Effect of temperature on alkane extraction from faeces and herbage

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SUMMARY

The solvent extraction of alkanes from faeces and herbage samples at two different temperatures (cold: 15–25 °C and hot: 65 °C) was studied in four samples of different matrix types (cattle faeces, sheep faeces, hill grass and heather), in two experiments performed at Villaviciosa, Asturias, Spain in 1994. Two internal standards (IS) of different chain length (C_{22} and C_{34}) were used to estimate alkane concentrations. Significant differences were detected in alkane extraction derived from temperature of extraction, IS and sample matrix. At the cold temperature, long-chain alkane extraction was not complete, resulting in errors in the estimation of alkane concentration when a long-chain alkane (C_{34}) was used as the only internal standard. However, under hot extraction, long-chain alkanes were completely extracted by the heptane, although estimates made with C_{22} or C_{34} as IS were not identical. These results suggest that it would be appropriate to use two internal standards with short and long carbon chain, such as C_{22} and C_{34} , in routine analyses to establish the completeness of alkane extraction, even under hot conditions, by calculating the relative ratio of both IS in extracts compared to the original $C_{22}:C_{34}$ ratio added to the samples. Any increase or decrease in expected peak areas could be adjusted for all the alkanes in the extracts, and the accuracy of alkane concentration measurements (and therefore the reliability of estimates of intake and especially of diet selection) would be improved.

INTRODUCTION

Mayes & Lamb (1984) and Gosden & Moseley (1984) proposed the use of n-alkanes, present in plant cuticular waxes, as markers for estimating herbage digestibility. Mayes *et al.* (1986) showed that herbage intake could be estimated by the use of natural odd-chain alkanes and dosed synthetic even-chain alkanes as internal and external markers, respectively. Accurate estimates were obtained using C_{32} and C_{33} alkanes, which have similar recovery values in faeces (Mayes *et al.* 1986). In addition, alkanes can be used as markers to estimate the diet composition of grazing animals. Since different plants tend to have different concentrations of each alkane, diet composition can be estimated from the alkane pattern (from C_{21} to C_{38}) found in the faeces of animals. This method has been used to estimate diet composition in free-ranging ruminants consuming diets with 2–10 botanical components (Dove & Mayes 1991; Mayes *et al.* 1994; Salt *et al.* 1994; Osoro *et al.* 1999). Dove (1992), Dove

& Moore (1995) and Hoebee *et al.* (1998) used this technique for the accurate estimation of the species composition of mixtures of plant species.

Both intake and diet composition calculations depend upon the accurate determination of alkane concentrations in faeces and herbage samples. The routine analysis developed by Mayes *et al.* (1986) was relatively slow, involving Soxhlet extraction of samples, saponification and alkane extraction with n-heptane. At present, the analysis is performed by the direct treatment of freeze-dried, ground samples with 1 M ethanolic KOH at 90 °C, over 3–16 h according to different authors (Laredo *et al.* 1991; Vulich *et al.* 1991; Dove 1992). Samples are then allowed to cool and subjected to liquid–liquid extraction with n-heptane (or n-hexane) and water. Extracts are passed through a silica-gel column, the eluates are concentrated and injected into a gas chromatograph. Alkane concentrations in the extracts are calculated by reference to a single internal standard (C_{34}) added to the sample at the beginning of the procedure.

Temperature could be an important factor affecting the extraction procedures. However, the effect of temperature on alkane extraction has not been

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determined. The objective of the present work was to examine the effect of temperature on the extraction of alkanes from samples in order to optimize the procedure.

MATERIALS AND METHODS

Samples

Four samples of different matrix type were analysed: cattle faeces, sheep faeces, hill grass (*Agrostis-Festuca*) and heather (*Calluna vulgaris*). These samples were obtained at random from a grazing experiment on hill communities of *Agrostis-Festuca-Nardus-Calluna*. Animals were dosed daily with C_{24} , C_{32} and C_{36} (each alkane: 1000 mg per cow, 50 mg per sheep).

Analysis

Routine alkane analysis was performed, with the only difference being that two internal standards (C_{22} and C_{34}) were used.

Freeze-dried and ground samples were weighed accurately (0.5 g of faeces and 1.5 g of herbage). Two alkanes not present in the samples (C_{22} and C_{34}) were added by weight (200 μ g) as internal standards (IS) for the quantitative estimation of alkanes by gas chromatography. Samples were heated in sealed tubes at 90 °C for 14 h in 1 M ethanolic KOH (7 ml for faeces samples, 14 ml for vegetation samples). Alkane extraction (liquid-liquid extraction) from faeces samples was performed by adding 7 ml of heptane-2 ml of deionized water, shaking, transferring the top solvent layer to a scintillation vial and repeating the extraction with a further 7 ml of heptane (14 ml heptane-4 ml water plus further 14 ml heptane for vegetation samples). Extracts were filtered through a silica-gel column (5 ml bed), eluted with 2+2+5+7 ml of heptane, concentrated to 500 μ l and injected into the gas chromatograph (GC).

Calibration

In order to calibrate the gas chromatographic method, a standard solution containing a mixture of synthetic

alkanes (C_{23} , C_{24} , C_{25} , C_{26} , C_{28} , C_{30} , C_{32} , C_{33} and C_{36}) was prepared with concentrations similar to those found in extracts from samples. The response factors for each of the alkanes studied were calculated from peak areas and the known concentrations. The detector response to varied concentrations of each solute was linear throughout the range of concentrations found in the injected samples.

Alkane concentrations in samples were calculated by reference to two IS (C_{22} and C_{34}), covering the wide range of alkanes studied (from C_{21} and C_{38}).

Gas chromatography

Alkane extracts were taken up in 500 μ l of heptane. Samples of 0.5 μ l were injected by on-column injection on a 15 m $\times$ 0.530 mm DB-1 megabore column, in a Varian Model 3400 gas chromatograph fitted with flame ionization detection. A SPI temperature-programmable injector was used with autoinjection.

The carrier gas (He) flow rate was 15 ml/min. Gradients of temperature were used for the injector (80 °C for 0.2 min; 200 °C/min to 380 °C), and the column (200 °C for 1 min, 6 °C/min to 300 °C; 6 min at 300 °C). The detector oven was maintained at 350 °C.

Experiment 1

The experiment compared alkane extraction at cold and hot temperatures in four samples of different matrix types (cattle faeces, sheep faeces, grass and heather). In order to determine the alkane extraction efficiency at both extraction temperatures, a recovery study was carried out. Known amounts of various alkanes (C_{23} , C_{25} , C_{30} , C_{33} and C_{36}) were added to the samples at two concentrations (Table 1) and alkane recoveries were calculated as a percentage of the alkane amount found in 'spiked' samples (sample + addition concentration 1 or 2) with respect to the alkane content recorded in the 'original' samples (sample + no addition) plus the 'addition' (synthetic alkanes). Alkane content in the 'original' samples was estimated for each treatment combination, as the alkane concentration found on the natural sample (without any addition of synthetic alkanes).

Table 1. Natural alkane concentration in samples and synthetic alkane amounts added in recovery experiments

Alkanes	Original amount in samples (μ g/g dry matter)				Amount added (μ g)	
	Cattle faeces	Sheep faeces	Grass	Heather	Concentration 1	Concentration 2
C_{23}	10.5	7.9	3.5	11.7	4.6	9.3
C_{25}	24.2	29.7	13.5	14.0	19.1	39.0
C_{30}	42.5	52.9	18.2	45.0	13.1	26.8
C_{33}	383.5	425.7	113.6	771.5	70.2	143.1
C_{36}	195.9	384.3	4.8	4.1	99.1	201.8

Fig. 1. Analytical procedure for recovery experiments. IS: internal standard.

A preliminary analysis of alkane content in the different sample matrix (with two replicates and two injections into the GC) was performed, to calculate the alkane amount to be added at each addition concentration. Table 1 shows the alkane content in samples ($\mu\text{g/g DM}$) found in this preliminary analysis, where extraction was performed as in routine analysis (hot extraction and only C_{34} as IS) and the mean alkane amount (μg) added to samples at the different treatment combinations.

'Alkane addition' was added to the samples at the beginning of the procedure, prior to the treatment with ethanolic KOH (step 1) and the resulting spiked samples were subjected to the entire analytical sequence (Fig. 1). Internal standards (C_{22} and C_{34}) were also added at the beginning of the procedure, as in routine analysis. The liquid-liquid alkane extraction from the resulting spiked samples was performed on cold samples (extraction temperature 15–25 °C) or after reheating the samples (extraction temperature 65 °C).

To avoid the influence of sample variability on the estimation of the accuracy of the analytical procedure, the effects of extraction temperature, IS and addition concentration on alkane recoveries were studied on one original sample of each of the four sample matrix types studied. With the aim of determining the accuracy and the reproducibility of alkane recovery calculations, the alkane extraction for each treatment combination was replicated twice and each extract was injected twice into the GC. The statistical analysis was based on the mean of these four values.

Experiment 2

The effect of cold and hot extraction at the different steps of the procedure was also examined in two samples of different matrix types (cattle faeces and heather) by adding the synthetic alkanes at the beginning of the procedure (step 1) and the internal standards (C_{22} and C_{34}) either at the beginning (step

1), after extraction with heptane (step 2) or after passage through the silica-gel column (step 3). As in Expt 1, one sample of each of the two different sample matrix types was analysed and subsamples were extracted at both cold and hot temperatures (Fig. 1). Alkane recoveries were calculated as for Expt 1. Alkane extraction for each treatment combination was replicated twice and each extract was injected twice into the GC, for estimating the accuracy and reproducibility of the analytical procedure.

Data analysis

Experiment 1

Alkane recoveries were analysed using analysis of variance. The statistical design was based on a $2 \times 2 \times 2 \times 4$ factorial (extraction temperature $\times$ IS $\times$ addition concentration $\times$ sample matrix), with a single value for each treatment combination. Since there were no replicates for the different sample matrix types, the model was a crossed factorial, the error being the interaction between the four factors.

Experiment 2

The design was a $2 \times 2 \times 2 \times 2 \times 3$ factorial (extraction temperature $\times$ IS $\times$ addition concentration $\times$ sample matrix $\times$ step), with a single value for each treatment combination. Alkane recoveries were analysed using analysis of variance to test the effects of temperature $\times$ IS $\times$ addition concentration $\times$ sample matrix $\times$ step, the error term being the interaction of the five factors.

RESULTS

In both experiments, the accuracy of the recovery calculations (estimated at the relative standard deviation of two replicates and two injections) was acceptable for analytical purposes (R.S.D. < 9%) and the reproducibility of data between two injections was high (R.S.D. < 5%), because an automatic injection system was used for the GC.

Experiment 1

A preliminary statistical analysis showed no significant effect of addition concentration, therefore this factor was eliminated and the model fitted the effect of extraction temperature $\times$ IS $\times$ sample matrix.

The temperature of extraction significantly affected alkane recoveries for the whole procedure ($P < 0.001$ for C_{23} , C_{25} , C_{33} and C_{36} ; $P < 0.01$ for C_{30}), as observed when synthetic alkanes (addition) and internal standards (C_{22} and C_{34}) were added at the beginning of the procedure (step 1) (Table 2). Under hot extraction, alkane recoveries were complete, ranging from 88 to 105%; however, in cold extracts, recoveries ranged from 32 to 127%. Thus, under cold extraction, recoveries calculated with C_{22} as IS were complete (102%) for C_{23} alkane, with similar chain

Table 2. Effect of temperature (hot: 65 °C or cold: 15–25 °C) and internal standard (C_{22} or C_{34}) on alkane recoveries

	Hot		Cold		S.E.	D.F.	Significance						
	C_{22}	C_{34}	C_{22}	C_{34}			T	IS	M	T×IS	T×M	IS×M	T×IS×M
C_{23}	101.4	105.0	102.4	126.7	1.82	16	***	***	***	***	***	***	***
C_{25}	92.1	97.6	92.2	123.3	1.42	16	***	***	***	***	***	***	***
C_{30}	95.5	91.4	82.7	104.2	2.83	16	**	***	***	***	***	*	**
C_{33}	97.1	97.7	52.9	100.0	1.14	16	***	***	***	***	***	***	***
C_{36}	88.3	93.7	32.1	80.8	1.2	16	***	***	***	***	***	***	***

T: temperature of extraction; IS: internal standard; M: sample matrix.

Fig. 2. Alkane recoveries under hot extraction (65 °C), calculated with C_{22} (—) or C_{34} (.....) as internal standard (● cattle, □ sheep, ◆ grass, ▲ heather).

Fig. 3. Alkane recoveries under cold extraction (15–25 °C), calculated with C_{22} (—) or C_{34} (.....) as internal standard (● cattle, □ sheep, ◆ grass, ▲ heather).

Table 3. Effect of temperature (hot: 65 °C or cold: 15–25 °C), step (1, 2 or 3) and internal standard (C_{22} or C_{34}) on alkane recoveries

	Hot						Cold						Significance				
	Step 1		Step 2		Step 3		Step 1		Step 2		Step 3						
	C ₂₂	C ₃₄	C ₂₂	C ₃₄	C ₂₂	C ₃₄	C ₂₂	C ₃₄	C ₂₂	C ₃₄	C ₂₂	C ₃₄					
C ₂₃	102.8	103.4	100.2	101.3	85.6	87.9	103.8	136.6	96.0	93.4	78.1	81.3	4.05	26	**	***	NS
C ₃₅	91.4	95.7	89.1	93.6	75.1	79.7	91.5	140.1	86.1	91.2	71.4	78.0	4.37	26	*	***	NS
C ₂₆	96.0	96.4	91.0	91.6	76.8	78.1	73.4	125.6	77.4	76.0	65.3	67.1	3.69	26	***	***	*
C ₃₀	100.5	98.1	95.1	93.1	80.7	79.7	57.1	101.7	78.4	73.9	60.4	63.9	2.85	26	***	***	NS
C ₂₃	87.1	91.9	83.6	88.4	70.8	75.7	36.5	71.1	47.4	50.1	41.0	43.2	2.71	26	***	***	***

T: temperature of extraction; IS: internal standard; M: sample matrix; S: step.

These results indicate that alkane extraction was complete when performed under hot conditions, while in cold extracts, the amount of alkane obtained decreased as the alkane carbon chain length increased.

Figures 2 and 3 show the alkane recoveries in hot and cold extracts, respectively, for both IS (C_{22} and C_{34}) and for the different sample matrix types. There were significant differences between both IS on the calculation of alkane recoveries ($P < 0.001$, Table 2), because any variation of the completeness of alkane extraction along the alkane range studied produced differences on the concentration of either C_{22} or C_{34} in extracts and hence on the estimation of alkane recoveries by the short (C_{22}) or long (C_{34}) carbon chain IS. This effect was especially marked in cold extracts (Fig. 3); however, under hot extraction, alkane recoveries calculated with both IS were complete, although they showed small differences.

There was a significant interaction (Table 2) between all the treatment combinations: temperature $\times$ IS, temperature $\times$ sample matrix, IS $\times$ sample matrix and temperature $\times$ IS $\times$ sample matrix, because in hot extracts it was estimated that alkane recoveries were complete for both IS and on the four types of sample; however, under cold extraction, with incomplete recoveries of long chain alkanes, marked differences were found between IS and sample matrix. Alkane extraction was particularly incomplete in the heather sample (when calculated with C_{34} as IS) and in the sheep faeces sample (when calculated with C_{22} as IS), which could indicate that the extraction of alkanes from ligneous material (heather or sheep faeces with a high proportion of heather in the diet) could be more difficult than from herbaceous material. Data from the heather sample showed a large overestimation of short chain alkanes compared to C_{34} . However, the effect when estimates were based on C_{22} were less marked. This shows that the loss of C_{34} when the extraction is not complete is higher than for C_{22} . This is one of the main reasons for suggesting the use of two internal standards: it provides an opportunity to compare the amount of each internal standard in the extract with respect to the ratio added at the beginning of the analytical procedure and hence to check the completeness of alkane extraction.

Experiment 2

As in Expt 1, there was no significant effect of addition concentration on alkane recoveries, therefore

Fig. 4. Alkane recoveries at steps 1 □, 2 ▨ and 3 ■ under hot extraction (65 °C), calculated with (a) C₂₂ and (b) C₃₄ as internal standard.

the statistical analysis was based on the interaction between extraction temperature × step × IS × sample matrix.

The results are shown in Table 3. Extraction temperature and step significantly affected recoveries for all alkanes. There was also a significant effect of IS on alkane recoveries for C₂₅ ($P < 0.001$), C₃₀ ($P < 0.5$) and C₃₆ ($P < 0.001$) and of sample matrix on C₃₀ ($P < 0.5$) and C₃₆ ($P < 0.001$). The most significant interactions between factors were temperature × step, step × IS, temperature × step × IS and temperature × step × sample matrix, which produced a complex set of results. Under hot extraction (Fig. 4), alkane recoveries at step 2 (synthetic alkanes added at the beginning of the procedure but IS introduced into the samples after the extraction with heptane) were complete for both IS (84–100% calculated with C₂₂ as IS, 88–101% with C₃₄ as IS), but slightly lower (2–5%) than at step 1. These lower recoveries recorded at step 2 compared with step 1 suggest that, even at hot temperatures, alkane extraction with heptane can be incomplete.

However, in cold extracts (Fig. 5), recoveries at step

Fig. 5. Alkane recoveries at step 1 □, 2 ▨ and 3 ■ under cold extraction (15–25 °C), calculated with (a) C₂₂ and (b) C₃₄ as internal standard.

2 were complete for short-chain alkanes (C₂₃ and C₂₅) when calculated with both IS (96–86% calculated with C₂₂ as IS and 93–91% with C₃₄ as IS), but decreased as the alkane chain length increased (recoveries for C₃₆ were 47% when calculated with C₂₂ and 50% with C₃₄). This shows that alkane extraction was poor for long-chain alkanes at step 1 (when saponified and extracted with heptane). The drop of recoveries from step 1 to step 2 was higher when calculated with C₃₄ as IS than with C₂₂. The reason is that when using C₃₄ as IS, the recoveries at step 1 were overestimated for short-chain alkanes. On the other hand, when using C₂₂ as IS, the alkane recoveries were higher at step 2 than at step 1 for long chain alkanes, because at step 1 the alkane recoveries were underestimated for long-chain alkanes.

Alkane recoveries calculated after elution through the silica-gel column (step 3) decreased compared to the estimates calculated at step 2 in both hot (13.7%) and cold (11.0%) extracts, probably because some losses of alkanes occurred when passing through the silica-gel column. This decrease in recoveries between step 2 and step 3 depended on the type of sample. Thus, for the faecal sample, recoveries decreased

16–19 points in hot extracts and 8–28 points in cold extracts, but for the heather sample the reduction was significantly ($P < 0.01$ for C_{23} , C_{25} , C_{33} , C_{36} ; $P < 0.001$ for C_{30}) lower (8–13 points in hot extracts and 2–15 points in cold extracts), although there seems to be no analytical reason for this difference.

DISCUSSION

The results obtained in this study show that temperature significantly affects alkane extraction. Alkane extraction from faeces and herbage samples must be performed at a hot temperature (65 °C) for long-chain alkanes to be completely extracted by heptane.

However, different authors have performed the extraction process under different temperature conditions. Mayes *et al.* (1986) proposed that alkanes be extracted from partially-cooled samples (after saponification). Laredo *et al.* (1991) extracted alkanes from cooled samples after initially heating them to 45 °C. Dove (1992) allowed samples to cool (until able to be handled) before adding the heptane for alkane extraction.

The main reduction in recovery occurs when alkanes are transferred from saponified samples to the solvent layer at the cold temperature (15–25 °C). At this step, samples must be hot for alkanes to be completely transferred to the heptane layer. At cold temperatures, alkanes are not extracted well, the error being higher for long-chain alkanes, probably because their solubility in n-heptane is greater when heated.

At the subsequent stages of the procedure, some losses of alkanes occurred when passing through the silica-gel column, in the same proportion for all the alkanes, and to the same extent in hot and cold extracts. Some differences were observed between different types of sample matrix (faeces or heather), but this was not a problem for estimating alkane concentration accurately if the internal standard is added at the beginning of the procedure and alkane extraction is complete, because all the alkanes present in the extracts would be lost to the same extent.

The significant interaction observed between temperature, IS and sample matrix suggest that it would be appropriate to use two IS covering the range of alkanes studied to control the completeness of alkane extraction, because, even under hot conditions, alkane extraction can be different for short and long-chain alkanes and for different types of sample matrix. These differences have been found to be particularly marked in cold extracts, but even under hot extraction estimates made with C_{22} or C_{34} as IS were slightly different. The lower values of alkane recoveries found at step 2 compared to step 1 under hot extraction also suggest that, even at hot temperatures, alkane extraction is not always complete. In routine analyses, only one internal standard (usually C_{34}) is added to the samples to estimate alkane concentrations by GC. If alkane extraction is not complete and only a long-carbon-chain alkane is used as IS, short-chain alkane content would be overestimated and long-chain alkane slightly underestimated. This could cause considerable errors in diet composition estimates (using alkane contents from C_{21} to C_{36}). Errors in intake and digestibility estimations would be smaller, since these calculations use only C_{32} , C_{33} and C_{36} alkanes, with chain lengths similar to that of C_{34} .

Errors could be reduced, particularly in diet composition studies, if two internal standards are used, one at each extreme of the spectrum of the chain lengths found in samples (e.g. C_{22} and C_{34}). This would allow the calculation of the relative ratio $C_{22}:C_{34}$ in extracts compared with the original ratio added to the samples. Any increase or decrease in expected peak areas in chromatograms (expected concentrations) could be calculated and applied to all the alkanes in the extracts for correcting any loss of alkanes during the analytical procedure.

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REFERENCES

- DOVE, H. (1992). Using the n-alkanes of plant cuticular wax to estimate the species composition of herbage mixtures. *Australian Journal of Agricultural Research* **43**, 1711–1724.
- DOVE, H. & MAYES, R. W. (1991). The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. *Australian Journal of Agricultural Research* **42**, 913–952.
- DOVE, H. & MOORE, A. D. (1995). Using a least-squares optimization procedure to estimate botanical composition based on the alkanes of plant cuticular wax. *Australian Journal of Agricultural Research* **46**, 1535–1544.
- GOSDEN, A. F. & MOSELEY, G. (1984). Naturally occurring forage hydrocarbons as intake markers. *Welsh Plant Breeding Station Bulletin Annual Report*, pp. 109–110.
- HOEBEE, S. E., DOVE, H. & OFFICER, D. I. (1998). Using plant wax alkanes to estimate the species composition of subtropical grass mixtures. *Proceedings of the Australian Society of Animal Production* **22**, 264 (Abstract).
- LAREDO, M. A., SIMPSON, G. D., MINSON, D. J. & ORPIN, C. G. (1991). The potential for using n-alkanes in tropical forages as a marker for the determination of dry matter intake by grazing ruminants. *Journal of Agricultural Science, Cambridge* **117**, 355–361.
- MAYES, R. W. & LAMB, C. S. (1984). The possible use of n-alkanes in herbage as indigestible faecal markers. *Proceedings of the Nutrition Society* **43**, 39A.
- MAYES, R. W., LAMB, C. S. & COLGROVE, P. M. (1986). The use of dosed and herbage n-alkanes as markers for the

- determination of herbage intake. *Journal of Agricultural Science, Cambridge* **107**, 161–170.
- MAYES, R. W., BERESFORD, N. A., LAMB, C. S., BARNETT, C. L., HOWARD, B. J., JONES, B-E. V., ERIKSSON, O., HOVE, K., PEDERSEN, Ø. & STAINES, B. W. (1994). Novel approaches to the estimation of intake and bioavailability of radiocaesium in ruminants grazing forested areas. *Science of the Total Environment*, **157**, 289–300.
- OSORO, K., OLIVÁN, M., CELAYA, R., MARTINEZ, A. (1999). Effects of genotype on the performance and intake characteristics of sheep grazing contrasting hill vegetation communities. *Animal Science* (in press).
- SALT, C. A., MAYES, R. W., COLGROVE, P. M. & LAMB, C. S. (1994). The effects of season and diet composition on the radiocaesium intake by sheep grazing on heather moorland. *Journal of Applied Ecology* **31**, 125–136.
- VULICH, S. A., O'RIORDAN, E. G. & HANRAHAN, J. P. (1991). Use of n-alkanes for the estimation of herbage intake in sheep: accuracy and precision of the estimates. *Journal of Agricultural Science, Cambridge* **116**, 319–323.