Short communication

Preservation in 70% ethanol solution does not affect $\delta^{13}C$ and $\delta^{15}N$ values of reindeer blood samples – relevance for stable isotope studies of diet

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Abstract: We compared duplicate samples of whole blood samples from 18 reindeer that were preserved either by immediate freezing or by immersion in 70% ethanol. All samples were dried at 60 °C, powdered, treated with 1:1 chloroform:methanol, and dried again before isotope analysis. There were no differences in the values of $\delta^{13}C$ and $\delta^{15}N$ between the methods of preservation. Isotopic differences were absolutely small ($\delta^{13}C = 0.1 \pm 0.1^{0}/_{00}$; $\delta^{15}N = 0.2 \pm 0.2^{0}/_{00}$), random in direction, and within the limits of analytical precision for the mass spectrometer. Preservation in ethanol thus appears to be an effective and efficient method for preserving blood samples for stable isotope analysis under field conditions.

Key words: caribou, ethanol, methodology, *Rangifer tarandus*, reindeer, sample preservation, stable isotope.

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Introduction

Stable isotope analysis of carbon and nitrogen from animal tissues has proven an accurate, reliable, and cost-effective method for investigating the assimilated diet of animal species from a wide range of taxonomic groups, including ungulates, at varying timescales (DeNiro & Epstein, 1978, 1981; Ben-David *et al.*, 2001; Halley & Minagawa, 2005; Halley *et al.*, 2006). The technique has obvious application to the analysis of reindeer diet, which is divided into three main classes of forage (C3 plants, lichens, and mushrooms), each of which have distinct isotopic signatures (Körner *et al.*, 1991; Kohzu *et al.*, 1999; Hobbie *et al.*, 1999; Maguas *et al.*, 1995; unpubl. data).

A practical difficulty with the technique is that reindeer typically live in remote locations, where immediate freezing of tissue, and maintaining samples in a frozen state on the journey to the laboratory, may be difficult. Here we report the results of an experiment comparing duplicate samples of reindeer blood immediately frozen, and preserved in 70% ethanol, which indicate that ethanol preservation does not affect the $\delta^{13}C$ and $\delta^{15}N$ values of reindeer tissue samples. Preservation in 70% ethanol is

thus a practical and robust method of preserving reindeer blood samples for stable isotope studies of reindeer diet.

Material and methods

We collected 18 duplicate samples of whole blood (1 ml) from domestic reindeer. Ten samples (5 adult males, 5 adult females) were collected on 15th and 16th September 2006, at the summer grazing grounds of Reindeer herding district 37 Skárfvággi, West-Finnmark reindeer herding area, at Kåfjord in northern Troms, Norway (hereafter D-37). A further eight samples (all adult males) were collected at FGFRI, Reindeer Research Station, Finland (hereafter FGFRI) on 11th April 2007.

One sample from each animal was immediately frozen to c. -5 °C, while the other was preserved in 1-2 ml of 70% ethanol. Samples were kept for at least 2 months, frozen samples in a freezer and ethanol-preserved samples at room temperature. Samples were then dried in an oven at 60 °C for 24 hours to evaporate all ethanol and water from the samples. Completely removing the ethanol in this way is required as otherwise the carbon and nitrogen in the ethanol would affect the values for the sample. Dried samples were ground to powder, and treated with 10 ml of 1:1 chloroform: methanol for 12 hours to remove any lipids (which may reflect stored and recently metabolised food rather than the recent diet). The mixture was centrifuged at 2000 rpm for 10 minutes, and the supernatant discarded. The residue was flushed with a further 10 ml 1:1 chloroform, centrifuged and supernatant discarded as before, dried in air in a draft chamber overnight, and then in an oven at 60 °C for at least 12 hours.

0.5-1.0 mg of the resulting powder were loaded into tin cups and analysed for variations in the proportion of the ^{12}C and ^{13}C, and ^{14}N and ^{15}N, isotopes. Analysis was carried out using a Elemental Analyzer N1500 fitted by a ConflowII interface to a Finnegan MAT 252 mass spectrometer housed at the Graduate School of Environmental Earth Sciences at the University of Hokkaido, Japan. Results are expressed in standard delta (δ) notation as deviations in parts per thousand ($^{0}/_{00}$) from the isotopic ratios of the relevant standards, PeeDee Belemnite for carbon and atmospheric nitrogen for nitrogen (Brand, 1996); the apparatus is normally accurate to within $0.2^{0}/_{00}$ for both carbon and nitrogen.

Results

Males and females from the D-37 sample varied slightly in absolute $\delta^{13}C$ and $\delta^{15}N$ values. This was probably due to slight differences in diet between sexes (Halley *et al.*, *in prep.*). The D-37 and FGFRI sample groups also showed differing absolute $\delta^{13}C$ and, especially, $\delta^{15}N$ values. This was due to the differing diets of the two groups, the Skárfvággi animals consuming a natural diet while the FGFRI animals had been fed a uniform artificial diet of commercial livestock feed pellets for the previous 92 days.

Neither males and females from D-37, nor samples from D-37 and FGFRI, varied significantly in either the size, or direction, of differences in δ13C and δ15N values between frozen and ethanol-preserved sample pairs (M vs. F, δ13C: Mann-Whitney, U=10.0, n=10, P=0.68 n.s.; δ15N: Mann-Whitney, U=10.0, n=10, P=0.68 n.s.; D-37 vs FGFRI, δ13C: Mann-Whitney, U=26.0, n=18, P=0.24 n.s.; δ15N: Mann-Whitney, U=37.5, n=18, P=0.83 n.s.). The sample sets were therefore combined for further analysis.

The δ13C and δ15N values for each frozen and ethanol-preserved sample pair are presented in Table 1.

Mean differences in isotope values between sample pairs were small for both elements: mean difference $\delta^{13}C = 0.1 \pm 0.1^{0}/_{00}$; $\delta^{15}N = 0.2 \pm 0.2\ ^{0}/_{00}$. Moreover, there was no

Table 1. Comparison of $\delta^{13}C$ and $\delta^{13}C$ values of reindeer whole blood samples preserved by freezing and in 70% ethanol. Values are expressed in ‰, relative to the appropriate standard (see methods).

Sex	Collection date	Place	$\delta^{13}C$ - Frozen	$\delta^{13}C$ - Ethanol	$\delta^{15}N$ - Frozen	$\delta^{15}N$ - Ethanol	Difference, $\delta^{13}C$	Difference, $\delta^{15}N$
M	15-16/9/2006	D-37	-25.2	-25.1	0.8	0.8	-0.1	0.0
M	15-16/9/2006	D-37	-25.4	-25.4	1.1	1.0	0.0	0.1
M	15-16/9/2006	D-37	-25.6	-25.5	0.5	0.5	-0.1	0.0
M	15-16/9/2006	D-37	-25.4	-25.4	0.7	0.8	0.0	-0.1
M	15-16/9/2006	D-37	-25.6	-25.6	0.9	0.9	0.0	0.0
F	15-16/9/2006	D-37	-25.5	-25.7	1.7	1.7	0.1	0.1
F	15-16/9/2006	D-37	-25.2	-25.3	0.2	0.4	0.0	-0.2
F	15-16/9/2006	D-37	-25.2	-25.4	0.5	0.4	0.3	0.1
F	15-16/9/2006	D-37	-25.4	-25.3	0.5	0.5	0.0	0.0
F	15-16/9/2006	D-37	-25.3	-25.5	0.4	0.2	0.2	0.1
M	11/04/2007	FGFRI	-24.6	-24.7	6.4	6.3	0.2	0.1
M	11/04/2007	FGFRI	-24.7	-24.8	6.6	6.7	0.1	-0.1
M	11/04/2007	FGFRI	-25.1	-24.9	6.2	5.9	-0.1	0.2
M	11/04/2007	FGFRI	-25.0	-25.0	6.0	6.0	-0.1	0.0
M	11/04/2007	FGFRI	-25.2	-25.0	5.8	6.4	-0.2	-0.6
M	11/04/2007	FGFRI	-24.7	-24.9	5.7	6.1	0.1	-0.5
M	11/04/2007	FGFRI	-24.9	-24.8	6.3	6.2	-0.1	0.1
M	11/04/2007	FGFRI	-25.0	-24.9	6.2	6.2	-0.1	0.0

tendency for one preservation method to be consistently higher or lower in value than the other, for either carbon or nitrogen. $\delta^{13}C_{frozen}$ was greater than $\delta^{13}C_{ethanol}$ in 6 cases; smaller in 7 cases, and in 5 cases they were equal. $\delta^{15}N_{frozen}$ was greater than $\delta^{15}N_{ethanol}$ in 7 cases; smaller in 5, and in 6 cases values were identical. Paired samples tests for both $\delta^{13}C$ and $\delta^{15}N$ demonstrate that values for frozen and ethanol preserved pairs were highly significantly correlated (Pearson correlation coefficient $\delta^{13}C$: 0.899, n=18, P<0.001; $\delta^{15}N$ 0.998, n=18, P<0.001); and that sample pairs did not vary significantly in values (paired sample t-test: δ13C t=0.392, 17d.f., P=0.70; δ15N t=0.744, 17d.f., P=0.47).

Discussion

The results show clearly that differences in $\delta^{13}C$ and $\delta^{15}N$ values between paired samples where one sample was frozen and the other preserved in ethanol were both very small in magnitude, and not biased in direction. The deviations from identical values appear generally to be within the range of experimental error; the mass spectrometry equipment used is by itself normally considered to be accurate to within ca. $0.2\,^{0}/_{00}$. Moreover, the scatter in values, positive or negative, appears random when a systematic effect should produce a bias in one direction.

Several studies have investigated the effect

of ethanol preservation in various animals, including tissues of invertebrates, fish, birds, and sheep (Tillberg *et al.*, 2006; Hobson *et al.*, 1997; Gloutney & Hobson, 1998; Sarakinos *et al.*, 2002 for review). In general, vertebrates show no significant change in values, while some invertebrates can be slightly but consistently enriched in $\delta^{13}C$ values. Our data is consistent with this pattern, and adds to the overall body of data indicating that ethanol preservation is suitable for use as a preservative in stable isotope studies of vertebrate tissues.

In summary, the evidence indicates that preserving reindeer blood in ethanol does not affect the final values of $\delta^{13}C$ and of $\delta^{15}N$, relative to freezing samples immediately. The method therefore offers a convenient method for field preservation of reindeer blood intended for stable isotope analyses of diet.

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Konservering av blodprøver fra rein i 70% etanolløsning påvirker ikke verdiene av δ13C and δ15N–verdiene og er en fullgod metode for analyse av stabile isotoper

Abstract in Norwegian / Sammendrag: Vi sammenlignet to og to prøver av blodprøver fra 18 reinsdyr. Prøvene var enten konservert ved umiddelbar frysing eller ved bruk av 70% etanol. Alle prøver ble tørket ved 60 °C, pulverisert og behandlet med kloroform:metanol i forholdet 1:1. Til slutt ble de tørket på nytt før gjennomføring av isotopanalysen. Vi fant ingen forskjell i verdiene av δ13C and δ15N mellom de to konserveringsmetodene. I absolutte verdier var isotopforskjellene små (δ13C = 0.1±0.1 0/00; δ15N=0.2±0.2 0/00). Forskjellene var tilfeldige og innenfor grensene for massespektrometerets presisjon. Bruk av etanol framstår som en effektiv og fullgod metode til konservering av blodprøver for analyse av stabile isotoper under feltforhold.