THE USE OF THE N-ALKANE TECHNIQUE FOR MEASURING HERBAGE DRY MATTER INTAKE IN HORSES

BY

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DECLARATION

I hereby declare that the whole of this thesis except where otherwise indicated in the text, is my own original work,and that the results obtained have not been previously submitted by me in respect of a degree at any other University.

D.M STEVENS

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ABSTRACT

The use of n-alkanes as indigestible markers for the estimation of herbage dry matter intake in grazing ruminants, is reported to have significant advantages over other markers used for this purpose (Dove and Mayes, 1991). The use of n-alkanes to estimate herbage intake in horses has not previously been reported, and was therefore investigated in this study.

A preliminary trial, carried out in order to investigate possible practical problems in applying the technique to horses, showed that administration of the external marker (C_{32}) in the form of coated grass pellets was satisfactory. Practical difficulties likely to be encountered in carrying out further indoor feeding/faecal collection trials were highlighted during this trial.

Four, mature, thoroughbred geldings were used in an indoor feeding and total faecal collection trial to determine the accuracy of intake estimates made using the C_{31}:C_{32} and C_{32}:C_{33} n-alkane pairs. The faecal recovery of C_{31},C_{32}, C_{33} and C_{36} as well as the ability of these n-alkanes to provide estimates of diet digestibility were also investigated.

Estimates of intake made using the C_{32}:C_{33} pairs were not significantly different ($P<0.05$) from measured intake when the horses were consuming fresh *Lolium perenne* or *Pennisetum clandestinum*, *P. clandestinum* hay or a mixture of concentrates and hay. Overall, error of intake estimate using this n-alkane pair in the total collection trial was 4.8 ±7%. The C_{31}:C_{32} n-alkane pair gave estimates of intake for individual animals that gave significant differences ($P<0.05$) from measured values when the horses were consuming *P. clandestinum* hay and the hay+concentrate diet. However, mean intakes were not significantly different ($P>0.05$) from measured intakes for the fresh *L. perenne* or *P. clandestinum*, or hay diet. Overall error in intake estimate was 8.5 ±16%.

Faecal grab samples taken twice daily gave mean estimated intake values which were not significantly different from measured intakes However, estimates of the intake of certain individual animals were found to be significantly different from measured values, using either of the n-alkane pairs. The overall error in intake estimate was 15.1± 14% and 4.0± 14% for the C_{31}:C_{32} and C_{32}:C_{33} estimates respectively.

The faecal recovery of the n-alkanes was significantly lower ($P<0.05$) when horses consumed *L. perenne* than when consuming the *P. clandestinum* grass, grass hay or a mixture of hay and concentrates. The recovery of the C_{31}, C_{32} and C_{33} n-alkanes were similar to those reported for ruminants (Dove and Mayes, 1991). However, there was no significant increase in n-alkane recovery with increasing chain length, and the recovery of C_{36} was significantly lower than reported in previous studies with ruminants, suggesting differences in behaviour of n-alkanes in the digestive

tracts of ruminants and horses.

Single doses of C_{32} resulted in peak faecal concentrations of C_{32} being reached between 20 and 32 hours after marker administration, after which the levels of the n-alkane decreased rapidly. Complete C_{32} excretion appeared to be complete 56-64 hours after final dose administration. Single, daily doses of C_{32} appeared to be insufficient to produce a steady state of faecal C_{32} marker excretion in the horse, the extent of which may have been influenced by the diet being consumed, resulting in marked diurnal variation in faecal n-alkane concentrations.

The results of this trial showed that reasonable estimates of herbage intake in the horse may be obtained using the C_{32}:C_{33} n-alkane pair. The C31:C_{32} n-alkane pair was a less reliable indicator of intake but may still be used to provide intake estimates. However, more frequent administration of external n-alkanes than once daily dosing, may be necessary to improve the accuracy of intake estimates made using faecal grab samples, due to the diurnal variation found in this study. The low faecal recovery of the n-alkanes observed in this study made limited their use as digestibility indicators. However, no comparable data is available to confirm the results of this trial.

Further investigation is needed with regard to the difference in behaviour of the n-alkanes in the digestive tracts of ruminants and horses. The frequency of external n-alkane marker administration, and the impact of diurnal variation in faecal marker concentration, needs further investigation if the n-alkane technique is to be used successfully with grazing horses.

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INTRODUCTION

The horse is a large, non-ruminant herbivore which, in the wild, is dependent on forage plant species to meet it's nutritional requirements. The equine digestive tract is capable of efficient extraction of nutrients from herbage, by both intestinal, enzymic digestion and bacterial fermentation, enabling the horse to utilize natural forage, or cultivated pastures (Frape, 1986).

Domesticated equines may not have access to a sufficient abundance of herbage, or herbage of sufficient quality to meet the demand for all its nutrient requirements, particularly when production demands are high (Frape, 1986). Nevertheless, where available, pasture may be capable of providing a significant proportion of the horses nutritional needs, and in an economically beneficial manner (Frape, 1986). Fresh herbage intake has also been reported to have beneficial effects on the health of horses due to the presence of vitamins and minerals which may be lacking in preserved forages and processed feeds.

In order for the stud breeder or horse nutritionist to utilize this source of nutrients efficiently, both the quality of the herbage, and the quantity of the herbage consumed must be known. The evaluation of herbage quality, by examination of the individual nutrients present in representative samples of the diet, is well documented. However, intake of plant material by grazing animals is more difficult to measure, particularly as intakes may vary according to forage availability, forage characteristics, climate season, animal factors and external factors such as management or feeding systems (Mayes and Duncan, 1986; Houpt, 1990).

The difficulty of obtaining accurate estimates of pasture intake has important practical consequences for both researchers and horse owners. Dietary imbalances have been associated with growth disorders in performance horses (Thompson *et al.,* 1988; Kronfeld *et al.,* 1990). As the intake of herbage by the horse at pasture is often unknown, the extent of imbalances in the diet due to pasture intake is also unknown. As a result of this, pastures may deliberately be under-utilised as a source of nutrients by horse producers, in order to maintain greater control over the horses nutrition (Stevens *et al.,* 1994).

The use of markers to provide estimates of pasture intake by grazing ruminants has been well established (Langlands, 1975). Markers have also been utilized in horses (McMeniman *et al.*, 1990; Barbisan *et al.*, 1993) to estimate herbage intake, but only infrequently. Moreover, ruminant studies have highlighted inadequacies of the marker substances used in obtaining these intake estimates (Langlands, 1975).

The use of n-alkanes, which are components of the cuticular wax of plants (Tulloch, 1976), reportedly have advantages over other markers used to estimate intake in grazing ruminants (Dove and Mayes, 1991). The use of these substance may be equally effective in the measurement of intake by the horse at pasture. However, reports concerning the use of these substances for this purpose had not been published at the time of writing.

The aim of the following study was therefore, to evaluate the use of n-alkanes for the estimation of herbage dry matter intake by the horse.

CHAPTER 1

LITERATURE REVIEW: THE N-ALKANE TECHNIQUE AND POSSIBLE USE OF N-ALKANES IN ESTIMATING HERBAGE DRY MATTER INTAKE IN HORSES

1.1 *Introduction*

The use of markers for estimating herbage intake is a method which derives from the feed:faecal ratio method (Raymond, 1954). This ratio method involves measuring the total faecal production of an animal, in an indoor digestibility experiment, fed a known amount of cut herbage. Simultaneously, a total faecal collection takes place with the grazing animal. Using the feed:faecal ratio, the amount of herbage consumed by the grazing animal can then be estimated.

In order to obviate the need for a total faecal collection, an external (dosed) substance, which is completely recoverable in the faeces is used. The concentration of this marker in the sample can be used to calculate total faecal output. To enable the dismissal of an indoor digestibility trial, an internal marker (plant substance) is used in conjunction with the external marker, and the herbage consumed is calculated by the following equation (Raymond ,1954) :

$$\text{Herbage intake/day} = \frac{\text{wt of marker fed per day}}{\text{wt of marker per gram faeces}} \times \frac{\%\text{internal marker in faeces}}{\%\text{ internal marker in herbage}}$$

or, as summarized by Dove and Mayes (1991) :

Intake = faecal output /1-digestibility

The main criterion for the successful use of markers is that they must be indigestible and chemically discrete. Chromium Sesquioxide (Cr_2O_3) is considered one of the most satisfactory external markers for ruminants as it conforms to these requirements (Langlands, 1975), although many other substances have been evaluated (Kotb and Lutkey, 1972; Le Du and Penning, 1982) for use in ruminant studies. The use of Cr_2O_3 has also been evaluated in the equine (Haenlein *et al.*, 1966; Vander Noot *et al.*, 1967) and has been used to measure digestibility, rate of passage (Parkins *et al.*, 1982) and faecal output (McMeniman *et al.*, 1990). Chromium-mordanted fibre has been evaluated as a digestibility indicator (Cuddeford and Hughes, 1990) and been included in equine diets to evaluate digestibility and faecal output (Barbisan *et al.*, 1993; Dugan *et al.*, 1993).

Many plant components have been evaluated for use as internal markers in both ruminants and equine diets including lignin (Swift *et al., 1947*), plant chromogens (Reid *et al.,* 1952), cellulose-indigestible fibre (Penning and Johnsen, 1983b) and indigestible, neutral-detergent fibre, to name but a few. The most commonly reported plant fraction used in recent equine studies is acid insoluble ash (AIA), which may be preferred to chromium markers in equine digestibility studies (Cuddeford and Hughes, 1990). However, many of the plant components studied have proved to be unsatisfactory, due mainly to difficulties with analysis as chemically discrete entities (Dove and Mayes, 1991). This results in markers apparently giving accurate estimates under certain conditions and poor results in others (Dove and Coombe, 1992).

Due to the unreliability of these internal (plant) markers, herbage digestibility is often calculated using *in vitro* digestibility procedures calibrated with *in vivo* estimates (Tilly and Terry, 1963; Trevor-Jones *et al.*, 1991). These *in vitro* estimates are frequently made, using mature animals at maintenance, and may therefore not be applicable to animals of different physiological status. Additionally, only a single estimate of digestibility is applied to all the test animals regardless of differences in intake, supplement intake or possible health differences (Dove and Mayes, 1991).

The use of long-chained plant n-alkanes may overcome these sources of error and improve the accuracy of intake measurements in grazing animals.

1.2 What are plant alkanes?

The plant n-alkanes form part of the unsaponified wax component of plant cuticles. These n-alkanes form between 3 and 40% of this component in the *Poacea* family (Tulloch, 1976) and, while often not the major component of plant waxes, have received much attention due to their widespread distribution in cuticular wax and the ease with which they can be analysed (Dove and Mayes, 1991). The composition of the n-alkane fraction of cuticular wax varies between plant species, examples of which are given in Table 1.1. Dove and Mayes (1991) noted several features that become obvious from this table, namely:

(1) The carbon chain lengths of alkanes detected in most plants are usually in the range C_{25} (Pentacontane) to C_{35} (Pentratriacontane). While alkanes of shorter chain-length are present, these are usually detected in smaller quantities.

(2) Odd-numbered carbon chains are present in far higher concentration in all species than even-chained alkanes. However, in certain Eucalypts this may not be true (Horn *et al.,* 1964). The dominant alkanes in these species are C_{29}, C_{31} and C_{33}. However, there are marked species differences in pattern and level of alkane.

Table 1.1: The n-alkane levels of some plant species (adapted from Dove and Mayes, 1991)

Plant species	Alkane level (mg/kg DM)[A]								
	C_{25}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{35}
MONOCOTYLEDONS									
Lolium perenne		19	5	73	9	137	9	116	18
		29		93		119		79	14
		36	6	142	12	220	7	99	9
		26	7	163	14	261	8	110	7
	6	20		109		215		141	12
	6	29	11	159	18	317	10	149	11
L. multiflorum		105	8	260	11	250	4	43	0
	10	40		230	12	242		57	7
L. rigidum	33	83		196		298		47	0
	14	38	11	187	15	263	8	122	1
Phalaris aquatica	31	41		50		35		41	0
	8	8	3	21	2	48	2	24	3
Dactylis glomerata		20	2	38	2	58	2	21	0
Holcus lanatus	41	55		101		98		20	
Vulpia sp.	29	39	3	89	4	29	1	10	0
Phleum pratense	32	24		15	0	17		14	7
Paspalum dilatatum	1	4	2	14	6	104	6	39	2
Chloris gayana		24		51		95		89	0
Brachiaria decumbens		8	2	23	7	126	14	223	77
Digitaria decumbens		60	10	103	13	323	12	278	40
Pennisetum glaucum		12	2	62	5	91	3	59	24
Setaria sphacelata		131	9	110	2	37	T	7	T
Monachather paradoxa	4	11	2	50	15	408	12	39	0
Eragrostis eriopoda	3	9	4	55	14	395	27	466	18
Aristida jerichoensis	10	14	8	48	17	364	11	122	7
DICOTYLEDONS									
Trifolium repens		38	7	109	5	67	1	7	0
		19		75		66		5	0
	4	12	6	88	4	55	3	26	5
cv. Halfa	6	19	7	48	4	50	2	16	4
T. subterraneum cv. Larisa	4	16		250		74		10	0
Dinninup	4	15		118		26		5	0
Mt Barker	2	7	6	65	2	14	1	4	0
T. pratense		30	11	408	5	57	1	11	0
	15	34		376	3	42		8	2
T. balansae	21	67	8	98	3	52	1	8	0
Medicago sativa		36	9	202	12	324	7	21	0
cv. Siriver	13	55	6	207	13	104	3	8	0
Ornithopus compressus	69	140		104		163		39	0
Leucaena leucocephala		10	5	37	4	29	3	18	2
Stylosanthes scabra		T	T	58	11	241	21	198	1
Stylosanthes hamata cv. Verano				19		112		128	0
Bassia diacantha	11	19	8	52	8	73	3	36	0
Acacia aneura	226	119	9	126	17	1197	87	1646	11
Dodonea attenuata	4	27	20	828	60	1498	25	55	0
Duboisia hopwoodii	11	55	12	351	137	4208	148	383	0

[A] Gaps in Table indicate that alkane not determined or reported.
[B] Mean of summer and winter values.
[C] Summer values, leaf only.
[D] Leaf only.

1.3 The use of alkanes in measuring herbage intake

1.3.1 Theory

Oro *et al.* (1965) noted a similarity in alkane patterns between the herbage consumed and faeces excreted by cattle. However, the implications of these findings for intake studies were not immediately noted.

Body and Hansen (1978) compared branched-chain fatty acid (C_{13}-C_{31}) levels in perennial ryegrass and in the faeces of ryegrass-fed sheep and discovered that the concentration of alkanes in the faeces was markedly higher than that in the grass, suggesting the indigestibility of these substances. Later work by Grace and Body (1981) suggested that long-chained fatty acids (C_{19}-C_{32}) may be suitable as indigestible markers in grazing studies. While this was the first study to postulate the possible role of cuticular-wax components for intake studies, little further work has been published concerning the use of long-chained fatty acids for this purpose. This may be due to difficulty of analysis of these plant components (Dove and Mayes, 1991). Mayes and Lamb (1984), following on from the work of Grace and Body (1981) suggested that the n-alkane fraction of cuticular waxes may be useful in digestion studies.

Mayes *et al.* (1986a) reported the incomplete recovery of alkanes from the faeces of experimental animals, which is contrary to the proviso that the internal marker should be indigestible. However, Mayes *et al.* (1986a) suggested that using an external even-chained alkane may overcome this problem.

Taking into consideration the observation illustrated in Table 1.1, that even-chained alkane levels in most species are relatively low, Mayes *et al.* (1986a) argued that the incomplete recovery of alkanes would not matter, provided the dosed (even-chained) and internal (odd-chained) alkanes were recoverable to the same extent in the faeces. This led to the derivation of the equation used by Mayes *et al.* (1986a) to predict herbage intake using n-alkanes (see Appendix 1). This equation allows the errors associated with the incomplete recovery of the alkanes to cancel each other out in the numerator and denominator (Dove and Mayes, 1991).

1.3.2 Behaviour of n-alkanes in the digestive tract of herbivores

Data presented by various scientists (Mayes and Lamb, 1984; Mayes *et al.*, 1986a; 1986b; Dillon and Stakelum, 1990) indicates that the faecal recovery of alkanes increases with increasing chain length. As the chain length increases, the difference in recovery between adjacent alkanes decreases, thus improving the accuracy of the equation given by Mayes *et al.* (1986a). Similarly, intake estimates using

short chain lengths have reduced accuracy (Dove & Mayes, 1991). Mayes *et al.* (1995) suggested that the principle of increased faecal n-alkane recovery associated with increasing chain length may not apply to non-ruminant herbivores, such as horses (Cuddeford and Mayes, unpublished data), pigs (Ganon and Mayes, unpublished data) and hares (Hulbert, 1993). Mayes *et al.* (1988) examined the recovery of alkanes in different segments of the ovine intestine. The results are seen in Table 1.2.

Table 1.2: Recovery (mean ± s.e) of alkanes from the duodenum, terminal ileum, and in the faeces of sheep fed fresh perennial ryegrass (n=8) (Mayes *et al.*, 1988)

Alkane	Duodenum	Terminal ileum	Faeces
C_{27}	1.037 ± 0.0387	0.626 ± 0.0250	0.594 ± 0.0174
C_{28}	0.877 ± 0.0424	0.759 ± 0.0446	0.786 ± 0.0210
C_{29}	0.997 ± 0.0354	0.745 ± 0.0224	0.697 ± 0.0144
C_{31}	0.965 ± 0.0340	0.815 ± 0.0214	0.779 ± 0.0095
C_{32}	0.821 ± 0.0433	0.819 ± 0.0329	0.859 ± 0.0101
C_{33}	0.988 ± 0.0348	0.875 ± 0.0209	0.839 ± 0.0127
C_{35}	1.013 ± 0.0387	0.977 ± 0.0219	0.953 ± 0.0090
C_{36}	0.841 ± 0.0415	0.876 ± 0.0373	0.922 ± 0.0115

Mayes (1988) observed that, while naturally occurring alkanes are associated with the particulate digesta phase, 30-40% of dosed alkanes are associated with the liquid phase. This may account for some of the higher faecal recoveries observed with dosed alkanes (even-chain), than plant n-alkanes (Dove and Mayes, 1991). However, providing recoveries are similar, the difference in behaviour of dosed and natural alkanes will not effect the intake estimates. The work of Mayes (1988) suggested that much of the unrecovered alkane is absorbed from the small intestine although ruminal loss of n-alkanes has been reported in cattle (Palmquist *et al.*, 1991). Once absorbed, radioactively labelled short-chained n-alkanes appear to be lost as carbon dioxide and, in the case of the rat, found in the fatty-acid moiety of the liver phospholipid (Kollatakudy and Hankin, 1966).

1.3.3 Selection of alkane pairs for intake estimation

Mean faecal recoveries of C_{32} and C_{33} for published ruminant data (Dove and Mayes, 1991) are found to be similar (0.868 and 0.872). For C_{35} and C_{36} the mean recoveries are given as 0.948 and 0.947 respectively. While C_{35} and C_{36} are more highly recoverable in the faeces, Table 1.1 shows that the occurrence of C_{35} in most species is too low to enable accurate estimates of intakes and digestibility (Dove and Mayes, 1991).

In a large number of *Poacea* species C_{33} is found in high concentrations. This fact, together with the similarity of faecal recovery of C_{32} and C_{33} found in sheep and cattle (Dove and Mayes, 1991), and the reasonably high faecal recovery (*ca.* 86%) has led to this pair of n-alkanes being most frequently used for intake studies. Trials using sheep (Dove and Mayes, 1991; Vulich *et al.,* 1991), goats (Mayes *et al.,* 1995) and cattle (Dillon and Stakelum, 1989) have validated the reliability of this n-alkane pair as intake indicators in ruminants.

1.4 Practical use of the n-alkane technique

1.4.1 Obtaining a representative sample of consumed herbage

A major area of concern in any intake study is to obtain a representative sample of the herbage consumed (Langlands, 1975). This may be even more important when using the alkane technique because, although the digestibility of two different plant species may be similar, and have little effect on the *in vitro* digestibility estimate, the alkane patterns may differ markedly and thus effect the estimates of intake (Dove and Mayes, 1991). This problem of sample collection may also occur in monospecific pastures, as differences in alkane concentration have been found to exist between the stems and the leaves of *Pennisetum clandestinum* (Marais and Escott-Watson, 1993) and probably most other species (Dove and Mayes, 1991). Vulich *et al.* (1993) examined the effect of different methods of herbage sample collection on n-alkane concentrations but found little difference between samples collected by hand-plucking, cutting or pooling the contents of oesophageal extrusa obtained from sheep. The errors in intake estimate due to herbage sample C_{33} variation are greater than those due to herbage C_{32} variation (Vulich *et al.,* 1993). Vulich *et al.* (1993) also reported that the approximate 80% confidence interval of proportional bias in estimated intake, from using 3 herbage samples was -0.07 to + 0.07, and calculated that 35 samples would yield a confidence interval of -0.02 to +0.02.

1.4.2 Administration of even-chained alkanes to grazing animals

Various methods of administering the synthetic or dosed alkane have been used. Dove *et al.* (1989b) compared paper pellets as used by Mayes *et al.* (1986a) with gelatin capsules as carriers of the alkane and found no significant differences in faecal alkane concentrations. Vulich *et al.* (1991) compared a different method of gelatin capsule preparation, but could not demonstrate any significant advantage over the paper pellets. More recently Marais *et al.* (1993a) demonstrated the usefulness of an alkane suspension, as a means of introducing the dosed alkane into the digestive tract. Controlled-release devices for n-alkane markers have been developed to obviate the need for repeated dosing in ruminants (Mayes *et al.*, 1995).

Studies involving sheep dosed with capsules and pellets have shown that the dosed alkane faecal concentration reached equilibrium within five to seven days (Mayes *et al.*, 1986a; Dove *et al.*, 1989b). The main concern in obtaining a representative faecal sample after this time is the possibility of diurnal variation in the faecal excretion of the alkanes.

Diurnal variation in faecal marker excretion in Cr_2O_3 studies, has been reported in ruminants (Langlands, 1975) as well as in horses (Haenlein *et al.*, 1966). However, while with Cr_2O_3 the variation in absolute faecal concentration of the marker has important consequences, the alkane technique relies rather upon the recovery ratio of the external:internal alkanes having little variation (Dove and Mayes, 1991).

Reports concerning the variation in n-alkane excretion vary. Mayes *et al.* (1986a) found no diurnal variation in faecal ratios, while Dove *et al.* (1991) found non-diurnal variation in faecal excretion of C_{35}:C_{36}. Malossini *et al.* (1994) reported that faecal diurnal variation in cattle was lower than variation between days and that one or two faecal samples taken daily provided the same information as four samples. Dillon and Stakelum (1989) found diurnal variation in faecal alkane concentration in cows. This variation was postulated to be due mainly to the variation in excretion of dosed alkane while the excretion of natural alkane remained fairly constant. Dillon and Stakelum (1989) suggested that this could be due to the alkane's association with either the liquid or particulate phase of digestion observed by Mayes (1986a). Further studies (Dillon and Stakelum, 1990; Stakelum and Dillon, 1990) showed less diurnal variation. Most of these studies involve stall-fed animals and therefore further work on grazing animals is needed to establish the extent of the variation problem (Dove and Mayes, 1991).

1.4.3 Sample preparation and extraction for alkane analyses

1.4.3.1 Oven drying versus freeze drying

The preparation of samples at present usually includes the freeze drying of both herbage and faecal samples (Dove and Mayes, 1991). This preparation could be simplified provided oven drying did not effect the alkane concentration of the samples.

Dove and Mayes (1991) reported reduced alkane concentrations from the same lucerne sample when using oven drying. Samples dried at lower temperatures (0°C versus 100°C) and over a longer period (<8 hours versus 24 hours) showed better agreement with the alkane concentrations measured with freeze-dried samples. Marais *et al.* (1993b) examined the effects of method of drying on n-alkane profiles of different tree and shrub species. This study suggested that method of drying effected the results in some species while not in others. Dove and Mayes (1991) also suggest that the alkane concentrations *per se* are not affected by the oven drying. However, in certain species this method may increase the difficulty of n-alkane extraction. Further research concerning the effects of oven temperatures and the possible interaction with sample type is needed, therefore freeze drying is the preferred method of sample preparation (Dove and Mayes, 1991).

1.4.3.2 Extraction and analysis

The extraction and analysis of samples to determine n-alkane profiles of herbage and faeces is relatively simple (Dove and Mayes, 1991). Originally (Mayes *et al.*, 1986a) alkanes were extracted in organic solvents followed by saponification in 1M KOH to remove fatty acids. However, later work by Dillon and Stakelum (1990b) revealed direct saponification of samples was possible (Dove and Mayes, 1991). However, the extraction and technical processes are constantly being refined to reduce the time taken (Vulich, 1994), and to improve the accuracy and cost effectiveness of the technique (Marais, personal communication). Following saponification, the extracts are passed through silica gel columns to remove plant pigments (Dove and Mayes, 1991). Alkane profiles are determined using gas-liquid chromatography, often using packed columns (Mayes *et al.*, 1986a; Dove and Mayes, 1991). However, capillary columns may offer better resolution over a greater range of chain lengths (Laredo *et al.*, 1991). The n-alkanes, tetratriacontane (C_{34}) or hexatriacontane (C_{36}), are often used as internal standards due to their low concentration in both herbage and faeces (Dove and Mayes, 1991). Duplicate extractions of samples have shown little advantage over single extractions, and duplicate injections of extracts into the gas chromatograph are unnecessary in most cases (Vulich and Hanrahan, 1990).

1.5 Accuracy of intake estimates using n-alkanes

An appraisal of the accuracy of the method is only possible when comparing actual and calculated intakes. This means that it is difficult to evaluate the technique under grazing conditions. However, results of published data involving sheep and cattle studies in this regard are summarized in Table 1.3.

Table 1.3: Comparisons of known herbage intakes of sheep and cattle, with those estimated using dosed C_{32} and herbage C_{33} n-alkanes (adapted from Dove and Mayes, 1991 and Mayes *et al.*, 1995)

Animals and diet	Mean true intake (DM)	Mean bias of estimate	Reference
Sheep, fresh perennial ryegrass	579 g/d	0 g/d	Mayes *et al.*, (1986a)
Lambs, milk + fresh herbage	112-273 g/d	0.4 g/d	Mayes *et al.*, (1986b)
Sheep, fresh herbage	778 g/d	20 g/d	Vulich *et al.*, (1991)
Cattle, beef, fresh herbage	4.0 kg/d	-0.07 kg/d	Mayes *et al.*, (1986c)
Cattle , Dairy,fresh herbage	13.27kg/d	0.1 kg/d	Dillon and Stakelum (1990)
Cattle, fresh herbage	12.39 kg/d	0.35	Dillon and Stakelum (1995)
Cattle, fresh herbage	13.7	0.05kg/d	Dillon and Stakelum (1995)
Cattle, fresh herbage	12.39	-0.96	Dillon and Stakelum (1995)

Based on these results, unbiased estimates of herbage intake can be obtained using C_{32}:C_{33} (Dove and Mayes, 1991). Vulich *et al.* (1991) when using sheep in similar trials to those reported by Mayes *et al.* (1986a), found a larger bias in estimated intake with n-alkanes than previously reported for these animals. This bias was lower (+3%) when using C_{32}:C_{33} as the markers than the -8% reported when using the C_{32}:C_{31} n-alkanes.

Under grazing conditions, as the herbage intake is unknown, the technique can only be evaluated by comparison with other techniques. Dove *et al.* (1989a) used grazing ewes to compare the estimation of intake using C_{33}:C_{32} and Cr_2O_3/*in vitro* procedures. In this, study different stocking rates were used

to achieve different intakes. Additionally, one group of ewes at each stocking rate was allowed a supplement while the others received no supplement. The results of this study showed, that at low intakes (high stocking rate) the alkane-based intakes were significantly higher than Cr_2O_3 intakes, while the reverse was true at high intakes. The authors (Dove *et al.*, 1989a) suggested this tendency was due to levels of intake not accounted for by the Cr_2O_3 intake method. Figures for apparent digestible organic matter and efficiency of microbial protein production were found to more closely correspond with ARC (1984) estimates when using alkanes rather than *in vitro* based estimates of intake (Dove *et al.*, 1988).

Dove and Mayes (1991) showed that when using the equation given by Mayes *et al.* (1986a), a difference in recovery of natural and dosed alkanes of 3%, the error in intake estimation will be 4.9%. Comparing this with a 13% error expected using the Cr_2O_3 intake method when digestibility is 0.8 it is apparent that the alkane-based estimates of intakes are more accurate than the Cr_2O_3 intake estimates. However, gross overestimation of intake using n-alkane markers have been reported in grazing deer (Mayes *et al.*, 1995) while more moderate overestimates of intake have been recorded in studies with grazing cattle (Wilkinson and Mackie, 1993; Horne, unpublished data).

1.5.1 Combining intake and digestibility measurements with alkanes

A separate estimate of digestibility is not a requirement of the alkane technique of intake estimation. However, it is often desirable to have a digestibility estimate in order to predict accurately, the nutritive value of the grazed herbage, or the calculation of digestible organic matter, to estimate DE or ME intake (Dove and Mayes, 1991). The alkane C_{35} is consistently 95-97% recoverable in sheep and may be a potential internal digestibility marker (Dove *et al.*, 1990). However, as the levels of this marker are low in many pastures a further external marker (Cr_2O_3 or C_{36}) is recommended for digestibility estimate (Dove & Mayes, 1991). A later study (Dove and Coombe, 1992) reported obtaining good estimates of digestibility when using C_{31} and C_{33} n-alkane markers.

1.6 Further uses of n-alkanes in herbivore nutrition

Due to the difference in alkane profiles of different plant species it may be possible, by the use of simultaneous equations, to estimate the botanical composition of consumed herbage (Dove and Mayes, 1991). This estimate of botanical composition could prove valuable particularly in estimating the nutritive value of grass/legume mixed pastures. The accuracy with which composition is estimated is effected by the similarity or difference between the alkane profiles of the species in question, the more different these profiles the greater the sensitivity of the estimation (Dove and Mayes, 1991).

1.7 Marker techniques applied to the horse

At the time of writing very little information is available concerning the specific use of markers in estimating herbage intake in the horse. However, similar markers to those used in ruminants for this purpose, have been used in rate of passage and digestibility studies in the horse.

1.7.1 Rates of passage of markers in the horses digestive tract

The anatomical differences in the digestive tract of the horse may be expected to confer different rates of passage of digesta and markers to those of other monogastric and ruminant species.

1.7.1.1 External markers

Vander Noot *et al.* (1967) measured Cr_2O_3 recovery in horses fed lucerne or timothy hay supplemented with either corn, barley or oats depending on treatment. Faecal recovery of the marker was reported as 83% for the first 48 hours and 99.6% after 96 hours. Hintz and Loy (1966) using styrofoam particles, recovered 995 of the marker after 63 hours with both pelleted and non pelleted rations. Alexander (1946) reported complete recovery of carbon granule markers after 48 hours while Haenlein *et al.* (1966) in contrast to Vander Noot *et al.* (1967) claimed 98% recovery of Cr_2O_3 within 48 hours. In these trials, the form of the ration did not appear to effect the rate of passage of the marker (Robinson and Slade, 1974). Based on these studies Vander Noot *et al.* (1967) proposed that a total faecal collection period of 96 hours (four days) should be adequate when using Cr_2O_3 markers but due to a large difference in recovery from individual animals this period may need to be extended. Cuddeford and Hughes (1990) observed that daily dosing with chromium-mordanted hay (96% recovery) produced a plateau in equine faecal marker levels within two days.

The use of external markers in horses has been mainly restricted to digestibility or rate of passage studies. However, Cr_2O_3 (McMeniman *et al.*, 1990), and chromium-mordanted fibre (Cr-fibre) (Barbisan *et al.*, 1993) have been used as faecal output indicators in recent intake studies with horses.

Diurnal variation in faecal samples, which has been a limiting factor in ruminant marker studies (reviewed by Langlands, 1975), has also been reported in horses when using Cr_2O_3 (Haenlein *et al.*, 1966; Parkins *et al.*, 1982) and for Cr-fibre (Cuddeford and Hughes, 1990).

1.7.1.2 Internal markers

While the physical form of the diet apparently does not effect the rate of passage of external markers, in many studies the physical form of the diet will effect the rate of passage of the diet, and hence the

passage of plant markers (Robinson and Slade, 1974). Pelleted diets move faster in the digestive tract than long hay (Haenlein *et al.*, 1966). Different sized particles move at different rates through the digestive tract (Hintz, 1975) while liquids move very rapidly through the stomach and small intestine to reach the caecum within two hours (Argenzio *et al.*, 1974). Alexander (1946) suggested that digesta in the equine tract spend only a third of the time that digesta spends in the ruminant tract.

The most commonly reported plant (internal) marker-substance used in recent equine studies is 4N-HCl insoluble ash (AIA). This marker has been used primarily as a digestibility indicator in the horse (Sutton *et al.*, 1977; Cuddeford and Hughes, 1990; McMeniman *et al.*, 1990; Cuddeford *et al.*, 1992; Barbisan *et al.*, 1993), and has been shown to give satisfactory estimates of the digestibility of equine diets in the above studies. McMeniman *et al.* (1990) found that for accurate digestibility estimates to be obtained using this marker, a minimum of 25g AIA/kg DM should be present in the diet.

AIA

Cuddeford and Hughes (1990) reported that the problem of diurnal variation in faecal AIA excretion was much less than when using Cr-fibre and was therefore a more reliable indicator of digestibility. Cr-based estimates of digestibility are generally underestimates while AIA was reported to give slight overestimates (Sutton *et al.*, 1977; Parkins *et al.*, 1982; Cuddeford and Hughes, 1990).

AIA

1.7.1.3 Combined external:internal marker studies to estimate intake in horses

McMeniman *et al.* (1990) used Cr_2O_3 and AIA as faecal output and digestibility indicators respectively, in order to estimate herbage intake in grazing horses. Although it is difficult to determine the accuracy of estimates made with grazing animals, the authors reported that individual estimates were obviously wrong. The reasons for these poor estimates was thought to be related to herbage sample variation in levels of AIA. The use of Cr-fibre and AIA to estimate DM intake in stabled horses showed that there was no significant difference between measured and estimated intakes (Barbisan *et al.*, 1993). In the same study the faecal output estimate made using a 15g dose of Cr-fibre gave an accurate estimate while a 30g dose of the marker produced a significantly different faecal output value than measured values.

Cuddeford and Mayes (1995) reported that the C_{32}:C_{33} combination of n-alkanes gave accurate estimates of intake in horses and ponies. However, no published data was available regarding the use of n-alkanes in the equine.

1.8 Discussion of literature

The fundamental difference between the n-alkane, and other marker techniques used to measure intake, appears to be in the chemical similarity of the external and internal n-alkanes. The advantage

of this similarity is that a single analytical procedure yields information concerning both internal and external marker concentrations. Exactly the same sample material is used in the analysis of the indicators, and both external and internal markers within a sample are subjected to exactly the same procedure. This similarity between the n-alkane markers also leads to the reportedly equivalent faecal recoveries of adjacent n-alkane markers, and therefore is vital to the success of the technique.

The faecal recoveries of n-alkanes are generally lower than that reported for other faecal markers. However, the similarity in faecal recovery of adjacent n-alkane pairs, ensures that the errors associated with these lower recoveries in the digestibility and faecal output estimates are equivalent. These errors, being equivalent, cancel each other out in the intake equation given by Mayes (1986), and therefore the intake estimate is not effected by the less than complete faecal recovery of the n-alkanes. Dove and Mayes (1991), in reviewing the use of n-alkanes reported that the errors associated with faecal recovery of n-alkanes in intake estimation are lower than errors obtained using markers with reportedly higher recoveries than those shown for the n-alkanes.

The C_{32} n-alkane is favoured in many of the reviewed ruminant studies as an external marker, due to the low concentration of this marker in many herbage types and the similarity in faecal recovery with that found for C_{33}, the most commonly used internal n-alkane. The C_{33} n-alkane is found in high concentrations in many herbage and in sheep trials the faecal recoveries of C_{33} and C_{32} were similar and consistent, even between trials.

The C_{35} n-alkane is highly recoverable (± 95%) in the faeces of sheep, and therefore may be expected to provide an accurate estimate of herbage intake when used with an adjacent even-chain n-alkane. However, the occurrence of this n-alkane in many herbage species is reportedly low, and therefore the C_{33}:C_{32} n-alkane pair is preferred in intake estimation trials. Due to the high recovery of C_{35} from the faeces of sheep this n-alkane has possibilities as an internal digestibility indicator. C_{33} and C_{31} can give good estimates of digestibility, provided the loss of marker in the faeces is taken into account. The consistent faecal recovery (± 86%) recorded for these markers in sheep would allow corrected digestibility estimates to be made.

The majority of the reviewed literature has shown that the C_{33}:C_{32} pair gives consistently accurate estimates of herbage intake in indoor ruminant trials. However, reports of obvious overestimates of intake in grazing trials are cause for concern when applying the technique in the field.

A problem under field conditions may be in obtaining a representative sample of herbage. The n-alkane profiles of plant species differs markedly and this may be responsible for errors where a representative sample of the diet is not available. The collection of such samples is made even more difficult by the fact that different parts of the plant, and plants of different maturity, may contain different n-alkane

profiles. It is also possible that individual animals within a species, will feed more selectively than others, leading to increased errors in intake estimates. However, this sampling problem is apparent with the use of nearly all plant markers and is not confined to n-alkanes.

Diurnal variation in faecal marker excretion has been well documented for chromium markers in both ruminants and horses. Although conflicting reports as to the extent of diurnal variation of n-alkane markers were apparent, total faecal collection and rectal grab samples taken during ruminant trials gave similar intake estimates. The extent of the diurnal variation problem under grazing conditions is unknown but if less than that observed with other markers, then the n-alkane technique would have a significant advantage over other marker techniques. The extent of the diurnal variation may be modified by the method of external marker application.

The extraction and analysis of n-alkanes is apparently simple and accurate. The main disadvantage of the process at present is that the use of n-alkanes in marker studies is relatively new and therefore preferred methods of extraction are evolving rapidly. As mentioned previously, only a single analytical procedure is necessary to quantify both the internal and external marker concentrations in a sample, an advantage over other marker techniques. This can be extended to include even more marker pairs if desired, and where possible, a highly recoverable n-alkane can be included to estimate herbage digestibility. While no published data was available regarding the use of n-alkanes to estimate herbage intake in horses, the data reported for ruminants showed no obvious reason why the technique should not be valid in this species.

A single unpublished study, reported that the C_{33}:C_{32} pair of n-alkanes gave accurate estimates of intake in horses and ponies (Cudderford and Mayes, unpublished data). However, this same study, and unpublished swine data did not support the principle observed in ruminants, that the longer the chain length of the n-alkane, the higher the faecal recovery of that alkane. The behaviour of the n-alkanes may differ slightly in non-ruminants than observed in ruminants. No data concerning the faecal recovery of n-alkanes from horses was available but one assumes that the recovery of the n-alkanes in the unpublished equine study was acceptable or an accurate intake estimation would not have been possible. Longer chain-length n-alkanes, whose high recoverability in ruminant faeces makes them suitable as digestibility markers, may not be as suitable for non-ruminant digestibility indicators if faecal recoveries are not sufficiently high.

Marker techniques applied to the horse have predominantly been concerned with evaluation of digestibility of diets rather than herbage intake and few studies in this regard have been reported. However, the markers used in ruminant studies are similar to those reported in equine studies and the problems encountered with these markers appear to be similar for both ruminants and horses. Diurnal variation in faecal excretion has been observed in both equine and ruminants when chromium markers

have been used. These markers are also recovered to a similar extent in both species. These similarities of marker behaviour suggest that provided the behaviour of n-alkanes is not markedly different in the equine digestive tract, the advantages of the n-alkane technique in ruminants should also be applicable to the horse. However, the diurnal variation problem, which deserves more attention in ruminant studies, has the potential to be a problem in the horse due to more rapid rates of passage of digesta. However, the possible reduced diurnal variation reported in ruminant studies with n-alkanes does suggest that a similar trend for horses is not improbable.

Internal markers used in studies with both ruminants and the horse have reportedly proven unreliable under certain conditions and the use of n-alkanes may improve intake estimates in both types of animals.

From the reviewed literature it appears that the n-alkanes may be used to provide an accurate estimate of herbage dry matter intake in ruminants and possibly horses. The technique has advantages over other marker techniques used in ruminant studies. These advantages include the simplicity of sample analysis to yield marker concentrations, the n-alkanes are readily recoverable as discrete entities, there is reduced diurnal variation in faecal marker excretion, and the estimates obtained are reportedly more accurate over a wide range of conditions than other techniques.

A comparison of common marker technique used for ruminants as well as horses, suggest that these advantages obtained using the n-alkanes in ruminants, may be applicable to the horse as well. However, studies similar to those carried out in ruminants are necessary to confirm this.

Despite the majority of studies confirming the good performance of the n-alkanes in providing intake estimates in indoor ruminant studies, certain studies, particularly under field conditions have reported results which were felt to have been overestimates. The lack of information regarding the technique, relative to other marker methods commonly used, suggests that more data should be made available before any definite conclusions concerning the use of the technique be made. This problem concerning the lack of information is also compounded by the fact that the majority of published work extolling the use of the technique has been compiled by only a handful of authors.

As information directly concerning the use of n-alkanes as intake indicators in the horse are almost non-existent it is evident that few conclusions in this regard can be made by referring to literature. The information available concerning the behaviour of other markers in the horse, and the similarity of these markers behaviour in ruminants, does infer that the n-alkane technique may be of use in the horse.

Studies to investigate the validity of the technique as means of estimating herbage dry matter intake in the horse are needed and are undertaken in the following study. Provided the technique proves to be valid in the horse, information regarding the herbage intake of horses at pasture may be more easily and accurately obtained, facilitating research in this field.

CHAPTER 2

LABORATORY PROCEDURES

2.1 Introduction

The basic approach used to extract and analyse the n-alkane content of herbage and faecal samples summarized by Dove and Mayes (1991), was used in the n-alkane trials of this study. This approach involves the addition of an internal standard (an alkane found in negligible quantities in herbage), to dried samples. This is followed by solvent extraction and saponification (Mayes *et al.*, 1986a), or direct saponification (Dillon and Stakelum, 1990). The saponified extracts are then cleaned and the n-alkanes separated according to chain-length by gas chromatography (Mayes *et al.*, 1986a).

While these basic procedures have been used with success in animal trials (Dove and Mayes, 1991), modification of certain elements of the procedure may improve the efficiency and accuracy of the technique (Vulich, 1994) as more published information becomes available. The method described below is that of (Marais, unpublished data). The method used however, is still evolving (Marais, personal communication) and slight modifications in technique were made during this study. These changes are discussed under the relevant headings.

2.2 Extraction procedure

The extraction procedure was initially carried out in the laboratories of the Department of Animal Science, University of Natal, Pietermaritzburg. During the course of the trial the site of extraction procedure was moved to the laboratories at the Department of Biochemistry, Cedara Agricultural Development Institute, due to problems with space, and to avoid the contamination of samples noted at the former facilities.

2.2.1 Preparation of internal n-alkane standard

Originally the preparation of the internal standard consisted of the addition of hexatriacontane (C_{36}) (0.0176g) to 50ml of hexane (Univar) so that 1ml of solution contained 352 μg of the n-alkane. The procedure was modified during the course of the trial (Chapters 3 and 4), but samples from these trials were re-extracted using the new procedure. Only the data collected in the preliminary trial were obtained using the above internal standard. The modified internal standard preparation involved the addition of 0.2g of C_{36} to an amount of undercane (Univar). After the n-alkane dissolved the undercane solution was made up to 100g, so that the 0.2 g of solution, added to each sample to be extracted, contained 4mg of C_{36}.

2.2.2 Extraction of n-alkanes from samples

Faeces (1g) and herbage samples (1.5g), which had been previously dried in a forced-draught oven (three days at 70° C), were added to 50ml, glass test tubes containing 0.2g of internal standard. Petroleum ether (80-100°C, BP) was added (40ml) to the test tubes and the tubes placed in a water bath at a temperature of 70° C for a period of two hours. The contents of the tubes were shaken at intervals during this period. Following removal from the water bath, the supernatant containing the n-alkanes, was decanted into 50ml glass beakers and the solvent removed by evaporation. The evaporation process was accelerated by using a warm air blower in conjunction with the fume cabinet.

The dried extract was reconstituted using hexane, or warmed petroleum ether (60-80°C, BP), and applied to a disposable polypropylene column (Supelco. Sue., Bellafonte. Pa., U.S.A). The column contained a 5ml bed volume of silica gel (Kieselgel 60, 70-230 mesh, Merck, Dormstadt, W. Germany). The n-alkanes were eluted from the column using 10ml of hexane and collected into test tubes. The eluted samples were then evaporated to dryness and re-dissolved in 0.3 ml hexane prior to gas-chromatographic separation.

2.3 Gas-chromatographic analysis of n-alkanes

2.3.1 Alkane calibration standards

The appropriate n-alkanes (Sigma) as well as the internal standard (0.006g of each), were dissolved in 6ml of hexane so that 1μl contained 1μg of each component. The n-alkane calibration standard was injected into the gas-chromatograph (Varian), and the machine calibrated to express the concentration of the n-alkanes relative to the internal standard.

2.3.2 Sample injection and analysis

The sample vials containing the dissolved n-alkanes were placed into an automated sampler (Varian). The sample (1μ) was applied to the column (megabore, 30m, 100% polysiloxine, 1.5 microns). The temperature programme was 120°C for three minutes rising to 240°C at 11°C per minute and reaching a final temperature of 298°C at 8°C per minute. Hold time was five minutes.

2.3.3 Calculations

The calculations and factors used in the determination of the n-alkane concentrations are listed below:

The concentration factor of the peaks, relative to internal standard (taken as one), was calculated as follows: Factor = area of unknown/area of internal standard.
In 1g faeces there is 0.4mg of internal standard and therefore 0.4 x 1000 mg of this in 1000 g of faeces. Thus the concentration of the unknown:
Concentration (mg/kg) = 0.4 x 1000 x area of unknown/area of internal standard.
Therefore 400 x factor for faecal samples (1g) and 266.67 x factor for herbage samples (1.5g).

2.4 External marker (C_{32}) preparation

The external n-alkane doses have successfully been administered to ruminants in the form of coated paper, formed into pellets (Mayes *et al.,* 1986), gelatin capsules (Vulich *et al.,* 1991), liquid suspensions (Marais *et al.,* 1993a), and reportedly by using controlled release devices (Mayes *et al.,* 1995).

The force-feeding of markers to horses, either in the field or in indoor studies may be difficult (Parkins *et al.,* 1982) and in preparing the n-alkane doses this factor was taken into consideration. The dosed marker was included in grass pellets, which were intended to be acceptable to the horses as they were, and easily incorporated in concentrate or supplement diets when necessary.

2.4.1 Preparation of (C_{32}) coated grass

The grass was coated using the same method given by Marais *et al.* (1993b) for preparing external marker doses in suspension form. Dried grass, Kikuyu (*Pennisetum clandestinum,* Hochst. Ex Chiov) was milled using a 0.5mm sieve. The n-alkane C_{32} (3g) was added to a rotary evaporator flask containing petroleum ether (60-80°C, BP). When the n-alkane had completely dissolved, 30g of the milled grass was added to this solution.

The solvent was removed by slow evaporation, under reduced pressure at a temperature of 60°C, allowing the attachment of n-alkane to the grass. All traces of the solvent were removed from the grass by leaving the grass in an oven at 60°C overnight, following which the grass was passed through a 1mm sieve to remove any lumps. The coated grass was then compressed using a hand press, into pellets of approximately 2g each. The daily doses for each horse was then weighed out (± three pellets) and placed into separate containers labelled with the exact mass of each dose.

2.4.2 Analysis of coated grass

Due to the very high n-alkane content of the coated grass pellets the extraction methods used differed slightly from that of normal samples. Again, the methods used are those of Marais, unpublished data.

The appropriate amount of internal standard was weighed out and dissolved in undercane (made up to 50g) so that 0.05g of n-alkane (C_{36}) was in 10g of standard. Coated grass (0.5g) was added to 10g of internal standard solution and 30ml of petroleum ether (60-80°C,BP), and subjected to the extraction procedure. The concentration of n-alkane (mg/kg) was calculated using 10000 × factor.

2.5 Discussion of the n-alkane extraction, analysis and administration procedures

The n-alkane analysis methods used in this study are theoretically simple, and in most cases easy and practical to apply. However, problems with the application of the methods did occur, and were a major limiting factor as regards the scope of this study. These factors are discussed below.

The major problem encountered was not caused by the methodology used in the extraction procedure but rather in the operation of the gas-chromatograph (G.C.). Samples from the preliminary trial were injected manually, which was time consuming but the results revealed no problems in the quantification of n-alkanes. However, due to a high volume of sample throughput, an automatic injection system was added to the G.C. This addition led to complications which resulted in failure of the machine to integrate the results (Escott-Watson, personal communication), and caused a lengthy delay in the throughput of samples.

Analysis of the samples from the main trial led to disappointing results. It was later discovered that this was attributable to two factors. A slight leak in the G.C. apparatus was discovered, which had interfered with resolution of the n-alkane peaks (Escott-Watson, personal communication) and contamination of the samples during the extraction process was suspected. The suspected contamination involved very high concentrations of the C_{25} and C_{27} n-alkanes (Marais, personal communication), and although these n-alkanes were not used in any of the calculations it was not known to what extent the contaminants effected the n-alkane concentrations used when making intake estimates. Numerous attempts to identify the contaminant were unsuccessful, but re-extraction of the samples at a different venue resulted in the apparent absence of the contamination.

The lengthy delay due to the G.C flaw was thus compounded by the time consuming re-extraction of samples. Further periodic delays in data collection, due primarily to problems with the G.C, were apparent throughout all of the trials, resulting in the delayed presentation of these results.

The method of external marker administration was a modification of that used by Marais *et al.* (1993b). Coated grass (C_{32}) was made into pellets by compression rather than included in the suspension used by Marais *et al.* (1993b). Administering the dose in suspension form, or even in capsule form may prove difficult with some horses (Parkins *et al.*, 1982). It was hoped that the coated-grass pellets would be accepted voluntarily by the horses, or that the dose could be included in a small amount of concentrate fed without the loss of the external marker. The pellets were readily accepted by all the horses in the indoor trials and the addition of maple syrup to the pellets helped to prevent marker loss. However, when the horses were returned to the pasture, or when the animals were receiving a large amount of concentrate, the pellets were not accepted, even by the same horses who had accepted the pellets in the indoor trials. In these cases the addition of the pellets to a small amount of concentrate feed (double handful) appeared to overcome this problem.

The process of compressing the coated grass into pellets was time consuming. It was also impossible, under the circumstances, to make the pellets exactly uniform in mass. The exact mass of each dose had to be recorded and taken into account when making the intake calculations, thereby making the calculations slightly more labourious. However, the acceptability of the pellets in the indoor trials ensured that little difficulty in administering the dose was encountered, an important factor to consider, as individual horses may object to other methods of dosing. The unacceptableness of the pellets under certain circumstances was overcome by inclusion of the pellets in a small amount concentrate feed. Providing the concentrate contains little of the dosed n-alkane, the intake estimates should not be effected. Pellets of coated grass appear to be a simple, and in most instances, a practical method of n-alkane administration.

Although apparently giving acceptable results in this study, the methods used for the extraction and analysis of n-alkanes in these trials are still evolving (Marais, personal communication). The extraction process, while theoretically and practically simple to perform, proved to be labourious and time consuming. The source of contamination of samples found in the trials, which appeared to be due to contact with a substance that was susceptible to the solvents used, (for example plastics, parafilm) was not discovered. However, re-extraction of the samples at a different venue (Cedara Agricultural Development Institute) resolved this problem. The facilities at the latter venue were dedicated to the purpose of n-alkane analysis, while the initial extractions had taken place in facilities used daily for a variety of tasks.

The contamination of samples, and the possible sources of contamination, are not often mentioned in the literature but must be considered if extractions are not carried out at an existing n-alkane analysis laboratory.

The delays encountered due to the G.C. analysis of the extracted samples were unfortunate as the main reason for these delays was that new equipment was being used. Even after the initial problems appeared to be resolved, further problems with the G.C throughout this study were encountered, despite the regular attention of a technician. The running of a G.C is a fairly specialised task, therefore if similar studies are to be undertaken it is suggested that an existing, fully functional G.C. unit be made available for the analysis of the n-alkanes.

CHAPTER 3

A PRELIMINARY TRIAL DESIGNED TO EVALUATE THE N-ALKANE INDICATOR METHOD OF ESTIMATING HERBAGE INTAKE IN THE HORSE

3.1 Introduction

Having little information on the use of the technique in the horse and the inexperience of staff in carrying out research on these animals at the University's facilities, it was decided to perform an exploratory experiment. The aims of the experiment were to test whether the methodology employed was applicable to the horse and to identify any practical difficulties in carrying out such experiments with the horse under the given conditions, prior to the undertaking of more detailed trials.

3.2 Materials and methods

The experiment was carried out at Ukulinga research farm (University of Natal, Pietermaritzburg).

3.2.1 Animals and Housing

Outbuildings which were previously unused, were made suitable for the housing of the horses. The stables contained large stone mangers and were provided with large water toughs for the period of the trial. Bedding during the trial period consisted of broad strips of thick rubber matting to facilitate faecal/ort separation while providing a stable and comfortable surface for the animals .

The two animals used in this preliminary trial were mature thoroughbred geldings (514kg and 440kg) previously adapted to a Kikuyu diet.

3.2.2 *External marker administration*

Previously prepared pellets (3x2g) containing the external marker n-dotriacontane (C_{32}) were fed to each horse immediately prior to the morning meal. The exact mass of this daily dose was determined prior to each application and a sample of each days dose was retained for n-alkane determination.

3.2.3 Feeding

Kikuyu grass was harvested each moming using a sickle bar mower. Approximately 8kg of fresh grass was weighed out and fed to each horse immediately, with the remainder of the harvest being stored in a cold room for the noon and evening feeds. The noon feed consisted of approximately 8kg and the evening feed 10kg at the beginning of the trial but these figures were adjusted as the voluntary intake patterns for the two horses became apparent. Leavings (orts) were collected, and weighed

immediately prior to each fresh meal to enable measurement of fresh herbage intake. Samples of grass and orts were taken each day for dry matter (DM) determination and n-alkane analysis.

3.2.4 Faecal collection

From the second day of the experiment a total faecal collection was carried out. A sample was taken from each horse's daily output for n-alkane analysis. As fairly large amounts of wet faecal matter were produced, pooling of the faeces consisted of taking a sample of no specific mass from each distinct defecation.

3.2.5 Sample treatment

All samples were dried in a forced-draught oven (70°C) for three days before being milled (0.5mm screen) and subjected to n-alkane analysis.

3.2.6 Calculations

The equation to calculate herbage intake using n-alkanes was taken from Mayes *et al.* (1986a).

$$HI = \frac{Fi/Fj \times D}{Hi - Fi/Fj \times Hj}$$

where: HI = herbage intake (kg DM/day)

Hi and Fi are the respective concentrations of natural odd-chain n-alkanes in the herbage and faeces (mg / kg DM).

Hj and Fj are the respective concentrations of even-chain n-alkanes in the herbage and faeces (mg/kg DM).

D is the amount of n-alkane dosed by pellet (mg/day)

Faecal recovery of n-alkanes reported in Table 2 was calculated as follows :

$$\text{Recovery} = \frac{\text{Faecal DM output (kg)} \times \text{faecal n-alkane concentration (mg/kg)}}{\text{Herbage DM intake(kg)} \times \text{herbage n-alkane concentration (mg/kg)}} \times 100$$

3.3 Results and discussion

Table 3.1: Daily fresh grass and dry matter (DM) intakes recorded over the seven day collection period

	Horse 1		Horse 2	
Day	Fresh (kg)	DM (kg)	Fresh (kg)	DM (kg)
1	24.2	5.9	11.3	2.87
2	21.4	5.7	14.2	4.1
3	14.3	3.8	13.1	3.7
4	17.9	4.8	19.8	5.6
5	18.4	5.0	18.9	5.1
6	16.9	4.5	15.1	4.2
7	11.4	3.2	11.7	3.3

The wet grass and DM intakes were lower than was expected for horses of these masses (NRC, 1989). Observation of their behaviour may reveal the reason for this. The horses ate steadily for approximately 20 minutes on receiving fresh or chilled grass, after which only periodic visits to the manger occurred, and only a few mouthfuls of grass were taken in during these latter visits. After the first hour since feeding very little grass appeared to be consumed and the remains were collected as orts. Only the evening feed resulted in few orts the next morning due to a long between-meal period (16 hours). A possible reason for the decreased intake after the initial bout of eating (20 min) may have been due to decreasing palatability of the grass when left unchilled. High gut fill may be partially responsible for the initial decline in intake post-feeding but does not appear to be the major factor as even during the longer period after feeding (evening) some grass was left. Smaller, more frequent feeding would be preferable to infrequent large meals but would require intensive labour. The horses were also unaccustomed to living indoors and this may have contributed to poor intake.

The initial period showed that after only two days the excretion of the external marker appeared to reach a maximum and faecal levels remained fairly constant hereafter. Cuddeford and Hughes (1990) observed that chromium-mordanted hay fed to horses resulted in a similar plateau in faecal chromium levels to that observed in this trial using an n-alkane marker. However, few other studies report this sort of information. These faecal concentrations of external marker are similar to those of faecal internal marker concentrations as can be seen in Table 3.2.

This similarity in faecal concentration is encouraging as it suggests that the chosen concentration of external marker was in fact adequate. This concentration was chosen on the basis of preliminary n-alkane trials performed with cattle (Marais, personal communication). While the dose was applicable

to the horses in this trial, the level of dosing may well have to be adjusted if intakes were to significantly change in future trials.

Recovery of markers in the faeces is of paramount importance to the success of the technique. The markers should ideally be completely indigestible in the digestive tract. However, due to similar behaviour of the closely related markers a slight loss of marker is acceptable provided this loss is equivalent to both the external and internal n-alkane markers. The equation given by Mayes *et al.* (1986a) allows errors in recovery to cancel out provided the above condition is met. Recovery of the considered n-alkanes is included in Table 3.2. The markers C_{31} to C_{35} were highly recoverable in this study and the C_{33} and C_{32} n-alkanes had almost identical recoveries. The recovery of the n-alkanes were higher than those reported in ruminant studies (Dove and Mayes, 1991) and were almost complete. However, the very high recovery of the C_{34} n-alkane, and the fact that the data was collected using only two animals suggests that further studies are needed before any conclusions can be drawn with regard to n-alkane recovery in the faeces of horses. The apparent similarity in recoveries of the C_{32} and C_{33} n-alkanes is encouraging in that the conditions for the valid use of the intake equation of Mayes *et al.* (1986a) appear to exist in the case of the horse.

The comparisons between actual intake of the horses and the estimates of intake calculated using the n-alkane marker technique are presented in Table 3.3 In the case of both horses, the discrepancy between mean intakes was negative. In other words, the technique overestimated the actual amount consumed. However, within-day discrepancies were found to be both positive and negative in the case of both test animals. Large negative discrepancies at the beginning of the trial may have been partially aggravated by the method of dosing. The form of the external marker application was chosen with ease of use in mind. Daily force-feeding of capsules or naso-gastric intubation with prepared solutions is both difficult and stressful to the animals (Parkins *et al.,* 1982) and would as such be unpopular if valuable animals participating in on-farm studies were to be used. The prepared pellets in this study were voluntarily accepted and consumed when hand-fed, but on occasion small fragments of pellet escaped from the mouths of the horses. This occurred during the first two days of the trial, but was apparently overcome by the addition of maple syrup to the pellets.

Table 3.2: Mean concentration (mg/kg DM) of n-alkanes in herbage (*P. clandestinum*) and faeces of individual horses, and percentage recovery of the n-alkanes

	C31	C32	C33	C34	C35
Grass	99.5 ±12.2	14.5 ±1.4	154.5 ±4.5	8.2 ±1.6	126.9 ± 13
Horse 1 Faeces	178. 5 ±46.4	233.0 ±85.9	284.3 ±78.1	13.6 ±3.12	233.5 ± 61.0
Horse 2 Faeces	195.5 ±32.5	269.5 ±52.1	295.1 ±47.0	12.28 ±2.07	208.2 ± 41.3
mean recovery %	97.3 ±11.2	100.4 ±14.0	100.1 ±1.4	115.3 ±2.3	100.4 ± 3.0

The mean, daily overestimate of intake using the technique was 12.43% and 10.93% for horses one and two respectively. The differences between these mean intakes and intake estimations were not statistically significant ($p>0.05$). This suggests that the technique may give a reasonable estimate of herbage intake in the horse. The overestimation of intake may be explained in part by the choice of n-alkane pairs used in this trial. Mayes *et al.* (1986a) suggests that, due to the slightly higher recovery of the longer chain n-alkanes found in some studies (Mayes and Lamb, 1984), an overestimate of intake may occur when the external marker is of lower chain length than the internal marker.

Table 3.3: Mean herbage DM intakes and intakes estimated using the C_{33}:C_{32} n-alkane pair. The daily variation in actual and measured intakes, as well as the difference (discrepancy) between hem are listed

Horse1	Day	Actual Intake (Kg)	Estimated Intake (Kg)	Discrepancy (Kg)
	1	5.9	6.5	-0.6
	2	5.7	7.7	-2.01
	3	3.8	5.5	-1.62
	4	4.8	4.1	0.7
	5	5.0	4.9	0.08
	6	4.5	4.3	0.26
	7	3.2	4.1	-0.92
	mean	4.72±0.958	5.31± 1.38	-0.587
Horse2	1	2.8	5.8	-3.07
	2	4.1	4.6	-0.55
	3	3.7	4.5	-0.787
	4	5.6	4.1	1.56
	5	5.1	4.5	0.62
	6	4.2	4.0	0.15
	7	3.3	4.4	-1.07
	mean	4.12 ±1.01	4.55±0.608	-0.449
overall		4.14±0.998	4.93±0.99	-0.518

As observed in similar equine studies (Dugan *et al.*, 1993), horses can be, and in this case were, 'messy eaters' in that they would scatter grass during each meal while searching for the more palatable fractions of grass. The grass scattered in this way was often trampled and while much effort was directed at the complete collection of orts, a small wastage factor was present. The problem of ort collection was compounded by use of large water troughs, as the horses deposited grass into the water while feeding and drinking. The use of rubber matting as bedding proved satisfactory for ort collection but the removal of urine and faecal residue from the matting required the daily hosing down and drying of the stables. This was both time and labour consuming even with only two horses. The use of more absorptive bedding may increase the difficulty of ort collection but may have time and labour saving benefits.

Faecal collection could be made more readily using special harnesses and bags (Parkins *et al.,* 1982), but the horses used in these trials would need to be docile and not inclined to remove such bags. Ideally, metabolic crates contained in a suitable building should be used for these experiments although by the end of the stable confinement the horses were showing signs of irritability, which may be compounded by the very close confinement of metabolic crates.

In this trial only sufficient grass was coated (with C_{32}) initially to cover a third of the trial, with the rest being prepared in two further batches, during the trial. Samples from each batch were taken and n-alkane concentrations within and between batches compared (Table 3.4).

Table 3.4: Comparison of concentrations of C_{32} found in different batches of pellets (n = 5)

	Batch1	Batch2	Batch3	Overall
	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Mean	92474.5[a]	76656.2[b]	94146.2 [a]	87758.9
s.e	9705.60	4897.90	6760.20	7121.231
C.V %	10.49	6.38	7.18	8.01

* Concentrations of pellets reported without superscripts in common are significantly ($p<0.05$) different from one another.

Analysis of the prepared pellets revealed that no significant differences in n-alkane concentration within a batch of prepared grass was evident, and that the coefficients of variation within batches was small. However, significant ($p<0.05$) differences between batches did exist. This had to be taken into account when calculating estimated intakes. Preparation of sufficient coated grass to cover the entire trial period, and thorough mixing of this prior to the pellet preparation should overcome this problem.

3.4 Conclusions

The n-alkane technique using the C_{33}:C_{32} chained pair appears to give a reasonable estimate of fresh grass intake in the horse using the described methodology. The marker levels in the faeces suggest that the preliminary dosing period need take place only two to three days prior to the beginning of sample collection. The faecal recovery of markers, while suspiciously high, was also encouraging as recovery of the external and internal markers used were identical, a prerequisite for the validity of the technique (Mayes *et al.,* 1986a). The above trial was conducted primarily to gauge the practical implications of applying the technique to the horse under the given conditions. The low number of animals used in this trial, and the lack of available data concerning the horse and n-alkanes, prohibits the drawing of any

definite conclusions concerning the use of n-alkanes in determining herbage inta results, however, were encouraging and led to more detailed experiments aimed validity of the n-alkane techniques ability to provide accurate estimates of herbage i

The experiment was a success as it confirmed the potential of the technique with regard to the horse. This trial also highlighted practical problems and ways in which these could be overcome, as well as confirming the adequacy of certain aspects of the methods used which were unknown prior to this. The results and observations made in this trial allowed smoother and more accurate execution of the following experiments.

CHAPTER 4

N-ALKANE MARKER PASSAGE THROUGH THE DIGESTIVE TRACT OF THE HORSE

4.1 Introduction

Information describing the behaviour of n-alkane markers in the digestive tract of ruminants has become available (reviewed by Dove and Mayes, 1991) but at the time of writing no similar information regarding these substances was available for the horse.

The aim of this study was to describe the faecal excretion of the markers, in particular the dosed (C_{32}) marker, in order to obtain information which would enable increased precision of the technique in the field. It was hoped that the rate of passage of the n-alkane marker dose could be determined and that the extent of diurnal variation in marker excretion could be observed.

The data collection occurred in two experiments. Part A consisted of data collection during the preliminary and post treatment phases of a total faecal collection trial (reported in Chapter 5) while Part B consisted of a separate, dedicated trial following on from the total collection trials. The results of these trials are reported separately yet discussed as one.

4.2 Part A

4.2.1 Materials and methods

4.2.1.1 Animals

Four mature geldings were stabled at the University of Natal's research farm in Pietermaritzburg in the accommodations described previously.

4.2.1.2 Feeding

The data collection was carried out when the animals were consuming either fresh Kikuyu or Kikuyu hay. The feeding regimen was carried out as described in the preliminary trial.

4.2.1.3 Dosing and sample collection

Initially a single dose containing approximately 600mg of C_{32}, in the form of coated grass pellets (described previously) was administered to each horse at the time of the first meal. Faecal samples from the horses were collected at three hourly intervals for the following 24 hours and analysed for

n-alkane content. Following the 24 hour period daily dosing continued until the end of the total faecal collection trials.

On the fourth and fifth days of the total faecal collection trials, faecal samples were taken at 08:00, 12:00 and 16:00 to gauge diurnal variation in n-alkane content.

Faecal samples were collected 24 hours after the last dose of C_{32}, and further samples were taken at regular intervals up until 57 hours after the administration of the final dose, in order to determine the time taken for faecal C_{32} to return to a baseline level.

4.3 Results

4.3.1 Single dose

The levels of C_{31}, C_{32} and C_{33} and relative proportions of C_{32}:C_{31} and C_{32}:C_{33} were plotted over time for each of the individual horses and for each diet (Figures 4.1 and 4.2 and Figures 4.3 and 4.4 respectively). In the fresh grass trial (Figures 4.1 and 4.2) the C_{32} levels remained at baseline for up to fifteen hours following dose administration. Thereafter, levels appeared to increase slowly for the following three hours after which faecal C_{32} and proportion of C_{32} relative to the internal n-alkane levels increased more rapidly up to 24 hours. However, the C_{32} concentrations in the faeces did not appear to reach a maximum during the 24 hour period.

A similar trend in C_{32} levels was observed when the diet consisted of hay (Figures 4.3 and 4.4). The faecal concentrations of C_{32} increased as did the proportion of C_{32} to internal markers during the 24 hour period. However, the mean faecal concentrations (mg/kg) and proportions of C_{32} to the internal n-alkanes were lower ($p<0.05$) when horses were consuming hay than those observed when the animals were consuming fresh grass

In the case of both the fresh grass and hay diets, the levels of faecal C_{33} were consistently higher than C_{31} levels. While the levels of both internal markers fluctuated during the measurement period, it was evident that the correlation between faecal concentrations of C_{31} and C_{33} were high ($r^2=0.872$ for grass and $r^2=0.906$ for hay).

4.3.2 Excretion after termination of C_{32} marker applications

The rate of C_{32} excretion following the final C_{32} marker dose given in the total collection trials is plotted for individual horses in Figure 4. 5 (fresh grass) and Figure 4. 6 (hay).

In the case of the fresh grass the levels of C_{32} and proportion of C_{32} to internal markers appeared to rise slightly up to 32 hours after the final dose was given. The levels then gradually declined to approach baseline at approximately 56 hours. Horse three (Figure 4.5), was kept confined longer than the other horses and faecal levels of C_{32} were monitored for a total of 108 hours after the final dose had been given. In this case the baseline level of C_{32} was reached between 48 hours and 56 hours and remained at these levels thereafter.

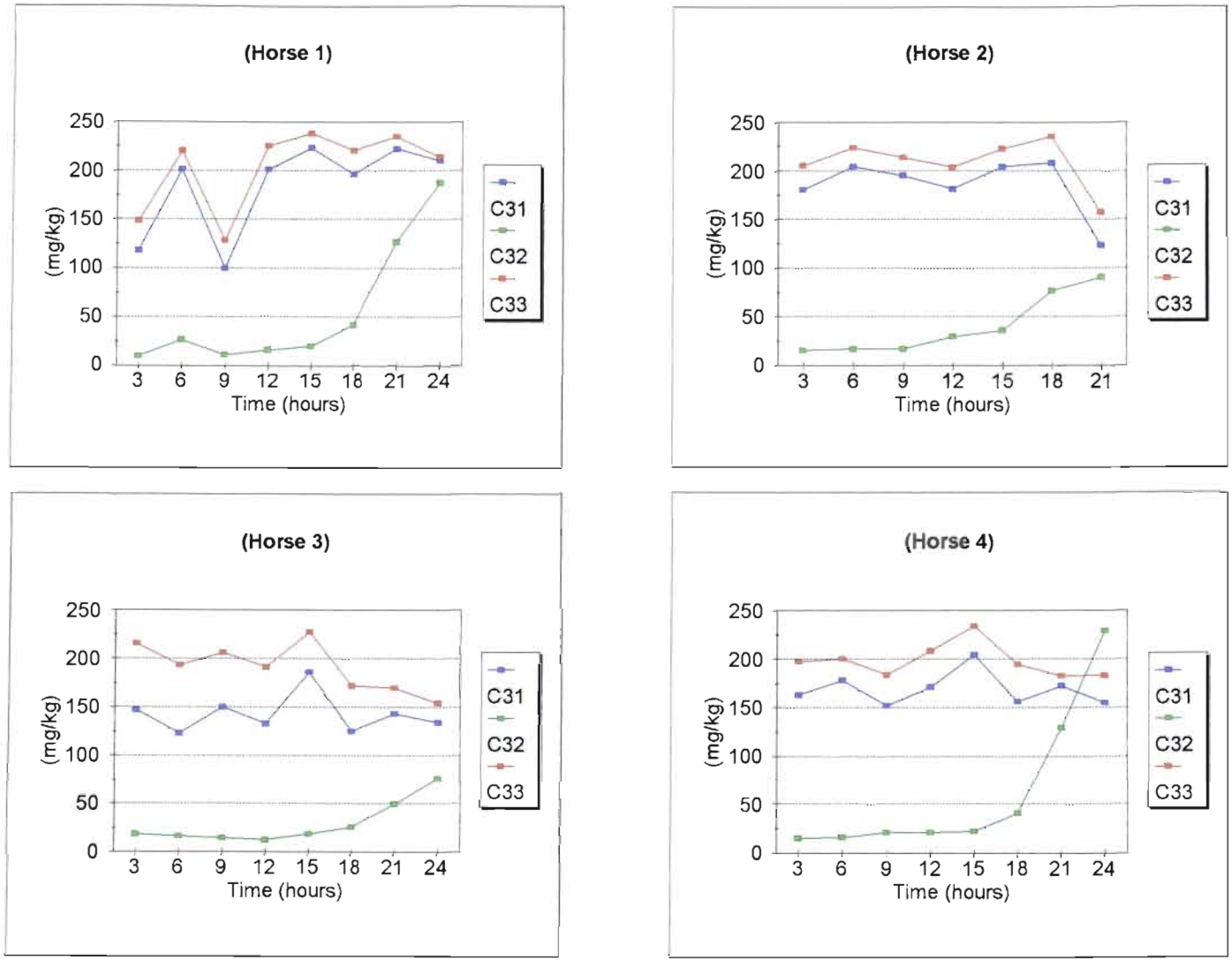


Figure 4.1: Concentration (mg/kg DM) of external marker C_{32} in the faeces of horses (1-4), following a single C_{32} marker dose. The horses were consuming fresh grass (*P. clandestinum*).

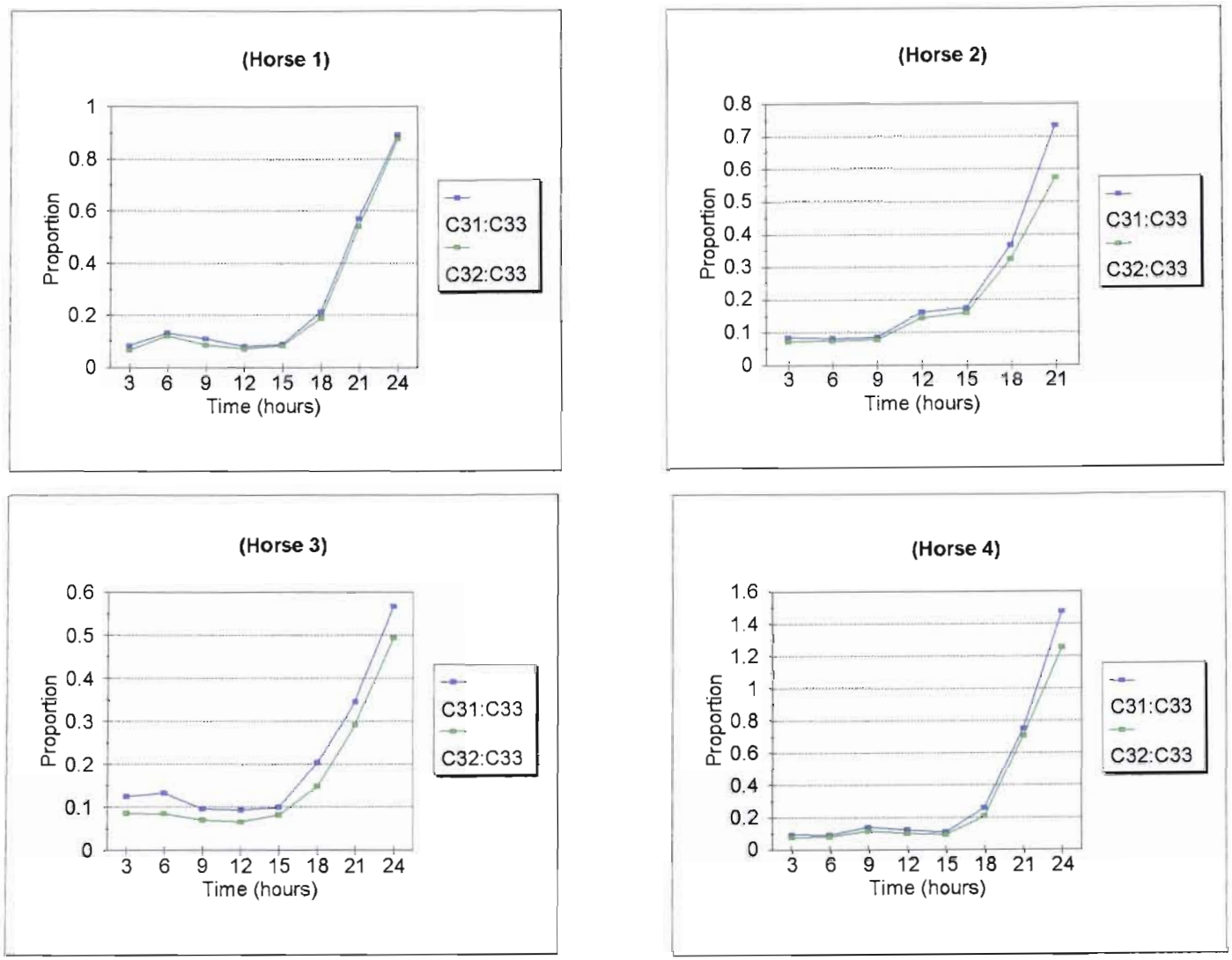


Figure 4.2: Proportion of external marker C_{32} relative to internal n-alkane markers (C_{31} and C_{33}) in the faeces of horses following a single C_{32} marker dose. The horses were consuming fresh grass (*P. clandestinum*).

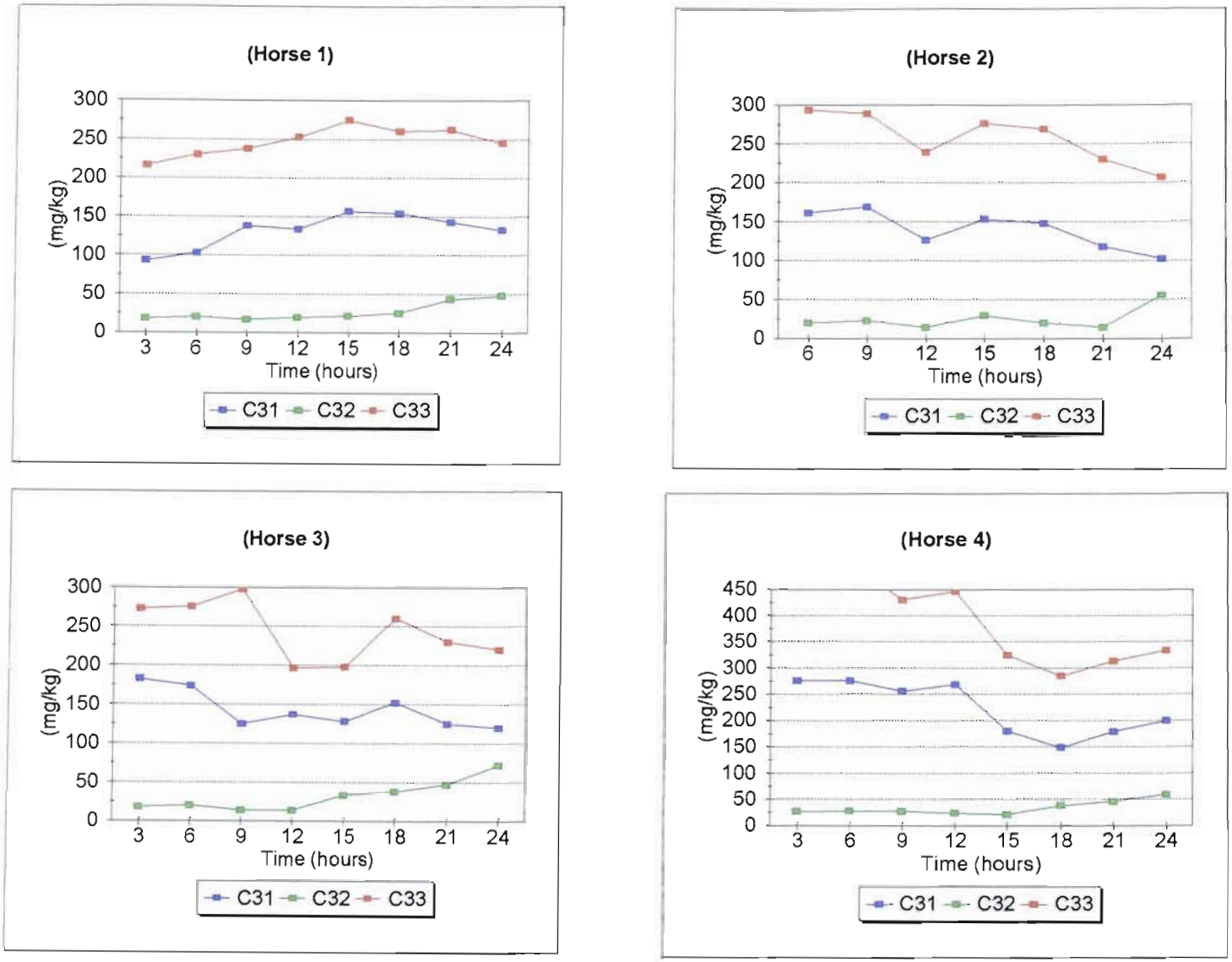


Figure 4.3: Concentration (mg/kg DM) of external marker C_{32} in the faeces of horses (1-4), following a single C_{32} marker dose. The horses were consuming grass hay (*P. clandestinum*).

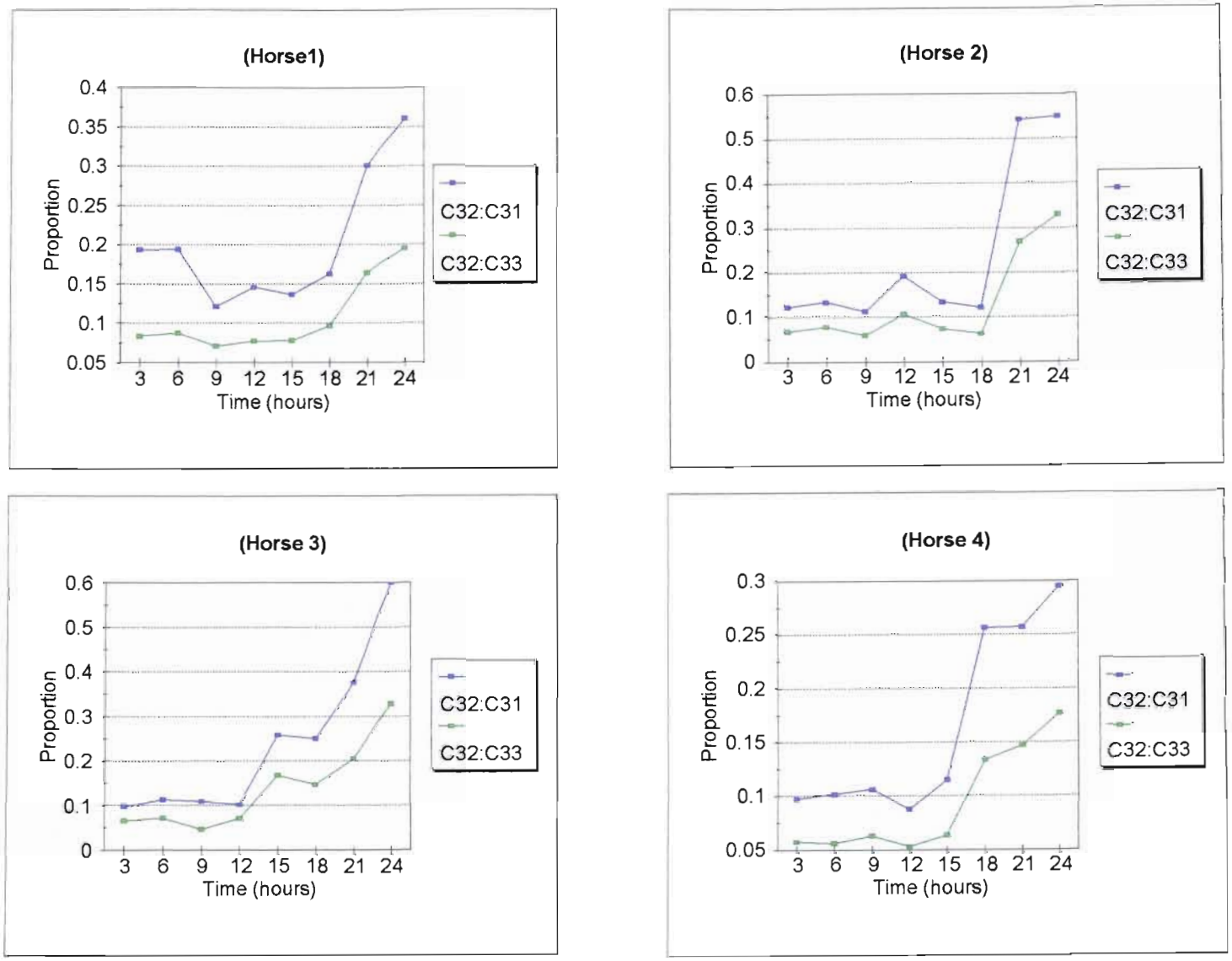


Figure 4.4: Proportion of external marker C_{32} relative to internal n-alkane markers (C_{31} and C_{33}) in the faeces of horses following a single C_{32} marker dose. The horses were consuming grass hay (*P. clandestinum*).

The pattern of faecal C_{32} excretion in the hay trial was similar to that when the horses were consuming fresh grass. However, the rise in C_{32} levels up to 32 hours was only observed in one of the animals. The remaining animal's faecal C_{32} levels showed a gradual decrease continuing from 24 hours after final dosing to 56 hours where levels, although approaching baseline, may not have completely reached these values.

4.3.3 Diurnal variation

The differences in proportion of C_{32}:C_{33} measured in faecal samples taken at different times of the day is shown in Figures 4.7(a) and 4.7(b). The proportion of C_{32}:C_{33} in the faecal samples varied from day to day and according to the time of day at which the samples were taken. On the first sampling day of the fresh grass trial (Figure 4.7 (a)), the proportion of C_{32}:C_{33} in faecal samples appeared to vary little between the 8h00 and 16h00 collections while the 12h00 C_{32}:C_{33} ratio differed to a greater degree. The variation in C_{32}:C_{33} at the different times was lower during the second day of collection than the first.

In the hay trial (Figure 4.7(b)) the situation was similar in that the samples taken on the second day of collection showed less variation in C_{32}:C_{33} ratios than those from the first day. As with the fresh grass trial, the 08h00 C_{32}:C_{33} ratios corresponded more closely to the 16h00 samples than samples taken at 12h00.

In both the hay and fresh grass treatments the difference in proportions of C_{32}:C_{33} was greater between days than within days. The mean coefficients of variation for the n-alkane proportions in daily samples were less than 10% except on day one of the hay trial (13.8%).

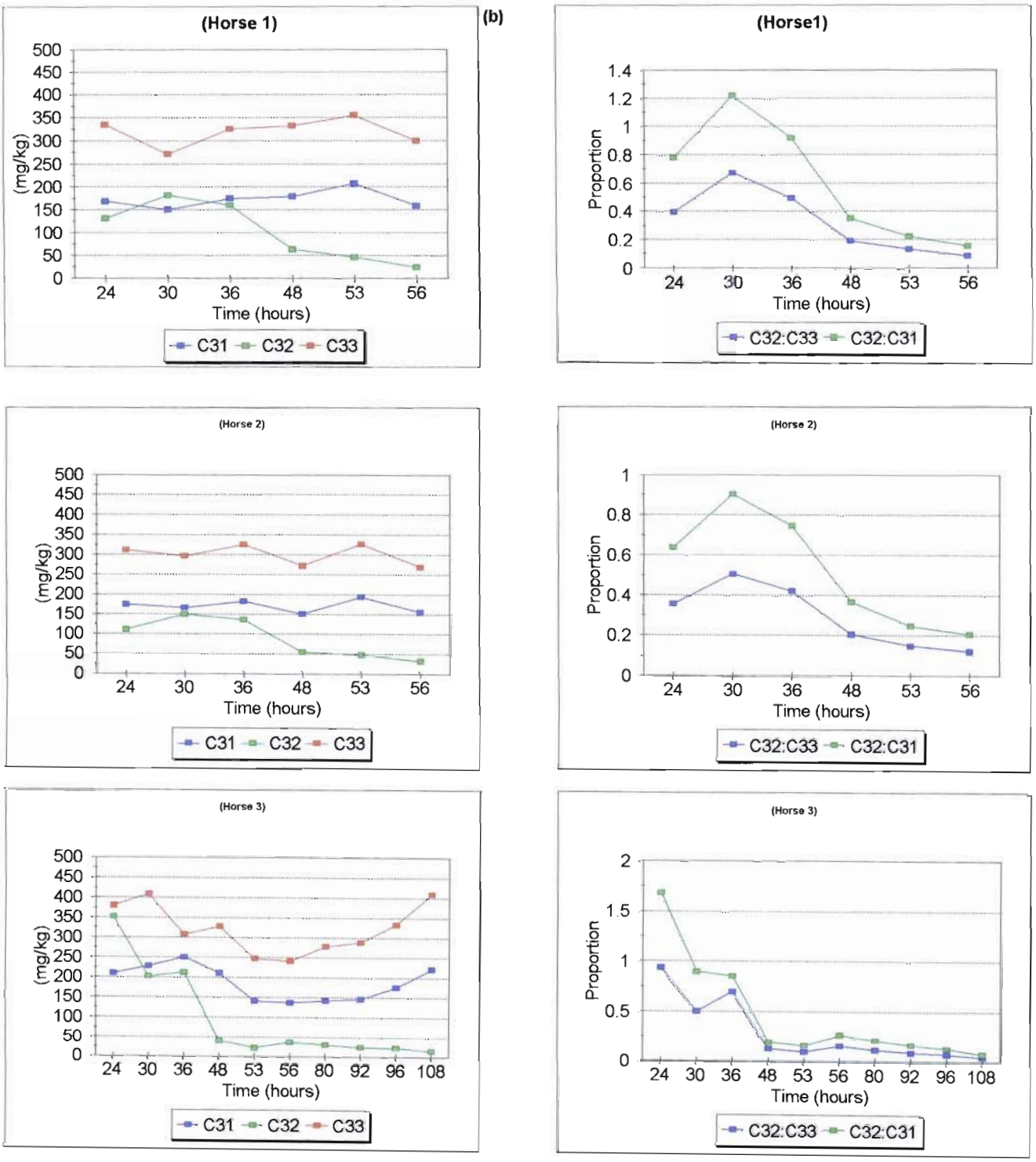


Figure 4.5: Concentration of external (C_{32}) marker and internal (C_{31} and C_{33}) markers in faecal samples (a) and ratio of C_{32} to internal markers (b). The horses (consuming fresh grass) were given the last of a series of daily C_{32} doses at 0 hours and collection began 24 hours later.

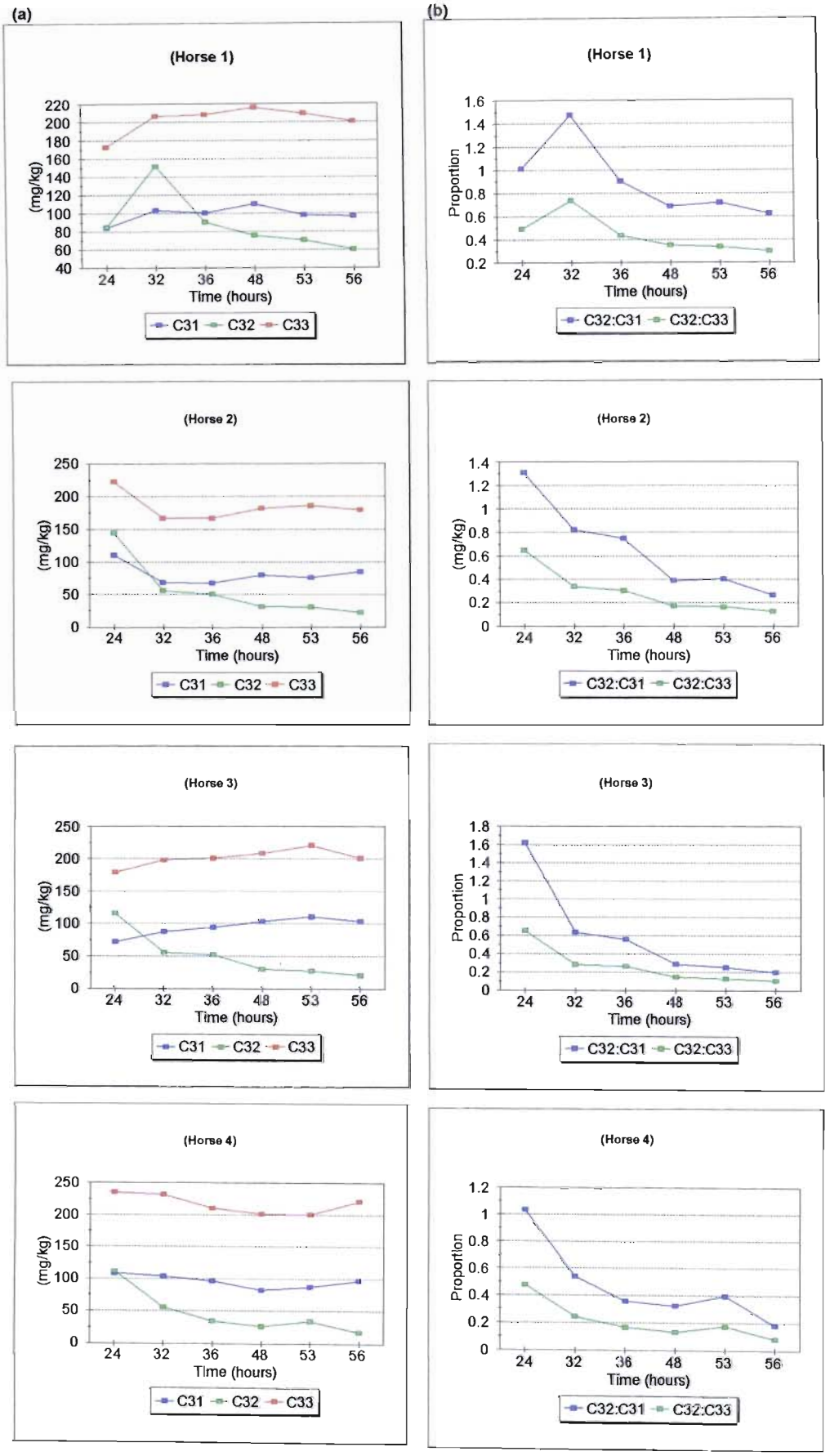
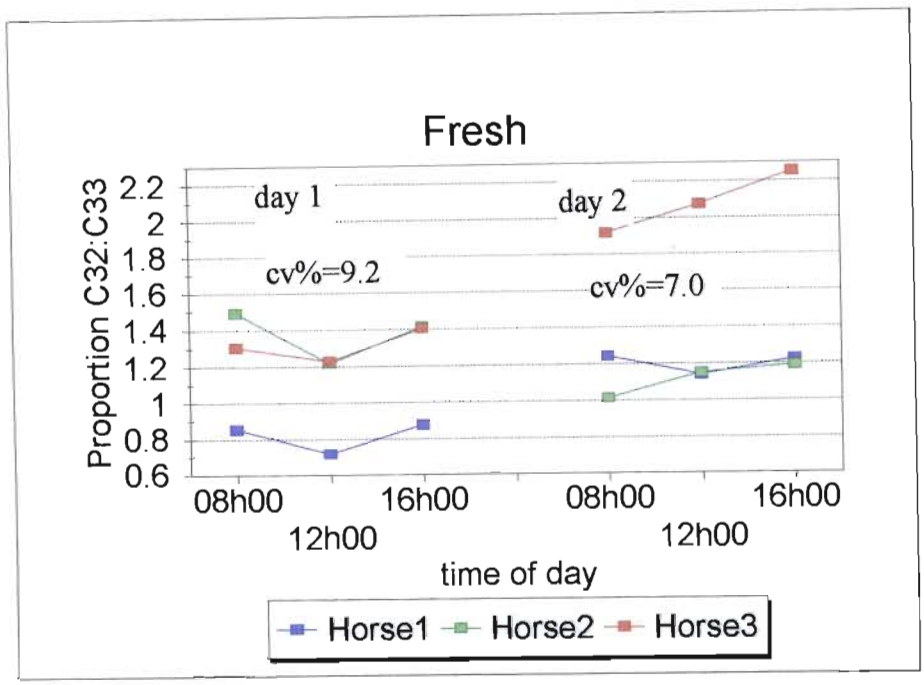


Figure 4.6: Concentration of external (C_{32}) marker and internal (C_{31} and C_{33}) markers in faecal samples (a) and ratio of C_{32} to internal markers (b). The horses (consuming grass hay) were given the last of a series of daily C_{32} doses at 0 hours and collection began 24 hours later.

(a)



(b)

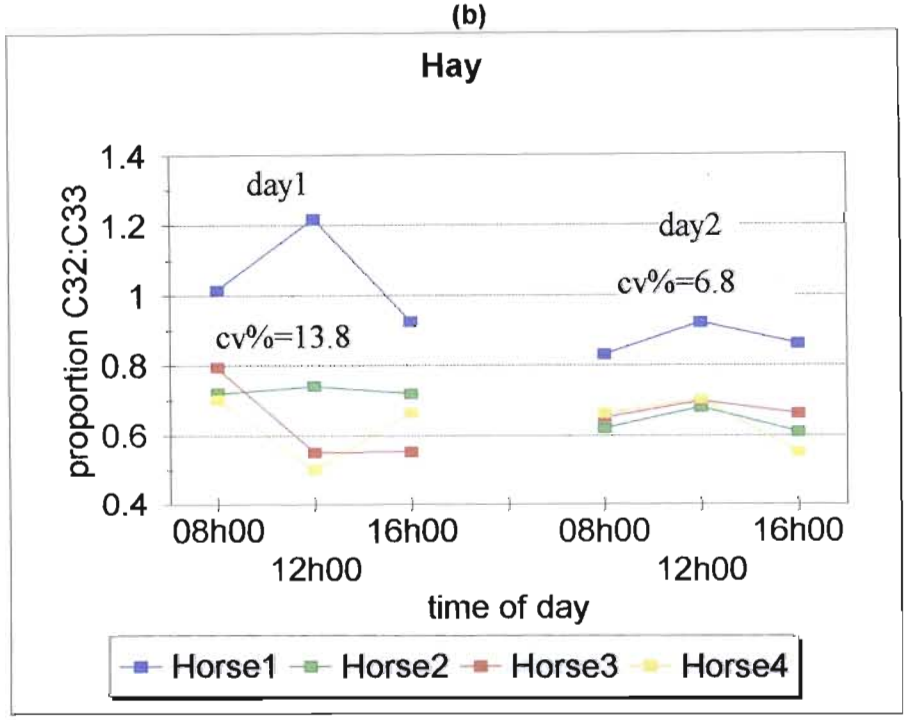


Figure 4.7: Proportion of C_{32}:C_{33} in faecal samples taken at 3 different times during two consecutive days. (a) Represents samples collected from horses consuming fresh grass while (b) represents data collected when the horses were consuming hay. Mean coefficients of variation (cv%) for proportion of C_{32}: C_{33} in daily faecal samples were 8.08% and 10.33% for the fresh grass and hay diets respectively.

4.4 PART B

4.4.1 Introduction

The data collected in Part A was collected during the course of a trial which required daily dosing of the external marker and as such prevented a more lengthy following of the appearance a single dose of C_{32} in the faeces. It was also felt that more information regarding the effect of continuous C_{32} daily dosing, and diurnal variation in faecal-grab sample C_{32} levels relative to internal markers would be valuable, and therefore a further experiment was implemented.

4.4.2 Materials and methods

The experiment took place on a small thoroughbred stud farm in Cape Town.

4.4.2.1 Animals and feeding regimen

Six thoroughbred horses (four colts and two fillies), were divided into two groups of three for these separate investigations. Both prior to, and during the trials, the horses were stabled during the night, exercised in the morning, after which they were placed in paddocks and allowed to graze for the remainder of the day. The horses in these experiments were in training, and were receiving between four and six kilograms of a commercial concentrate meal in two separate meals, as well as having access to lucerne hay at meal times and pasture (predominantly Kikuyu) throughout the day.

4.4.2.2 Dosing and feeding

The horses were divided into two groups for dosing. Group 1 horses were given one single dose of the external marker C_{32} (at 0 hours), while Group 2 horses were given a single, daily dose of the marker for the period of the trial. The doses consisted of the coated grass pellets described earlier mixed in with the concentrate feed at meal times.

4.4.2.3 Sample collection and analysis

Faecal sample collection for horses in group one began 4 hours after a single administration of C_{32} pellets and continued at four hourly intervals for 72 hours. Faecal grab samples were collected from group two horses, sixteen hours following the first dose, and then at four to eight hour intervals for a total of 120 hours. After 90 hours, further samples were taken throughout the day at four hour intervals to observe diurnal variation in faecal excretion of the markers.

4.5 Results

4.5.1 Single dose

The levels of C_{32} and proportion of C_{32}:C_{33} over the collection period are presented in Figure 4.8 and shows that both the concentration (mg/kg) of C_{32} and proportion of C_{32}:C_{33} increased in the faeces over time following the introduction of the marker pellets into the digestive system. The increase in faecal concentration occurs slowly over the first eight hours in all horses after which more rapid increases occurred. Peak levels were different for each horse and occurred at different times after receiving the marker dose. However, peak levels in all horses occurred at between twenty and 32 hours post-marker administration. Both the concentration of C_{32} and proportion of C_{32} to C_{33} declined rapidly after reaching peak levels and began to level off to approximately baseline levels between 48 and 64 hours. The cumulative excretion curve (Figure 4.9) was obtained by expressing, for each individual horse, the faecal concentration of C_{32} in the timed samples as a percentage of the total collected marker, and plotting these percentages over time (Graham and Williams, 1982). Due to the naturally occurring levels of C_{32} in faeces, corrections of percentages recovered were made on the assumption that faecal C_{32} levels at 0 hours were representative of normal baseline levels. Mean passage time of the marker was calculated using the equation of Blaxter *et al.* (1956) namely:

Mean passage time (hrs) = $1/\text{Total marker} \sum[½\ n\ (t' + t)]$

where :

$\sum$ signifies the sum of n collected between time intervals until $n = 0$ and

n is the concentration of C_{32} collected between times t and t' and $(t'-t)$ = hours.

The mean passage times of the marker are reported in Table 4.1.

Table 4.1: Mean passage time of the marker for individual horses, calculated using the equation of Blaxter *et al.* (1956)

Horse no.	Mean passage time (hours)
1	27.20
2	28.35
3	28.06
Mean ± s.e	27.87 ± 0.59

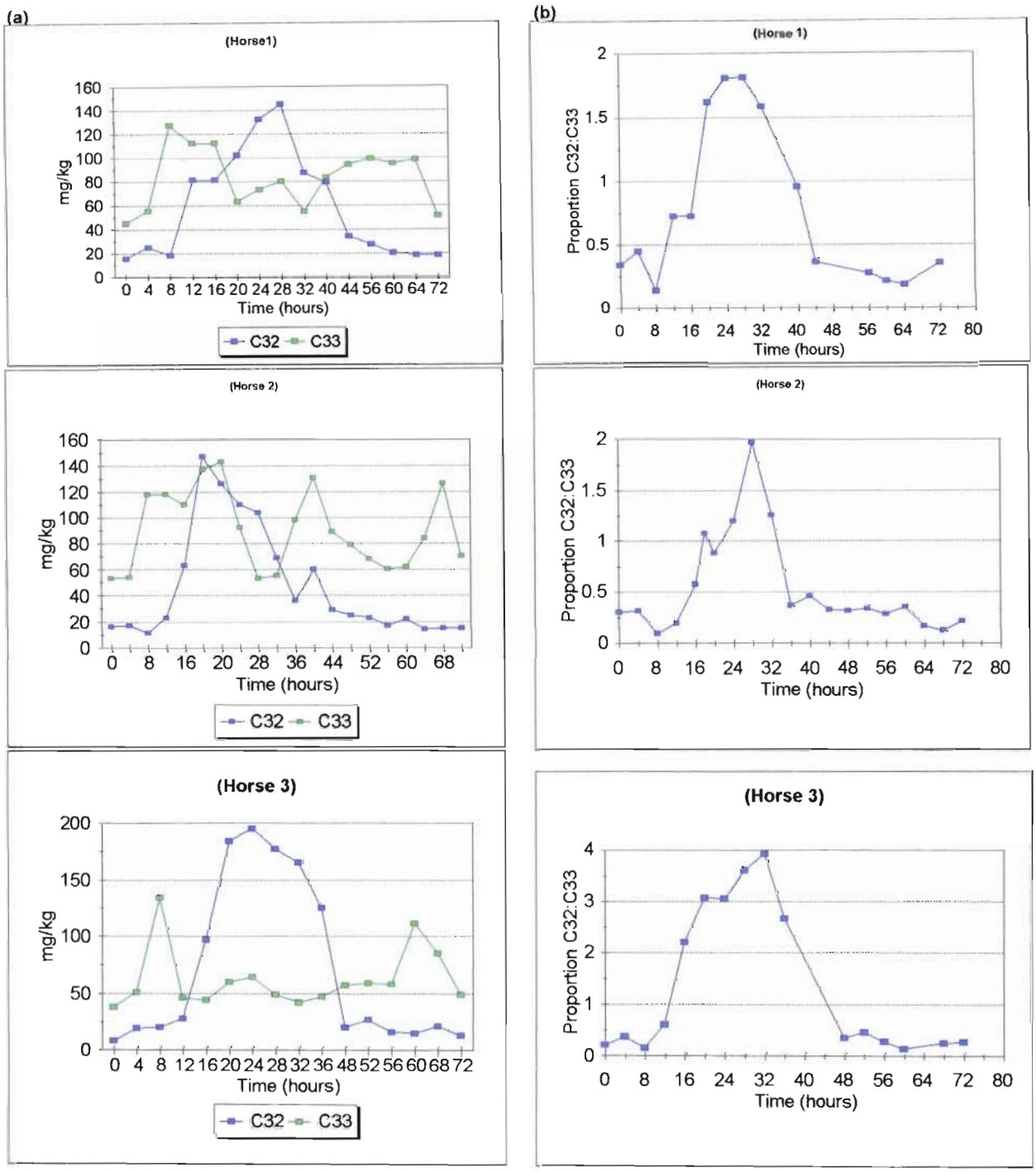


Figure 4.8: Concentration (mg/kg DM) of C_{32} and C_{33} (a) and proportion of these alkanes in faecal samples following a single C_{32} dose. The dose was administered at 0 hours.

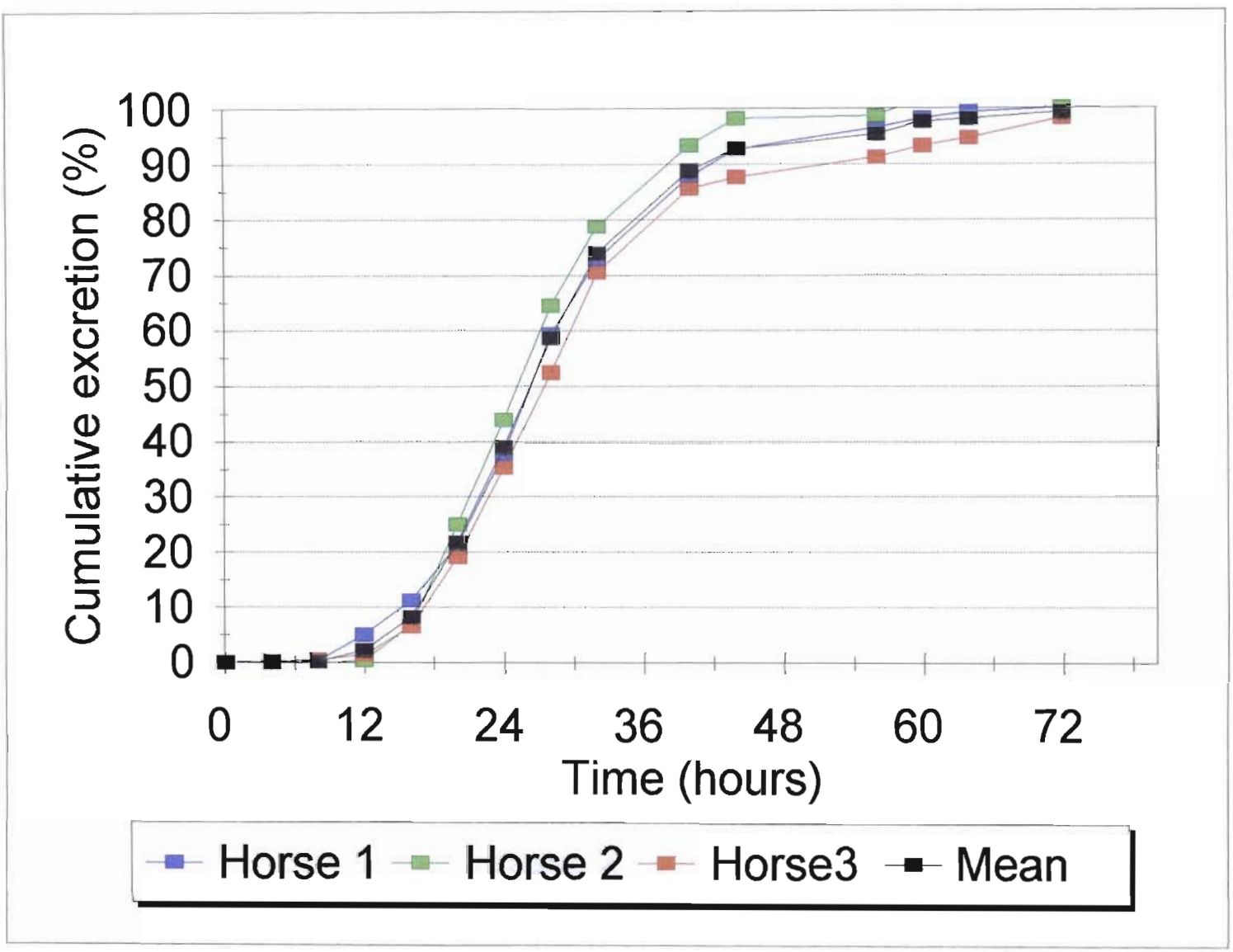


Figure 4.9: Cumulative excretion of C_{32} over time

4.5.2 Multiple doses

The concentrations of C_{32} and proportions of C_{32}:C_{33} in the faecal samples over the period of the trial are presented in Figure 4.10. The levels of the external marker in the faeces increased rapidly between the first dose (0 hours) and the second dose (24 hours). Further doses at 24 hour intervals resulted in levels being maintained at those above or similar to C_{33} levels and above those of baseline C_{32} levels. However there was large variation in excreted levels of the external marker and proportion of external:internal marker throughout the trial period.

4.5.3 Diurnal variation

The ratios of faecal C_{32}:C_{33} over a period of time (30 hours), after three days of continuous daily dosing with C_{32} n-alkane pellets are presented in Figure 4.11. The proportion of C_{32}:C_{33} in samples collected over seven consecutive four hour periods showed differing amounts of variation depending on the horses involved.

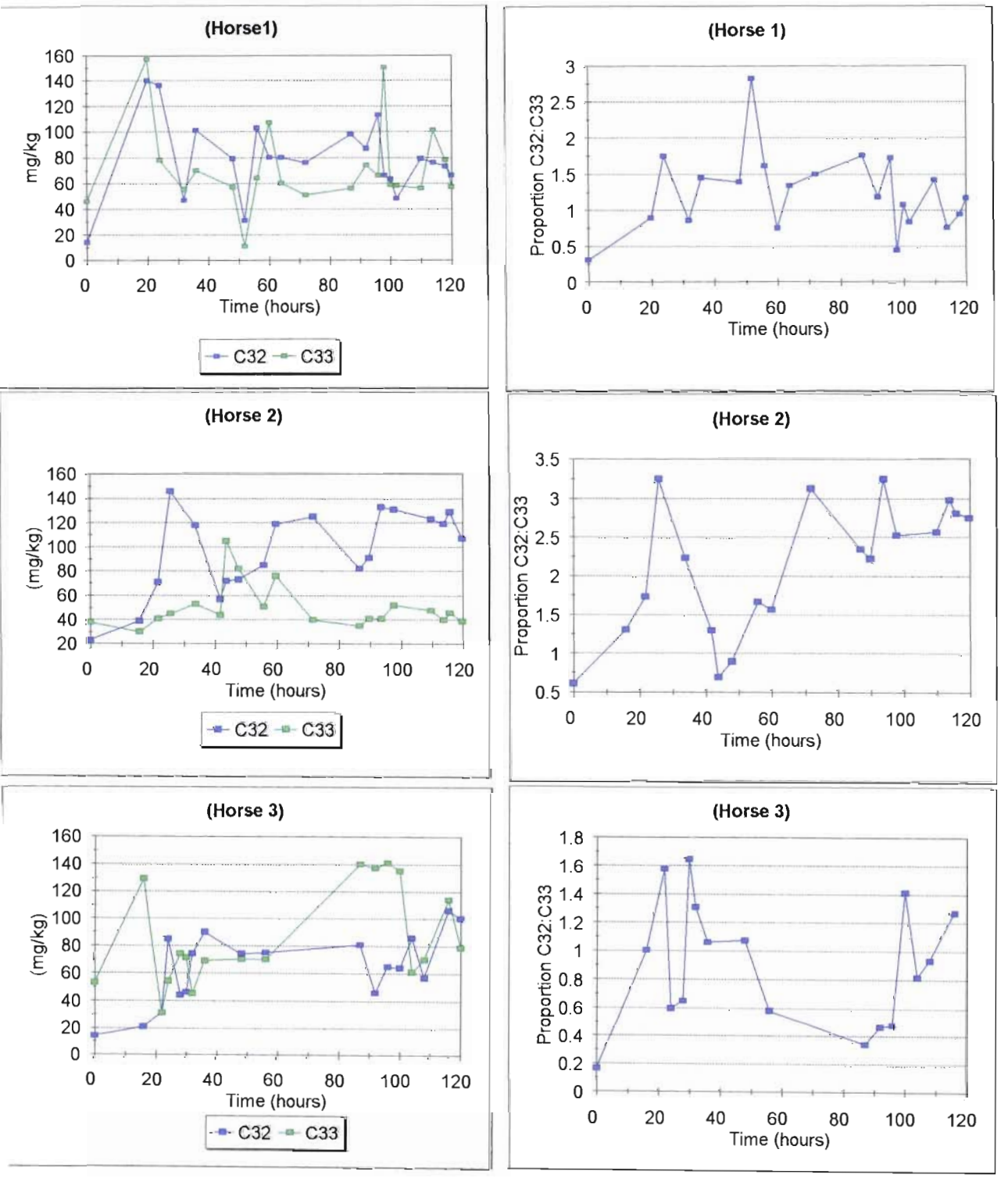


Figure 4.10: Faecal levels of C_{32}, C_{33} (a) and ratio of C_{32} to C_{33} (b) over time. The C_{32} marker was dosed at 0 hours and at every 24 hours thereafter

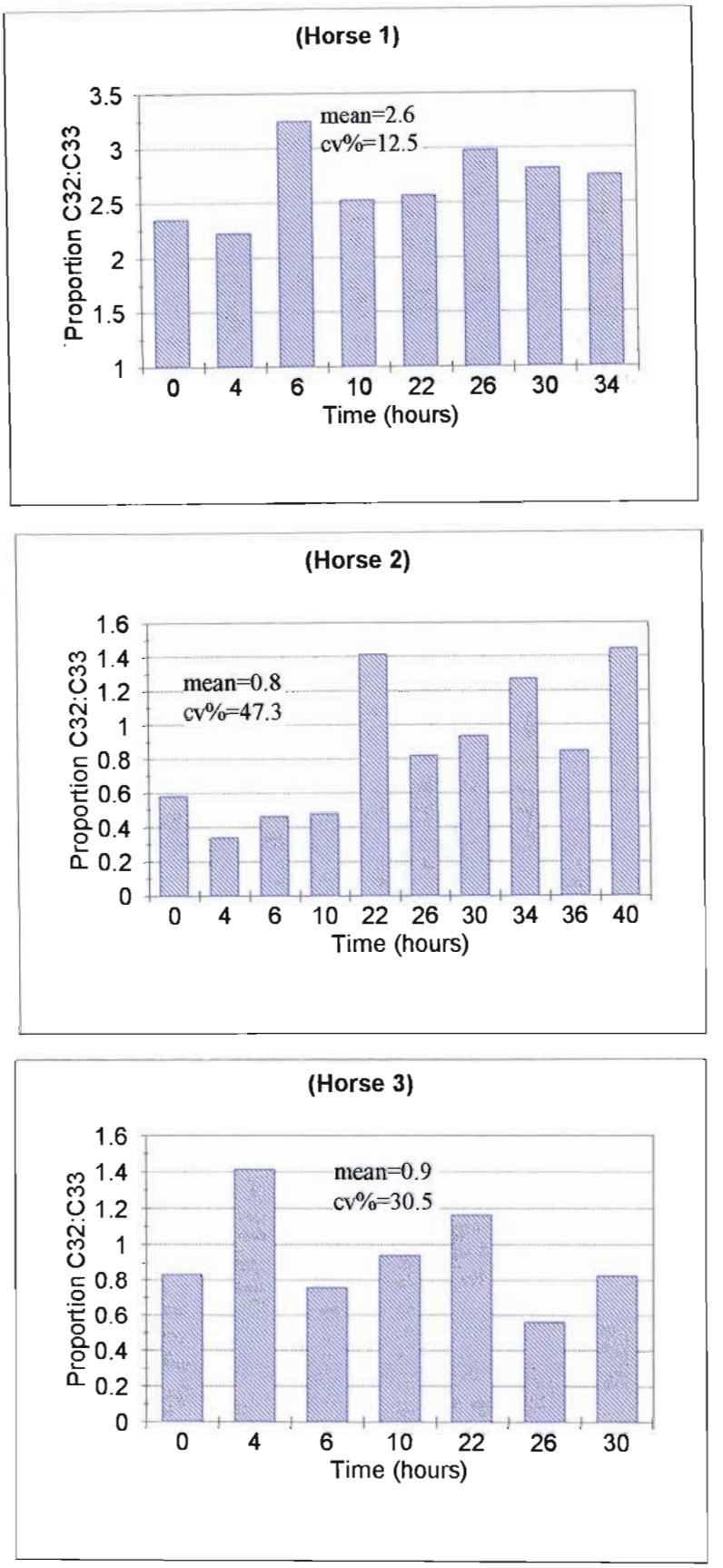


Figure 4.11: Variation in faecal C_{32}:C_{33} ratios following daily C_{32} dosing. Daily dosing commenced 3 days prior to sampling and was applied at 6 hours and 30 hours during the sampling period. The first sample (0 hours) was taken 18 hours after the previous dose

4.6 Discussion of results

The faecal recovery of a single dose of C_{32} appeared to follow a similar pattern for fresh grass, hay and the diet containing a large amount of concentrate meal. In Part A, where fresh grass and grass hay were fed, the faecal C_{32} concentration began to increase as early as twelve hours after the dose administration, but in the majority of horses the increase was apparent after fifteen hours. In Part B where the horses were consuming a mixed diet, the increase in faecal C_{32} levels occurred earlier (between eight and twelve hours after dosing) than with the herbage-only diets and increased rapidly hereafter.

The faecal collections in Part A were not continued for a sufficient length of time to enable an accurate comparison of mean passage rates of the marker due to diet type. However, the times taken for marker levels to increase, and for faecal C_{32} levels to reach baseline after withdrawal of the marker dose, were similar for both the fresh grass and hay diets. The more rapid increase in faecal C_{32} levels observed in Part B may have been caused by the more rapid rate of passage of concentrates through the digestive tract (Robinson and Slade, 1974). Frape (1986) reported that due to the relatively small stomach size of the horse, a large proportion of a meal may have only limited exposure to gastric secretions, due to a high rate of passage through this organ. This becomes more evident with a large meal (Frape, 1986). The high rate of passage may result in a small proportion of the digesta reaching the caecum within 45 minutes (Frape, 1986) while markers associated with the liquid phase of digesta are reported to reach the caecum within two hours of ingestion (Argenzio *et al.*, 1974).

While a large proportion of a meal spends only a short time in the stomach, the cessation of meal consumption results in arrested expulsion of digesta from the organ (Frape, 1986). A significant proportion of the meal may therefore remain in the stomach for two to three hours. The timing of dose administration, (before, during or after a meal) in indoor trials, where discrete meals are fed to animals, may have a significant effect on the rate of passage of the dosed marker and the extent to which the marker becomes dispersed within the digesta. The faecal levels of C_{32} began to increase as early as twelve hours after administration, suggesting a rapid rate of marker passage. The latter portion of the meal may therefore have had only limited exposure to the dose, resulting in non-uniform excretion of the dose. Udén *et al.* (1982) while examining digesta retention in the equine, noted that the passage of digesta, and the passage of markers inserted into the caecum of these animals, was less continuous than observed in ruminants. Reports concerning chromium marker recoveries in equine studies suggested that the time for total faecal recovery of a dosed marker may vary from 48 hours (Haenlein *et al.*, 1966) to more than 96 hours (Vander Noot *et al.*, 1967). In all the diets considered, the C_{32} appeared to have been completely excreted 64 hours post-dosing, although the time recorded for baseline levels to be reached varied for individual horses.

In the preliminary trial reported earlier (Chapter 3), where total faecal collection took place, the faecal concentrations of the C_{32} marker (and proportion of C_{32}:internal markers) appeared to reach a plateau after only two days (48 hours). A similar plateau was observed by Cuddeford and Hughes (1990), when Cr-fibre was used as a marker in equine trials. However, when the levels of C_{32} in samples taken at timed intervals for 120 hours (Figure 4.10) were measured, it was apparent that there was large variation in the both the faecal marker concentrations and the proportion of C_{32}:C_{33} both within, and between days. The variation between days may be aggravated by the variation in daily DM intake.

Diurnal variation in n-alkane ratios, which is presumably due to variation in the excretion of the dosed (external) marker (Dove and Mayes, 1991), has been observed in cattle (Dillon and Stakelum, 1989), but not in certain studies with housed sheep (Mayes *et al.*, 1986a). Samples taken at three different periods within a day (Figure 4.7) show that the ratio of external:internal markers varied, although this variation did not appear to be considerable. Diurnal faecal marker (external:internal ratio) variation for horses consuming a mixed diet (Figure 4.11) was high over the period of sample collection. Cattle studies using n-alkanes (Malossini *et al.*, 1994) have shown a reduced variation in diurnal n-alkane excretion, with once daily n-alkane dosing, when concentrates were added to the diet, but this was not evident in this trial.

The single and multiple dose data collected in Part B suggests that dosing once daily with C_{32} was insufficient to produce a steady state of excretion of this marker. The rapid increase in faecal marker following a single dose, and the pattern of marker excretion (Figure 4.8) suggests that first order digesta kinetics, as described in ruminants (Grovum and Williams, 1973) did not apply in this study, and that marker mixing with digesta was incomplete, (Pienaar, personal communication). The observations of Udén *et al.* (1982) that the passage of digesta, and the passage of markers inserted into the caecum of horses was less continuous than observed in ruminants, may also have contributed to variation in faecal marker excretion.

The incomplete mixing of the dosed marker may explain the diurnal variation observed in both Parts A and B. The greater variation observed in Part B may be due to different rates of passage of marker resulting from different diet types (Robinson and Slade, 1974), and the incomplete mixing of the marker may have been amplified due to the low concentration of the marker dose. The marker pellets, which were to have contained approximately 600mg of C_{32}, were found to contain just above half of this (later found to be due to a fault in the rotary evaporation process). However, lower concentrations of chromium markers fed to horses have reportedly decreased diurnal variation in comparison to higher doses (Cuddeford and Hughes,1990), which would indicate that the low concentration of C_{32} did not increase the diurnal variation, unless the low levels had an effect on the accuracy of the extraction of n-alkanes from the samples. The presentation of figures representing ratios of external markers relative to internal markers have relevance in that it is the change in the ratio of the markers which will effect

intake estimates. However, it was also noted that the fluctuation of the internal marker concentrations in samples, also appeared to effect the concentrations of the external marker. This may be due to the extraction or analysis process where the internal standard (C_{36}) may be present naturally in small, but varying quantities in the samples, resulting in inaccurate reading of alkane concentrations. This would not effect the relative concentrations of the n-alkanes.

The diurnal variation problem evident in this study, may be reduced by more frequent dosing of the external n-alkane. Dillon and Stakelum (1990) reported diurnal variation in n-alkane ratios in cattle faeces when the animals were dosed twice daily, but that the variation was lower than that observed when animals were dosed only once a day. Chromium markers used in horse studies reportedly caused similar problems with diurnal variation when animals were dosed once or twice daily. In some instances a pattern of marker variation was apparent (Haenlein *et al.*, 1966), which may have been affected by diet type or meal frequency (Parkins *et al.*, 1982; Cuddeford and Hughes, 1990). Controlled-release devices for external n-alkane marker application have been developed for ruminants (Mayes *et al.*, 1995) to enable the reduction of this problem. Until such devices are developed for use in non-ruminants, it appears that more frequent external marker dosing must be considered in these animals.

CHAPTER 5

ACCURACY OF N-ALKANE BASED ESTIMATES OF HERBAGE DRY MATTER INTAKE AND DIGESTIBILITY IN HORSES FED DIETS OF FRESH RYEGRASS (*Lolium perenne*), KIKUYU (*Pennisetum clandestinum*), KIKUYU HAY OR A COMBINATION OF CONCENTRATES AND HAY

5.1 Introduction

The results obtained in the preliminary trial were encouraging in that reasonable estimates of intake were obtained for horses, using the n-alkanes. However, more data was needed to confirm these results, and to investigate more fully the precision with which herbage intake estimates could be made using the n-alkanes. The extent to which the n-alkanes could be recovered in the faeces of horses, the effect of different diet types, and the possibility of using an n-alkane to provide accurate digestibility estimates of herbage consumed by the horse needed further investigation. The following experiment were therefore aimed at providing more information in this regard.

5.2 Materials and methods

5.2.1 Animals and housing

Four mature geldings (513 kg, 490 kg, 450 kg, and 509 kg), were stabled at the University of Natal research farm "Ukulinga" in Pietermaritzburg in the facilities described previously. The horses were previously adapted to a forage/mineral-lick diet and were free-ranging prior to the onset of the feeding trials.

5.2.2 Dosing procedures

In all cases the horses were dosed each morning with the prepared C_{32} pellets, (approximately 6g/horse/day), prior to the morning meal. The exact mass of each daily dose was recorded. The dosing procedure commenced three to four days prior to the start of the total faecal collection, and continued as above for the duration of the trial.

5.2.3 Feeding procedures

Four diets were used in different trials. Each trial consisted of feeding the same diet to all four animals for a preliminary period (three to four days) followed by the period of the experiment itself (six days). After each trial, the horses were placed onto pastures and brought back into the stables the day before the onset of the next trials preliminary period.

Diets in trial one and two consisted of fresh grasses. Diet one *(L. perenne)* and diet two (Kikuyu) were harvested from the University farm's pastures using a sickle-bar mower. The approximate mass of fresh grass needed for a single day was harvested and bagged early each morning.

At 08:00 each morning, immediately after dosing with the external marker, an amount (8-10 kg) of fresh grass was weighed out and fed to each horse. The remaining harvest was placed in a cold room, until further feeds of similar amounts to the first were fed at 12:00 and 17:00. The amounts fed varied throughout the trial according to the individual horses intake. However, it was ensured that forage was available to each animal at all times.

Diet three consisted of cured Kikuyu hay, which was fed to each animal, following the same routine as for diets one and two. Diet four consisted of a commercial concentrate feed (Romix) as well as the Kikuyu hay. The hay was fed as previously described, while the concentrate was fed in two equal meals at 08:00 and 17:00. The concentrate feed was weighed out and moistened prior to feeding to prevent the animals from bolting the food, which may have resulted in digestive disturbances. A salt/mineral lick was made available with all diets and clean water was provided *ad libitum*.

5.2.4 Sample collection

5.2.4.1 Feeds and left-overs (orts)

The mass of each individual meal was recorded prior to feeding. Samples of daily feeds were collected from each bag/bale of harvested herbage prior to each meal. The samples for each meal were pooled and the samples immediately weighed and placed in a drying oven at 70°C for 72 hours for dry matter determination and later determination of n-alkane content. Unconsumed herbage, was collected at least three times per day with all traces of the previous meal removed prior to the following meal. These orts were pooled and weighed for individual animals. The daily herbage DM intake of each horse was obtained by subtracting the mass of the orts from the mass fed per day and correcting for dry matter.

5.2.4.2 Faecal collection

After the three to four day preliminary period a total faecal collection began for the following six days. All traces of faeces were removed at least three times per day and the mass of wet faeces produced by each horse recorded .

Faecal samples for n-alkane analysis were obtained by removing a small amount of non-specific weight, from each individual defecation and pooling these amounts to attempt to provide a representative

sample of daily faecal output. The resultant samples were treated in exactly the same manner as the herbage samples to obtain DM and n-alkane values. Faecal grab samples were taken directly from the anus of each horse in the fresh grass (diets one and two). These samples were taken for four days at each meal time (08:00, 12:00 and 17:00) and subjected to DM determination and n-alkane analysis.

5.2.4.3 Sample analysis and calculations.

The n-alkane extraction and quantification of herbage and faecal samples took place at the University of Natal and the Cedara Agricultural Development Institute by the methods described earlier.

The estimated dry matter intake of the horses was calculated using the appropriate formulae for herbage and herbage/concentrate diets as described by Mayes *et al.* (1986a).

The mean measured values of DM intake were then compared with the calculated mean intake values and digestibility estimates compared with values obtained with total faecal collections.

Digestibility (DM) = DM in/ DM out

Estimated Digestibility (DM) = 1-(faecal n-alkane/herbage n-alkane)

The faecal recovery or indigestibility of the n-alkanes was calculated from the ratio of:

Faecal n-alkane (mg/kg) × output(kg):Ingested n-alkane(mg/kg) × intake (kg)

5.3 Results

5.3.1 Faecal recovery of n-alkanes

Table 5.1: Mean faecal recoveries (proportion of ingested recovered in faeces) of $_{31}$-C_{35} n-alkanes for each diet type. Means and s.e's are based on four animals over six days for each diet

Diet type	C_{31}	C_{32}	C_{33}	C_{35}
	mean ±s.e	mean ±s.e	mean ±s.e	mean ±s.e
L. perenne (fresh)	0.73^{a} ±0.06	0.86^{b} ±0.05	0.76^{a} ±0.03	0.76^{a} ±0.12
P.clandestinum (fresh)	0.79^{ab} ±0.07	0.87^{bc} ±0.02	0.84^{bc} ±0.07	0.91^{bc} ±0.12
P.clandestinum (Hay)	0.88^{b} ±0.10	0.93^{b} ±0.05	0.86^{b} ±0.08	0.88^{b} ±0.08
Hay + conc *	0.84^{b} ±0.08	0.82^{b} ±0.24	0.88^{b} ±0.03	0.86^{b} ±0.01
General means and s.e	0.810^{b} ±0.06	0.87^{b} ±0.04	0.84^{b} ±0.05	0.85^{b} ±0.05

* Figures corrected for intake and n-alkane content of concentrate feed.

Note: Numbers in columns and across rows having superscripts in common are not significantly different ($p<0.05$) to each other.

The faecal recovery of C_{32} was higher than that of the adjacent n-alkanes in the herbage-only diets, but was lower when concentrates were included in the diet. However, these differences were not significant ($p>0.05$) except in the *L. perenne* diet treatment. Recovery of the odd chain length n-alkanes was lowest in the fresh grass trials and in particular with the *L. Perenne* diet where C_{31}, C_{33} and C_{35} recoveries were significantly lower than in the other diet treatments.

Over all diets, mean recovery of C_{32} was higher than that of the adjacent n-alkanes while C_{31} was recovered to a lesser extent than C_{33} and C_{35}, although these differences were not significant.

5.3.2 Herbage dry matter intake estimates

The mean estimates of herbage DM intake made using the C_{32}:C_{33} n-alkane pair are compared with measured intakes (Table 5.2). The mean estimates of herbage DM intake did not significantly ($p>0.05$) differ from measured intakes in any of the individual animals. There were also no significant differences between mean estimated intake and measured intakes, when the overall intakes for each separate trial were considered. There were also no significant differences when overall (all four diet measurements considered together) evaluation of the C_{33}:C_{32} performance was considered.

Discrepancies, calculated as the measured intake minus the estimated intake, were large in some cases. The discrepancies in the hay + concentrate trial were the highest with mean discrepancy over the trial representing 10.13% of actual intake. Mean discrepancy was lowest (0.012 ± 5.5 %) when the horses consumed fresh kikuyu and when consuming hay alone (1.67 ± 8.5%), while the mean discrepancy in the *L. perenne* diet was 9.8 ± 5.2%. Over all the diet treatments the mean discrepancy was 0.31kg, which represented 4.8 ± 7% of the mean measured intake.

The discrepancies in the *L. perenne* trial were all positive, suggesting intake was underestimated in this trial. Discrepancies in the remaining trial were both positive and negative but estimates of intake appeared to be predominantly underestimated (Figure 5.1). Mean estimated intakes using C_{33}:C_{32} were highly correlated with measured intakes ($r^2 = 0.96$ overall). The mean estimates of herbage DM intake made with the C_{31}:C_{32} n-alkane pair are presented in Table 5.3. In the hay + concentrate, and hay-only diet treatment, the C_{31}:C_{32} estimate of intake significantly ($p<0.05$) differed from measured values in individual animals. Mean C_{31}:C_{32} estimates for the hay + concentrate trial differed significantly ($p<0.05$) from mean measured values, but no significant difference was apparent between mean estimates and measured values for any other diet. Overall estimated DM intake was not statistically different from measured DM intake.

Mean discrepancies were highest in the hay + concentrate diet (25.7 ± 2.6%) and hay only (37.0 ± 15%) trials. Mean discrepancies were lower in the *L. perenne* (2.2 ± 10.4%) and *P. clandestinum* (5.0 ± 9.9%) trials. Over all treatments the mean discrepancy between measured and estimated intake was -0.5 ± 1.03 kg which represents 8.5 ± 16% of measured intake. Discrepancies were predominantly negative (Figure 5.1) suggesting an overestimate of intake was obtained using the C_{31}:C_{32} n-alkane pair.

Table 5.2: Estimates of herbage DM intake (kg/day) made using the C_{32}:C_{33} n-alkanes, compared with measured intakes

(a) ***L. perenne***

Horse	Mean intakes(kg/d)				Discrepancies	Correlation
	Estimate	s.e	Measured	s.e	Measured-est	Est : measured
1	4.124	1.448	4.976	0.610	0.852	0.987
2	10.197	2.878	11.064	1.445	0.867	
3	7.494	2.333	8.786	0.620	1.292	
4	9.457	4.991	9.688	1.197	0.231	
Trial Mean	7.818	2.913	8.628	0.968	0.811	
				se	0.437	

(b) ***P. Clandestinum (hay)***

Horse	Mean intakes(kg/day)				Discrepancies	
	Estimate	s.e	Measured	s.e	Measured-est	Est : measured
1	3.263	0.670	2.740	1.054	-0.523	0.989
2	5.325	2.071	5.652	0.549	0.327	
3	5.895	1.638	6.180	0.461	0.285	
4	4.094	1.669	4.320	1.224	0.226	
Trial Mean	4.644	1.512	4.723	0.822	0.079	
				se	0.403	

(c) ***P. clandestinum***

Horse	Mean intakes(kg/day)				Discrepancies	Correlation
	Estimate	s.e	Measured	s.e	Measured-est	Est : measured
1	10.033	2.046	10.470	2.230	0.437	1.000
2	8.087	1.405	8.110	1.369	0.023	
3	6.677	1.502	6.220	1.722	-0.457	
4						
Trial Mean	8.266	1.651	8.267	1.774	0.001	
					0.447	

(d) **Hay and concentrate**

Horse	Mean intakes(kg/day)				Discrepancies	
	Estimate	s.e	Measured	s.e	Measured-est	Est : measured
1	6.330	2.344	7.380	1.446	1.050	0.994
2	3.076	0.612	3.125	0.330	0.049	
3	2.906	1.038	2.840	0.297	-0.066	
4	4.132	1.116	4.950	0.723	0.818	
Trial Mean	4.111	1.278	4.574	0.699	0.463	
				se	0.554	

(e) **Overall**

	Estimate (kg/day)	Measured (kg/day	Discrepancy (kg/day)	Correlation
mean	6.07	6.43	0.31	0.98
s.e	2.53	2.73	0.50	

Table 5.3: Estimates of herbage DM intake (kg/day) made using the C_{31}:C_{32} n-alkanes, compared with measured intakes

(a) ***L. perenne***

Horse	Mean intakes(kg/day) Estimate	s.e	Measured	s.e	Discrepancies Measured-est	Correlation Est : measured
1	4.442	1.959	4.976	0.610	0.534	0.960
2	11.053	6.790	11.064	1.445	0.011	0.93
3	7.587	2.586	8.786	0.620	1.199	
4	10.654	6.334	9.688	1.197	-0.966	
Trial Mean	8.434	4.417	8.628	0.968	0.195	
				se	0.914	

(b) ***P. Clandestinum (hay)***

Horse	Mean intakes(kg/day) Estimate	s.e	Measured	s.e	Discrepancies Measured-est	Est : measured
1	4.478	0.936	2.740	1.054	-1.738	0.998
2	7.433	2.805	5.652	0.549	-1.781	
3	8.108	2.399	6.180	0.461	-1.928	
4	5.950	2.928	4.320	1.224	-1.630	
Trial Mean	6.492	2.267	4.723	0.822	-1.769	
				se	0.123	

(c) ***P. clandestinum***

Horse	Mean intakes(kg/day) Estimate	s.e	Measured	s.e	Discrepancies Measured-est	Est : measured
1	10.015	5.741	10.470	2.230	0.455	0.931
2	9.285	5.254	8.110	1.369	-1.175	
3	6.743	3.602	6.220	1.722	-0.523	
4						
Trial Mean	8.681	4.866	8.267	1.774	-0.414	
				se	0.820	

(d) **Hay and concentrate**

Horse	Mean intakes(kg/day) Estimate	s.e	Measured	s.e	Discrepancies Measured-est	Est : measured
1	8.554	7.104	7.380	1.446	-1.174	0.948
2	5.244	1.148	3.125	0.330	-2.119	
3	3.333	1.182	2.840	0.297	-0.493	
4	5.868	1.693	4.950	0.723	-0.918	
Trial Mean	5.750	2.782	4.574	0.699	-1.176	
				se	0.689	

(e) **Overall**

	Estimate (kg/day)	Measured (kg/day)	Discrepancy (kg/day)	Correlation
mean	7.25	6.43	-0.55	0.97
s.e	2.38	2.73	1.03	

Measured intakes in red are significantly different to estimated intakes ($p<0.05$).

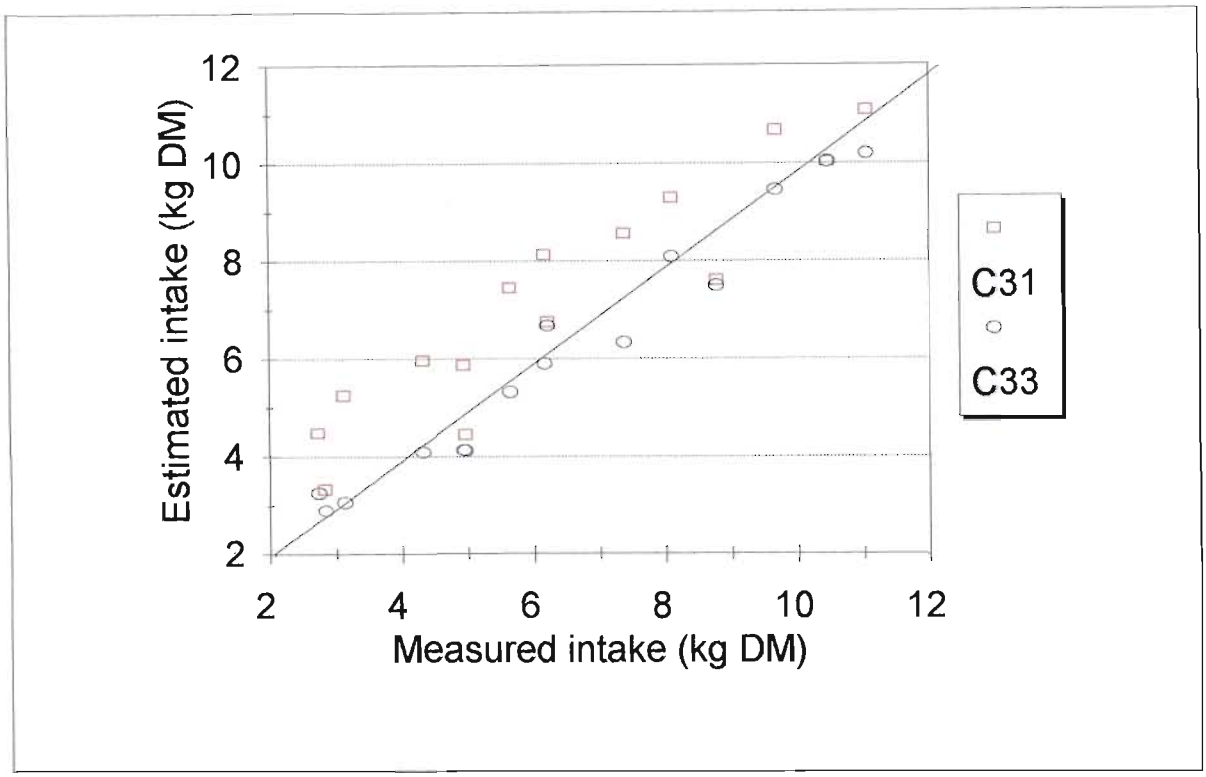


Figure 5.1 : Deviation of mean estimated intakes (kg DM) from actual intakes, over all diets, using either internal indicator. The solid line represents actual intakes or 100% accuracy

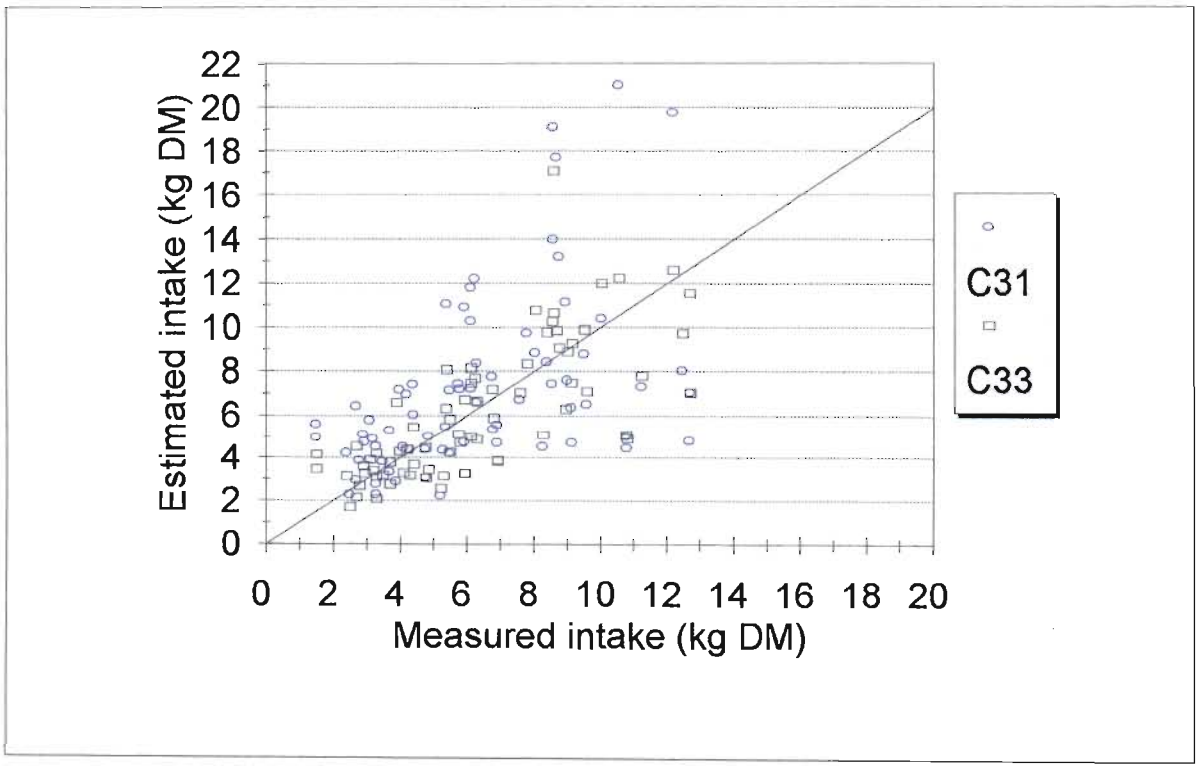


Figure 5.2: Deviation of daily estimated intakes (kg DM) from actual intakes using either n-alkane marker pair. The solid line represents actual intakes. Note that the deviations of intake estimates made using the C31 internal marker increase with increasing DM intake

Mean intake (over or under) estimates (kg DM) for all diets using the C_{31}:C_{32} and C_{33}:C_{32} n-alkane pairs are represented in Figure 5.1.

Samples taken directly from the anus of the animals twice daily were pooled and analysed for n-alkane concentrations (mg/kg), and used to calculate herbage dry matter intake. The comparisons between measured and grab sample estimates of intake are presented in Table 5.4.

Mean dry matter intake estimates in individual animals, using these grab samples, differed significantly ($P<0.05$) from measured values in the *L. perenne* trial using both C_{31} or C_{33} as the internal indicator. There were no significant differences in estimated and measured herbage dry matter intakes using either of the marker pairs when *P. clandestinum* was the diet being consumed. Mean measured herbage dry matter intakes considered for all animals, within each diet, were not significantly different nor were overall means (including both diets) different from estimates calculated using the n-alkane concentrations of faecal grab samples.

Discrepancies for the C_{31}:C_{32} estimates in the *L. perenne* diet were $10.2 \pm 12.5\%$ and $22.7 \pm 9.2\%$ for the *P. clandestinum* diet. The mean discrepancies were $6.2 \pm 16\%$ and $0.15 \pm 11\%$ for the *L. perenne* and *P. clandes*tinum diets respectively. Overall, mean differences between measured intakes and estimated intakes using faecal grab samples were $15.1 \pm 14\%$ for the C_{31}:C_{32} and $4.0 \pm 14.6\%$ for the n-alkane pairs. Mean discrepancies were of a positive magnitude in both diets and therefore overall mean discrepancies were positive. However, in some instances individual means were found to be negative.

Table 5.4: Measured mean intakes (kg DM) compared with estimated intakes calculated using the concentrations of C_{31}:C_{32} (a) and (c), and C_{32}:C_{33} (b) and (d) n-alkanes.

(a) L. perenne

	Mean (kg DM/day)	Intake				
Horse	C31:C32 (kg/day)	se	Measured (kg/day)	se	Discrepency (kg/day)	Correlation
1	5.026	0.569	5.220	0.380	0.194	0.717
2	8.637	0.997	10.580	0.534	1.943	
3	7.801	2.829	10.670	0.049	2.869	
4	10.143	3.499	8.960	0.185	-1.183	
Trial	7.902	1.973	8.858	0.287	0.956	
				se	1.806	

(b) L. perenne

	Mean (kg DM/day)	Intake				
Horse	C33:C32 (kg/day)	se	Measured (kg/day)	se	Discrepancy (kg/day)	Correlation
1	5.833	0.864	5.220	0.380	-0.613	0.833
2	9.777	0.646	10.580	0.534	0.803	
3	8.159	3.435	10.670	0.049	2.511	
4	9.436	0.536	8.960	0.185	-0.476	
Trial	8.301	1.370	8.858	0.287	0.556	
				se	1.451	

(c) P. Clandestinum

	Mean (kg DM/day)	Intake				
Horse	C31:C32 (kg/day)	se	Measured (kg/day)	se	Discrepancy (kg/day)	Correlation
1	5.912	0.084	7.810	0.295	1.897	0.857
2	3.801	0.810	5.570	0.603	1.769	
3	5.428	0.655	6.220	1.358	0.792	
Trial	5.047	0.516	6.533	0.752	1.486	
				se	0.605	

(d) P. Clandestinum

	Mean (kg DM)	Intake				
Horse	C33:C32 (kg/day)	se	Measured (kg/day)	se	Discrepancy (kg/day)	Correlation
1	7.072	0.835	7.810	0.295	0.737	0.739
2	5.466	0.977	5.570	0.603	0.104	
3	7.031	1.008	6.220	1.358	-0.811	
Trial	6.523	0.940	6.533	0.752	0.010	
				se	0.779	

(e) Overall Means (kg DM)

	Estimate (kg DM/day)	se	Measured (kg/day)	se	Discrepancy	se	Correlation
C31:C32	6.678	1.349	7.861	0.486	1.183	1.354	0.787
C33:C32	7.539	1.186	7.857	0.567	0.318	1.157	0.786

Measured intakes in red are significantly different ($p<0.05$)to estimated intakes.

5.3.3 Digestibility estimates

The estimates of herbage digestibility calculated using C_{31}, C_{33} or C_{35} as internal digestibility markers are represented in Table 5.5.

Table 5.5 : Estimated digestibility of diets using herbage n-alkanes. The measured and estimated digestibility are means of four animals over five days for all diets

Diet	Measured*	C_{35} Estimate**	C_{33} Estimate**	C_{31} Estimate**
L. perenne fresh	0.535 ± 0.087	0.411 ± 0.127	0.454 ± 0.151	0.397 ± 0.053
P.clandestinum fresh	0.586 ± 0.046	0.523 ± 0.088	0.490 ± 0.054	0.445 ± 0.09
P.clandestinum Hay	0.338 ± 0.084	0.298 ± 0.089	0.212 ± 0.027	0.273 ± 0.0454

Figures in red differ significantly ($P<0.05$) from measured digestibility in the same row.

* Calculated from total faecal collection values and measured intakes.

** Calculated from concentrations (mg/kg) in the herbage and in the faeces.

Table 5.6: Herbage nutrient concentrations (%) on a DM basis

Herbage	Protein	Calcium	Phosphorus	Ash	NDF	ADF
P.clandestinum (fresh)	15.7 ±0.64	0.65 ±0.06	0.28 ±0.03	8.4 ±0.1	64.0 ±3.35	30.05 ±0.84
P.clandestinum (hay)	9.16 ±2.35	0.54 ±0.01	0.18 ±0.08	6.04 ±1.9	70.62 ±4.19	38.66 ±1.04
L. perenne (fresh)	11.71 ±2.33	0.88 ±0.04	0.23 ±0.01	7.50 ±0.30	61.88 ±0.88	36.46 ±0.82

Digestibility estimates, using any of the indicators, were lower than digestibility calculated using total faecal collections. However, only the C_{31} estimate of digestibility in the *L. perenne* diet trial was significantly different ($p<0.05$) from the total collection estimates.

The C_{33} n-alkane gave digestibility estimates corresponding to 85%, 84% and 62% of measured digestibility of *L. perenne*, fresh *P. clandestinum* and *P. clandestinum* hay respectively. Estimates made using C_{31} were lower than those obtained using C_{33} for the *L. perenne* (74%) and fresh *P. clandestinum* (76%) diets but were significantly ($p<0.05$) higher in the hay trial (80%). The C_{35} estimates of digestibility corresponded to 77%, 88% and 88% of measured digestibility for the respective herbages. Overall estimates of digestibility were underestimated with figures for C_{31} being 77% of measured digestibility. The C_{33} marker gave digestibility estimates of 77% of actual digestibility while those for C_{35} averaged 85%.

5.4 Discussion

The overall mean faecal recovery of n-alkanes were $81 \pm 0.064\%$, $87.2 \pm 0.044\%$, $83.6 \pm 0.051\%$ and $85.5 \pm 0.60\%$ for C_{31}, C_{32}, C_{33} and C_{35} respectively. When compared with other equine studies using Cr_2O_3 and chromium mordanted hay, which reported recoveries of 94.8% (Haenlein *et al.*, 1966; Parker *et al.*, 1982) and 96.5% (Cuddeford and Hughes, 1990) for these markers respectively, the present recoveries are low. Mayes *et al.* (1986a) however, argued that, provided the recovery of the external and internal n-alkane markers were the same, the bias in intake created by the incomplete recovery of markers would cancel each other out in the intake equation. While these n-alkane recoveries are not significantly different from each other, the equation used to calculate intakes requires that the markers are equally indigestible (Mayes *et al.*, 1986a). Therefore, the slight differences in recoverability of the markers in these trials, while not significant, may contribute to the overall differences in intakes calculated using the marker pairs.

The faecal recovery of the n-alkanes was comparable to those reported in studies with sheep. Mayes *et al.* (1988) reported faecal recovery of $77.9 \pm 0.95\%$, $85.9 \pm 1.0\%$ and 83.9 ± 1.2 % for C_{31}, C_{32} and C_{33} respectively, which corresponds closely to the figures obtained in this study. However, Mayes *et al.* (1986a) and Vulich *et al.* (1991) reported a closer relationship between the recovery of the external and internal markers than recorded in this study. Dove and Mayes (1991) and Mayes *et al.* (1995) reported that in almost all ruminant studies the faecal recovery of n-alkanes increased as chain length increased. In this study there was a small, yet non-significant increase in the recovery of odd-chain (natural) n-alkanes. The C_{35} marker has been reported to be highly recoverable (95%) in the faeces of sheep (Dove and Mayes, 1991) but was not recovered to this level in the present study. In non-ruminant studies with horses (Cuddeford and Mayes, unpublished data), pigs (Gannon and Mayes, unpublished data) and hares (Hulbert, 1993) the increased recovery of n-alkanes with increasing chain length was, as with this study, not substantial, which suggests that the behaviour of the alkanes in the monogastric digestive tract may differ from that observed in ruminants. The faecal recovery of C_{35} has not been reported for horses prior to this study. The lack of significantly increased recoveries with increasing n-alkane chain length reported in monogastric trials, together with the data reported in this

study, suggests that the recovery of this n-alkane may be lower in the horse than in ruminants.

In the individual diet treatments, estimated intakes using the markers were not found to be significantly different from measured values. The *L. perenne* diet displayed the highest discrepancy between measured and estimated mean intakes. This may be explained by looking at the faecal recoveries of the n-alkanes. The recovery of both C_{31} and C_{33} differed from that of C_{32} to a larger extent in this than in other diets, and these differences were found to be significant. Although intake estimates using the C_{33}:C_{32} n-alkanes were not significantly different from measured values the recovery differences may be partially responsible for the large discrepancies. Mean discrepancies between intake and estimates of intake were found in the fresh *P. clandestinum* diet where the differences between C_{32} and C_{33} faecal recoveries were lowest.

The C_{31}:C_{32} n-alkane pair also gave an overall estimate of intake which was not statistically significant to measured intakes. However, in the individual diet treatments significant differences did exist. The mean intake estimate for the hay+concentrate diet obtained using the C_{31}:C_{32} n-alkanes was significantly different ($P<0.05$) to the measured intake. Additionally, although for the hay only diet, the measured mean versus estimated intakes were not significantly different, individual animal mean intakes did differ significantly ($P<0.05$) from C_{31}:C_{32} estimates.

The faecal recovery of C_{32} (the dosed or external marker) was fairly consistent regardless of diet, and with the exception of the hay concentrate diet, was recovered to a greater extent than the internal (herbage) n-alkanes. The higher recovery of the C_{32} relative to the adjacent n-alkanes may be due to the association of the alkane with the liquid rather than the particulate phase of the digesta. Mayes *et al.* (1988) observed that natural n-alkanes were predominantly (95%) associated with the particulate phase of digesta while 30-40% of dosed n-alkanes were associated with the liquid phase (Udén *et al.*, 1982). This may result in a more rapid rate of passage of the C_{32} and a subsequent increase in faecal recovery (Dove and Mayes, 1991). The influence of diet type on the uniformity of marker mixing in the digestive tract of the horse may need further investigation.

Dove and Mayes (1991) explained that a 3% unit difference in marker recovery would, using the equation given by Mayes *et al.* (1986a), result in a 4.9% error in intake estimation. Overall intake error, in these trials, using C_{31}:C_{32} was 8.55% and 4.82% for C_{33}:C_{32} while recovery discrepancy was 6.2% and 3.6% between C_{31}-C_{32} and C_{33}-C_{32} respectively. Using these figures, an error of 10.12% for C_{31}:C_{32} and 5.8% for C_{33}:C_{32} based estimates may be explained by the differences in recovery of the markers in question, the errors in this study falling within this range. While marker recovery may help to explain the large discrepancies in mean measured and estimated intake, it is unlikely that marker recovery is the sole responsible factor.

The mean discrepancies for the overall trial and for individual diet treatments were higher than those reported for sheep 0 % (Mayes *et al.,* 1986a) and cattle 0.8% (Dillon and Stakelum, 1989) but similar to those reported by Vulich *et al.* (1991) when using sheep (8% for C_{31}:C_{32} and 3% for C_{33}:C_{32}). In the study by Vulich *et al.* (1991) DM intake estimates made using the C_{31}:C_{32} pair were underestimates while the C_{33}:C_{32} gave slight overestimates. In contrast, this study showed the overall C_{33}:C_{32} estimates were slight underestimates while the overall C_{31}:C_{32} tended to overestimate intake. A recent study in monogastrics (Cuddeford and Mayes, unpublished data), showed that the C_{33}:C_{32} led to a slight overestimate of intake in horses and a slight underestimate of intake in ponies and pigs (Gannon and Mayes, unpublished data), although this n-alkane pair gave what was considered to be satisfactory representations of actual intake with these animals (Mayes *et al.,* 1995).

The observation that the alkane pairs gave estimates of different direction cannot be explained in terms of faecal marker recovery, as similar recoveries of C_{31} and C_{33} were recorded. However, within diet treatments using either of the marker pairs, individual animal intake estimates gave both positive and negative discrepancy figures. Thus, both underestimates and overestimates of intake were recorded using either marker pair.

Mean discrepancies were calculated for individual animals by subtraction of estimated (n-alkane based) intake from measured values on a daily basis and mean values over the trial period. Voluntary intakes varied from day to day over the experimental period and the standard errors of the mean for both estimated and measured intake reflect this. The fact that intake varied from day to day and the n-alkane estimate of intakes reflect intake over a period of time which may not correspond exactly to the 24 hour day in question, may be responsible for the high standard errors observed, and also the calculated discrepancy between actual and estimated intake.

The complicating effect of the voluntary intake variation is cause for concern as it effects the accuracy of the measured versus estimated intake comparisons. Initially the trials were planned to incorporate measures of intake control y restricting the amount of herbage made available, by feeding only a percentage of daily voluntary intake. However, even when the animals were being fed amounts that were below suggested (NRC, 1989) intakes the horses daily intake varied to a large degree. Individual horses consumed considerably less than suggested levels, even when forage was on offer at all times. Therefore it was felt that restriction of intake below these levels would be unacceptable.

While the lack of control of intake influences the comparison of actual vs estimated herbage intakes, the intake variation observed within these trials reflects more accurately the variation in intake found with the grazing animal, and therefore the response of the n-alkane technique to this variation. Increasing the length of the collection period in these trials may have resulted in more accurate mean measured intake values, but would not have overcome this variation problem.

Estimated individual animal intake deviations (Figure 5.2) from measured intakes were higher in the case of C_{31} estimates than C_{32} estimates. These deviations appeared to increase as intake increased, especially for the C_{31} estimates. Individual intake estimations on a daily basis, articularly at higher intakes may have contributed to high mean discrepancies.

Dove *et al.* (1989) observed that in sheep, where intakes were low, n-alkane based estimates of DM intake in sheep were higher than those obtained by Cr_2O_3/*in vitro* estimates, and where intakes were higher, n-alkane estimates were lower. It was argued that differences in herbage digestibility and rates of passage, associated with differences in intake were accommodated by the n-alkane technique while not so using the Cr_2O_3/*in vitro* procedure. While intake estimates appeared to agree with this, in giving reasonable estimates of intake, the C_{31}:C_{33} estimates were more erratic at all levels of intake than C_{33}:C_{32} estimates, particularly at high intakes. To some extent more erratic discrepancies obtained with the C_{31}:C_{32} n-alkane pair may be partially explained by the lower recovery of C_{31} relative to C_{32} but rate of passage data (see previous chapter) suggests similar behaviour of C_{31} and C_{33} n-alkanes in the digestive tract. The reasons for the more erratic behaviour, and also the mean overestimate of intake rather than the expected underestimate, relative to estimates, are therefore unclear.

The addition of concentrates to the diet had no apparent effect on the precision of herbage intake estimation in studies with sheep (Mayes *et al.*, 1986a), nor in this study with horses.

Overall dry matter intake estimates obtained using pooled faecal grab samples taken twice daily were not significantly different ($p>0.05$) from measured values using either n-alkane pair. However, significant differences in individual mean estimates in the *L. perenne* trial were observed using the C_{31}:C_{32} n-alkane pair. These differences were not apparent in the total faecal collection trial. Using faecal grab samples, mean estimates of intake were lower than measured intakes, fitting in more with ruminant data than was apparent in the total faecal collection trial. The diurnal variation in proportion of the markers was discussed in the previous chapter. Malossini *et al.* (1994) reported that only two faecal grab samples taken each day are sufficient for accurate estimates in cattle studies. However, due to the diurnal variation evident in this study, more frequent sampling may have allowed more accurate estimates to be made.

Overall intake discrepancies were again higher for the C_{31}:C_{32} pair than those obtained with C_{33}:C_{32} n-alkanes. However, the high standard errors associated with both the estimated and actual intake values observed with the comparative data, obtained for both total and grab sample faecal collections, makes it difficult to determine any real difference between actual or estimated intakes, nor differences between the estimates made using the different n-alkane pairs.

The equation of Mayes *et al.* (1986a) does not require that an independent estimate of digestibility be obtained in order to calculate intake. However, in ruminants the faecal recovery of C_{35} has been reported to be highly (95%) recoverable (Dove and Mayes, 1991) and to give better digestibility estimates than those obtained using *in vitro* or lignin based methods (Dove *et al.*, 1990; Dove and Coombe, 1992). The value of the C_{35} digestibility estimate is however reliant on the recovery of this marker in the faeces of the animal and the level of the marker in the herbage (Dove and Mayes, 1991).

In this study the faecal recovery of C_{35} was lower than that reported in ruminant studies (85% vs 95%) and was only marginally higher (not significant) than the recovery of the lower order odd-chain alkanes. The digestibility estimates made with this marker had no advantage over estimates made with C_{31} or C_{33}. Dove and Coombe (1992) found that C_{31} and C_{33} gave accurate estimates of diet digestibility in sheep provided correction for the recovery of these markers, which is consistent in all sheep studies (Dove and Mayes, 1991), was made before calculation. A similar correction made for the faecal recovery in this study would have resulted in more accurate digestibility estimates being made with the n-alkanes. However, insufficient data relating to the consistency of faecal n-alkane recovery is available in the equine. Faecal recovery of the n-alkanes did differ according to herbage species consumed by the horses but was not significantly effected by the addition of concentrates. Casson *et al.* (1990) also reported a difference in faecal n-alkane recovery in sheep consuming different pastures, which contradicts the assumption made by Dove and Coombe (1992) that faecal recovery of the n-alkanes are always consistent. Digestibility estimation in the practical situation, or in grazing trials, would require that situation-specific faecal n-alkane recoveries were obtained prior to the onset of the trial, unless further studies in the horse show the consistent levels of recovery of the n-alkanes reportedly (Dove and Coombe, 1992) seen in sheep studies.

The use of chromium markers and acid insoluble ash (AIA) have been successfully used to estimate the digestibility of equine diets (Cuddeford and Hughes, 1992). The recovery of n-alkanes in this study suggest that digestibility estimates based on AIA and chromium markers may be more reliable than those based on n-alkanes. The measured digestibility coefficients for the three herbage types were lower than expected for the horse, particularly the very low value for the grass hay, and the poor digestibility of the *L. perenne*. While no comparable figures for the fresh grass DM digestibility in horses could be found for the different species, various grass hays were reported to have DM digestibility, in the equine (*in vitro* analysis), of between 46% and 56% (Fonnesbeck *et al.*, 1967). The fresh grass DM digestibilities were only marginally higher than these values while the grass hay was substantially lower. The analysis of herbage quality (Table 5.6) reveals that the herbages consumed were of poor quality, particularly the grass hay. However, the dry matter digestibility of diets consumed by horses grazing pastures containing *P.clandestinum*, *Lotonis bainesaii*, *Trifolium repens* and *Paspalum plicatum* were reported to range from 42% to 59% (McMeniman *et al.*, 1990) which agrees with the estimates made here.

The feeding of concentrates did not significantly effect the amount of hay being consumed by the horses. McMeniman *et al.* (1990) reported a substitution effect in grazing horses when fed additional concentrates. The substitution effect being that a 1kg increase in pelleted concentrate feed intake led to a 0.7 kg decrease in grass intake. However, in this study the effect of adding concentrates to the diet of horses consuming grass hay resulted in mean DM intakes increasing with little or no decrease in hay DM intake. This lack of substitution may be due to an increased rate of passage of the total diet with the concentrate feed and also to its organoleptic qualities. A study with horses (Smoulders and Houbiers, personal communication) found that, contrary to the hypothesis that a horse would increase DM intake to compensate for low herbage energy content, horses consumed more DM when on a higher energy herbage, at least over a short period of time. Certainly in this study, the poor digestibility and quality of the grass hay did not result in a higher DM intake. Observation of the horses at meal times during the trial periods, while purely subjective, appears to confirm that the presence of the concentrate had a stimulatory effect on appetite. The length of the meal and the frequency of visits to the manger appeared to increase when concentrates were fed in conjunction with grass hay.

The use of the long chain n-alkane pairs in this study provided estimates of herbage dry matter intake which did not differ significantly from measured intake. These accuracy of these estimates did not appear to be effected by the inclusion of concentrate feeds in the diet, or by the type/species of the herbage used in this study.

The intake data corresponds reasonably well with ruminant data (Dove and Mayes, 1991) and unpublished non-ruminant studies. However, the complicating effect of feed intake variation, which was rarely reported in these other studies, may have limited the precision of the measured versus estimated intake comparisons. Nonetheless, by applying the methods described in this study, it appears that n-alkanes, in particular the C_{33}:C_{32} pair, may give reasonable estimates of herbage dry matter intake for grazing horses and those consuming both forage and concentrates. The faecal recovery of long chained n-alkanes reported in this study were similar to those found in ruminants with the exception of C_{35}. The recoveries of these n-alkanes did not, unlike in ruminant studies, increase significantly with increasing chain length. The low recovery of C_{35} found in this study results in this marker being of limited use as a digestibility indicator in horses, as is the case with the C_{31} and C_{33} n-alkanes, unless faecal recovery of these markers is known, and corrected for, in calculation of the digestibility estimate.

GENERAL DISCUSSION

The primary objective of this study was to determine whether the n-alkane technique was capable of providing an accurate estimate of herbage intake in horses. Further objectives included the collection of data which may enable improvement in the application of the technique to these animals, and to highlight areas where further research may be beneficial in this regard. These objectives were apparently fulfilled during this study.

The data collected in the preliminary trial indicated that reasonable estimates of herbage intake may be obtained with the C_{32}:C_{33} n-alkane pair. This observation was confirmed during the remaining total faecal collection trials, where the technique was applied to animals consuming different herbage types, or when concentrates were included in the diet. The ability of n-alkanes to provide an accurate estimate of herbage intake when concentrates are being fed to grazing horses is important for the practical application of the method, as such feeding systems are commonly applied in the thoroughbred industry.

The ability of the n-alkane pairs (both C_{32}:C_{33} and C_{32}:C_{31}) to give reasonable estimates of herbage DM intake when different species or different forms of the grass were fed is also important. However, the grass types used in these horse trials contained relatively high concentrations of these n-alkanes. The analytical techniques used at present may result in decreased precision of intake estimates when very low levels of the n-alkanes are present in the herbage (Dove and Mayes, 1991) and the use of these n-alkanes for herbage diets other than those used in this study still needs to be determined. An example of this may be seen in the case of the horse. Lucerne (*Medicago spp.*) is a commonly used source of high quality forage in equine diets and is fed both fresh and as a hay on many South African thoroughbred stud farms. This forage contains low levels of C_{33} (Table 1.1) which would limit the use of this marker in intake estimations. However, this plant species contains high levels of C_{31} which may be a useful alternative in this case.

The estimates of herbage DM intake using this n-alkane (C_{31}) were, in this study, found to be more inconsistent than those made using the C_{32}:C_{33} pair, particularly when intakes were high. The reasons for this observation are not clear and may require further investigation. Nevertheless, overall estimates made using the C_{32}:C_{31} n-alkanes were not statistically different to measured intakes in this study. The lack of an imposed restriction of intake in this trial makes comparison of the intake estimate discrepancies with those reported for ruminant studies, where daily intake variation was not reported, more difficult to assess. While studies with sheep (Mayes *et al.*, 1986a) and cattle (Dillon and Stakelum, 1990) have reported lower discrepancies than found in this study, other studies (Vulich *et al.*, 1990) have discrepancies of a similar magnitude to those observed in the horses. This would suggest that the technique has similar validity in both ruminants and the horse.

A lack of published data concerning the accuracy of the n-alkane technique in horses makes comparisons between this and other equine studies difficult. However, unpublished data cited by Mayes *et al.* (1995) confirms the general observation that the C_{32}:C_{33} n-alkane pair may give an accurate estimate of intake in the equine.

The use of markers to specifically provide an estimate of herbage intake in horses has been reported only rarely, again making comparisons between this technique, and other marker techniques more difficult. The combination of AIA as a digestibility indicator and chromium markers to estimate faecal output have reportedly given reasonable estimates of herbage intake in horses in indoor trials (Barbisan *et al.,* 1993), and with grazing horses (McMeniman *et al.,* 1990). The n-alkane technique has advantages over these and other markers used in ruminant studies (Dove and Mayes, 1991; Mayes *et al.,* 1995), which may well apply to the horse. Certain problems encountered with these markers, such as diurnal variation in faecal marker excretion, were also encountered in the n-alkane study. The main advantage in using n-alkanes to estimate intake may be due to the reduction in the number of laboratory analyses to be performed, and the relative simplicity and accuracy of the n-alkane extraction/analysis procedure.

While the faecal recovery of the n-alkanes used in the intake estimation calculations were similar in horses and ruminants, certain important differences were apparent in this trial. Dove and Mayes (1991) suggested that the increasing faecal recovery of n-alkanes with increasing chain length is consistent in all ruminant studies, with the result that the long-chained C_{35} is highly recoverable ($\pm$95%) in these animals. This trend was not found to be significant in this trial, although small increases in recovery with increasing chain length were apparent. The lack of significant increase was also observed in unpublished non-ruminant studies (Mayes *et al.,* 1995), and may indicate a difference in behaviour of the n-alkanes in the digestive tract of ruminants and monogastric animals, which deserves further investigation.

The observation that adjacent n-alkanes are recovered to an equal extent in the faeces of ruminants, enabling the validity of the equation given by Mayes *et al.* (1986a), was confirmed in this study. The differences in recovery of C_{32} and the adjacent n-alkanes were however, greater than those reported in studies with sheep (Vulich *et al.,* 1990; Dove and Mayes, 1991). The n-alkane C_{35} was recoverable to a lesser extent in the faeces of horses, which is a further indication that differences in the behaviour of the n-alkanes in these animals and ruminants may exist. Future studies in horses may or may not confirm the reduction of faecal recovery of C_{35} seen in this study over that observed for ruminants.

The low recovery of the n-alkanes C_{31}, C_{32}, C_{33} and in the case of the horse, C_{35}, resulted in the use of these markers as digestibility indicators, being limited. The n-alkanes which are incompletely recovered have been used in studies with sheep (Dove and Coombes, 1992) to determine herbage

digestibility. To enable accurate estimates, the loss of n-alkanes had to be taken into account, and in the sheep study, these losses were assumed to be constant. In this trial, the recovery of the n-alkanes differed according to the grass species being consumed, and though not significantly, according to the form in which the herbage was fed. This would suggest that faecal recoveries would have to known for the specific situation, before these n-alkanes could be used to give accurate estimate of herbage digestibility in the horse. Acid insoluble ash has reportedly given accurate and repeatable estimates of diet digestibility in the horse (Sutton *et al.*, 1977; Cuddeford and Hughes, 1990), and based on this study, may be a preferred digestibility indicator to the n-alkanes used in this study.

Despite the accuracy of intake estimates reported during the indoor trials where total faecal collections took place, the value of the technique depends on its ability to provide accurate intake estimates in the grazing horse, using only a few faecal sample to make these estimates.

The overall intake estimates made using the n-alkane pairs, when n-alkane concentrations in faecal grab samples (pooled sub-samples of three samples) were used in calculations, were not significantly different from measured values. These results would suggest that similar dosing and sampling procedures applied to the grazing horse would yield accurate herbage DM intake estimates.

Diurnal variation in faecal n-alkane concentrations (proportion C_{32}:C_{33}) did not appear to have significant influence on the intake estimates made with faecal grab samples. However, data collected at a later date when horses were consuming a mixed diet revealed two important factors which need to be considered. The first observation was that diurnal variation in n-alkanes under the given conditions were large and individual faecal samples taken during the day may result in poor intake estimation. The second observation was that single samples taken at intervals, contradicted the observation made in the preliminary trial, that a steady state of C_{32} n-alkane excretion was achieved after only two days of dosing. The "plateau" observed in the preliminary trial, and the intake estimates made in chapter 5 were based on pooled (mean) samples rather than single samples and did not reveal the extent of the diurnal variation. This variation in excretion may have been more exaggerated when the animals were consuming diets containing both herbage and concentrates, due to the increased rates of passage of the marker, when dosed with or prior to, the concentrate meal. The effect of the timing of marker dosing may have influenced the extent to which the marker was able to mix with the digesta, although no published literature could be found to confirm this. Patterns of variation in marker excretion, observed in studies with chromium markers (Haenlein *et al.*, 1966; Parkins *et al.*, 1982) have been explained by differing rates of passage of dietary ingredients (Cuddeford and Hughes, 1990), although the possible effect of the timing of the marker dose was not previously considered.

The incomplete mixing of the external marker, observed in this study, and diurnal variation in faecal excretion has been reported in other marker studies with horses. As with ruminant studies, a more

frequent dosing regimen may reduce the observed variation in faecal marker and improve intake estimates under grazing conditions. Unfortunately, the data concerning the variability of the n-alkanes in the faeces of horses was available only after the total collection trials had taken place. The frequency and timing of dose administration, as well as the factors influencing the ability of the administered dose to mix uniformly with digesta in the equine hindgut needs further investigation. An apparent advantage of the n-alkane technique over other marker methods is the simplicity of the analysis of the n-alkanes as discrete substances (Dove and Mayes, 1991). While this may be true when a smoothly running analysis system exists, the problems and delays encountered due the analysis procedures in this study cannot be ignored if similar studies are to be undertaken. However, the reduction in numbers of samples that need to be analysed to obtain the levels of both external and internal markers, and even more n-alkane pairs where desired, is an advantage over other techniques.

Conclusions

The data collected in this trial, and comparison of this data with published literature suggests the n-alkanes may have advantages over other markers used for herbage intake estimation, in both the equine and ruminant animals. The intake estimates made using the C_{32}:C_{33} n-alkane pair in this trial were comparable to data presented in ruminant studies, and while the C_{31}:C_{32} pair were more inconsistent indicators of intake, this pair may be useful intake indicators when the levels of C_{33} in herbage consumed by the horses is low. The addition of concentrates to the diet or herbage type consumed, did not result in significant changes in accuracy of the technique yet effected the recovery of the n-alkanes, which is important to consider under certain conditions. The use of the n-alkanes as digestibility indicators in horses appears to be limited, due the low recovery of these markers, the uncertainty as to whether these markers are consistently recoverable to the same extent, and the possible difference in behaviour of these n-alkanes in the digestive tracts of ruminants and horses.

The results of this trial also suggest that faecal grab sampling may enable accurate intake estimation in the horse under grazing conditions, but that the problem of diurnal variation observed in this trial needs to be addressed. Practically this problem may be overcome, to some extent, by more frequent dosing of the external n-alkane, but further research may be necessary to confirm this.

The lack of available information concerning the use of the technique in the horse is a major disadvantage of this technique. Moreover, as the majority of published work concerning the use of the technique in ruminants has originated from the work of only a few authors, certain practical problems regarding the application of the technique, as experienced in this study have seldom been reported.

The aim of this study was to assess the value of the n-alkane technique in providing estimates of herbage dry matter intake in horses as well as to suggest areas where the application of the technique

may be improved. Possibly the most important conclusion that can be drawn from this study is that further research is needed before the n-alkane technique, as described in this study, can be applied with confidence to the grazing horse. The main areas in which information is still required have been highlighted, and some of the problems which may be encountered in applying the technique identified, hopefully aiding future investigations of the technique in horses.

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Appendix 1: Derivation of the equation used to estimate herbage dry matter intake using the n-alkanes (Dove and Mayes, 1991)

Let the concentrations of the natural, odd-chain alkane in herbage and faeces be H_i and F_i respectively. Assuming that the faecal recovery of the herbage alkane is 1·0, the indigestibility of herbage is given by

$$\text{Indigestibility} = H_i/F_i.$$

Similarly, let the concentrations of the dosed, even-chain alkane in herbage and faeces be H_j and F_j respectively, and let the daily dose of this alkane be D_j. If herbage intake = I, faecal output (O) then is given by

$$O = \frac{\text{Dose + intake of even-chain alkane from herbage}}{\text{Faecal concentration of even-chain alkane}}$$

i.e.,

$$O = \frac{D_j + I^*H_j}{F_j}.$$

Now from equation (1) above

$$\text{Intake} = \frac{\text{Faecal output}}{(1 - \text{Digestibility})} = \frac{\text{Faecal output}}{\text{Indigestibility}}$$

$$I = \frac{D_j + I^*H_j}{F_j} \div \frac{H_i}{F_i} = \frac{D_j + I^*H_j}{F_j} * \frac{F_i}{H_i}$$

$$I^*F_j^*H_i = F_i^*D_j + F_i^*I^*H_j$$

$$I^*(F_j^*H_i - F_i^*H_j) = F_i^*D_j$$

$$I = \frac{F_i^*D_j}{(F_j^*H_i - F_i^*H_j)} = \frac{F_i^*D_j}{F_j^*(H_i - (F_i/F_j)^*H_j)}$$

$$I = \frac{F_i}{F_j}^*D_j \Big/ \left(H_i - \frac{F_i}{F_j}^*H_j\right).$$