The measurement of rate of passage using different pairs of alkane as markers for sheep fed hay or fresh grass

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ABSTRACT: This study used alkanes marker absorbed in the paper bung. Four pairs of alkanes: C_{30}:C_{32}, C_{28}:C_{34}, C_{26}:C_{36} and C_{24}:C_{38} were introduced to sheep by mouth, with a different time of dosing. The first group of sheep (n= 6) that were fed hay and second group of sheep (n = 4) were fed fresh ryegrass at similar dry matter intake (DMI), from the alkane marker excretion were calculated residence time of digesta in the digestive tract (mean retention time / MRT). MRT calculations were performed for each individual alkane on each individual sheep with two different feed. From the calculation, it was found that the MRT obtained in the group of sheep fed hay was 35.8 hours (C_{24}), 43.0 hours (C_{26}), 41.6 hours (C_{28}), 40.1 hours (C_{30}), 40.8 hours (C_{32}), 42.0 hours (C_{34}), 48.0 hours (C_{36}), and 40.3 hours (C_{38}). In the group sheep fed fresh ryegrass, the estimated MRT was 32.2 hours (C_{24}), 41.7 hours (C_{26}), 36.3 hours (C_{28}), 40.3 hours (C_{30}), 40.9 hours (C_{32}), 36.6 hours (C_{34}), 45.2 hours (C_{36}), and 34.5 hours (C_{38}). It can be seen that in general the value of MRT for sheep fed hay gave a higher value when compared with the MRT in the group fed fresh ryegrass, although statistically the MRT of the two groups did not differ significantly, except for MRT estimated using marker C_{38}. MRT which estimated using the C_{38} gave a significant difference ($P < 0.05$), i.e. 40.3 hours in sheep fed hay and 34.5 hours in sheep fed fresh ryegrass. The results of observations made, indicated that, for all types of markers and in all groups of sheep, in general there was no significant differences between MRT, except for C_{24}vsC_{36} ($P<0.05$) for groups of sheep fed hay and for C_{24}vsC_{36}, and C_{38}vsC_{36} ($P<0.05$) for the group of sheep fed fresh ryegrass. The use of these alkanes markers were absorbed in paper bung provides a good estimation of the MRT.

Key words: mean retention time, alkane, sheep, hay and fresh ryegrass

INTRODUCTION

Some breeds of ruminant livestock are able to utilise vegetation more efficiently than other breeds, probably due, at least in part, to morphological and physiological differences in the digestive tract. Animals with an inherent ability to digest food more efficiently are likely to have higher digestive tract volumes and slower passage rates than other animals. Such knowledge of gut passage rates in different genotypes could be useful in trying to help explain differences in animal behaviour and pasture utilisation in cattle, sheep or goats grazing in upland environments. Breeding animals to have larger gut volumes and slower passage rates could enable animals to digest roughage diets more effectively. If there were an easy method of determining gut passage rate it has the potential to be used as a selective breeding tool. Mould *et al* (1983) showed a large differences between Bos Indicus (Bangladesh cattle) and Bob taurus (beef cattle in the UK), in ability to consume low quality forage. Similarly, rumen volume is strongly influenced by the period (time) of gestation and lactation (Kay, 1990), whereas Hoffman (1989) showed that variation of the size of stomach was caused by the quality of the feed. Some times ruminants are selected to have a low rumen volume (Hoffman, 1989), as it gives higher carcass weight relative to body weight.

Studies on the feed passage rate continues to evolve. Observations on the flow rate has been made for about 50 years by using various kinds of markers, and also various equations to calculate flow rate. An alternative method based on the analysis of plant alkanes in herbage and feces has been used to estimate DMI in ruminants (Mayes *et al* 1986, 1995, Dove and Mayes, 1996). The n-alkanes are saturated straight-chain hydrocarbons located in the cuticular wax of plants (Dove and Mayes, 1991). Natural alkanes were developed as marker to estimate intake, diet composition and digestibility by herbivores (Dove and Mayes, 1991). Dove and Mayes (1991) proposed using alkanes as marker for

measuring rate of passage. Studies in ruminants indicated that fecal recovery of alkanes increased with increasing chain length (Mayes and Lambs, 1984, Mayes *et al*., 1986).

The extent to which dietary components are fermented in the rumen is a function of potential rate and extent of fermentation and residence in the rumen. The residence in the rumen, usually expressed as the mean retention time (MRT), and can be determined from the decrease in the faecal concentration of a non absorbable marker in rumen. The faecal marker excretion technique (Grovum and William 1973) overcame the problem of representative sampling and has the advantage that fistulated animals are not necessarily required. Giradez *et al* (2004) have examined the rate of feed passage using carrier matrix different. Marker C_{24}: cellulose powder, C_{26}: paper bungs, C_{28}: Gibson paper filters, C_{30}: Cellulose powder, and C_{32}: Shredded paper. While's Bulang *et al* (2008). Were studied rate of passage use different marker. Different carrier matrices (lucerne fiber vs coarse maize meal) were labelled with synthetic alkanes C_{28} and C_{36}.

The objective of this research was to investigate utilisation of pairs of alkanes as markers for measuring rate of passage by single dose for two differents groups of animals, one group of sheep were fed hay consisting of mixture of ryegrass and clover and the second group of sheep were fed fresh ryegrass.

MATERIALS AND METHODS

Preparation of Marker Doses

Each alkane dose consisted of a pair of even-chain alkanes absorbed into tissue paper bungs ('Cellucotton filters'- Carl Roth gmbh, Germany). The alkane pairs for the doses were as follows: Dose 1- C_{30}/C_{32}; Dose 2 – C_{28}/C_{34}; Dose 3 – C_{26}/C_{36}; Dose 4 – C_{24}/C_{38}. The doses were prepared in a fume cupboard, by dispensing a hot solution containing both alkanes of the pair (about 10% w/v in *n*-heptane, 60^oC) on to each paper bung, after the bungs had been preheated to 130^oC. The bungs were left in the fume cupboard over night to allow the solvent to evaporate. They were then placed in a oven at 100^oC for 5 min, so that the alkane melted and was absorbed into the bungs.

Animal and Diets

Twelve adult Scottish Blackface sheep (average live weight 26.75 kg) were maintained on either hay consisting of mixture of ryegrass and clover (six animals) or fresh perennial ryegrass (six animals) in individual pens for two weeks as an acclimatisation period. The hay treatment was medium-quality grass hay collected from a single 'big bale' and chopped and mixed prior to feeding. After the acclimatisation period the sheep were transferred to metabolism cages and maintained on the same diet and feeding level throughout. The animals had constant access to water and were fed twice daily.

Marker Applications and Sampling Procedure

After two days in the cages animals on each dietary treatment were orally dosed as follows:

1. Artificial alkane Dose 1 (C_{30}/C_{32})
2. Artificial alkane Dose 2 (C_{28}/C_{34})
3. Artificial alkane Dose 3 (C_{26}/C_{36})
4. Artificial alkane Dose 4 (C_{24}/C_{38}).

Following the first dose, total faecal collection bags were changed as the experimental period progressed according to the timetable.

Analytical Procedure for n-Alkanes in Sheep Faeces (Modification of Dove and Mayes, 2006)

Samples of faeces collections were freeze-dried and milled before analysis. Individual samples for each sheep at each sampling time were analysed for *n*-alkanes using 0.1g samples run in duplicate. After weighing the samples into screw-capped tubes (4ml capacity) approximately 0.11g of internal

standard solution (*n*-docosane (C_{22}) and *n*-heptacosanol (C_{27}) in *n*-decane) was added by weight. Ethanolic potassium hydroxide solution (1.5ml, 1M) was added. The tubes were capped and heated for 16 h at 90^oC in a dry-block heater.

After partial cooling, (to 50 – 60^oC), 1.5 ml *n*-heptane was added; the tube was capped and shaken gently. Water (0.4ml) was added and the tube, after re-capping, was shaken vigorously. The tubes were reste to allow the contents have separated into two liquid layers; if separation was not complete, tubes were centrifuged at low speed (1000rpm) for 5 min. The top (non-aqueous) layer was transferred to a second 4 ml tube using polyethylene pasteur pipettes. A second aliquot (1.5 ml) of heptane was added to the tube and the extraction was repeated, adding the top layer to the first non-aqueous extract in another 4ml tube. The tubes containing the non-aqueous extracts were placed on a dry-block heater fitted with a sample concentrator blowing air into the tube. The dried extract was redissolved in 0.3 ml heptane, with warming, and gentkly applied to a small column containing silica gel with a bed volume of 1 ml. Heptane (0.1 ml) was added to the column to wash the extract into the silica-gel bed. The hydrocarbons (including the n-alkanes) were eluted from the column into a third 4 ml tube by the addition of a further 2.4 ml n-heptane. The heptane in the eluate was removed by evaporation to dryness on a dry-block heater. The hydrocarbon fractions obtained from the total collection faecal samples were dissolved in 0.5ml *n*-heptane, with warming; a small aliquot (50µl) was transferred to a GC autosampler vial and the hepatane removed by evaporation. The dried hydrocarbon aliquot was redissolved in 30µl *n*-dodecane and the autosampler vial was capped prior to analysis by GC.

Calculation of Faecal Alkane Concentrations

A data file produced by the Chromquest software, which contained peak areas was imported into Excel spreadsheet software; all subsequent data processing was carried out using Excel software.
For eacg GC run, the 'area %' of each alkane peak was calculated:

$$Area\%\ alkane_i = \frac{Peak\ area\ of\ alkane_i \times 100}{Peak\ area\ of\ IS\ alkane\ (C_{37})}$$

Next, the standard response factors (SRF) for each alkane (*alkane*$_i$) were calculated:

$$SRF_i = \frac{Area\,\%\,alkane_i\ in\ mixed\ standard}{Wt\%\ alkane_i\ in\ mixed\ standard}$$

where:

$$Wt\%\ alkane_i = \frac{100 \times Wt\ of\ alkane_i\ in\ mixed\ standard\ solutuion}{Wt\ of\ IS\ alkane\,(C_{37})\ in\ mixed\ standard\ solution}$$

The second internal standard (C_{22} alkane) was used to calculate a fractionation factor (FF), which enables a correction to be made for any chain length-dependent variation in degree of extraction of alkanes. This is based on the concept that without fractionation (discrimination) at the extraction stage, the ratio of the internal standard peak areas (i.e. C_{22}:C_{37}), after correction for GC responses, will be the same as the ratio of the amounts of each internal standard added to the sample. For C_{22} internal standard, the fractionation factor (FF_{22}) was calculated as follows, for each GC sample run:

$$FF_{22} = \frac{Area\%\ C_{22}\ in\ sample \big/ SRF_{22}}{100 \times C_{22} : C_{37}\ concentration\ ratio\ in\ IS\ solution}$$

Assuming that there was a linear change in fractionation factor with chain length, the fractionation factosr for the other alkanes were calculated as shown:

$$FF_i = (i-22) \times \frac{1-FF_{22}}{15} + FF_{22}$$

Having added sample and internal standard weight data, and dry-matter (DM) concentrations to the Excel spreadsheet, the concentrations of n-alkanes in faecal samples could then be calculated:

$$Concentration\ alkane_i\ (mg/kg\ DM) = \frac{10 \times Area\%\ alkane_i \times C_{37}\ IS\ wt\ (mg)}{Sample\ wt\ (g) \times DM\ content \times SRF_i \times FF_i}$$

where: $C_{37} IS\ wt = IS\ solution\ weight\ (g) \times C_{37}\ concentration\ in\ standard\ solution\ (mg/g)$.

6. Gut passage rate calculations

The following set of procedures were used in the processing of the data: Subtract ‘background’ faecal concentrations of markers from the marker concentrations in faeces samples collected after administration of the first marker dose. Calculate the mean retention time (MRT) for each marker in each sheep, using equation of Faichney (1975).

Statistical Analysis

Data for the MRT were analyzed using analysis of mean differences (T test), the correlation between the MRT and peak time analyzed using linear regression, while comparison of the mean of the individual alkane in the same feed, using one way ANOVA. All calculations of data using statistical program statistical product and service solution (SPSS) for windows version 17

RESULTS AND DISCUSSION

Dry Matter Intake

The average live weight of the sheep used in this study was 26.7 kg, the average of live weight was almost the same for both of group I and II used in the measurement of mean retention time of sheep fed hay and sheep fed fresh ryegrass. Dry matter intake was 379 ± 22 grams per head per day in groups of sheep fed hay and 376 ± 6 grams per head per day in groups of sheep fed fresh ryegrass (Table 1).

Table 1. Dry matter intake

	Feeds offered	
Sheep no	Hay	Fresh Ryegrass
1	378 ± 23	387 ± 21
2	399 ± 0.2	349 ± 35
3	381 ± 30	383 ± 27
4	391 ± 14	385 ± 23
5	336 ± 48	
6	387 ± 28	
Mean live weight (Kg)	26.75	26.75
Average DMI±StDev (gr/head)	379.08 ± 22.16	376.69 ± 6.12

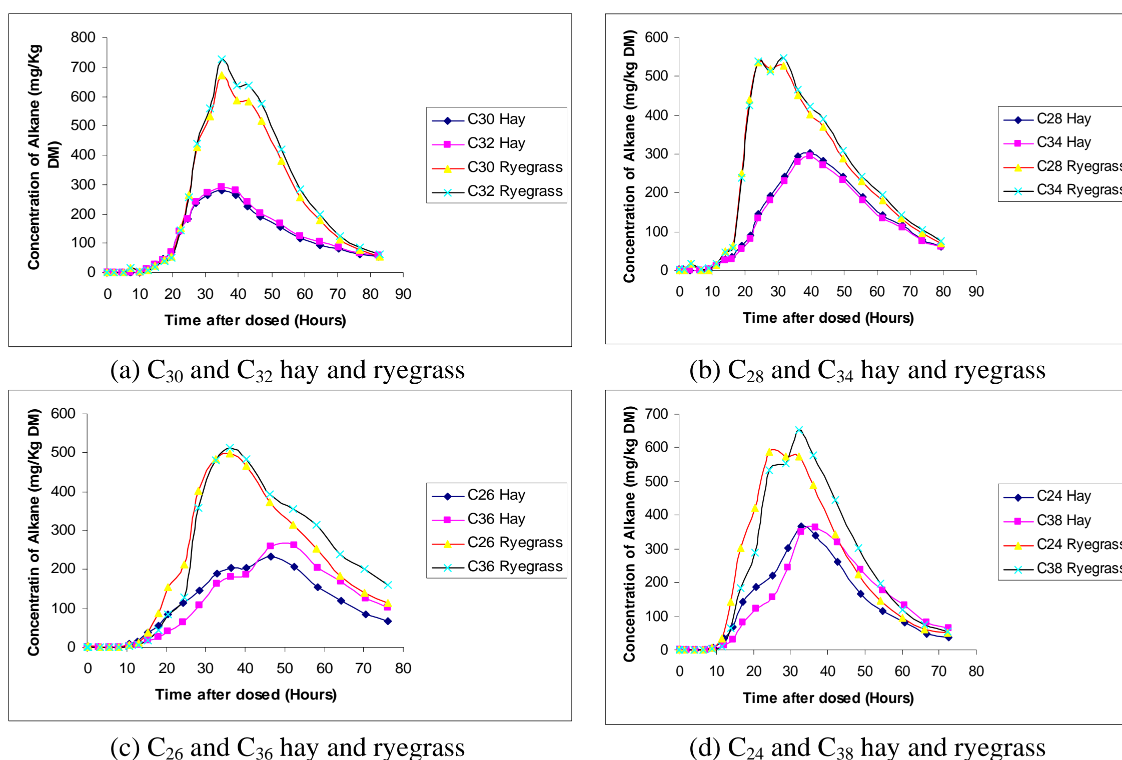
[a,b] Different superscripts in the same row indicate a significant difference (P<0.05)

Marker Excretion Curve

Marker excretion was observed during the 9 days, and the calculations take into account the excretion of fecal samples collected based on the time of collection. Marker excretion curve was observed for each pair of markers which is C_{30}/C_{32}, C_{28}/C_{34}. C_{26}/C_{36} and C_{24}/C_{38} for the group of sheep fed hay (Group I) and for the group of sheep fed fresh ryegrass (Group II). Overall, it was observed that, each pair of marker alkanes gave a similar curve patterns with one another, it was observed for both groups of sheep fed differently. In making the calculation of the curve, the concentrations of alkanes (g/kg DM) was reduced by the existing content of alkanes in the feces before the introduction of markers. Curves obtained, on all pairs were similar to the normal curve (Figure 1).

By calculating each MRT alkane markers that are not significantly different, then we have conducted adjustment curve for marker pairs C_{26}/C_{36} and C_{24}/C_{38} (Figure 1). Overall, the concentration curve of marker excretion (mg/kg DM) to ryegrass was always higher, when compared with the hay diet. Concentration highest in the ryegrass reached 700 mg/kg DM for the C_{30} and C_{32}, 500-550 mg/kg DM in the C_{26}, C_{28}, C_{34}, C_{36} and reached 600 mg / kg of DM at C_{24} and C_{38}. Different observations obtained for hay, which is overall marker excretion was approximately 300 mg/kg DM, this applies to all alkane markers (Figure 1).

If all marker pairs were observed together with observed excretion markers individually, it was found that the marker pairs C_{24}/C_{38}, is the earliest marker excreted both for sheep fed hay and for sheep fed fresh ryegrass, and pairs of C_{26}/C_{36} out the most latest marker, for both of sheep fed differently.



(a) C_{30} and C_{32} hay and ryegrass

(b) C_{28} and C_{34} hay and ryegrass

(c) C_{26} and C_{36} hay and ryegrass

(d) C_{24} and C_{38} hay and ryegrass

Figure 1. Faecal total collection sample concentration (mg/Kg DM) plotted against time (h) after marker dosed for sheep fed hay (n=6) and for sheep fed ryegrass (n=4)

Table 2. Mean Retention Time (MRT) calculated from difference marker (Mean ± S.E.M)

Feeds	Total Mean Retention Time (hours)							
	C_{24}	C_{26}	C_{28}	C_{30}	C_{32}	C_{34}	C_{36}	C_{38}
Hay (n=6)	35.8 ^{a}	43.0^{a}	41.6 ^{a}	40.1 ^{a}	40.8 ^{a}	42.0^{a}	48.0^{a}	40.3 ^{a}
(SEM)	(0.3)	(0.6)	(0.6)	(0.8)	(0.8)	(0.6)	(0.7)	(0.4)
Fresh ryegrass (n=4)	32.2^{a}	41.7^{a}	36.3^{a}	40.3 ^{a}	40.9 ^{a}	36.6^{a}	45.2 ^{a}	34.5 ^{b}
(SEM)	(0.2)	(0.9)	(1.2)	(0.5)	(0.5)	(1.1)	(1.2)	(0.2)

$^{a, b,}$ Different superscripts in the same column indicate a significant difference (P<0.05)

The calculation of the MRT showed that in the same feed, the MRT calculated from each individual alkanes, the lowest MRT was 35.8 ± 0.4 hours (estimated based on C_{24}) and the highest MRT was 48.0 ± 0.7 hours, (estimated from the C_{36}) (Table 2). These value were obtained in sheep

fed hay (n = 6). While in the group of sheep fed fresh ryegrass, the MRT estimates ranged from 32.2 ± 0.2 hours when estimated using marker C_{24} and MRT of 45.2 ± 1.1 hours when estimated using C_{36}. It can be stated that, for all markers in group of sheep fed hay, in general there was no significant differences between MRT, except for C_{24}vsC_{36} ($P<0.05$), and in group of sheep fed fresh ryegrass there was no significant differences between MRT, except for C_{24}vsC_{36}, and C_{38}vsC_{36} ($P<0.05$).

Dry Matter Intake

Feed offered to sheep in this study was hay consisting of mixture of ryegrass and clover or fresh ryegrass or commonly called *Lolium perenne*. Naturally, ryegrass already contains the n-alkane (Cortes *et al*, 2005, Dove *et al*, 1996, Stevens *et al*, 2002). For example (Ferreira *et al*, 2009) showed that even-alkane concentration of *L. perenne* was 2.2 mg/Kg DM for C_{26}, 9.4 mg/Kg DM for C_{28}, 11.1 mg/Kg DM for C_{30}, and 6.1 for C_{32}. The grass naturally contain an alkanes, which was substracted from the faeces to separate alkane marker from the basal feeds.

The quantity of feed given was restricted, rather than ad libitum. This was to avoid bias, which may be obtained if the feed is ad libitum, because as described above, that ryegrass naturally, already contain alkanes. So, with the restricted diet, the initial alkane content, was not expected to significantly different for each individual animal. Although the feed offered was limited, the DMI of both groups of sheep were 379 g/head/day (hay) and 376 g/head/day (fresh ryegrass). No significant differences between the two DMI. DMI was approximately 1.4% of body weight.

Excretion Marker Curve

Visible curve for each pair of markers gave the same pattern, as well as providing value of peak time and MRT did not differ statistically, meaning that in fact when used alkane as a marker for the measurement of the MRT, then only needed one of these alkanes in pairs (Figure 3). In this study fecal alkane recovery has not measured, but from previous research, it was found that the faecal recovery of n-alkane increased with the C-chain length (Duncan *et al*, 1999, Mayes *et al*, 1986 and Giraldez *et al*, 2006). For example, Giraldez *et al* (2004) found that the faecal recovery of alkanes was 77% for C_{24}, 81% for C_{26}, 92% for C_{30} and 95% for C_{32}, while Ferreire *et al* (2009) showed that the faecal recovery of mixture of herbage was 40% for C_{28}, 75% for C_{30}, 92% for C_{32}.

Alkane excretion patterns for shep fed hay and fresh ryegrass were different, for peak excretion of sheep fed hay was reached at concentrations around 300 mg/kg DM, while in the sheep fed ryegrass, peak excretion was reached at concentrations above 500 mg/kg of DM (Figure 3). This excretion pattern similar to that obtained by Duncan *et al* (1999) who compared Spinach and cabbage or a mixture of grass-dominated pasture-phleum pratense Timothy.

Mean Retention Time (mean±**s.e.m)**

Alkane pair data, provided no significant differences of MRT when compared in pairs C_{30}/C_{32} different feed, either observed for the pair C_{28}/C_{34}, C_{26}/C_{36} and C_{24}/C_{38}.

The digesta flow or the residence time of food particles can be used to predict fibre digestibility (Veira *et al*, 2008a, Veira *et al*, 2008b, Stensig *et al*, 1999., Huhtanen *et al*, 1995) so that the flow rate or residence time will be reflected in dry matter intake (DMI). Giraldez *et al*, (2006) stated that there be an increase in the level of intake is generally related to flow rate.

MRT was calculated from pair of alkane C_{30}/C_{32} were 40.4 and 41.5 hours, consecutively for hay and ryegrass and pairs of alkane C_{28}/C_{34} were 42.1 hour for hay and 36.4 hour for fresh ryegrass, and pairs of alkane C_{26}/C_{36} were 45.6 hours for hay and 43.4 hours for fresh ryegrass, for the pairs of alkane C_{24}/C_{38} were 38.3 hours for hay and 33.3 for fresh ryegrass. No significant differences were found between the feed.

MRT values in this study, was 10 points lower when compared to the MRT as measured using marker mordanted Cr-fiber (Giraldez *et al*, 2006), with similar diet, but the value in this study was 10 points higher if MRT estimated using alkanes sprayed on leaves or on stems, (Giraldez *et al*, 2006). Similar value obtained of MRT when estimated using the absorbed C_{26} paper bung (Giraldez *et al*,

2004). While Bulang *et al* (2008) found high values for the estimated of MRT when estimated using marker Cr mordanted in Lucerne. It appears there was a consistency of usage of Cr is to give value always higher when compared to estimates using alkanes.

MRT estimated from the mean of each marker pairs, showed that the type of feed was not making any differences on the MRT, the same observation was found to peak time, when observed in one feed, apparently in sheep fed hay, pair marker C_{30}:C_{32}, C_{28}:C_{34}, C_{26}:C_{36} and C_{24}:C_{38}, did not make a difference significantly. Whereas for sheep fed ryegrass, MRT estimated from marker pairs C_{30}:C_{32}, C_{28}:C_{34} and C_{26}:C_{36}, provided no difference significantly, but the MRT for sheep fed on ryegrass, which are estimated pair alkanes C_{26}:C_{36} differ significantly from the MRT which are estimated using the alkane pair C_{24}:C_{38} ($P < 0.05$).

Alkanes absorbed in the paper bung, apparently were released faster and better mixed with the digesta in sheep fed ryegrass than in sheep fed hay.

CONCLUSIONS

In general, the marker excretion curve, for each individual alkane showed the same pattern for each type of feed, so that the group of sheep fed hay gave a similar pattern of excretion curve to the group of sheep fed ryegrass, but with different peaks.

Marker excretion curve for pairs of markers C_{30}:C_{32}, C_{28}:C_{34}, C_{26}:C_{36} and C_{24}:C_{38}, gave a similar pattern for each type of feed, although the sheep fed ryegrass gave higher peak of the curve when compared to sheep fed hay .

There was no significantly difference between MRT in sheep fed hay and sheep fed ryegrass when estimated from individual each marker.

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