C until SIRA of lipid free muscle was performed as previously described. Data were analysed by (Multivariate) Analysis of Variance.

Results and Discussion

The stable isotope composition of the feeds is shown in Table 2. The $\delta^{13}C$ of maize silage was less negative than the other feeds. The $\delta^{15}N$ was highest for grass silage and lowest for the concentrate. Muscle SI composition is summarized in Table 3. Feed and muscle $\delta^{13}C$ were linearly related: Muscle = 0.41 (feed) – 14.02, se = 0.59, $P<0.05$, $R^2 = 0.95$. Beef from maize silage-fed cattle had the least negative $\delta^{13}C$ reflecting the SI composition of the feed. The $\delta^{13}C$ value distinguished ($P<0.05$)

between beef from concentrate/wheat silage, grass silage, grazed grass and grazed grass/clover-fed cattle, but not between beef from alkalage and wheat silage-fed cattle or between beef from alkalage and concentrate-fed cattle. The relationship for $\delta^{15}N$ was: Muscle = 0.29 (feed) + 6.36, se = 0.61, P = 0.05, R^2 = 0.47. Using the $\delta^{15}N$ value resulted in a poorer discrimination of beef samples compared to using the $\delta^{13}C$ value.

A scatter plot of the individual data is shown in Figure 4. Beef from maize silage-fed cattle was clearly distinguished from other samples. The combined isotopic composition of carbon and nitrogen did not improve the discrimination of beef from wheat silage or concentrate-fed cattle beyond that achieved by considering $\delta^{13}C$ lone.

Table 2. Carbon and nitrogen stable isotope composition of feed ingredients

Ration	**Delta ^{13}C**	**sd**	**Delta ^{15}N**	**sd**
Alkalage	-28.20	0.10	4.37	0.73
Concentrate	-27.48	0.30	2.95	0.36
Grass silage	-30.65	0.11	9.31	0.73
Maize silage	-12.74	0.30	6.32	0.26
Wheat silage	-28.02	0.25	3.11	0.36
Grass	-30.37	0.56	5.88	1.14
Grass/clover	-30.24	0.64	4.88	0.97

Table 3: Carbon and nitrogen stable isotope composition of *Longissimus* muscle from cattle fed various diets pre-slaughter[1]

Ration	Delta ^{13}C	Delta ^{15}N
Alkalage	-25.36^{b,c}	6.93^{a}
Concentrate	-25.65^{c}	7.16^{a}
Grass silage	-26.18^{d}	8.82^{d}
Maize silage	-19.42^{a}	7.76^{b}
Wheat silage	-25.28^{b}	7.19^{a}
Grass	-27.51^{e}	9.05^{d}
Grass/clover	-27.19^{f}	8.17^{c}
Sed	0.145	0.167
Significance	P<0.001	P<0.001

[1]Within a column means with different superscripts differ (P<0.05)

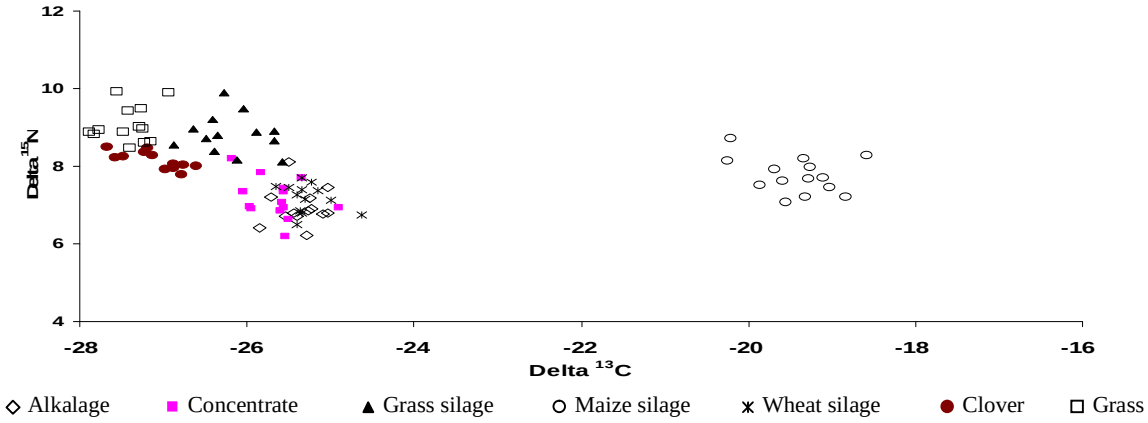


Figure 4. Carbon and nitrogen stable isotope ratios in beef from cattle fed different diets.

Conclusions

The differences in the carbon and nitrogen stable isotope composition of the feeds examined were reflected in the muscle of cattle thereby allowing SIRA to be used as a component, at least, of a scheme to authenticate the dietary history of beef from cattle consuming these feeds.

Experiment 5: Temporal change in the carbon stable isotope ratio of beef following a change in ration composition

Introduction

The potential of SIRA to distinguish between beef from cattle fed a range of feedstuffs was demonstrated in Experiment 3 and Experiment 4. Moreover, the seasonal impact on the stable isotope composition of beef was shown in Experiment 1. For general application, information is needed on the pattern of change in stable isotope composition when cattle experience a change in ration composition. The specific objectives of this experiment to determine the temporal variability in the stable isotope composition of C ($\delta^{13}C$) in feed materials over one growing season, and 2) to determine the speed at which temporal changes in the isotopic composition of beef muscle tissue occur following a change in diet.

Materials and Methods

From a group of 63 heifers, 15 were fed concentrates for 220 days and 36 were grazed for 97 days, then housed, and offered in groups of 12 one of three diets differing in the proportion of concentrates, silage and grass (Figure 5). Heifers were slaughtered at approximately monthly intervals (n=3 per date per diet) and feed samples were collected regularly from June to November. Two control groups (n=6) were slaughtered at day 30 and day 101 (see Figure 5). Samples of the striploin (*M. longissimus dorsi*) were collected, freeze-dried and milled. The $\delta^{13}C$ of bulk muscle and bulk feed materials was determined by continuous flow isotope ratio mass spectrometry.

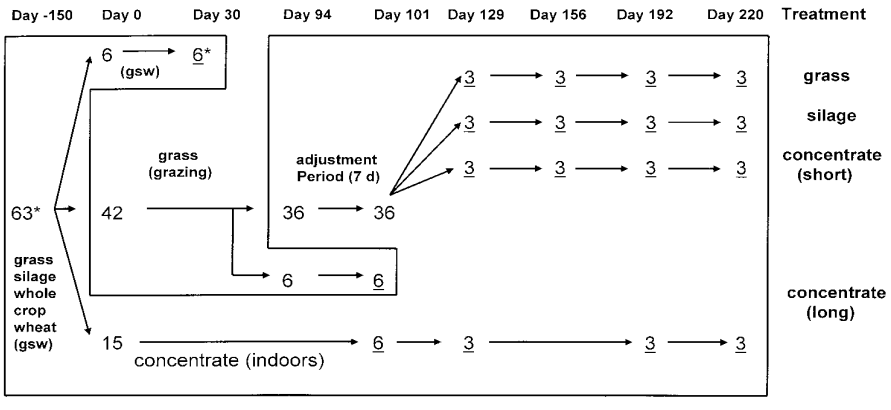


Figure 5. Experimental design.

Results and Discussion

The isotopic composition of the concentrate and grass silage showed little temporal variation (range <0.6‰ $\delta^{13}C$) over a 5-month period. Grass, by contrast, showed marked temporal trends over this period with monthly values ranging from –29.6 to –31.0‰ in $\delta^{13}C$. There was an isotopic difference (about 3‰ $\delta^{13}C$) between concentrate and grass/grass silage feed materials (Figure 6).

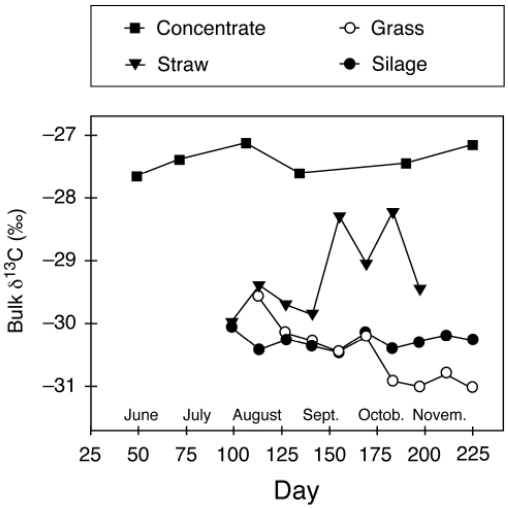


Figure 6. Isotopic composition of feed materials.

The isotopic composition of muscle tissue changed only slowly after diets were switched. However, even small isotopic differences between grass- and concentrate-based diets were consistently reflected in bulk muscle tissue (Figure 7). The final difference in muscle $\delta^{13}C$ between the grass and 'long concentrate' treatments was 1.9‰.

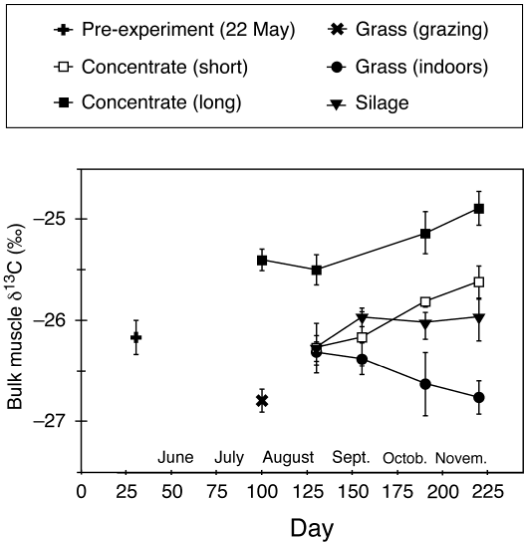


Figure 7. Isotopic composition of muscle tissue.

Conclusions

While $\delta^{13}C$ showed little temporal variation in concentrate and silage feed materials, it was more variable in fresh grass over one growing season. Muscle reflected small isotopic differences between dietary components after about 100 days of consumption.

Experiment 6 (a): How fast are dietary carbon and nitrogen replenished in bovine muscles?

Introduction

The findings of Experiment 1-5 demonstrated the potential of SIRA as a tool to authenticate meat from beef cattle. However, a fundamental knowledge gap that can confound or fail this authentication approach is the rate at which bovine muscles turn over. For example, the time lag between a diet switch and its manifestation in muscle tissue of cattle is unknown and this could potentially be exploited fraudulently to manipulate the short-term pre-slaughter diet of cattle in order to claim life-long premium feeding histories. While Experiment 5 demonstrated that the stable carbon isotope composition changes over time after a change in ration, the absolute change was small reflecting the similarity in the stable carbon isotopic composition of the feeds. The primary objective of this study was to quantify the isotopic turnover of C, N and S in bovine skeletal muscle (*Longissimus dorsi* and *Psoas major*) and to assess the implications of the turnover for meat authentication.

Materials and Methods

Seventy steers were offered a C_3-based pre-experimental diet consisting of grass silage *ad libitum* and 1-kg barley d^{-1} per animal from mid-December to mid-February. This was followed by a gradual adaptation to a barley-based high concentrate diet with *ad libitum* access to wheat straw (C_3) mid-February to mid-March. From mid-March all animals were fed a control diet until the appropriate time for switching onto the experimental diet was reached. The major dietary sources of C, N and S in the experimental ration were maize, urea with elevated near-natural abundance ^{15}N levels, and seaweed.

A group of ten animals slaughtered on March 30 served as a pre-experimental control (**T0**). Ten animals received the control diet for the duration of the whole experiment (168 d) and served as an experimental control (**T6**). The remaining 50 animals were switched from the control diet to an experimental diet in groups of 10 either 168 (**T5**), 112 (**T4**), 56 (**T3**), 28 (**T2**) or 14 (**T1**) d prior to slaughter. The average whole body weights of the animals at the beginning of the experimental period was 493.0 ± 38.0 kg. The experimental and control concentrate rations were formulated to be equivalent in terms of energy and protein. Accordingly, an allowance of 0.07 kg concentrate plus

0.016 kg straw per kg **BW** $^{0.75}$ was fed to achieve a target growth rate of 0.9 kg d^{-1} per animal. Animals were fed individually by allowing them into the feeding pen through individual, electronically controlled gates. Concentrate rations were offered in two, approximately equal meals and the straw was offered in one meal. The allowance for each animal was determined based on the four-weekly body weight gain and adjustments of the allowances were made to achieve a constant growth rate throughout the experiment. Following slaughter, samples of the striploin (*M. longissimus dorsi*) and fillet (*M. psoas major*) were collected, freeze-dried and milled. The stable isotope compsoition of bulk muscle and bulk feed materials was determined by continuous flow isotope ratio mass spectrometry.

Results and Discussion

Turnover of C

For both *L. dorsi* and *P. major* muscles, the $\delta^{13}C$ values of the different treatment groups changed significantly ($P < 0.001$ for both muscles) due to feeding of the experimental diet containing maize for different periods of time. Compared to T6, in T1, T2, T3, T4 and T5, the shifts were 0.9‰, 1.8‰, 2.9‰, 5.0‰ and 7.0‰, respectively, for *L. dorsi* (SED = 0.16‰, $P < 0.001$) (Figure 8A). For *P. major,* the corresponding values were 1.1‰, 2.1‰, 3.3‰, 6.0‰ and 8.0‰, respectively (SED = 0.21‰, $P < 0.001$) (Figure 8B). Feeding of the experimental diet for 168 d was insufficient to attain an isotopic equilibrium of C in both muscles. This was evident, firstly, from the diet-tissue spacing for the control (+ 3.0‰ in *L. dorsi* and + 2.8‰ in *P. major*) and experimental diets (– 4.2‰ for *L. dorsi* and – 3.4‰ for *P. major*). Secondly, the C turnover curve had not reached a steady state plateau even after feeding on the experimental diet for 168 d (T5).

Turnover of N

For both *L. dorsi* and *P. major* muscles, the $\delta^{15}N$ values of different treatment groups changed significantly ($P < 0.001$ for both muscles) due to feeding of the experimental diet. Compared to T6, in T1, T2, T3, T4 and T5, the shifts were 0.6‰, 0.8‰, 1.4‰, 2.6‰ and 3.9‰, respectively, for *L. dorsi* (SED = 0.25‰, $P < 0.001$) (Figure 9A). For *P. major,* the corresponding values were 0.8‰, 1.0‰, 1.4‰, 3.4‰ and 4.6‰, respectively (SED = 0.27‰, $P < 0.001$) (Figure 9B). Feeding of the experimental diet for 168 d was insufficient to attain an isotopic equilibrium of N in both muscles. This was evident, firstly, from the diet-tissue spacing for the control (+ 5.2‰ in *L. dorsi*

and + 4.7‰ in *P. major*) and experimental diets (– 0.6‰ for *L. dorsi* and – 0.5‰ for *P. major*). Secondly, the N turnover curve had not reached a steady state plateau even after feeding on the experimental diet for 168 d (T5).

Turnover of S

In *L. dorsi* muscle, feeding of the experimental diet containing seaweed resulted in a significant difference ($P > 0.001$) in the $\delta^{34}S$ values. Compared to T6, in T1, T2, T3, T4 and T5, the shifts were 1.7‰, 1.9‰, 2.3‰, 3.0‰ and 3.6‰, respectively (SED = 0.25‰, $P < 0.001$ (Figure 10). Isotopic equilibrium of $\delta^{34}S$ was not reached after 168 d (Figure 10). Compared to the $\delta^{34}S$ values (excluding the intake of straw) of the control diet (3.7‰) and of the experimental diet (10.3‰), *L. dorsi* had a $\delta^{34}S$ diet-tissue spacing of + 1.4‰ and – 1.6‰ after 168 d on the control and experimental diets, respectively. Because of the high standard deviation of $\delta^{34}S$ value in T6 and a small increase in S isotope values, the exponential regression model failed to fit the data. However, when T6 was excluded, the T1-T5 groups could be fitted to the exponential regression model ($R^2 = 78$).

Conclusion

The turnover of the light elements (C, N and S) in bovine skeletal muscles is a slow process and therefore, skeletal muscles contain isotopic information on dietary inputs integrated over a long period of time (months to years). Inferring dietary histories of animals fed an isotopically distinct diet for a long period is possible through analysis of bulk skeletal muscle. However, for the detection of short-term dietary shifts, isotopic analysis of bulk skeletal muscle alone is inadequate; it may require the analysis of different biochemical fractions of meat, or of other soft (blood, liver) or incremental (hair, hoof) tissues, if available.

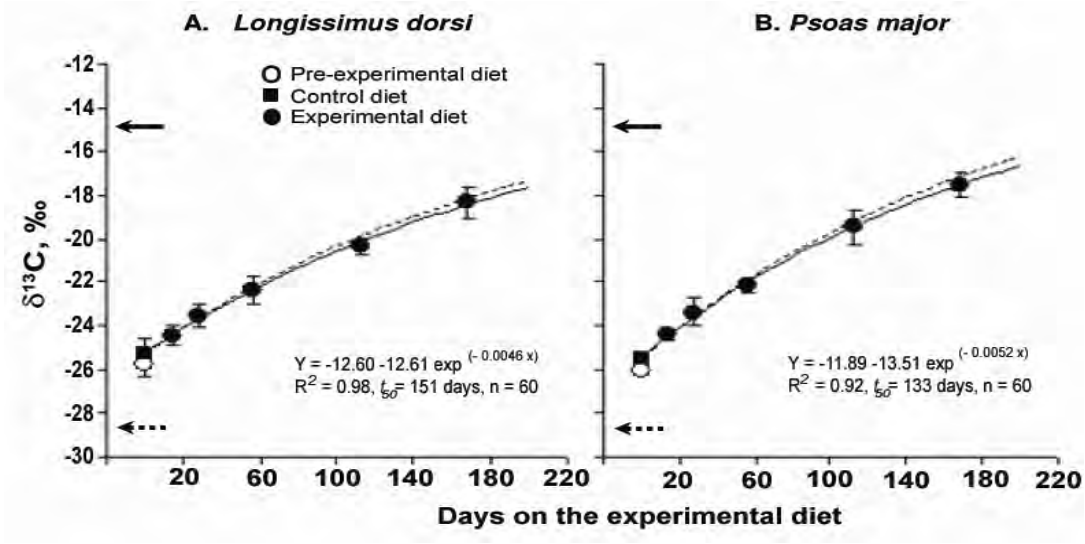


Figure 8. Turnover of ^{13}C in bovine *L. dorsi* (A) and *P. major* (B) muscles in two experimental feeding scenarios; Case 1: 168 d (T5), 112 d (T4), 56 d (T3), 28 d (T2) and 14 d (T1) (solid line) and Case 2: 158 d, 102 d, 56 d, 28 d and 14 d (dotted line). Mean values of muscle from animals on the pre-experimental (T0) (○), control (T6) (■), and experimental (●) diets are shown with error bar (1 SD). The composite feed isotope values for the control (dotted arrow) and experimental diets (solid arrow) are also shown. Stable isotope ratios are expressed in delta notation [δ per mille (‰)]

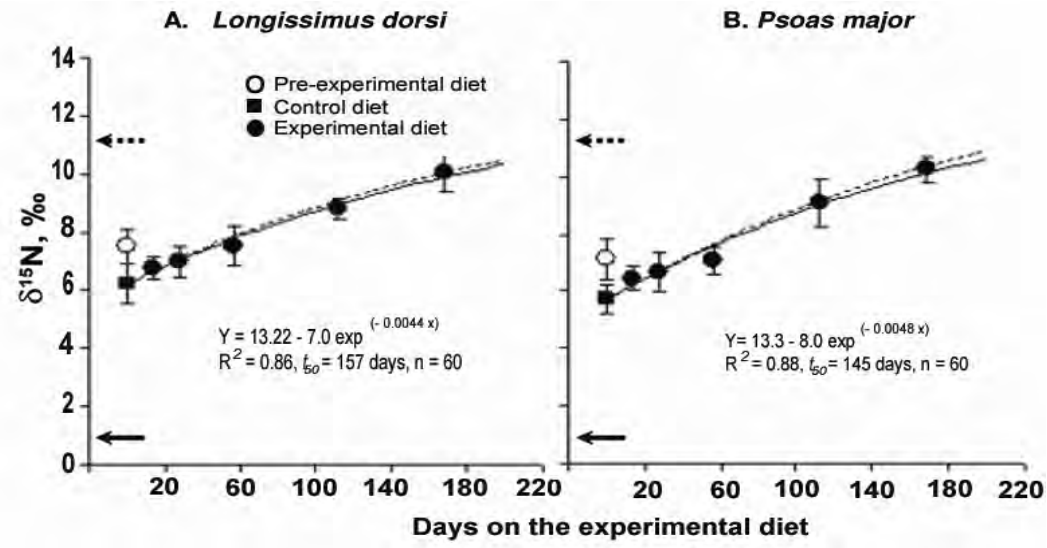


Figure 9. Turnover of ^{15}N in bovine *L. dorsi* (A) and *P. major* (B) muscles in two experimental feeding scenarios Case 1: 168 d (T5), 112 d (T4), 56 d (T3), 28 d (T2) and 14 d (T1) (solid line) and Case 2: 158 d, 102 d, 56 d, 28 d and 14 d (dotted line). Mean values of muscle from animals on the pre-experimental (T0) (○), control (T6) (■), and experimental (●) diets are shown with error bar (1 SD). The composite feed isotope values for the control (dotted arrow) and experimental diets (solid arrow) are also shown. Stable isotope ratios are expressed in delta notation [δ per mille (‰)]

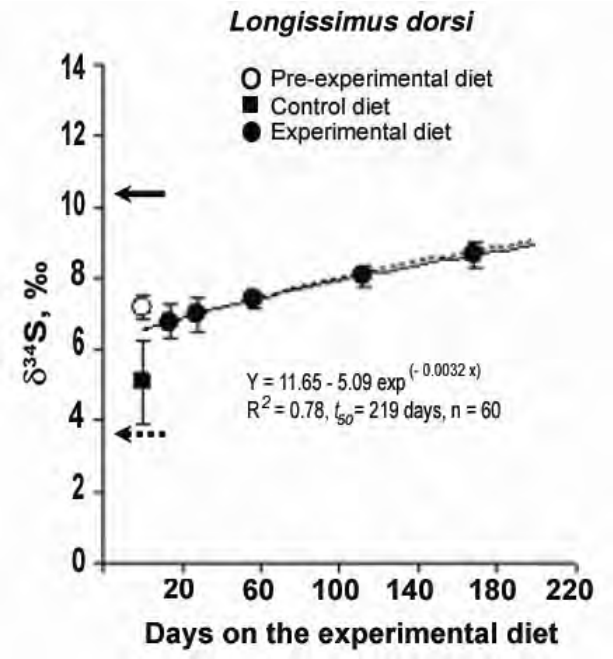


Figure 10. Turnover of ^{34}S in bovine *L. dorsi* muscle in two experimental feeding scenarios Case 1: 168 d (T5), 112 d (T4), 56 d (T3), 28 d (T2) and 14 d (T1) (solid line) and Case 2: 158 d, 102 d, 56 d, 28 d and 14 d (dotted line). Mean values of muscle from animals on the pre-experimental (T0) (○), control (T6) (■), and experimental (●) diets are shown with error bar (1 SD). The composite feed isotope values for the control (dotted arrow) and experimental diets (solid arrow) are also shown. Stable isotope ratios are expressed in delta notation [δ per mille (‰)]

Experiment 6 (b): Experimental determination of dietary carbon turnover in bovine hair and hoof

Introduction

It was shown in Experiment 6a that bulk muscle SIRA, while suitable for inferring dietary history of cattle fed an isotopically distinct diet for a long period was unsuitable for detection of short-term dietary shifts. Tissues such as muscle integrate dietary information over time. In contrast, SIRA of animal keratinized tissues including hair, hoof, claw, feather, nail, and horn, has potential as a high-resolution recorder of an individuals dietary history. The use of keratinized tissues offers several advantages. They are composed of 90% keratin, a protein which contains all the major light elements: H, C, N, O and S. Hair, for example, can be sampled non-invasively and like tooth enamel, keratinized tissues such as hair, nails or hooves are metabolically inert once formed, can grow continuously, and thus are able to record time series up to several years. Finally, a significant fraction of dietary carbon is rapidly incorporated into keratin. Accurate estimation of the turnover rate requires that the studied tissue is initially in isotopic equilibrium with its first diet, and then approaches equilibrium with the second diet at the end of the experiment. The objective of this study was to determine if hair and hooves provide a similar record of the kinetics of carbon turnover following a diet-switch.

Materials and Methods

Following slaughter of the cattle described in Experiment 6(a), one tail hair from seven different individuals was selected for isotope analysis. Sampled animals comprised 1 animal from the experimental control group and 6 animals who received the experimental diet for 168 d, respectively. One heavy (>15 mg) and long (>250 mm) tail hair was chosen per animal, cleaned using soapy water, then defatted following published protocols for other keratin-based tissues. Each hair was sonicated for 30 min, twice, in a solution of methanol and chloroform (2:1 v/v), rinsed with distilled water and oven-dried overnight at 60°C. Individual hairs were serially sectioned into 2-5 mm sub-samples and weighed into ultra-light tin capsules for stable isotope analysis. Up to 50 sub-samples were taken from each hair and over 300 samples were analysed in total.

The hooves from five individuals that received the experimental diet for 168 days and one control animal were selected for stable isotope analysis. Of these, three (animal 2, 6 and one control) correspond to individuals whose hair was also selected for stable isotope analysis. The left front hoof was separated from the rest of the leg of the animal. Then, a 15 mm thick slice of hoof was cut parallel to the growth axis using a band saw and the hoof wall was detached from adhering tissues. The hoof wall was sonicated twice for 30 min in a solution of methanol and chloroform (2:1 v/v), rinsed in distilled water and oven-dried at 60°C overnight. Up to 41 samples per hoof were collected from the surface of the hoof wall using a drill equipped with a diamond bit. Samples were taken from the top to 1 cm from the bottom of the hoof, following the hoof growth axis, and weighed into tin cups. Samples had mean dimensions of 3.0±0.6 mm, 1.2±0.1 mm and 0.8±0.2 mm (length x width x depth), respectively (±SD, n= 188). Average sample mass was 0.84 ±0.06 mg (±SD, n= 188). The distance between two consecutive samples was less than 1 mm. $\delta^{13}C$ of all samples was determined using continuous flow isotope mass spectrometry.

Growth rate calculation

Different hairs may grow at different rates within and between individual steers. To compare the hair chronologies established for different individuals, it was necessary to convert hair length measurements to time. Growth rates (mm . d^{-1}) were calculated for each hair by dividing the distance between the position where the change from control to experimental diet was first recorded in the hair and hair base, by the time elapsed between diet-switch and slaughter. The centre point of the tail hair segment that first showed a significant change in $\delta^{13}C$ from baseline values was assumed to mark the commencement of the C_4 experimental diet. This point was assigned the value of zero days plus a lag factor of 12 h, to account for the time taken for ingested food to be digested and laid down as keratin in the hair follicle. A similar approach was followed for calculating growth rates in hooves. In doing so, growth rate was assumed to be linear during the time of the experiment (168 d).

Results and Discussion

Figure 11 shows the $\delta^{13}C$ profiles for the tail hairs and the hooves; the carbon isotope profile from the control animal is also presented for comparison. The x-axis was transformed from length units to time units using the growth rates calculated as described above, and t=0 is the time of diet-switch from control to experimental diet. The tail hair exhibited a distinctly non-linear response to the diet isotope switch, with a rapid increase in $\delta^{13}C$ values in the first days after the diet-switch, followed by a much slower increase from approximately day 10 to day 72-90 (Figure 11A). It was calculated that 25-32% of the total change in hair $\delta^{13}C$ values occurred within one day. Accordingly, the change in $\delta^{13}C$ values observed was ~63±4% 7 d after the diet-switch, and ~88±3% 70-90 d after the diet-switch. After 70-90 d on the experimental diet, 88±3% of the expected change in hoof $\delta^{13}C$ values had taken place, which is identical to hair (Figure 11B). The rate of initial increase in $\delta^{13}C$ values was much lower in hoof: it took approximately 20 d for the hoof to record 60% of the expected change, which is three times longer that in the hair.

After 70-90 d on the experimental diet, all the animals recorded a sharp decrease in their $\delta^{13}C$ values, ranging from 2.5 to 6.8‰ in hair, and from 1.8 to 2.9‰ in hoof. This episode was followed by an increase of similar magnitude a few weeks later. Dates were calculated for these two unplanned diet-switches assuming a constant growth rate for both hair and hoof between the time of the switch to the experimental diet and slaughter. All animals received this unknown diet for 20 to 40 d, starting late June to early July 2004, except for animal 3 whose diet was switched later in July. Isotopic analysis of seven individual C_4 pellets coming from the batch of food that was offered to the animals during this period showed that this food was incorrectly formulated. However, these two additional diet-switches occurred at a time when the hair and hooves were close to equilibrium with the C_4 diet. Therefore, it did not prevent us from modelling the hair and hoof response to the first diet-switch.

Multi-component model

The modelling approach outlined in Ayliffe et al. (2004) and Cerling et al. (2007) was applied to the hair and hoof datasets (Table 4). Only data points prior to the July diet-switch were included. For hair, the model suggested the existence of three pools with half-lives of 1.7, 7.7 and 69.1 days, respectively. These pools accounted for 53±1%,

20±8% and 28±4% of the total dietary change of 14‰. By contrast, the isotope record in hoof could only be decomposed into 2 pools. The first pool had an intermediate rate constant of 11.7 d and accounted for 52±4% of the total change, whereas the second pool has a longer half-life of 34.0 d and accounted for 45±7% of the total change. Because the shape of the isotope profile in hoof was sigmoid rather than exponential, and because of inter-individual differences in turnover rates, the model fit was not as good as for hair. This is reflected by the pools accounting only for 97% of the total variation in hoof.

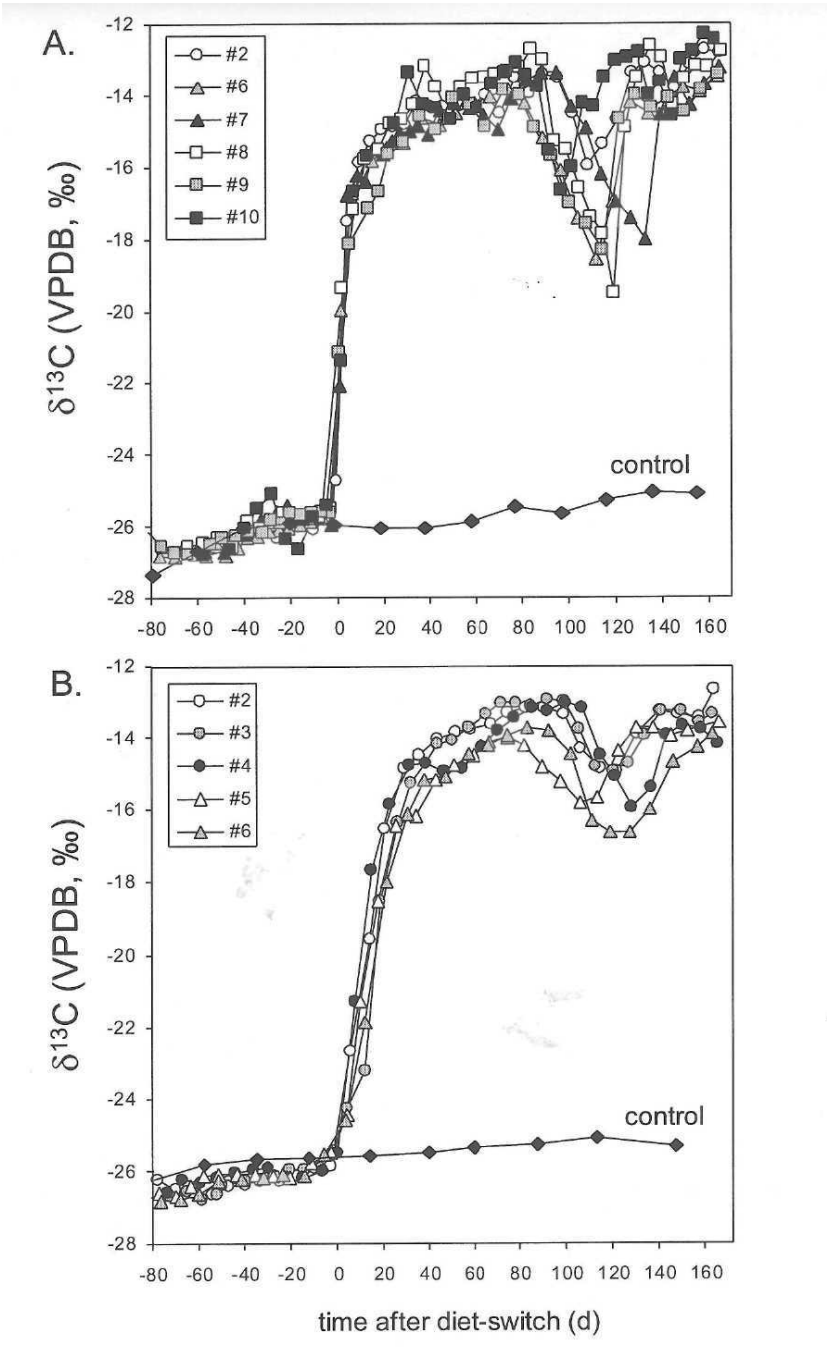
Conclusion

Sequential sampling and stable isotope analysis of bovine tail hair and hoof revealed that the two tissues can provide a detailed and continuous record of animal dietary history. Temporal resolution, which is mainly determined by the tissue growth rate and current analytical limits is 5 d per sample for both tissues. Although our dataset only covers the last 7 months of the animal's life, there is sufficient material in hair and hoof of steers to obtain dietary information for up to 15 months. Because hair can be sampled repeatedly and non-invasively, we anticipate that this approach will prove useful for the investigation of short-term wildlife movements and changes in dietary preferences.

Table 4 – Half-lives and fractions for steer hair and hoof carbon pools

		Pool 1	Pool 2	Pool 3
Hair	**Half life (days)**	1.67±0.03	7.74±0.85	69.1±3.6
	Fraction	0.53±0.01	0.20±0.08	0.28±0.04
Hoof	**Half life (days)**	-	11.74±1.00	34.04±2.5
	Fraction	-	0.52±0.04	0.45±0.07

Figure 11 – Stable carbon isotope profiles in 6 hairs (A) and 5 hooves (B) expressed as time after the switch from control to experimental diet, in days. Result for one control animal is also shown for comparison Zazzo et al.



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