Analysis of n-alkanes in kidney fat for tracing feeding systems in meat producing animals

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Abstract
There is a need for chemical markers that allow distinguishing animal products derived from different production systems. We examined the potential of n-alkane analysis by gas chromatography in kidney fat. N-alkanes are components of vegetable wax and are absorbed and deposited in fat depots to a small extent. In trial 1, kidney fat was analysed from three groups of bulls that had been fattened on diets differing in the content of grass and grass silage versus maize silage and concentrate. In trial 2, samples from lambs that had been fattened for two or three months on different concentrate/hay diets were compared with samples from lambs slaughtered at the beginning of the trial. All lambs had been similarly reared with their mother on exclusive pasture feeding before the trial. In both trials, there were differences between feeding groups in the profile of 21 linear (odd- and even-numbered) n-alkanes. However, the largest differences were seen for the content of 4 unknown peaks (near C18), that significantly increased with increasing proportions of grass or grass silage in the diet. After identification, these compounds could be potential markers for grass feeding.

Introduction

There is an increasing search for methods that allow tracing back animal food products along the production process chain for various reasons, e.g. the growing consumer concern on the origin of these foods and the feeding history of farm animals. In this context, end-product markers to distinguish meat production and feeding systems could be worthwhile. The potential of various analytical and spectroscopic methods has been reported for this purpose, e.g. stable isotope ratio analysis, NMR-techniques, atomic emission spectrometric and chromatographic techniques to determine various compounds and parameters.
The n-alkane profile of adipose tissue could also be a marker for the feeding background of farm animals. N-alkanes are lipid soluble components of plant cuticula wax layers (Dove and Mayes, 1991). In most higher plants, odd-numbered n-alkanes in the range C21-C37 comprise most of the hydrocarbons of cuticular wax. The n-alkane profile is plant species and plant part dependent, e.g. the ratio C29/C33 is much higher in temperate legumes than in temperate grasses (Dove and Mayes, 1991). During digestion, n-alkanes are not modified and are

absorbed to a small extent with shorter chain n-alkanes being absorbed more easily and being deposited preferentially in adipose tissue (Lintas et al., 1979; Bernardini et al., 1982; Dove and Mayes, 1991). The n-alkane profile of faeces has been used to measure the dietary nutrient intake and to estimate diet composition in free-ranging herbivores (Mayes and Dove, 2000; Ali et al., 2003; Valiente et al., 2003).

Tejeda et al. (1999, 2001a, 2001b) analysed the n-alkane profile of subcutaneous and intramuscular fat from fresh Iberian hams in relation to the feeding system, and Petron et al. (2004, 2005) studied the linear and branched hydrocarbon fraction in dry-cured Iberian hams. These studies demonstrated that there is some potential for n-alkane analysis on end-products to trace back diets and to guarantee authenticity. Similar studies on ruminant tissues showing sufficient discriminating power to distinguish different dietary backgrounds are lacking to our knowledge.

The aim of this study was to evaluate the use of the n-alkane profile of adipose tissue from bulls and lambs fed different diets as a possible tool for traceability purposes.

Material and methods

Animal samples and diets

Additional kidney fat and feed samples were taken from two feeding trials for n-alkane analysis. Kidney fat samples were taken at slaughter and frozen at -20°C until analysis.

In trial 1, samples were taken from a feeding trial with Belgian Blue young bulls described by Raes et al. (2003). Results for three of the four groups are presented here. Group 1 (n=8) was fattened indoors (209 d) and was fed a diet based on concentrate / maize silage (80/20 ratio on DM). Groups 2 (n=7) and 3 (n=8) were pastured for 70 days before being fattened indoors. Group 2 then first received a concentrate / whole-crop triticale silage diet (92 d) followed by an all-mash fattening concentrate during the last phase before slaughter (139 d). Group 3 was fed both during the pre-fattening (98 d) and the fattening phase (134 d) a concentrate / grass silage (70/30 ratio on DM) diet. Average live weight and age at slaughter were respectively 680 kg and 22.3 months.

In trial 2, samples were taken from a lamb fattening trial. All lambs had a similar genetic background and originated from an organic farm. They were weaned at onset of the trial and had been exclusively grazing with their mother prior to the trial. Four male lambs were slaughtered immediately for comparison (pasture group; average live weight and age at slaughter respectively 23.3 kg and 2.7 months). Twenty-four female lambs were fattened indoors on different concentrate/hay (approximately 50/50 ratio on DM) diets. Diets differed in the origin of the feed ingredients (conventional versus organic) and in the type of oil and grain in the concentrate. Half of the animals were slaughtered after two months and half after three months on the diets. Only the effect of conventional versus organic origin of the feed compounds across the other treatments will be considered here (n=12 lambs per group). The hay for these two groups differed in botanical composition, but the concentrates were formulated similarly and differed only in the origin of the compounds. The average live weight and age at slaughter were respectively 26.1 kg and 5.3 months for these 24 lambs.

n-alkane analyses

N-alkanes were extracted and isolated from kidney fat according to a slight modification of the method described by Tejeda et al. (2001a). Kidney fat (8 g) was saponified by refluxing for 2 hours with 280 ml of a 15% KOH in ethanol solution. After adding 60 µg internal standard (dotriacontane, C32) n-alkanes were extracted using 3 volumes of 70 ml hexane. After washing and drying with anhydrous Na_2SO_4 the extract was concentrated by evaporation, loaded on a silica 60 gel column and eluted with 50 ml hexane. The effluent was re-dissolved in 200 µl hexane after evaporation and was analysed by gas chromatography (HP6890) using a HP-5 1909091J-413 column (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness). The injector and detector temperature were 260 °C and 290 °C respectively. The temperature program was as follows: (1) 4 min at 100 °C; (2) increase at 6 °C/min to 160 °C, keep for 3 min; (3) increase at 6 °C/min to 270 °C, keep for 18 min. A total of 25 peaks were quantified, of which 21 were linear n-alkanes (C12 till C31 and C33) and four unknown peaks around the C18 peak. Peaks were identified by comparison of their retention times with n-alkane standards. The values are expressed as µg C32/g fat.

Although the unknown peaks were not characterised, they are very likely branched-chain or unsaturated hydrocarbons (n-alkenes, phytane, phytenes), as can be deduced from Lintas et al. (1979) and Tejeda et al. (2001b). Another significant branched-chain hydrocarbon in the chromatograms was squalene, that appeared after the C28 peak. This peak was not confirmed by a standard, but was identified earlier in the laboratory of one of the co-authors (Tejeda et al., 2001b; Petron et al., 2005) and was therefore also listed.

For feed samples, a similar procedure was followed but fat was first extracted with petroleum ether and nonadecane (C19) was used as internal standard. The values are expressed as µg C19/g dry matter.

Statistics

Several composite variables were calculated from the individual n-alkane concentrations, i.e. overall sum, sum of odd-numbered n-alkanes, sum of even-numbered n-alkanes and sum of 4 unknown peaks. The effect of feeding treatments was assessed by one-way analysis of variance, followed by post-hoc Tukey test for comparison of the means. Discriminant analysis using the stepwise method and cross-validation was performed to test for correct classification (SPSS version 11.0).

Results and discussion

Feed n-alkane profiles

From trial 1, only grass samples from two pastures and from the grass and maize silage were analysed for their n-alkane content, but not from the concentrates, hence no total feed n-alkane profile is available. The shorter chain n-alkanes (C12-C22) were rather equally distributed in the different roughages. On the other hand, the contents of the long-chain odd-numbered n-alkanes (C25, C27, C29, C31 and C33), that were also the most abundant ones, were much higher in the grass and grass silage samples compared to the maize silage sample. Similarly, the concentration of the unknown peak 3 was highest in the grass and grass silage samples.

Hence, the total content of n-alkanes, odd-numbered n-alkanes and unknown peaks were higher in grass and grass silage compared to maize silage. However, there were also large differences between the two grass samples and between the grass and the grass silage samples, probably reflecting an effect of differences in botanical composition or stage of maturity. The squalene content was highest in the maize silage sample.
From trial 2, samples were analysed from the concentrates and the two hay types, but not from the grass of the pasture group. Overall, the long-chain odd-numbered n-alkanes were by far the most abundant ones. The contents of the unknown peaks were negligible in as well the concentrates as the hays. The differences in n-alkane profile between the different concentrates were rather small, except for a higher content of C27, C29, C31, sum of odd-numbered n-alkanes and overall sum of n-alkanes in the conventional versus the organic concentrates. On the contrary, the sum of odd-numbered n-alkanes was higher for the organic hay compared to the conventional hay. However, marked differences for individual n-alkanes were found between the two hay types, e.g. the C29 content was factor 1.5 higher whereas the C33 content was threefold lower in the organic versus the conventional hay (C29/C33 ratio 4.8 and 0.8 respectively). Since the organic hay was probably richer in legumes than the conventional hay, our data correspond with the higher C29/C33 ratio reported by Dove and Mayes (1991) for temperate legumes versus temperate grasses.

Kidney fat n-alkane profile (trial 1, bulls)
There were significant differences between the three feeding groups for several n-alkanes and for the composite variables (Table 1). However, the differences were most outspoken for the unknown peaks, that were much higher in group 3 that received grass silage in the fattening period compared to groups 1 and 2. These 4 peaks also made up the largest contribution to the total n-alkane content in group 3, whereas in group 1, that received no grass or grass silage at all, the linear even-numbered n-alkanes contributed most. Group 2 was intermediate for the contents of most n-alkanes, reflecting their higher intake of n-alkanes from their pasturing period. The squalene content was significantly different between the three groups with group 3 having the highest and group 2 the lowest content.
When stepwise discriminant analysis was applied, three variables only (unknown peak 2 and 3 and C15) were withheld in two canonical discriminant functions (Figure 1). A 100% correct classification was obtained both for the original groups and after cross-validation.

Kidney fat n-alkane profile (trial 2, lamb)
The values for the lamb kidney fat samples were of similar order as those of the bull samples. The mean total n-alkane content was significantly higher for the pasture group slaughtered at the beginning of the trial compared to the organically fed group, but not compared to the conventionally fed group (Table 2). All three groups differed significantly for the sum of the four unknown peaks, that was highest for the pasture group, intermediate for the conventionally fed group and lowest for the organically fed group. Differences for the sum of even n-alkanes were not significant, but the sum of odd-numbered n-alkanes was significantly lower for the pasture group compared to the organically fed group. There were significant differences for several individual n-alkanes, particularly in the group of long-chain odd-

numbered n-alkanes, but these differences were smaller than the differences in the unknown peaks. The squalene content was significantly higher for the pasture group compared to the two other groups that were not significantly different.
When stepwise discriminant analysis was applied, five variables (unknown peak 1 and 3, C27, C31 and C16) were withheld in two canonical discriminant functions (Figure 1). A 100% correct classification was obtained both for the original groups and after cross-validation.

General considerations
Analysis of the n-alkane profile of kidney fat from slaughter bulls and lambs allowed discrimination between feeding groups. Although total diets were not analysed for their n-alkane profile, the differences in n-alkane profile of kidney fat samples could be related to differences in the n-alkane profile of analysed feed components. In both trials, the most discriminating variables were two or three unknown peaks, that are very probably unsaturated or branched-chain hydrocarbons and that were highly abundant in the grass and grass silage samples, but that were marginally present in the concentrates and the hay samples analysed in this study. In the lamb trial, differences between the conventional and organic feeds for some of the long-chain odd-numbered n-alkanes were also reflected in the kidney fat values.
However, the n-alkane profile of kidney fat was clearly not a full reflection of the feed n-alkane profile. Kidney fat was relatively enriched in shorter versus longer chain linear n-alkanes, reflecting the increased rate of absorption with decreasing chain length (Dove and Mayes, 1991). Kidney fat was also clearly enriched on a relative basis in the unknown peaks compared to the feed samples. In the feeding groups that had received grass or grass silage in this study, two or three of these unknown peaks were the most abundant ones, whereas they made up at maximum one quarter of the total n-alkane content in grass or grass silage and thus only represented a small fraction of the total dietary n-alkane content. It thus appears that these compounds are more easily absorbed and deposited than the linear n-alkanes and are good markers for grass intake. Squalene also offered some potential for discriminating between feeding groups and was also relatively enriched in kidney fat compared to feeds, but was not necessarily higher in grass or grass silage versus other roughages, and did not provide additional discriminating power in this study.

Conclusion
More research is needed to assess the value of analysing the n-alkane profile of animal fats as a tool for tracing back diets, but it appears that a few number of compounds offer potential in this respect. Particularly discriminating the intake of grass or grass silage versus other diets seems feasible.

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Table 1. Mean concentration of n-alkanes and squalene in kidney fat of bulls (µg C32/g fat) (trial 1)

Group	1 (n=8)	2 (n=7)	3 (n=8)	RMSE	P
Sum even-numbered n-alkanes	25.6 ^{A}	33.4 ^{B}	29.8 ^{AB}	4.7	0.016
Sum odd-numbered n-alkanes	17.0 ^{A}	22.4 ^{A}	28.1 ^{B}	4.3	0.000
Sum 4 unknown peaks	11.8 ^{A}	19.9 ^{A}	85.4 ^{B}	15.7	0.000
Total	54.4 ^{A}	75.7 ^{A}	143.4 ^{B}	21.5	0.000
Squalene	63.1 ^{A}	43.9 ^{B}	80.7 ^{C}	13.7	0.000

Group 1 = fattened indoors on concentrate/maize silage diet; group 2 = pastured + fattened indoors on all-mash concentrate diet; group 3 = pastured + fattened indoors on concentrate/grass silage diet
^{A,B,C} Means with different superscript are significantly different at $P<0.05$

Table 2. Mean concentration of n-alkanes and squalene in kidney fat of lamb (µg C32/g fat) (trial 2)

Group	1 (n=4)	2 (n=12)	3 (n=12)	RMSE	P
Sum even-numbered n-alkanes	28.0 ^{A}	31.8 ^{A}	26.2 ^{A}	5.9	0.086
Sum odd-numbered n-alkanes	13.6 ^{A}	22.0 ^{B}	21.1 ^{B}	2.7	0.000
Sum 4 unknown peaks	79.4 ^{A}	56.2 ^{B}	26.9 ^{C}	11.7	0.000
Total	121.0 ^{A}	109.9 ^{A}	74.3 ^{B}	17.2	0.000
Squalene	90.5 ^{A}	54.6 ^{B}	45.5 ^{B}	17.7	0.002

Group 1 = lambs slaughtered at beginning of trial, exclusive grazing with mother; group 2 = fed conventional hay/concentrate diet; group 3 = fed organic hay/concentrate diet
^{A,B,C} Means with different superscript are significantly different at $P<0.05$

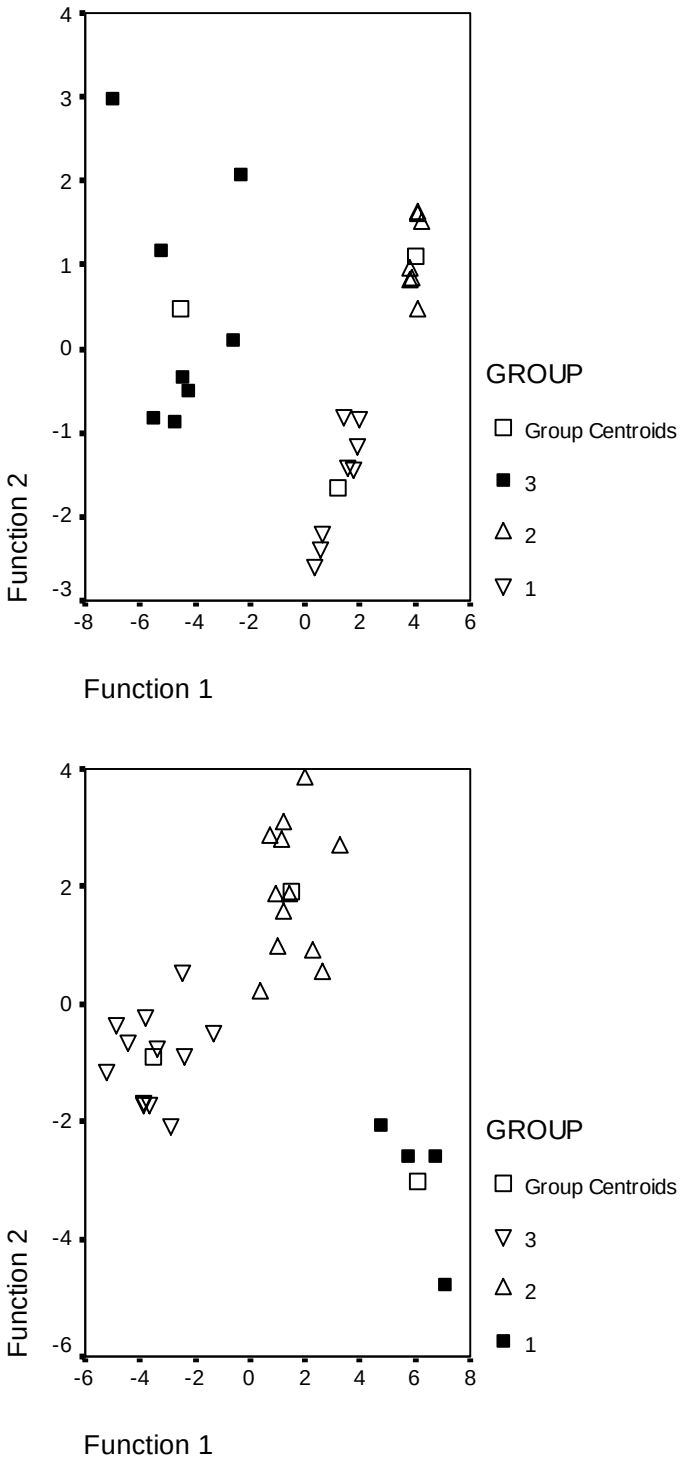

Figure 1. Canonical discriminant functions separating the feeding groups in trial 1 (upper graph) and trial 2 (lower graph)