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Stable isotope ratios of blood components and muscle to trace dietary changes in lambs

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Multielemental stable isotope ratio (SIR) analysis was used in lamb plasma, erythrocytes and muscle to detect the switch from a pasture- to a concentrate-based diet, with the aim of verifying the possibility to trace the change of feeding in animal tissues. During 89 days of experimental feeding, lambs were subjected to four dietary treatments: pasture (P), pasture followed by concentrate in the stall for either 14 days (P-S14) or 37 days (P-S37) or concentrate in the stall (S). Pasture and concentrate diets comprised C_3 plants only and had different values of $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{2}H/^{1}H$ and $^{34}S/^{32}S$ ratios. Muscle $^{13}C/^{12}C$ and $^{34}S/^{32}S$ and plasma $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios in P, P-S14 and P-S37 lambs were significantly different. A multivariate analytical approach revealed that $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios in plasma were the most powerful variables for the discrimination among the dietary treatments.

Keywords: IRMS, lamb muscle, lamb plasma, traceability, diet

Implications

Multielemental stable isotope ratio (SIR) analysis was used in lamb plasma, erythrocytes and muscle to detect the switch from a pasture- to a concentrate-based diet. Lambs that were moved from a pasture to a concentrate diet were discriminated from those fed exclusively pasture or concentrate. The carbon and oxygen SIR in plasma were among the most reliable parameters able to discriminate the dietary treatments. This highlights the possibility of using blood as a noninvasive substrate, alternative to muscle, for traceability purposes.

Introduction

In the past few decades, efforts have been made to identify objective tools to trace the dietary background of animals directly from their products (Prache *et al.*, 2005). The great interest in pasture-based feeding systems (as opposite to stall feeding) lies in its implication for organic and sustainable production systems, as well as for the health properties of products. Several potential markers have been evaluated as tracers of pasture feeding in ruminants: carotenoid pigments (Prache and Theriez, 1999), terpens and other volatile compounds (Vasta and Priolo, 2006), fatty acids (Aurousseau *et al.*, 2004), vitamin E isomers (Röhrle *et al.*, 2011) and stable isotopes (Moreno-Rojas *et al.*, 2008). However, one of the main problems in tracing the animal dietary background in milk and meat products is that, in common practice, the animals during their life are exposed to different feeding systems. Typically, for meat production, finishing diets are based on concentrates even for those animals that previously were kept on pasture. The detection of the change from a pasture- to a concentrate-based diet has been investigated in few studies that focused on carotenoid pigments (Prache *et al.*, 2003) and on fat volatiles (Priolo *et al.*, 2004).

The SIR of the bioelements C, N, H, O and S ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) in animal tissues or products (muscle, hooves, hair, blood, milk, etc.) is dependent on the isotopic composition of the plants and water ingested by the animals (Kornexl *et al.*, 1997; Bahar *et al.*, 2005; Perini *et al.*, 2009). Moreover, the isotopic composition of tissues reflects dietary variations and the rate of change depends on the specific turnover rate of each tissue, with muscles having a lower turnover rate compared with blood plasma and liver (Phillips and Eldridge, 2006). Consequently, in the case of animals grown at pasture and finished on concentrates, it is reasonable to expect that SIR may be affected by both the length of the finishing period and the animal tissue.

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Therefore, the aims of this study were to assess the possibility of using C, N, H, O and S SIR analysis of different animal tissues (muscle, plasma and erythrocytes) to detect the change from herbage- to concentrate-based diets and to identify the most suitable animal tissue and isotope ratio.

Material and methods

Animal management

The study was conducted in a farm located in southern Italy (38°38′N, 16°04′E) from December 2008 to March 2009. Thirty-eight single male Italian Merino lambs were fed maternal milk and received a commercial weaning concentrate starting from the 35th day of age. At 60 ± 10 days of age, lambs were weaned and divided into four groups consisting of nine or 10 lambs each. During the 18-day transition period, lambs were gradually adapted to the experimental diets by receiving a decreasing amount of the weaning concentrate, 100 g/day of natural pasture hay and increasing proportions of the experimental diet. Starting from day 1 of the experimental feeding trial, and for the whole 89-day experimental period, animals were fed pasture (group P), pasture followed by an abrupt switch to concentrate in the stall for either 14 days (i.e., pasture for 75 days and stall for 14 days; group P-S14) or 37 days (i.e., pasture for 52 days and stall for 37 days group P-S37) or concentrate in the stall (group S).

Each day, the lambs in the P group and those in the P-S37 and P-S14 groups (during the pasture feeding phase) were allowed to graze from 1000 h to 1600 h on a natural non-fertilized pasture (80% Gramineae and 20% Leguminosae, approximately, with a C_3 metabolism) in accordance with a continuous grazing system. In the remaining hours of the day, the lambs were kept indoors in multiple boxes without receiving any feed supplementation. For the whole experimental period, the lambs in the S group were individually penned and were fed a concentrate (64% barley, 34% chickpea and 2% mineral and vitamin mix; the seeds were roughly ground and mixed) and natural pasture hay in a ratio of 80/20 of the diet on an as-fed basis. After the period spent at pasture, the lambs in the P-S37 and P-S14 groups were individually penned and were fed the same diet as the S group. All the animals were weighed weekly and feed allowances for the S lambs were adjusted to obtain similar growth rates compared with the animals from the P group. The daily ration for animals in the S treatment and for those in the P-S14 and P-S37 groups (during the stall phase) was distributed once a day in the morning. All the animals had an *ad libitum* access to water exclusively in the stall. All the experimental procedures, both *in vivo* and at slaughtering, were approved by the University of Catania.

Feed, blood and muscle sampling

Hay and concentrate were sampled four times during the trial. One pooled sample was used for the analysis because a unique batch of hay and concentrate was used for the whole trial. Seven samples of pasture (≈2 kg each) were collected throughout the experimental period (first and last sampling being at day 10 and 88, respectively). All the feed samples were oven-dried at 60°C and were ground with a mill using a screen of 1 mm. On the last day of the experiment, before feeding time, blood samples were taken individually from the jugular vein, collected in EDTA tubes and centrifuged (3000 × **g** for 15 min at 4°C) in order to separate plasma and erythrocytes. Blood fractions were stored in Eppendorf vials at −20°C until analysis (Norman *et al.*, 2009). The lambs were slaughtered in a public slaughterhouse 15 km away from the farm. The carcasses were stored for 24 h at 4°C and then halved. The *longissimus dorsi* muscle (50 g) was sampled from the left side between the 10th and the 13rd vertebrae, wrapped in aluminium foil, vacuum packed and stored at −20°C until analysis. The *longissimus dorsi* muscle was also taken from the right side for intramuscular total lipid determination (Folch *et al.*, 1957).

Analytical procedures

Erythrocytes, plasma and muscle sample preparation. For each animal, erythrocyte samples and one subsample of plasma destined for the analysis of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios (or $\delta^{13}C$ and $\delta^{15}N$, respectively) were lyophilized for 48 h to remove water and were stored at room temperature in small glass sealed bottles put in a vacuum desiccator, pending analysis. The other subsample of plasma, destined for the analysis of $^{18}O/^{16}O$ isotopic ratio (or $\delta^{18}O$), was stored at −20°C. Muscle samples were lyophilized; the defatted muscle protein fraction (destined for the analysis of the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotopic ratios) and the fat fraction (destined for the analysis of the $^{13}C/^{12}C$ isotopic ratios) were obtained as described in Perini *et al.* (2009). Both muscle protein and fat fractions were stored at room temperature in small glass sealed bottles put in a vacuum desiccator, pending analysis.

Stable isotope analysis. The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios in feeds (≈1.5 mg), defatted meat (≈0.5 mg) and blood components (≈0.5 mg) were measured simultaneously using an Isotope Ratio Mass Spectrometer (DELTA V, Thermo Scientific, Germany) following total combustion in an elemental analyser (EA Flash 1112, Thermo Scientific). The $^{13}C/^{12}C$ ratio of fat was measured with the same instruments, as well as the ratio $^{34}S/^{32}S$ of feeds and defatted meat. For $^{34}S/^{32}S$ ratio determination, around 8 mg of the oxidation agent V_2O_5 was added to the capsule in order to optimize the combustion of the sample. The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios in feed (≈0.8 mg) and defatted meat (≈0.5 mg) were measured using an Isotope Ratio Mass Spectrometer (DELTA XP, Thermo Scientific) coupled with a Pyrolyser (TC/EA, high temperature conversion elemental analyzer, Thermo Scientific). Further details of the analytical procedures adopted are reported in previous papers (Perini *et al.*, 2009). The ratio $^{18}O/^{16}O$ in plasma was determined in 2 ml of defrosted samples after equilibration with CO_2 using an ISOPREP 18 (VG Isotech, Middlewich, UK) online preparation system that allows CO_2/H_2O equilibration, interfaced with an Isotope Ratio Mass Spectrometer (SIRA II, VG Isogas,

Middlewich, UK), according to the water equilibration method described for wine in the OIV method (MA-AS2-12: R2009).

The values were expressed in δ‰ ($=[(R_{sample}-R_{standard})/R_{standard}]\times 1000$, where R is the ratio between the heavier isotope and the lighter one) referenced to international standards: Vienna-Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, Air for $\delta^{15}N$, Vienna-Standard Mean Ocean Water (V-SMOW) for $\delta^{2}H$ and $\delta^{18}O$, Vienna-Canyon Diablo Troilite (V-CDT) for $\delta^{34}S$. For the calculation of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$, we used casein and water working standards, calibrated against international reference materials: L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA) for $^{13}C/^{12}C$; L-glutamic acid USGS 40 for $^{15}N/^{14}N$; V-SMOW (IAEA) and benzoic acid (IAEA-601) for $^{18}O/^{16}O$ in water and in casein, respectively. The $\delta^{34}S$ values were calculated against barium sulphates IAEA-SO-5 and NBS 127 (IAEA) through the creation of a linear equation. The $^{2}H/^{1}H$ values were corrected against the same casein standard with an assigned value of $\delta^{2}H$, according to the 'comparative equilibration technique' (Wassenar and Hobson, 2003, quoted by Camin *et al.*, 2007). The uncertainty of measurements (2σ obtained by analysing ten times the same sample) was <0.3‰ for the $\delta^{13}C$ and $\delta^{15}N$ analysis and for $\delta^{18}O$ of plasma water, <0.8‰ for $\delta^{34}S$, <0.6 ‰ for $\delta^{18}O$ and <3‰ for $\delta^{2}H$.

Statistical analysis

All the variables considered in this study were subjected to a stepwise canonical discriminant analysis (CAN. DISC) in order to select the variables that better discriminate the different groups and to derive canonical functions (CAN) that summarize the variation between groups. On one hand, this procedure was adopted to assess whether the selected variables were able to effectively discriminate between the dietary treatments. The discrimination efficiency was tested by the Mahalanobis distance test of significance and was validated using the 'leave-one-out' cross-validation test. On the other hand, the CAN. DISC. was used as exploratory approach to assess the correlations between the selected variables and the canonical functions in order to attribute a meaning of the variables in the discrimination between the groups. Discriminant analysis was performed using the statistical software SPSS (18.0, 2009).

In addition to the multivariate approach, each variable was subjected to univariate ANOVA. Specifically, ANOVA on blood $\delta^{13}C$ and $\delta^{15}N$ included dietary treatment (P, P-S14, P-S37 and S) and blood component (plasma or erythrocytes) as fixed experimental factors and the dietary treatment × blood component interaction. Data of $\delta^{18}O$ in plasma and of all the isotopic ratios in defatted muscle and fat were analyzed with a monofactorial ANOVA including the dietary treatment as fixed factor. Pairwise comparisons were performed using Tukey's test.

Results and discussion

Feeds

The samples of pasture offered to the lambs presented lower $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{2}H$ and higher $\delta^{34}S$ values compared with concentrate (Table 1). Less negative values for $\delta^{13}C$ in C_3 concentrates compared with C_3 forages have already been observed (De Smet *et al.*, 2004; Schwertl *et al.*, 2005). According to Bahar *et al.* (2005), the different $\delta^{13}C$ values between C_3 concentrate and C_3 forage could be due to the different proportion of photosynthetic and non-photosynthetic tissues, which have different $\delta^{13}C$ values. The results obtained for the nitrogen isotopic ratio agree with those previously reported for forages (Camin *et al.*, 2008) and concentrates (Schwertl *et al.*, 2005). In the present experiment, the concentrate given to lambs was composed by barley and chickpea. Although the latter ingredient represented only 1/3 of the mixture supplied to lambs, it is reasonable to hypothesize its great contribution to the overall $\delta^{15}N$ value of the concentrate because of its much higher protein level than barley. Regarding the hydrogen and oxygen ratios, our results are in line with previous reports (Camin *et al.*, 2008). The $\delta^{18}O$ in the pasture samples increased throughout the whole experimental period ranging from 21.2‰ at starting to 23.8‰ at the end. The passage from winter to spring seasons during the experiment could have increased plant evapotranspiration, a process that produces an ^{18}O-enriched water in the plant tissue (Kornexl *et al.*, 1997). Data on $\delta^{34}S$ in forages have seldom been reported. According to Richards *et al.* (2003), the main factors affecting sulphur isotopic ratio in growing plants are related to local bedrock, microbial processes active in the soil and atmospheric deposition of sulphate on the forages in coastal areas (sea-spray effect). Considering that the farm is located ~3 km from the sea, the high $\delta^{34}S$ values of pasture could have been affected by the sea-spray effect.

Multivariate discrimination among dietary treatments

Multivariate statistical approaches have been shown to represent powerful tools in traceability studies (Camin *et al.*, 2007; Gonzalves *et al.*, 2009). Among the multivariate

Table 1 *Feed isotope ratios for C, N, O, H, S (for pasture mean ± s.d. are shown)*

	Sample no.	$^{13}C/^{12}C$	$^{15}N/^{14}N$	$^{18}O/^{16}O$	$^{2}H/^{1}H$	$^{34}S/^{32}S$
Pasture	7	−30.5 ± 0.4	1.1 ± 0.9	22.5 ± 1.0	−97 ± 6	6.3 ± 0.6
Hay	1	−28.1	1.0	24.8	−95	0.8
Concentrate[a]	1	−25.3	3.0	28.7	−58	−3.7

[a]Composition (as-fed basis): barley 64%; chickpea 34%; mineral and vitamin mix 2%.

Table 2 *Structure matrix and variance explained by each canonical function*

	Structure matrix[a]		
Variables[b]	CAN 1	CAN 2	CAN 3
δ^{13}C muscle	−0.303	−0.295	0.901
δ^{34}S muscle	0.282	0.582	0.328
δ^{2}H muscle	−0.010	0.580	0.350
δ^{18}O plasma	0.442	−0.265	0.138
δ^{13}C plasma	−0.752	0.072	0.378
	Variance explained by each canonical function		
Variance (%)[c]	94.5	5.4	0.1

[a]Correlations between each variable retained after the stepwise selection and each of the three canonical functions. The higher the absolute value of the correlation coefficient, the greater the contribution of the individual variable to the canonical function.
[b]Variables retained after the stepwise selection procedure.
[c]Percentage of the total variance explained by each canonical function.

Table 3 *Classification and distances between groups*[a]

Dietary treatments[b]	P	P-S14	P-S37	S
P	–	**<0.0005**	**<0.0005**	**<0.0005**
P-S14	148.91	–	**<0.0005**	**<0.0005**
P-S37	351.15	54.53	–	**<0.0005**
S	437.07	121.41	25.93	–
	Cross-validated correctness (%) of attribution to the groups[c]			
	100	100	100	100

[a]Mahalanobis distances and significances (in bold) between groups.
[b]P = pasture; P-S14 = pasture feeding followed by concentrates in stall for 14 days; P-S37 = pasture feeding followed by concentrates in stall for 37 days; S = concentrate in stall.
[c]Cross-validation achieved with the 'leave-one-out' procedure, in which each case is classified into a group on the basis of the function derived from the remaining cases.

approaches, the CAN. DISC. allows to simultaneously consider several measurements and to combine them to form canonical functions able to discriminate between animal products coming from different geographic areas (Heaton *et al.*, 2008) or from different feeding systems (Perini *et al.*, 2009). Moreover, stepwise CAN. DISC. enables to assess the discriminating power of each variable in the multivariate structure, allowing to select those with the highest discriminating capacity. This can be useful in identifying variables that are worth being considered for specific research needs.

From the full set of variables considered in the present study, the stepwise procedure retained five variables (δ^{13}C in muscle and plasma, δ^{18}O in plasma, δ^{34}S and δ^{2}H in muscle) with high discrimination capacity. The CAN. DISC. combined the selected variables in three canonical functions (CAN), which, together, explained the total variance of the dataset ($P < 0.001$; Table 2). The Mahalanobis distances between the groups were all highly significant and, after cross-validation, each animal was classified in the original group (100% accuracy of the classification; Table 3). Considering

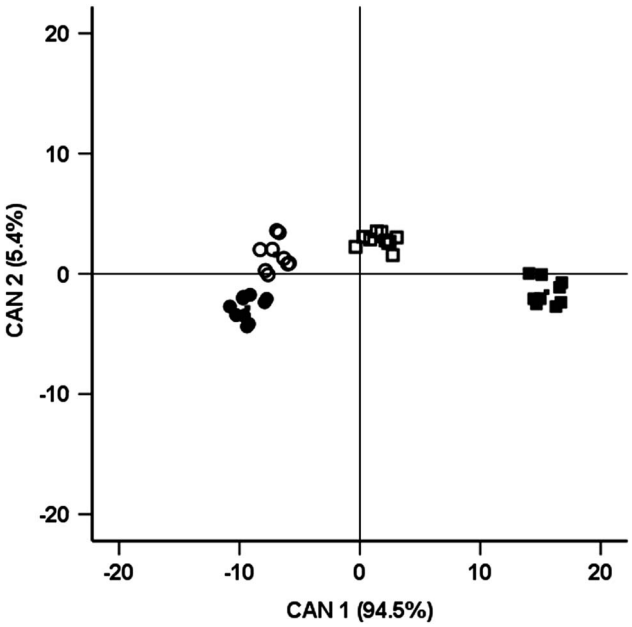

Figure 1 Discrimination of the dietary treatments achieved by plotting the first two canonical functions (cumulative variance explained: 99.9%). The treatments were: Pasture (P, ■), Pasture followed by concentrates in stall for 14 days (P-S14, □), Pasture followed by concentrates in stall for 37 days (P-S37, ○) or concentrate in stall (S, ●).

that the first two CAN together accounted for 99.9% of the total variability, the multivariate structure can be well represented by the first two CAN only. This is evident in the loading plot of the first two canonical functions (Figure 1) in which the four dietary treatments are totally discriminated, with the P and S groups being highly distant and the P-S14 and P-S34 groups placed between them. The absolute values of the bivariate correlation coefficients between each original variable and each canonical function were considered, as an exploratory approach, to assess the meaning of the different variables in the multivariate structure. It is evident, in Figure 1, that CAN 1 alone totally discriminated the P, P-S14 and S groups, whereas some overlapping appeared between P-S37 and S groups. As shown in Table 2, CAN 1 correlated mainly with δ^{13}C and δ^{18}O in plasma; δ^{13}C and δ^{34}S in muscle had a weak correlation coefficients with CAN 1. This result, together with the fact that δ^{13}C in erythrocytes was not retained in the multivariate discriminant structure, suggests that the blood carbon isotopic ratio could be successfully used to trace dietary treatment changes in ruminants, with plasma being a much more reliable substrate compared with erythrocytes. The different turnover rate of erythrocytes and plasma (Phillips and Eldridge, 2006), probably affected by their different origin, could explain these results. Different times of equilibration of carbon SIR in plasma and red blood cells following diet changes were indeed observed in bears (Hilderbrand *et al.*, 1996, quoted by De Smet *et al.*, 2004).

Interestingly, the CAN 2 showed that animals in the extreme dietary treatments (P and S) were not discriminated, but were separated from animals that switched from pasture to concentrates (P-S34 and P-S14 groups). This canonical

Table 4 *Effect of the dietary treatment on the stable isotope ratios in erythrocytes and plasma of lambs fed pasture (P, 9 lambs), pasture followed by concentrates in stall for either 14 days (P-S14, 10 lambs) or 37 days (P-S37, 9 lambs) or concentrates in stall (S, 10 lambs)*

	Erythrocytes				Plasma							
	P	P-S14	P-S37	S	P	P-S14	P-S37	S	s.e.m.	Diet (D)	Substrate (S)	D × S
$\delta^{13}C$	-25.32^{b}	-25.21^{b}	-23.97^{cd}	-23.87^{d}	-25.86^{a}	-24.36^{c}	-23.4^{e}	-23.23^{e}	0.109	*	*	*
$\delta^{15}N$	6.09^{bcde}	6.24^{cde}	5.84^{b}	5.22^{a}	6.46^{e}	6.27^{de}	5.92^{bcd}	5.88^{bc}	0.0501	*	*	*
$\delta^{18}O^{f}$					-0.21^{c}	-2.46^{b}	-3.50^{a}	-3.49^{a}	0.219	*		

[abcde]Values within the same row with different superscripts differ significantly ($P < 0.05$).
[f]$\delta^{18}O$ has been determined on plasma only, monofactorial ANOVA has been applied on analytical data.
*$P < 0.001$.

function showed rather strong correlations with both $\delta^{2}H$ and $\delta^{34}S$ in muscle, while only slight correlation coefficients were found with the other isotopic ratios. Therefore, it seems that the hydrogen and sulphur isotopic ratios in muscle could show the switch in the diet of animals, from herbage-based to concentrate-based diet. Interestingly, whereas $\delta^{34}S$ also showed a slight correlation with CAN 1, $\delta^{2}H$ in muscle was unrelated with CAN 1. This suggests that the change of the diet rather than the composition of the diet somehow affected the $\delta^{2}H$ in the muscle.

Effect of the dietary treatment on the blood components and muscle SIRs.

Blood components. The $\delta^{13}C$ and $\delta^{15}N$ in blood were affected by both the dietary treatment and the blood component and a significant interaction between the factors was also found (Table 4). The $\delta^{13}C$ gradually increased moving from the P group to the groups that received the concentrate either in erythrocytes and plasma. No overlapping was observed between the P and the P-S14 lambs in plasma. The higher values obtained in the S group were expected because C_3 concentrate presented less negative $\delta^{13}C$ values than pasture. In plasma from the P lambs, $\delta^{13}C$ was lower compared with erythrocytes ($P < 0.05$), whereas in the other groups it was always higher in plasma compared with erythrocytes ($P < 0.05$). In our experimental conditions, switching lambs from pasture feeding to a long period of a concentrate-based diet (37 days) produced different isotopic ratios in both blood components. However, the change of diet from pasture to a short period (14 days) on concentrates could be detected by SIR analysis of plasma only. These results are in agreement with those obtained with the multivariate discriminant analysis and further confirm that $\delta^{13}C$ determination in plasma allowed to detect diet changes better than in erythrocytes. Previous reports showed that diet $\delta^{13}C$ differently affected plasma or milk $\delta^{13}C$ depending on the magnitude of the diet carbon SIR variation. Abrupt changes from C_3 to C_4 plants (or vice versa) in the diet resulted in a prompt response, which was observed within three days in sheep plasma (Norman *et al*., 2009) and within four (Boutton *et al*., 1988) or seven (Wilson *et al*., 1988) days in cow's milk. Contrarily, when changes in diet involved plants with the same photosynthetic pattern, no significant differences in plasma were observed, even after 18 days of diet change (Norman *et al*., 2009). However, although in that trial lambs received C_4 forages with a narrow range of $\delta^{13}C$ values ($-14.9‰$ and $-12.6‰$), wider differences in $\delta^{13}C$ values were found in the present experiment between pasture and concentrate.

Nitrogen SIR in erythrocytes from pasture lambs (P group) was not significantly different compared with P-S37 and P-S14 groups (Table 4); this suggests that, in our experimental conditions, erythrocytes maintained the isotopic 'signature' of pasture feeding regardless of the length of the finishing concentrate-based diet. Lower $\delta^{15}N$ values were found in the erythrocytes from lambs in the S group compared with the grazing animals. The P and P-S14 groups showed higher values for plasma $\delta^{15}N$ compared with the S group ($P < 0.05$; Table 4), whereas the P-S37 and S groups were not significantly different. In this study, overlapping data among the three pasture groups makes $\delta^{15}N$ of plasma and erythrocytes an unreliable indicator of diet change from pasture to concentrate; this agrees with the results of the multivariate analysis in which the stepwise procedure did not select blood $\delta^{15}N$ as a discriminating variable.

As previously reported, the CAN. DISC. analysis retained the $\delta^{18}O$ values of plasma among the most discriminating variables. Indeed, ANOVA showed that the $\delta^{18}O$ values in plasma decreased in the order P > P-S14 > P-S37 and S ($P < 0.05$; Table 4), which highlights that the longest finishing period on concentrates (37 days) masked the previous period on pasture. The higher $\delta^{18}O$ values observed in plasma from the P and P-S14 animals compared with the S group could be explained considering: (1) the higher content in ^{18}O in fresh plant tissues compared with the ground water because of the plant evapotranspiration processes (Kornexl *et al*., 1997; Camin *et al*., 2008) and (2) the low drinking water intake in animals fed fresh herbage compared with animals fed hay and concentrates (Boner and Förstel, 2004).

The results observed for $\delta^{13}C$ and $\delta^{18}O$ suggest the high reliability of plasma as a noninvasive substrate for detecting pre-slaughter feeding system.

With regard to the comparison between animals fed exclusively pasture (P) or concentrate (S), all the studied isotopic ratios, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$, were significantly different regardless of the blood fraction considered. This is of interest because, in livestock species, only blood $\delta^{13}C$ was focused on (De Smet *et al*., 2004; Norman *et al*., 2009; Martins *et al*., 2012).

Table 5 *Effect of the dietary treatment on the stable isotope ratios in defatted muscle and fat of lambs fed pasture (P, nine lambs), pasture followed by concentrates in stall for either 14 days (P-S14, 10 lambs) or 37 days (P-S37, nine lambs), or concentrates in stall (S, 10 lambs)*

	P	P-S14	P-S37	S	s.e.m.	P
Defatted muscle						
$\delta^{13}C$	-24.5^{a}	-24.0^{b}	-23.2^{c}	-22.9^{d}	0.106	*
$\delta^{15}N$	6.3^{bc}	6.4^{c}	6.0^{b}	5.7^{a}	0.061	*
$\delta^{18}O$	15.5^{b}	15.4^{b}	14.0^{a}	13.5^{a}	0.18	*
$\delta^{2}H$	-89^{a}	-84^{b}	-84^{b}	-90^{a}	0.554	*
$\delta^{34}S$	5.4^{d}	4.7^{c}	3.4^{b}	1.8^{a}	0.235	*
Fat						
$\delta^{13}C$	-29.8^{a}	-28.2^{b}	-27.3^{c}	-27.3^{c}	0.202	*

[abcd]Values within the same row with different superscripts differ significantly ($P < 0.05$).
*$P < 0.001$.

Defatted muscle and fat. As shown in Table 5, ANOVA analysis revealed an effect of the dietary treatment on the muscle $\delta^{13}C$ values, with differences between the groups being all significant. In line with the carbon SIR values of pasture and concentrates, the P treatment resulted in the lowest values and the S treatment in the highest. To our knowledge, the effect of switching from a C_3 pasture to a C_3 concentrate diet on muscle $\delta^{13}C$ has never been investigated, whereas the discrimination between muscle from animals raised exclusively on C_3-pasture or on C_3-concentrates has been previously achieved (Moreno-Rojas *et al.*, 2008; Osorio *et al.*, 2011). Muscle $\delta^{13}C$ showed a positive Pearson correlation with $\delta^{13}C$ in both plasma ($r = 0.93$; $P < 0.001$) and erythrocytes ($r = 0.85$; $P < 0.001$).

The $\delta^{13}C$ in muscle lipid fraction was also significantly affected by diet (Table 5), with values for the P group being lower compared with the other experimental groups. The P-S37 and S groups showed similar $\delta^{13}C$ values in the intramuscular fat ($P > 0.05$). This means that, in our experimental conditions, 37 days of concentrate feeding were sufficient to mask the previous period of pasture feeding by the analysis of $\delta^{13}C$ in the intramuscular fat. The $\delta^{13}C$ value in muscle was higher compared with fat fraction ($-23.6‰$ *v.* $-28.0‰$, respectively, $P < 0.001$), in agreement with Moreno-Rojas *et al.* (2008) and Perini *et al.* (2009). The phenomenon was explained by the discrimination against ^{13}C, the heavier isotope, during lipid biosynthesis (DeNiro and Epstein, 1977, quoted by Perini *et al.*, 2009). The carbon isotopic ratio in fat extracted from muscle was not affected by muscle intramuscular lipid content ($r = 0.246$; $P > 0.05$; data not shown).

The $\delta^{15}N$ values of defatted muscle from animals in the S group were lower ($P < 0.05$) than those found in muscle from lambs in the P, P-S37 and P-S14 groups. This means that a pasture-based feeding system produces different muscle $\delta^{15}N$ values compared with a concentrate-based one, as previously observed by Moreno-Rojas *et al.* (2008) in lambs and Osorio *et al.* (2011) in beef. However, no differences were observed in the $\delta^{15}N$ values in the P group compared with the P-S37 and P-S14 groups. These results agree with the multivariate analysis in which muscle $\delta^{15}N$ was not retained among the most discriminating variables.

Regarding the $\delta^{18}O$ in defatted muscle, the P and P-S14 treatments resulted in higher $\delta^{18}O$ values compared with the P-S37 and S animals (Table 5). ANOVA analysis agrees with the multivariate analysis, which did not retain muscle oxygen SIR as a variable that allowed discrimination among the four groups. Nevertheless, it is of interest that the pasture-feeding system (P group) produced different muscle $\delta^{18}O$ values compared with the stall-feeding system (S group). The ingestion of vegetal water enriched in ^{18}O and the low amount of drinking water consumed by pasture-fed animals can justify the higher values in the P group compared with the stall-feeding system, as previously discussed for plasma. Furthermore, Harrison *et al.* (2011) found that drinking water represents the main source of oxygen in muscle from concentrate-fed lambs. To our knowledge, this is the first study comparing the effect of pasture- and concentrate-feeding systems on defatted muscle $\delta^{18}O$ in experimentally controlled feeding conditions. Similar results were previously observed by Perini *et al.* (2009) in an overview on lambs from different Italian production systems. An increase in the $\delta^{18}O$ value due to a diet based on fresh forages compared with preserved forages has also been observed in cow milk water fraction (Kornexl *et al.*, 1997; Renou *et al.*, 2004a; Engel *et al.*, 2007) and in beef meat water fraction (Boner and Förstel, 2004; Renou *et al.*, 2004b).

Hydrogen isotopic ratio seems to be unreliable in terms of dietary treatment traceability. Indeed, $\delta^{2}H$ values in muscle from lambs in the P and S groups did not differ and were lower ($P < 0.05$) compared with those measured in muscle from the animals switched from the pasture-based to the concentrate-based diet (P-S37 and P-S14 groups; Table 5). The univariate analysis confirmed the results of the multivariate discriminant analysis. Further research must be undertaken to better understand these findings, considering that around 70% of hydrogen in body proteins derives from feed (Hobson *et al.*, 1999; Ehleringer *et al.*, 2008).

Diet affected muscle $\delta^{34}S$ ($P < 0.001$). The four groups presented significantly different values, with the highest in muscle from lambs in the P group and the lowest in muscle from lambs in the S group (Table 5). Overlapping data were observed exclusively between P and P-S14 groups. These results suggest that S SIR of muscle could be used to trace the change of diet, confirming what was observed in the multivariate discriminant analysis, as well as the feeding system (pasture *v.* stall), in agreement with the results provided by Osorio *et al.* (2011) who obtained a good discrimination between concentrate and pasture fed beef.

In synthesis, both carbon and sulphur SIRs in defatted meat seem to be useful tools to trace the switch from pasture to stall-feeding system. These results disagree with Bahar *et al.* (2009) who consider muscle to be an unsuitable tissue for detecting short-term pre-slaughter feeding changes in beef because of the long time required to reach isotopic equilibrium with a new diet. However, Martins *et al.* (2012)

found that even if the isotopic equilibrium in plasma was not reached after 104 days on the experimental diets, different isotopic ratios were observed and allowed to distinguish the diets offered to the animals. Similarly, the results of the present experiment suggest that the renewal of chemical elements (and their isotopes) was sufficient to 'trace' the feeding change from pasture to concentrate even if the isotopic equilibrium in the different tissues was probably not reached due to the short duration of the experiment.

An intriguing challenge is the possibility to trace a pre-slaughter finishing period in grazing animals when the diet is unknown. The current literature allows to give some indications. A finishing diet based on corn should be easy to detect in animals kept on a C_3 pasture, thanks to the huge difference in carbon SIR ranges between C_3 and C_4 feedstuffs. Plasma represents a reliable tissue, owing to its prompt response to the dietary variations. If the finishing diet consists in C_3 concentrates, our data and results by Osorio *et al.* (2011) suggest that its detection should be feasible. However, some issues have to be considered: the magnitude of the difference between forages and concentrate SIRs, the different tissue's turnover rate, the effect of nutritive allowance on the elements turnover rate, as observed in muscle (Harrison *et al.*, 2011), the length of the finishing period, the source of water. We think that the implementation of a data bank showing the range of each isotopic ratio for each tissue, coupled with the respective diet isotopic composition, and the use of the right statistical methods, could probably allow a correct assignment to an unknown diet. The sequential SIR determination in incremental tissues such as hair, woof and wool, which represent an archive of temporal changes in the isotopic composition of the diet (Monahan *et al.*, 2012), could be considered as supplementary tool: when SIR determination in plasma or defatted muscle gives uncertain results, it could assess the existence of diet changes.

In conclusion, although the results of the present experiment are closely connected to our specific experimental conditions, it is possible to extrapolate that: (i) SIR analysis is able to detect short-time dietary changes; (ii) plasma seems to be a reliable substrate for detecting these changes and might be proposed as a substrate alternative to muscle.

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