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 (C₄₅ and C₆₇) 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 (C₆₇) was used as the only internal standard. However, under hot extraction, long-chain alkanes were completely extracted by the heptane, although estimates made with C₄₅ or C₆₇ 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 C₄₅ and C₆₇, 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 C₆₇/C₄₅ 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 deduced synthetic even-chain alkanes as internal and external markers, respectively. Accurate estimates were obtained using C₂₄ and C₃₂ 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 C₁₂ to C₄₅) 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 Hoebbe 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 % 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 (C₄₅) 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
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 analyzed: cattle faeces, sheep faeces, Hill grass (Agrostis-Poaceae) and heather (Calluna vulgaris). These samples were obtained at random from a grazing experiment on hill communities of Agrostis-Poaceae-Nardus-Calluna. Animals were dosed daily with C₁₈, C₂₄, and C₃₆ (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 (C₃₄ and C₄₈) were used.

Freeze-dried and ground samples were weighed accurately (0.5 g of faeces and 25 g of herbage). Two alkanes not present in the samples (C₄₄ and C₅₀) were added by weight (200 µg) as internal standards (IS) for the quantitative estimation of alkanes by gas chromatography. Samples were housed in sealed tubes at 90°C for 18 h in 1 mL ethanolic KOH (7 ml for faeces samples, 14 ml for vegetation samples). Alkane extraction (liquid-liquid extraction) from faecal samples was performed by adding 7 ml of heptane-2 ml of deionized water, shaking, transferring the top solvent layer to a centrifugation 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 µl 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 (C₁₈, C₂₄, C₃₂, C₄₀, C₄₄, C₅₀, C₅₄, and C₆₀) was prepared with concentrations similar to those found in extracts from samples. The response factor 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 (C₂₄ and C₃₆), covering the wide range of alkanes studied (from C₁₈ to C₆₀).

Gas chromatography

Alkane extracts were taken up in 500 µl of heptane. Samples (0.5 µl) were injected by on-column injection on a 15 m x 0.53 mm DB-1 megabore column, in a Varian Model 3400 gas chromatograph fitted with flame ionization detection. A SP1 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 type (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 (C₄₄, C₅₀, C₅₄, and C₆₀) 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 alkane). Alkane content in the 'original' samples was estimated for each treatment comparison, 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

<table>
<thead>
<tr>
<th>Alkanes</th>
<th>Original amount (µg/g dry matter)</th>
<th>Amount added (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in samples</td>
<td>Concentration 1</td>
</tr>
<tr>
<td></td>
<td>C18</td>
<td>C24</td>
</tr>
<tr>
<td>cattle</td>
<td>0.5</td>
<td>242</td>
</tr>
<tr>
<td>faeces</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>sheep</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>faeces</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>grass</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>heather</td>
<td></td>
<td>1.2</td>
</tr>
</tbody>
</table>


Effect of temperature on alkane extraction

**Addition of synthetic alkanes**

[Diagram showing the analytical procedure for recovery experiments. IS: internal standard.]

A preliminary analysis of alkane content in different sample matrices (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 (µg/g DM) found in this preliminary analysis, where extraction was performed as in routine analysis (hot extraction and only C₃₅ as IS) and the mean alkane amount (µg) added to samples at 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 (C₉₅ and C₃₅) were also added at the beginning of the procedure, as in routine analysis. The liquid-liquid alkane extraction from the 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 the analytical procedure 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 1

The effect of cold and hot extraction at the different steps of the procedure was also examined in two samples of different matrix types (cattie faeces and heather) by adding the synthetic alkanes at the beginning of the procedure (step 1) and the internal standards (C₉₅ and C₃₅) 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 x 2 x 2 factorial (extraction temperature x IS x addition concentration x 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 x 2 x 2 x 3 factorial (extraction temperature x IS x addition concentration x sample matrix x step), with a single value for each treatment combination. Alkane recoveries were analysed using analysis of variance to test the effects of extraction temperature x IS x addition concentration x sample matrix x 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 x IS x sample matrix.

The temperature of extraction significantly affected alkane recoveries for the whole procedure (P < 0.001 for C₉₅, C₃₅, C₂₅, C₁₅, P < 0.01 for C₉₅), as observed when synthetic alkanes (addition) and internal standards (C₉₅ and C₃₅) 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 C₉₅ as IS were complete (102%) for C₂₅, alkane, with similar chain.
Table 2. Effect of temperature (hot: 65 °C or cold: 15–25 °C) and internal standard (C23 or C34) on alkane recoveries

<table>
<thead>
<tr>
<th>Hot</th>
<th>Cold</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C_{23}</td>
<td>C_{24}</td>
</tr>
<tr>
<td>C_{23}</td>
<td>101.4</td>
<td>105.0</td>
</tr>
<tr>
<td>C_{24}</td>
<td>92.1</td>
<td>97.6</td>
</tr>
<tr>
<td>C_{25}</td>
<td>95.5</td>
<td>91.4</td>
</tr>
<tr>
<td>C_{26}</td>
<td>97.1</td>
<td>97.7</td>
</tr>
<tr>
<td>C_{27}</td>
<td>88.3</td>
<td>93.7</td>
</tr>
</tbody>
</table>

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

Fig. 2. Alkane recoveries under hot extraction (65 °C), calculated with C_{24} (—) or C_{34} (-----) as internal standard (● cattle, □ sheep, ◆ grass, ▲ heather).

Fig. 3. Alkane recoveries under cold extraction (15–25 °C), calculated with C_{24} (—) or C_{34} (-----) as internal standard (● cattle, □ sheep, ◆ grass, ▲ heather).
lengths to the IS, but recovery values decreased as the alkane chain length increased (from 92% for C₁₅ down to 32% for C₃₀). Likewise, when calculations were made with C₄₆ (the IS usually added to samples in routine analyses) recoveries were complete (100%) for alkanes of similar chain length (C₄₆) but increased for shorter chain alkanes (from 104% for C₄₄ up to 127% for C₃₀).

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 (C₄₆ and C₃₀) and for the different sample matrix types. There were significant differences between 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 difference on the concentration of other C₁₅ or C₃₀ is extracts and hence on the estimation of alkane recoveries by the short (C₄₆) or long (C₃₀) 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 × IS, temperature × sample matrix, IS × sample matrix and temperature × IS × 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 alkane, marked differences were found between IS and sample matrix.

Alkane extraction was particularly incomplete in the heather sample (when calculated with C₄₆ as IS) and in the sheep faeces sample (when calculated with C₃₀ as IS), which could indicate that the extraction of alkanes from ligneous material (heather or sheep faeces with high proportion of heather in the diet) could be more difficult than from heather or sheep faeces. Data from the heather sample showed a large overestimation of short chain alkanes compared to C₄₆. However, the effect when estimates were based on C₃₀ was less marked. This shows that the loss of C₄₆ when the extraction is not complete is higher than for C₃₀. 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 Exp 1, there was no significant effect of addition concentration on alkane recoveries, therefore...
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 alkanes. Recoveries for C₁₅ (P < 0.001), C₁₆ (P < 0.5) and C₁₈ (P < 0.001) and of sample matrix on C₁₉ (P < 0.5) and C₁₈ (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), alkanes recovered 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 C₁₄ as IS, 88–101 % with C₁₉ 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, alkanes extraction with heptane can be incomplete.

However, in cold extracts (Fig. 5), recoveries at step 2 were complete for short-chain alkanes (C₁₅ and C₁₈) when calculated with both IS (96–86 % calculated with C₁₄ as IS and 93–91 % with C₁₉ as IS), but decreased as the alkanes chain length increased (recoveries for C₁₉ were 47 % when calculated with C₁₄ and 50 % with C₁₉). This shows that alkanes 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 C₁₅ as IS than with C₁₉. The reason is that when using C₁₅ as IS, the recoveries at step 1 were overestimated for short-chain alkanes. On the other hand, when using C₁₉ as IS, the alkanes recoveries were higher at step 2 than at step 1 for long chain alkanes, because at step 1 the alkanes recoveries were underestimated for long-chain alkanes.

Alkanes 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 \( C_{29} \), \( C_{30} \), \( C_{31} \), \( C_{32} \), \( C_{33} \); \( P < 0.001 \) for \( C_{34} \) lower (8-13 points in hot extracts and 2-15 points in cold extracts), although these 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 \( C_{30} \) 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 \( C_{30} \)) 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 \( C_{30} \) to \( C_{34} \)). Errors in intake and digestibility estimations would be smaller, since these calculations use only \( C_{30} \), \( C_{32} \) and \( C_{34} \) 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. \( C_{30} \) and \( C_{34} \)). This would allow the calculation of the relative ratio \( C_{30}:C_{34} \) 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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