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SUMMARY - The results of several experiments which demonstrate varying aspects in the use of n-alkanes in animal feeding trials are presented. In particular, the following have been considered: the concentration of n-alkanes in forages and sources of variability, some analytical aspects, faecal recovery and the use of n-alkanes as markers for the estimation of digestibility and intake.

Key words: N-alkanes, markers, digestibility, intake.

RESUME - "Les n-alcanes comme marqueurs en essais d'alimentation". On expose les résultats de quelques recherches ayant pour objet différents aspects de l'utilisation des n-alcanes dans les études d'alimentation animale. En particulier nous avons considéré la concentration en des n-alcanes dans des fourrages, ainsi que quelques sources de variabilité, quelques aspects analytiques, leur récupération dans les fèces et leur emploi comme marqueurs pour l'évaluation de la digestibilité et de l'ingestion.

***Mots clés** : N-alcanes, marqueurs, digestibilité, ingestion.*

Introduction

The possibility of using the epicuticular wax components present on the surfaces of the aerial parts of higher plants, and in particular the n-alkanes, as markers in animal feeding studies was first suggested by Mayes *et al.* (Mayes and Lamb, 1984; Mayes *et al.*, 1986).

A complete review of the subject has recently been published (Dove and Mayes, 1991); for a detailed discussion of the chemistry of the plant cuticular waxes, we refer to the reviews by Kolattukudy (1976), Tulloch (1976) and Baker (1980).

We consider below some aspects regarding the presence of n-alkanes in forages and their use as markers, principally referring to the results of experiments at our Department.

Forage alkane concentrations and their variability

In a recent trial (Malossini *et al.*, 1990), the concentration of n-alkanes was studied in 3 species of gramineae (*Dactylis glomerata*, *Lolium multiflorum* and *Lolium perenne*) and 3 of leguminosae (*Medicago sativa*, *Trifolium repens* and *Trifolium pratense*). Each species was represented by 3 cultivars; the samples were collected to correspond with the 1st and 2nd vegetative cycle.

Table 1 shows that the n-alkanes measured were between C_{27} and C_{35} and, in all the species analyzed, those with odd carbon chain lengths were prevalent. The most frequently found homologues were C_{29} and C_{31}. The quantity of odd-chain alkanes present varied between 94.4% and 96.6% of the total C_{27}-C_{35} alkanes.

For nearly all the n-alkanes, considerable and statistically significant differences were observed between the species within family, but not between families.

Fig. 1 shows the difference of n-alkane concentrations of between the 2nd and 1st vegetative cycle. As can be seen from this figure and as confirmed by the analysis of variance, it is not possible to demonstrate any systematic differences linked to the cycle.

The results of an experiment conducted on samples of *Dactylis glomerata* harvested either fresh or as hay are presented in Table 2 (Piasentier *et al.*, 1989). Hay making resulted in a loss of n-alkanes, probably as a consequence of the detachment of parts of the plant, such as the leaves, which are rich in epicuticular waxes or of a loss of wax during the harvesting process.

Analysis of alkanes

According to the method proposed by Mayes *et al.* (1986), n-alkanes are determined by gas chromatography of the unsaponifiable fraction of the ether extract purified in silica gel, using C_{34} as an internal analytical standard.

The method permits a good level of repeatability (Table 3) and can be performed with instruments normally found in analytical laboratories (Piasentier *et al.*, 1989).

One limitation of the method is the time required for the extraction: Mayes *et al.*, (1986) recommend 6 hours in the Soxhlet apparatus for forages and 40 minutes in Soxtec for faeces. However, as Table 4 shows (Malossini *et al.*, 1991), independently of the initial substrate concentration, the n-alkane extraction in Soxhlet can be considered complete in decidedly shorter times.

It has recently been proposed that the Soxhlet extraction phase be omitted, with the sample being saponified directly (Dove and Mayes, 1991; Dillon and Stakelum, 1990).

Table 1. Forage n-alkane concentrations (mg kg^{-1} DM) in some forages

Alkane(1)	Gramineae				Leguminosae				Mean square(2)
	Dactylis glomerata	*Lolium multiflorum*	*Lolium perenne*	Mean	*Medicago sativa*	*Trifolium repens*	*Trifolium pratense*	Mean	
C_{27}	20^{b}	105^{a}	36^{b}	53	36	38	30	35	291.60
C_{28}	2^{b}	8^{a}	6^{a}	5	9^{b}	7^{b}	11^{a}	9	2.15
C_{29}	38^{c}	260^{a}	142^{b}	147	202^{b}	109^{c}	408^{a}	240	895.65
C_{30}	2^{b}	11^{a}	12^{a}	8	12^{a}	5^{b}	5^{b}	8	1.18
C_{31}	58^{c}	250^{a}	220^{b}	176	324^{a}	67^{b}	57^{b}	149	444.51
C_{32}	2^{c}	4^{b}	7^{a}	4	7^{a}	1^{b}	1^{b}	3	0.26
C_{33}	21^{c}	43^{b}	99^{a}	54	21^{a}	7^{b}	11^{b}	13	64.88
C_{35}	0^{b}	0^{b}	9^{a}	3	0	0	0	0	5.15
Total	143^{c}	681^{a}	531^{b}	452	611^{a}	234^{c}	523^{b}	456	2384.93
Odd-chain %	95.8	96.6	95.3	95.9	95.4	94.4	96.8	95.6	

On the same line, within family and between families, a, b, c: $P<0.05$
(1) C_{34} was not reported because it was used as an internal analytical standard
(2) Degrees of freedom = 24

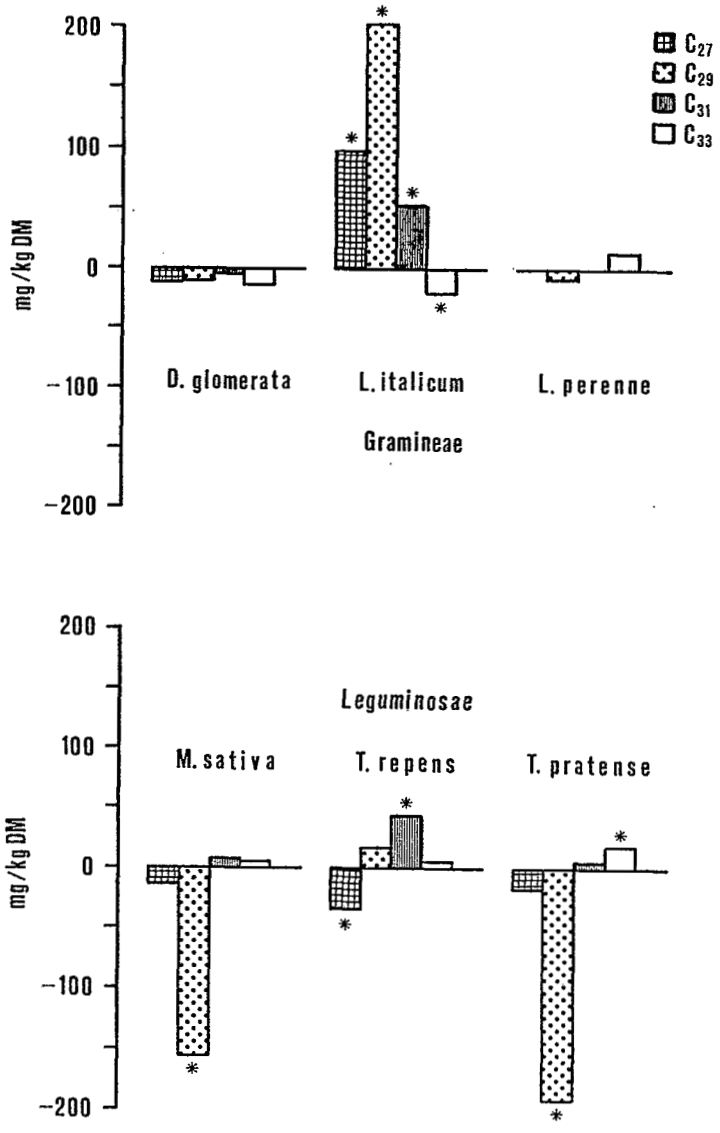

Fig. 1. Differences between n-alkanes contents in the 2nd and the 1st cut of forages (* = P<0.05).

Table 2. Natural n-alkanes in *Dactylis glomerata* (mg kg^{-1} DM, mean ± SE)

	Fresh	Hay	P
Nº samples	6	6	
Alkane:			
C_{27}	19.0 ± 2.77	11.3 ± 2.43	0.09
C_{29}	26.2 ± 3.34	17.0 ± 2.21	0.06
C_{31}	31.9 ± 4.03	25.1 ± 3.87	--
C_{33}	11.8 ± 2.21	10.1 ± 1.85	--

Table 3. Repeatability of n-alkanes determinations

Alkane	SD(1)mg kg^{-1} DM	r_I(2)
C_{27}	4.56	0.858 ***
C_{29}	2.20	0.984 ***
C_{31}	1.98	0.994 ***
C_{33}	0.91	0.995 ***

(1) Within samples standard deviation (DF = 20)
(2) Intraclass correlation coefficient
*** = P≤0.001

Table 4. Effect of extraction time on the determination of C_{31} in feeds with different concentrations of the n-alkane (mg kg^{-1} DM)

Extraction time in Soxlhet (h)	Feed			Mean
	A	B	C	
1	17	51	78	49
2	15	52	77	48
3	17	52	79	49
4	16	53	84	51
6	16	52	83	50
6Mean	16	52	80	49
SE	0.4	0.3	1.4	0.3

Administration of even-chain alkanes

Even-chain alkanes, present in minimal quantities in forages, are available as purified preparations and can be used as external markers in combination with the naturally present odd chain homologues, for the estimation of intake by grazing animals.

The original method (Mayes *et al.*, 1986) described the administration to the animals of shredded paper pellets which contained the even chain alkanes. The analysis of a batch of the pellets gave a coefficient of variation for their alkane content of between 1.5% and 2.5% (Malossini *et al.*, 1991).

As an alternative to the paper base, the use of cellulose powder contained in gelatine capsules (Dove et *al.*, 1989) further reduces the already low variability in the content of even-chain alkanes.

Recently, Dove *et al.* (1991) has proposed the use of intraruminal controlled release devices which avoid the need for daily dosing.

Faecal recovery of markers

Fig. 2 reports the average faecal recovery of the natural and dosed n-alkanes in 3 trials with sheep (Piasentier *et al.*, 1989; Piasentier *et al.*, 1991; Malossini *et al.*, 1991).

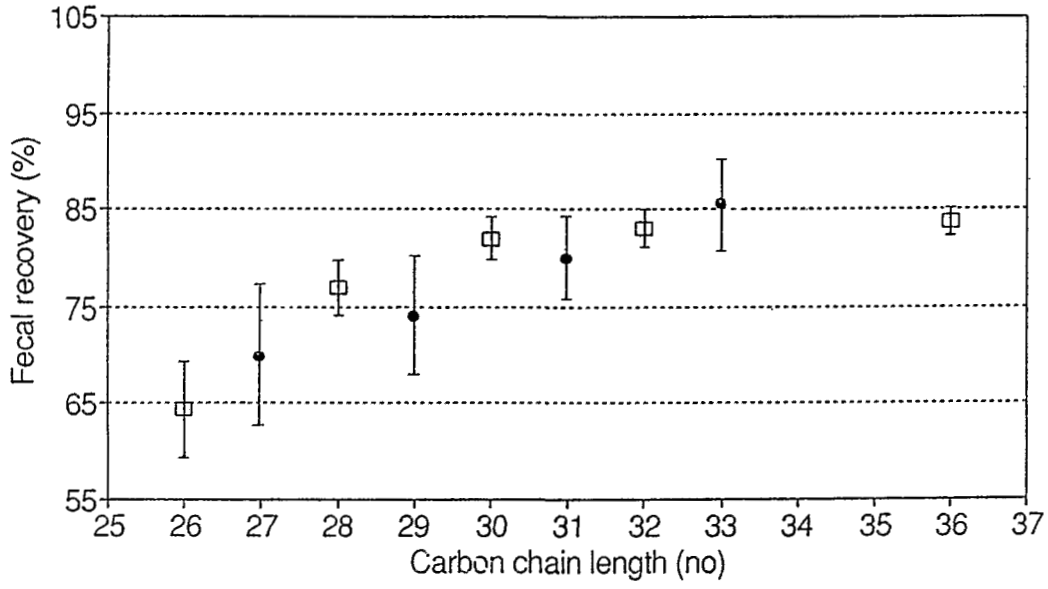

Fig. 2. Faecal recoveries of n-alkanes: effect of carbon chain length (mean ± SD).

The faecal recovery of n-alkanes, always incomplete, increases with increasing carbon chain length at an ever reducing rate; as a consequence, the difference in recovery between adjacent homologues tends to diminish.

In general, the recovery of dosed n-alkanes was less variable than that of the natural n-alkanes, probably due to a more precise evaluation of the quantity administered daily.

The variability of the recoveries tends to diminish with increasing carbon chain length; if the natural n-alkanes are used as internal markers for the estimation of digestibility, it follows that more correct estimates will be obtained for the longer

homologues. This aspect is demonstrated in Fig. 3 which shows the relationship between the direct measurement of digestibility (DD) and the estimate with index method (IMD); the RSD are between 3.38 for C_{27} and 2.64 for C_{33}.

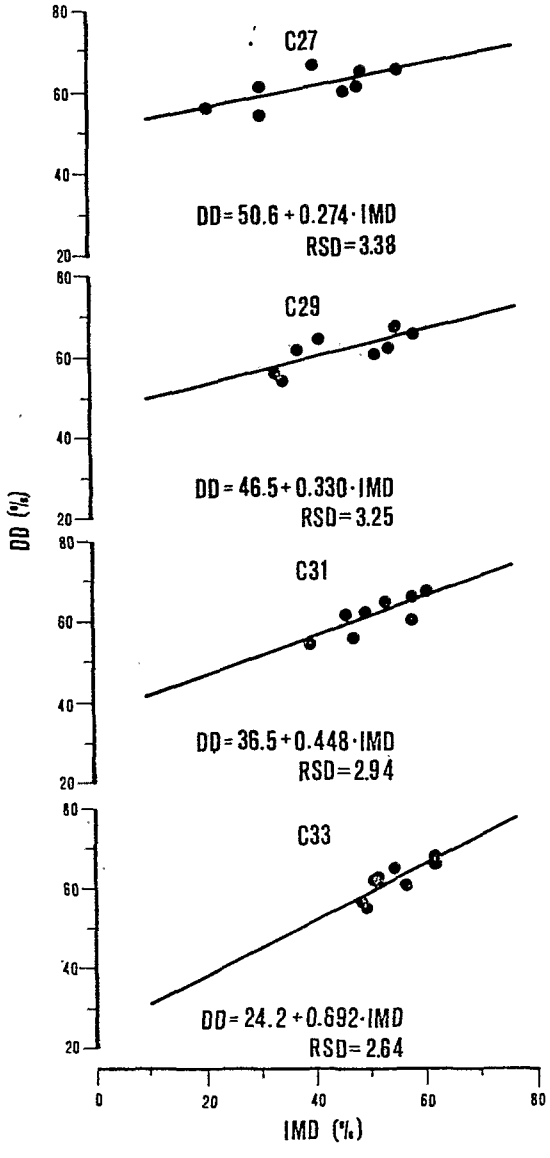

Fig. 3. Relationship between index method digestibility (IMD) and direct digestibility (DD).

Estimate of intake

The n-alkanes are extremely useful for the estimate of intake by grazing animals. In this case the odd-chain homologues function as internal markers while the even-chain homologues are administered as external markers.

If Dj is the quantity of external marker administered daily (mg), Fj its concentration in the faeces, Hi and Fi the respective concentrations of the internal marker in the forage and in the faeces, then the forage dry matter intake I (kg) is given by:

$$I = \frac{Dj}{Hi} \quad \frac{Fi}{Fj} \qquad [1]$$

If the content of alkanes in the concentrate and the concentration of even-chain alkanes in the forage are taken into account, the formula becomes:

$$I = \frac{\frac{Fi}{Fj}\,(Dj + Ic\ \ Cj) - Ic\ \ Ci}{Hi - \frac{Fi}{Fj}\ Hj} \qquad [2]$$

where Ic is the concentrate intake (kg DM day^{-1}), Ci and Cj are the respective concentrations of odd- and even-chain alkanes in the concentrate and Hj is the concentration of even-chain alkanes in the forage (all concentrations of n-alkanes in mg kg^{-1} DM).

If, as has been seen, the recovery of adjacent homologues is similar, the ratio Fi/Fj and hence the estimate of I are not influenced by the recoveries. In other words it is possible to obtain an unbiased estimate of intake even if the recovery of the markers is incomplete.

In a study performed with heavy lambs in digestibility crates fed with pelleted diets containing low concentrations of odd-chain alkanes (Fig. 4), the smallest discrepancies were obtained with the pairs C_{31}/C_{30} and C_{31}/C_{32} (Malossini *et al.*, unpublished data).

Some consideration must be given to the methodological aspects. Given that the intake is calculated from [1], all the elements which are involved in the formula must be considered.

Dj represents the content of dosed n-alkanes, which is not an important source of variability; however, administration for more days gives more precise average measures.

The precision of the evaluation of Hi depends most of all on the availability of herbage samples representative of what the animal eats; the use of animals fistulated at the oesophagus could be helpful in this respect. If these are not available, it becomes necessary to collect a number of samples adequate to describe the complexity of the pasture, which itself depends upon the grazing habits of the animals on trial.

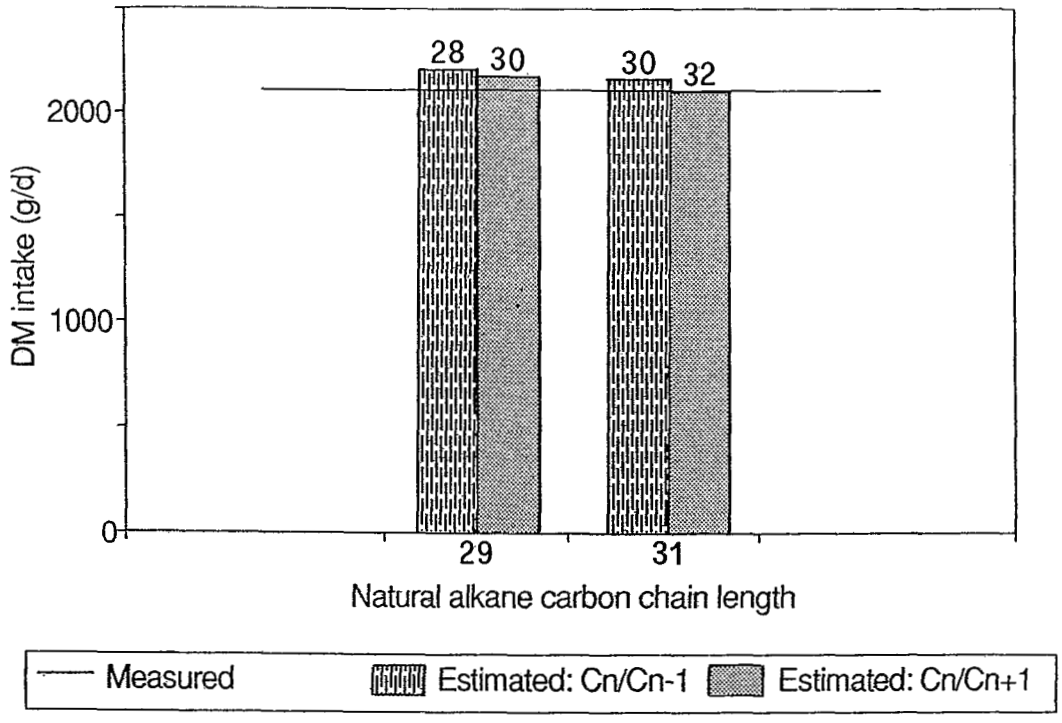

Fig. 4. Estimates of DM intake with different pairs of odd and even-chain alkanes compared to measured intake.

The Fi/Fj ratio remains to be considered. In general, the grab samples collected at different times during the day cause fluctuations, in the same ratio, independent of the sampling time with oscillations smaller than those recorded between days.

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