Nutrition of grazing cattle in the Mid Rift Valley of Ethiopia:

Use of an improved n-alkane method to estimate nutrient intake

Melkamu Bezabih Derseh

Thesis committee

Thesis supervisor

Prof. dr. ir. W.H. Hendriks

Professor of Animal Nutrition

Wageningen University

Thesis co-supervisors

Dr. ir. W.F. Pellikaan

Assistant professor, Animal Nutrition Group

Wageningen University

Prof. dr. A. Tolera

Professor of Animal Nutrition

Hawassa University, Ethiopia

Other members

Prof B. Kemp, Wageningen University

Prof G. P. J. Janssens, Ghent University, Belgium

Prof M. A. M. Rodrigues, UTAD, Portugal

Dr S. van Wieren, Wageningen University

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Thesis

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Melkamu Bezabih Derseh
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Abstract

Nutrient intake is an important factor that determines the performance of production animals. In free ranging animals, direct measurement of nutrient intake is difficult to conduct, and it is frequently estimated indirectly by the aid of markers. The aim of this thesis was to investigate the potential of using cuticular n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as markers to study the nutritional ecology of grazing animals under tropical conditions. In addition, this improved method was used to determine the seasonal patterns of nutrient intake and diet composition of grazing cattle in the Mid Rift Valley grasslands of Ethiopia. The first focus of the thesis was to quantify the interspecies variability in the n-alkane profile and $\delta^{13}C$ values of alkanes among commonly available pasture species in the Mid Rift Valley of Ethiopia. The analysis showed that the variability is sufficiently large to allow n-alkane and their $\delta^{13}C$ values to be used as diet composition markers, with a combined use of the two increasing the discriminatory power. Faecal recovery of dosed and natural alkanes in cattle consuming low-quality tropical roughages was investigated in an indoor balance study. The recovery of synthetic alkanes dosed in the form of molasses boluses was considerably higher than adjacent natural odd-chain alkanes, and correction appears necessary when intake is estimated with the double n-alkane method. The next focus of the thesis was to generate information on the nutritive value of pasture species and nutritional status of grazing cattle in the region. Large variability was observed in the nutritive value and methane production potential of pasture species as evaluated *in vitro*, with scope for selection of genotypes with high nutritive value and low methane production potential for a sustainable pastureland management. The nutritional status of grazing cattle measured using a combination of n-alkanes, their $\delta^{13}C$ values and visual observations showed that diet composition and nutrient intake of the animals is highly dependent on rainfall patterns, with a cyclic positive (wet period) and negative (dry period) energy and nutrient balance observed over the grazing seasons. Energy intake was more limiting than crude protein for body weight gain in most of the grazing seasons. While mature and non-producing animals appeared to tolerate nutritional restriction in the dry period and regain lost body condition in the following wet periods, young animals before the age of puberty may need supplementary feeding. Furthermore, concentrate supplementation of finishing animals needs to coincide with the onset of the wet season to take advantage of compensatory growth. In conclusion, the n-alkanes method coupled with isotope enrichment in n-alkanes and visual observations as used in the present study can provide realistic nutritional data for free-ranging cattle which correlates well with changes in body conditions.

Table of contents

Chapter 1	General introduction	1
Chapter 2	Evaluation of n-alkanes and their carbon isotope enrichments as diet composition markers	11
Chapter 3	Using n-alkanes and their carbon isotope enrichments to estimate the botanical composition of pasture mixes in the Mid Rift Valley grassland of Ethiopia	29
Chapter 4	Estimation of intake and digestibility of cattle consuming low-quality tropical roughages using molasses-based n-alkane boluses	45
Chapter 5	Chemical composition, *in vitro* total gas and methane production of grass and browse species from the Mid Rift Valley grasslands of Ethiopia	67
Chapter 6	Nutritional status of grazing cattle in the Mid Rift Valley grasslands of Ethiopia measured by use of cuticular hydrocarbons and their isotope enrichments	87
Chapter 7	General discussion	113
	References	129
	Summary/Samenvatting	143
	Acknowledgment	151
	Curriculum vitae	155

CHAPTER 1

General introduction

Grazing herbivores are the largest contributors of livestock output in tropical regions, and natural pasture forms the main source of their feed (Brown and Ash, 1996). The availability and quality of tropical pastures experience distinct seasonal fluctuations following the rainfall pattern (Schlecht *et al.*, 1999; Schlecht *et al.*, 2006; Cline *et al.*, 2009). During the dry periods, the availability and quality of pasture may decrease to a level that may not fulfil the animal's maintenance requirement, resulting in body weight loss and low condition score (Hornick *et al.*, 2000; Tolla *et al.*, 2003). During the wet periods, pasture condition improves progressively allowing grazing animals to consume energy and nutrients in excess of their maintenance requirements. The level of nutritional restrictions (in the dry period) and excesses (in the wet period) are also dependent on the grazing pressure and the productivity of the available grasslands (Miller and Thompson, 2007).

To optimize the productivity of grazing animals on tropical pastures, it is essential to accurately monitor their energy and nutrient intakes along the different grazing seasons (Bailey *et al.*, 1998; Schlecht *et al.*, 1999; Dziba *et al.*, 2003). Such knowledge is vital to develop effective strategies for grassland management such as predicting the production performance of the grazing animals, identifying limiting nutrients for a target production, adjusting stocking rates according to the carrying capacity of pasturelands, and improving the productivity of pasturelands through reseeding of selected pasture species.

Nutrient intake is a resultant of the amount of diet consumed, the nutrient density of the diet, and availability of the nutrients after digestion in the gut (Launchbaugh *et al.*, 1990; Mayes and Dove, 2000; Coleman and Moore, 2003; Ribeiro Filho *et al.*, 2005). The quality of the diet consumed varies according to stage of maturity, the botanical as well as morphological compositions of the diet consumed (Machado *et al.*, 2007). As a result, measurement of feed intake, diet composition and digestibility are important nutritional variables to describe the nutrition of grazing animals (Dove and Mayes, 2006).

Herbage intake measurement of grazing animals

In animals constrained in barns or stalls, intake can be measured relatively easily by weighing the amount of feed offered and refused. In grazing animals, however, measurement of feed intake is more complicated as it is nearly impossible to be measured directly. Several indirect methods of intake measurement of grazing animals have therefore been developed, which can

be broadly classified as plant-based and animal-based techniques.

The plant-based intake measurement technique relies on the measurement of sward biomass before and after grazing with corrections for sward re-growth during the measurement period (Walters and Evan, 1979). This method does not allow intake to be estimated from individual animals unless a single animal is kept on a separate plot. Moreover, differences in the cutting height before and after grazing and estimation of vegetal accumulation during the grazing period may induce large errors, especially if the sward is heterogeneous and the grazing duration is long (more than two days) (Frame, 1993).

The animal-based techniques may include observations of the grazing behaviour of animals or the use of markers. Short-term intake rates have been estimated from the measurement of biting rate and bite size and the total time spent on grazing (Chacon *et al.*, 1976; Forbes and Hodgson, 1985; Parker *et al.*, 1993). This technique involves observation of biting rate and simulation of bite mass by hand-plucking. The bite mass can alternatively be calculated from the daily dry matter intake measured by other techniques (Chilibroste *et al.*, 1997). Intake is then calculated by bite mass × biting rate × grazing time. Behavioural studies using different grazing data recorders have allowed the application of this technique to a wide range of animal species (Gordon, 1995). However, this technique is time consuming and liable to large biases, especially in simulating a bite mass that represents the entire grazing duration for which intake is estimated (Rook, 2000).

Measurement of body weight before and after grazing, with corrections for body weight changes due to faecal, urinary and insensible weight losses (respiratory and other obligatory weight losses) has been applied to estimate short-term intake rates (Penning and Hooper, 1985). The insensible weight loss can be measured using animals penned in an environmental condition similar to that of the grazing animal but without access to food and water (Penning and Hooper, 1985). Concurrent measurement of total bite during the grazing time allows the calculation of mean bite size. In order to estimate the dry matter intake, an estimate of the dry matter ratio of the forage needs to be obtained. The technique could give accurate estimate of short-term intake (usually 1 hour), but it requires capital investment in terms of high precision weighing scales (Penning and Hooper, 1985; Murray and Brown, 1993). The other limitation is that the insensible weight loss measured with penned animals may not be representative for grazing animals.

Measurement of rumen fills before and after grazing using rumen cannulated animals, and determination of dry matter clearance rate is another technique to estimate the intake rate (Chilibroste *et al.*, 1997). However, the measurement is laborious and may not be acceptable from an ethical point of view, as the animal's rumen physiology, micro-flora and normal grazing behaviour would be affected by the emptying and refill of the rumen contents (Gregorini *et al.*, 2007).

Measurement of faecal output and digestibility has been the most widely used method to estimate long-term forage intakes of grazing animals (Dove and Mayes, 1991), whereby intake is calculated as faecal output/(1-digestibility). As collection of total faecal output in free-ranging animals is inconvenient and can disrupt normal grazing behaviour, faecal output is estimated through a dilution in faeces of an indigestible external marker. When the concentration of the dosed marker stabilizes in faeces through continuous dosing, the daily dose of the marker is related with the faecal concentration of the same to yield the daily faecal output estimates. For several decades, chromium oxide ($Cr_2O_{3)}$ has been the most extensively used marker for feed intake studies. A major limitation of this marker, apart from recent concerns over its carcinogenic properties, has been the error associated with the diurnal variation in faecal marker concentration (Malossini *et al.*, 1996).

The digestibility of forage consumed by the grazing animal can be determined either *in vivo* or *in vitro*, both of which require representative sample of the diet selected by the grazing animal to be obtained. Because the *in vivo* method is time consuming and expensive for a routine application, the *in vitro* digestibility test has been widely used. However, mimicking the forage diet selected by the grazing animal has remained a challenge, particularly when animals graze on heterogeneous vegetation. Another limitation of this approach is that a single digestibility coefficient is used across animals, without giving allowances for between-animal variation in diet digestibility due to genotype, parasite burden, physiological status or level of feed intake (Mayes and Dove, 2000). If concentrate supplements are provided, this method also does not provide reliable estimation of whole diet digestibility, particularly when there are digestive interactions between diet components (Dove and Mayes, 1996).

Faecal crude protein (CP) concentration has been used as an index of diet organic matter digestibility (OMD) (Langlands *et al.*, 1963). This method of digestibility estimation is based on the positive relationship between faecal CP concentration and diet OMD resulting

from the increasing undigested microbial CP and decreasing faecal OM as OMD increases. The main advantage of this approach is that it is non-invasive and does not require sampling of forage selected by the grazing animal. However, it requires calibration of the relationship with a large dataset generated from conventional (indoor) *in vivo* digestibility trials, and the validity of the estimate usually remains limited to the forage species and geographical location for which the equation has been developed (Langlands, 1975).

The ideal approach to estimate forage digestibility in the free-ranging animals would be to use an indigestible marker naturally occurring in the forage. However, many of the internal markers considered fell short of the criteria required of an 'ideal marker' (Kotb and Luckey, 1972). Plant components such as lignin, indigestible acid detergent fibre, indigestible neutral detergent fibre, acid-insoluble ash and plant silica have been evaluated as internal markers although none of them has gained acceptance because of inconsistent faecal recovery rates and hence unreliable results. The main reason is that most of them are empirical measurements and that what is analyzed as the marker in the diet may be chemically different from that measured in the faeces. Although plant silica is a discrete compound, its use has been undermined by the high probability of soil contamination with the forage sample analyzed.

Overall, although errors are introduced from both the separate measurement of faecal output and digestibility of herbage when intake is estimated with this approach, a small error in the estimate of digestibility can lead to much larger error in the estimate of intake, especially when diet digestibility is high and the denominator (indigestibility) is small (Dove and Mayes, 1991).

Use of plant cuticular wax components as markers

Nutritional markers are entities measurable in the diet and faeces of animals, and they serve as a tool to estimate nutrient intake and digestibility where direct measurement is impractical or laborious. Markers that originate from the diet are referred to as internal markers, and those that are absent from the diet but are administered by oral dosing or through rumen cannula are referred to as external markers. The properties of an 'ideal marker' are complete recovery in faeces, accurate quantitative measurement, inertness in terms of having no effect on the animal and having physical characteristics that are similar to the contents of the digestive tract

(Kotb and Luckey, 1972). However, no single substance has so far been found to fulfil all the 'ideal marker' attributes (Dove and Milne, 2006)

Plant cuticular wax contains various discrete chemical components including n-alkanes, long-chain fatty acids, primary alcohols, secondary alcohols and ketones (Dove and Mayes, 1991). n-Alkanes are widely distributed in the cuticular wax and their analysis is easier than the other wax components. The use of n-alkane markers in nutritional studies has gained increasing acceptance since the development of the double n-alkane technique to estimate forage intake of grazing animals (Mayes *et al.*, 1986). The carbon-chain length of the main alkanes usually range between C_{25} (pentacosane) to C_{35} (pentatriacontane). Shorter-chain length alkanes could be detected, but in very small quantities which have, therefore, little value as internal markers. Generally, in flowering plants, odd-chain n-alkanes are found in much higher concentration than even-chain n-alkanes. The characteristics of these cuticular n-alkanes are that they exhibit distinct profiles between plant species, and that they have a high recovery in faeces (Dove *et al.*, 1996). These characteristics have long enabled researchers to use alkanes in chemotaxonomic studies (Herbin and Robins, 1968) and, more recently, in predicting diet composition of free-ranging herbivores (Fraser *et al.*, 2006; Ferreira *et al.*, 2007b).

Estimation of forage intake using n-alkanes

The recovery of cuticular n-alkanes in faeces is recognized to be incomplete, with the recovery generally increasing with increasing carbon-chain length and adjacent n-alkanes showing similar recovery rates (Mayes and Lamb, 1984). Significant progress in the use of n-alkanes in nutritional studies was achieved with the development of the double n-alkane method (Mayes *et al.*, 1986) to estimate feed intake of herbivores without the need to measure faecal output and diet digestibility separately. This approach requires continuous dosing of a synthetic n-alkane until the faecal concentration of the dosed alkane stabilizes, and then forage intake is estimated using the ratio in faeces of dosed even-chain and an adjacent natural odd-chain n-alkane. Besides the discrete nature of the markers, this method enables estimation of intake of individual animals by taking into account animal-diet interactions. The assumption that adjacent even and odd-chain alkanes have similar faecal recovery rates may not always hold true, and in such case considerable discrepancies may occur between actual

and estimated intakes. Knowledge of the faecal recovery of dosed and forage n-alkanes is thus important to select suitable alkane pairs and, if necessary, to correct for differences in faecal recovery of the alkane pairs. Overall, various validation experiments showed that the double n-alkane method is more robust compared to all other methods of feed intake estimation in free-ranging animals (Hameleers and Mayes, 1998; Mayes and Dove, 2000; Ferreira *et al.*, 2007a).

Estimation of diet composition using n-alkanes

In natural grasslands where the vegetation is complex, the diet of grazing animals comprises different botanical species. The n-alkane method has been applied to quantitatively determine the diet composition of individual grazing animals from the alkane patterns found in faeces. This approach is based on the fact that different diet components have different n-alkane profiles and that the faecal alkane pattern reflects that of the diet consumed (Dove and Mayes, 2005). Least square optimization algorithms have been used to find a solution of diet composition by minimizing the squared differences between observed alkane pattern in faeces (corrected for incomplete recovery) and that indicated by the predicted diet composition (Dove and Mayes, 1996). The main advantage of this method is that diet composition can be estimated with little or no interference to the normal grazing behaviour of the animals, which is particularly important under rangeland conditions and when the nutrition of wild herbivores is considered.

The main constraint with this approach is that the number of potential diet components identified is limited to the number of n-alkane markers available. In complex vegetation, the diet of the herbivore may contain more botanical species than the available alkane markers. Taking this into account, recent research has focused on the use of other cuticular wax components, mainly long-chain fatty acids and long-chain alcohols together with alkanes to increase the discriminatory power in estimating diet composition (Ferreira *et al.*, 2011). In addition, linear programming algorithms that can avoid the constraint that the number of potential diet components should be equal or less than the number of available markers have been developed (Barcia *et al.*, 2007). Generally, the accuracy with which diet composition is estimated is expected to decline as the number of dietary components increases, but there is a

scope for the cuticular wax to provide additional markers to increase the discriminatory power of n-alkanes (Dove and Mayes, 2006).

Application of the n-alkane method under tropical conditions

Although there is ample data on the validation and application of the n-alkane method for temperate grasslands (Mayes and Dove, 2000), comparable data for tropical grasslands is scarce (Hendricksen *et al.*, 2002; Molina *et al.*, 2004). Owing to the importance of accurately assessing the nutrient intake and diet composition of free ranging animals on tropical grasslands, the use of the n-alkane method appears to be ideal to improve the quality and quantity of data generated on the nutrition of grazing animals. The requirements for wider application of this technique include determining the marker profile of major botanical species, examining the discriminatory potentials of the n-alkanes and other potential markers of the cuticular wax to estimate diet compositions, and generating reliable faecal recovery data of the markers under different diet scenarios (Dove, 1992; Ferreira *et al.*, 2009). In this respect, more work is required to validate and apply the method under tropical conditions.

Grazing livestock in Ethiopia

Ethiopia has the highest ruminant livestock population in Africa and tenth in the world (FAO, 2010). Despite the large livestock population, however, the productivity has remained very low mainly due to genetic, nutritional and disease constrains coupled with poor service delivery systems. About 62% of Ethiopia's landmass is classified as arid- and semi-arid where grazing livestock are the main source of livelihood for pastoral and agro-pastoral communities. This arid and semi-arid part of the country produces approximately 90% of the meat and live animal supply to local and international markets (Berhe *et al.*, 1999). Assessments on the condition of natural grasslands in the arid and semi-arid regions of the country have shown that the condition of the grasslands is deteriorating rapidly due to the lack of proper management and high grazing pressures (Gemedo *et al.*, 2006; Solomon *et al.*, 2007). To optimize resource utilizations and, at the same time, protect environmental damages (land degradation and greenhouse gas emission), it is important that knowledge-based grassland management decisions are implemented in the country (Kamara *et al.*, 2005). As

such, accurate estimation of nutrient intakes, diet composition and nutritive values of dietary components of the grazing animals will provide the knowledge base to improve both animal and grassland productivity in these semi-arid grasslands of Ethiopia.

Aim and outline of this thesis

The aim of the research described in this thesis is to investigate the use plant cuticular n-alkanes and their carbon isotope enrichments in measuring the nutrient intake of grazing animals in tropical grasslands, and to assess the seasonal pattern of nutrient intake and diet composition of grazing cattle in the Mid Rift Valley of Ethiopia.

Chapter 2 describes the cuticular n-alkane profiles and the carbon isotope enrichments of the alkanes for commonly available pasture species in the Mid Rift Valley grasslands of Ethiopia. Using multivariate analysis, the chapter explores the potentials to use alkane profiles and their carbon isotope enrichment as diet composition markers. Chapter 3 describes a laboratory validation experiment evaluating the accuracy with which the botanical composition of a pasture mixture can be estimated using either n-alkanes or a combination of n-alkanes and their carbon isotope enrichment. Chapter 4 describes a feeding experiment to measure the faecal recovery rate of dosed and natural n-alkanes in cattle consuming tropical roughage feeds. The experiment evaluates the use of molasses-based alkane boluses to administer synthetic n-alkanes, and compares alternative faecal sampling methods to estimate intake and digestibility using the n-alkane method. Chapter 5 describes the nutrient composition, *in vitro* gas and methane production potential of grass and browse species harvested during the main pasture growth period in the Mid Rift Valley of Ethiopia. The nutritive values of the available forages are predicted using the *in vitro* gas and chemical composition data. Chapter 6 describes a study where the nutritional status of grazing cattle in the Mid Rift Valley of Ethiopia was assessed with the aid of n-alkanes and their carbon isotope enrichment as markers in combination with visual observations. This chapter compares the estimated energy and nutrient intakes with requirements of the animals and predicts seasonal animal performances on pasture. Chapter 7 discusses the major research findings presented in this thesis, provides recommendations for further research and lists the main conclusions.

CHAPTER 2

Evaluation of n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as diet composition markers

M. Bezabih[1,2], W. F. Pellikaan[1], A. Tolera[2], W. H. Hendriks[1,3]

[1]Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Box 338, 6700 AH Wageningen, the Netherlands

[2]Department of Animal and Range Sciences, Hawassa University, P.O. Box 5, Hawassa, Ethiopia

[3] Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.151, 3508 TD, Utrecht, the Netherlands

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Abstract

Plant cuticular n-alkanes have been successfully used as markers to estimate diet composition and intake of grazing herbivores. However, additional markers may be required under grazing conditions with botanically diverse vegetation. This study was conducted to describe the n-alkane profiles and the carbon isotope enrichment of n-alkanes of common plant species from the Mid Rift Valley rangelands of Ethiopia, and evaluate their potential use as nutritional markers. A total of 23 plant species were collected and analyzed for long chain n-alkanes ranging from heptacosane to hexatriacontane (C_{27}-C_{36}), as well as their carbon isotopic ratio ($^{13}C/^{12}C$). The analysis was conducted by gas chromatography/combustion isotope ratio mass spectrometry following saponification, extraction, and purification. The isotopic composition of the n-alkanes is reported in the delta notation ($\delta^{13}C$) relative to the Vienna Pee Dee Belemnite standard. The dominant n-alkanes in the species were C_{31} (mean±sd, 283±246 mg/kg dry matter) and C_{33} (149±98 mg/kg dry matter). The carbon isotopic enrichment of the n-alkanes ranged from -19.37 to -37.40‰. Principal component analysis was used to examine interspecies differences based on n-alkane profiles and the carbon isotopic enrichments of individual n-alkanes. Large variability among the pasture species was observed. The first three principal components explained most of the interspecies variances. Comparison of the principal component scores using orthogonal procrustes rotation indicated that about 0.84 of the interspecies variances explained by the two types of data sets were independent of each other, suggesting that use of a combination of the two markers can improve diet composition estimations. It was concluded that, while the n-alkane profile of the pasture species remains a useful marker for use in the study region, the $\delta^{13}C$ values of n-alkanes can provide additional information in discriminating diet components of grazing animals.

Introduction

In extensive agro-pastoral systems rangelands are the main sources of nutrition for domestic and wild herbivores. In Ethiopia, about 62% of the total land mass is classified as arid and semi-arid and mainly used for livestock production based on grazing (Kassahun *et al.*, 2008). Proper management of grazing animals is important to maintain sustainable range resource utilization in such areas (Bailey *et al.*, 1998). Naturally, free-ranging herbivores grazing on diversified plant communities exert different levels of selection to optimize their nutrient intake (Prache *et al.*, 1998). Understanding the type of plant species selected by the animal and the contribution of each species to the total intake could give insight into the nutritional status of the animal and offer a feasible range management strategy to optimize resource utilization (Dumont *et al.*, 2002). However, measurement of feed intake, diet composition and nutrient digestibility in free-ranging animal remains a challenge in nutritional studies because of the inherent errors associated with the presently used methods (Dove and Mayes, 1991; Mayes and Dove, 2000).

The use of plant wax components, mainly n-alkanes, as markers for estimation of intake and diet composition of herbivores evolved in the last two decades (Dove and Mayes, 2005; Ferreira *et al.*, 2007a,b). n-Alkane profiles of plants show distinct differences between species and to some extent between plant parts of the same species. In addition, they have high recovery rates in the faeces of herbivores (Ferreira *et al.*, 2009) offering an opportunity to reconstitute the diet of the herbivore from the faecal patterns of these compounds (Bugalho *et al.*, 2004; Dove and Mayes, 1996). Validation experiments revealed the potential of using n-alkanes to estimate intake, diet composition and nutrient digestibility of individual animals (Monks *et al.*, 2005; Oliván *et al.*, 2007a).

Although validation experiments using n-alkanes produced good estimates with less complex vegetation, grouping of species or use of other markers in addition to n-alkanes was necessary for correct estimation of diet composition of herbivores grazing botanically diverse vegetation (Oliván *et al.*, 2007b). Stable carbon isotopic (^{13}C) composition of plants has been used to estimate the proportion of C_3 and C_4 plants in the diet of herbivores (Bennett *et al.*, 1999). Garcia *et al.* (2000) reported that the use of a combination of the n-alkanes and ^{13}C composition of the organic matter of feeds could increase the accuracy of estimation of diet compositions in cows. However, so far, the ^{13}C of n-alkanes has not been evaluated as an

additional marker together with the alkane profiles themselves. Currently, the possibility of separating organic compounds of interest prior to isotope ratio analysis using gas chromatography-combustion isotope ratio mass spectrometry (GC-CIRMS) provides an opportunity to consider the ^{13}C of n-alkanes rather than the whole organic matter. The latter would improve the reliability of isotopic techniques, as plant compounds that are stable both in herbage and in faeces can be specifically targeted for isotope analysis.

There is little information about the plant wax profiles of native pasture species in Ethiopia for application in nutritional assessments of grazing animals. The aims of the present study were: 1) to describe the n-alkane profiles of pasture species commonly available in the Mid Rift Valley rangelands of Ethiopia, 2) to determine the stable carbon isotope enrichment of individual n-alkanes for each pasture species, and 3) to evaluate the potential for using the two markers to estimate the diet composition of free-ranging herbivores.

Materials and Methods

Description of study site

The research area lies in the Mid Rift Valley region of Ethiopia extending from 7°30´N to 8°00´N and from 38°35´E to 38°45´E. The area is classified as semi-arid with an annual rainfall ranging from 500 to 700 mm per annum (MoA, 2000). The rainfall pattern is bimodal with short rains from March through May, followed by the main wet season from July to October. The mean annual minimum and maximum daily temperature ranges between 11.4 and 26°C. The grazing lands exhibit typical savannah woodland vegetation with a scattered population of acacia trees and broadleaved shrubs. Cattle are the dominant livestock in the area followed by goats. Natural pasture is the main source of feed, supplemented by agricultural crop residues (CSA, 2007).

Plant sampling and processing

A total of 23 commonly available pasture species were collected from the study area during the months of July and August, 2008. For collection of samples several transect walks covering 15 km of length were conducted across both enclosed and communal grazing lands. Whole-plant pasture species were sampled from various locations along the transects by cutting at a height of 5 cm from the ground. The sampling was done at the time of the

flowering stage for all the species. After harvesting, the biomass sample of individual species was coded and stored in a pollen bag, while a specimen for each species was placed into a plant press for species confirmation in a herbarium. On average, a species was sampled from about 20 sites. Biomass samples of the same species collected from different sites were pooled to a sample before drying. The samples were dried in a forced air oven at 60^{o}C for 48 h. Dried samples were ground to pass through a 1mm sieve size (Thomas Wiley Lab mill, model 4, Philadelphia, U.S.A.), and afterwards pulverized using a bullet mill (MM 2000; 4 min at 80 Hz; Retsch Technology GmbH, Haan, Germany) before analysis of n-alkane concentrations and ^{13}C enrichment of alkanes.

Chemical analysis

The chemical analysis was conducted at the laboratory of the Animal Nutrition group of Wageningen University, the Netherlands. n-Alkane extraction and analysis was carried out as described by Mayes *et al*. (1986), with modifications by Salt *et al.* (1992) and tetratriacontane (C_{34}) used as an internal standard. The extracted samples were analyzed for n-alkanes (C_{27} to C_{36}) using a gas chromatograph (GC:Carlo Erba HRGC Mega 2 series, CE instruments, Milan, Italy) fitted to a flame ionizing detector (FID), using helium as the carrier gas. The column was a 40 m × 0.32 mm (i.d.) fused silica capillary (SPB-1) with 0.10 µm film thickness. A split type injector was used, with a split ratio of 1:10. The temperature for both the detector and injector was 340^{o}C (temperature program: 1 min at 210^{o}C, increase at 7.2^{o}C/min to a temperature of 300^{o}C, 6 min at 300^{o}C). Chrom Card Data System 2.2 (Thermo Finnigan, Waltham, MA) software was used to calculate peak areas. The data were transferred to an excel spread sheet to calculate alkane concentrations according to the following formula:

$$\text{Alkane}_i\,(\text{mg/kg DM}) = [10 \times \text{area \% alkane}_i \times \text{IS wt (mg)}] / \text{SDW} \times \text{SRF}_i$$

where IS wt is the weight of the internal standard, SDW is sample dry weight, and SRF_i is the standard response factor for $alkane_i$, calculated as area % $alkane_i$ in the mixed standard divided by weight % $alkane_i$ in the mixed standard.

Using the same alkane extracts, the carbon isotope composition of the alkanes was determined by fitting a GC (Finnigan_MAT, TraceGC Ultra, Milan Italy) with a split/ splitless injector operated in split mode (split ratio 1:10), to a combustion interface (Finnigan_MAT Combustion interface III, Bremen, Germany), which was connected to an elemental analyzer

isotope ratio mass spectrometer (Finnigan_MAT CN, Bremen, Germany). Full base line separation of all individual alkanes was achieved by fitting the TraceGC with a capillary column as described earlier and using helium as carrier gas. The temperature setting of the column was identical to that described earlier. The isotope ratio of the alkanes was calculated in terms of conventional delta values ($\delta^{13}C$) as follows:

$$\delta^{13}C = 1000\ (R_{sample} - R_{standard})/R_{standard}$$

where, R_{sample} is the abundance ratio of ^{13}C to ^{12}C in the plant sample, and $R_{standard}$ is the abundance ratio of ^{13}C to ^{12}C in the standard sample (Vienna Pee Dee Belemnite, PDB).

Data analysis

Principal Component Analysis (PCA) was used to explore the pattern of n-alkane profiles and ^{13}C enrichments of alkanes across the species. The correlation matrix was used for the calculation, after the data was mean-centred and standardized. The first two principal components (PC1 and PC2) were plotted graphically where points on the graph represent plant species. The distance between species in the scatter plots is an indication of the difference in marker profile between the species. The species which are positioned close together in the scatter plots are the ones with a similar marker profile. On the other hand the species that are placed far apart are expected to have large differences in their marker profiles.

The principal components for the two groups of markers were compared by Orthogonal Procrustes Rotation (OPR) to assess the similarity between the two data sets in describing the species identities. The PCA coordinates based on n-alkanes were used as fixed values, and those based on ^{13}C were used as fitted values. The OPR procedure rotates the fitted PCA axes to match the fixed axes, minimizing the residual sum of squares between the two PCA configurations. The magnitude of unexplained residual variance after OPR indicates the extent to which the two PCA configurations differ from one another. The two data sets were again examined together by employing Redundancy Analysis (RDA), in which the PCA based on the alkane profile was constrained by ^{13}C enrichments of n-alkanes and then the dispersion of species was presented in a two dimensional space. Data were analyzed using GenStat for Windows (11^{th} edition).

Table 1 n-Alkane concentration for pasture species collected from the Mid Rift Valley rangelands of Ethiopia.

Species	n-alkane concentration (mg/kg DM)								
	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{35}	Total
Cynodon dactylon	64	11	67	13	153	13	186	198	705
Pennisetum straminium	40	7	130	15	596	9	126	64	987
Cenchrus ciliaris	39	8	88	13	391	14	282	210	1043
Cymbopogon pospischilii	86	16	132	8	158	2	40	9	451
Indigofera spicata	23	24	76	7	202	6	20	6	363
Heteropogon contortus	68	5	46	10	266	11	238	98	742
Zaleya pentandra	14	33	35	8	1265	6	29	10	1398
Chloris gayana	116	18	125	11	318	11	258	165	1022
Eragrostis aspera	37	19	38	5	33	2	17	18	169
Eragrostis cilianensis	58	6	75	9	185	14	192	75	613
Cynodon ethiopicus	58	17	103	7	196	6	190	132	709
Eleusine mutiflora	59	11	75	9	186	12	170	51	574
Brachiaria lachnantha	30	17	45	6	129	11	171	158	569
Aristida adscensionis	31	8	81	7	225	5	103	32	493
Bracheria marlothii	429	31	281	18	174	6	72	23	1034
Sporobolus pellucisus	262	27	227	24	397	20	300	120	1378
Dactyloctenium aegyptium	84	7	86	11	313	12	208	33	754
Digitaria abyssinica	74	12	159	12	278	11	117	32	694
Pennisetum polystachion	40	6	36	5	73	3	13	7	182
Hyparrhenia anamesa	48	9	57	10	233	6	62	25	449
Snowdenia petitiana	28	39	81	10	245	11	160	84	657
Rhynchelytrum repens	91	30	76	21	356	22	363	91	1049
Melinis repens	78	24	54	11	127	7	104	53	457
Mean±sd	81±91	17±10	95±61	11±5	283±246	9±5	149±99	74±63	
Pooled SE[a]	1.27	1.07	1.52	0.60	1.84	0.54	0.94	1.65	

[a]Pooled standard error of measurement.

Results

Alkane concentrations

The n-alkane concentrations (C_{27} to C_{35}) in the pasture species collected from the grazing lands is shown in Table 1. The odd-chain alkanes were found in much higher concentrations than the even-chain alkanes. The even-chain alkane C_{36} was excluded from the results as the

values for most of the species were within the range of the analytical error of the GC. In most species, C_{31} was the most abundant odd-chain alkane, ranging from 33 mg/kg dry matter (DM) in *Eragrostis aspera* to 1265 mg/kg DM in *Zaleya pentandra* with a mean concentration of 283±246 mg/kg (mean±sd) DM across the species. This was followed by C_{33}, which ranged from 13 mg/kg DM in *Pennisetum polystachion* to 363 mg/kg DM in *Rhynchelytrum repens* with a mean concentration of 149±98 mg/kg DM for all the species. While alkane C_{35} was abundant in some species (e.g. *Cenchrus ciliaris*, *Cynodon dactylon)*, it was found in very low concentration in *Indigofera spicata, Cymbopogon pospischilii and Pennisetum polystachion*. The sum of C_{27} to C_{35} concentrations showed large between species variation, ranging from 169 mg/kg DM in *E. aspera* to 1398 mg/kg DM in *Z. pentandra*.

Carbon stable isotope composition of n-alkanes

The stable isotope enrichment of carbon ($\delta^{13}C$) for individual n-alkanes (Table 2) showed a wide variation, ranging from -19.37‰ (*Digitaria abyssinica*) to -37.40‰ (*I. spicata*). The lowest level of enrichment was observed in *I. spicata*, which was the only legume species in the collected samples, followed by that of *Z. pentandra* (a non-legume forb). The other grass species showed higher levels of enrichment, but differences could be observed between species. Regarding the delta values of individual alkanes, the odd-chain alkanes had a higher level of ^{13}C enrichment by at least one delta unit than the subsequent even chain alkanes (Table 2). The level of enrichment tended to decrease, in both even and odd chain alkanes with increasing carbon number.

Principal component and redundancy analyses

The results of the PCA are shown in Table 3, and Figures 1 and 2. When the PCA was based on n-alkane concentrations, about 91% of the variance between species was explained by the first three principal components (PC1-PC3). Similarly, when the analysis was based on $\delta^{13}C$ values of n-alkanes, 74% of the variance was explained by the first three principal components (Table 3).

Table 2 Carbon stable isotope (^{13}C) enrichment of n-alkanes for pasture species collected from the Mid Rift Valley rangelands of Ethiopia.

Species	$\delta^{13}C$ values (‰) of n-alkanes							
	C27	C28	C29	C30	C31	C32	C33	C35
Cynodon dactylon	-22.93	-27.36	-23.90	-25.79	-22.69	-27.84	-23.43	-23.66
Pennisetum straminium	-20.69	-23.09	-20.59	-22.26	-20.10	-24.50	-21.42	-21.71
Cenchrus ciliaris	-20.85	-23.41	-21.13	-23.01	-21.80	-23.65	-21.94	-22.87
Cymbopogon pospischilii	-20.61	-22.08	-20.39	-24.91	-20.81	-26.84	-22.55	-22.30
Indigofera spicata	-32.27	-34.00	-36.61	-36.68	-37.40	-35.67	-33.77	-34.58
Heteropogon contortus	-19.46	-23.47	-20.10	-23.17	-20.45	-22.51	-21.08	-21.94
Zaleya pentandra	-25.67	-27.21	-25.12	-25.47	-21.11	-27.83	-24.11	-25.22
Chloris gayana	-21.37	-22.83	-21.55	-23.07	-21.08	-24.75	-22.26	-22.91
Eragrostis aspera	-21.09	-23.46	-22.13	-25.73	-21.95	-23.15	-24.52	-23.15
Eragrostis cilianensis	-21.86	-24.67	-22.33	-24.61	-23.33	-25.18	-24.32	-25.72
Cynodon ethiopicus	-23.44	-25.20	-23.42	-25.11	-22.17	-25.09	-22.55	-23.71
Eleusine mutiflora	-22.25	-26.01	-23.11	-25.37	-23.65	-26.03	-24.97	-25.57
Brachiaria lachnantha	-20.81	-23.22	-21.88	-23.50	-21.34	-22.90	-22.94	-21.49
Aristida odscensionis	-22.49	-25.00	-22.68	-24.29	-22.69	-23.39	-23.31	-23.30
Bracheria marlothi	-20.72	-22.13	-21.41	-23.84	-22.45	-35.64	-26.36	-22.40
Sporobolus pellucisus	-21.05	-22.93	-21.50	-23.85	-22.50	-23.60	-24.22	-24.40
Dactyloctenium aegyptium	-21.94	-26.58	-23.12	-27.14	-22.48	-26.60	-23.55	-24.79
Digitaria abyssinica	-19.37	-20.87	-19.81	-20.56	-19.67	-22.88	-22.05	-22.46
Pennisetum polystachion	-22.52	-22.57	-23.04	-22.93	-23.80	-23.79	-27.21	-23.70
Hyparrhenia anamesa	-20.12	-25.02	-19.75	-23.93	-19.97	-22.03	-21.68	-22.42
Snowdenia petitiana	-21.97	-25.11	-23.08	-24.66	-23.68	-26.63	-25.18	-24.58
Rhynchelytrum repens	-21.70	-22.80	-22.55	-21.88	-22.97	-21.59	-23.54	-24.55
Melinis repens	-20.92	-22.94	-21.94	-22.37	-21.31	-22.09	-21.78	-23.34
Mean±sd	-22.00	-24.34	-22.66	-24.53	-22.58	-25.03	-23.77	-23.95
	±2.62	±2.69	±3.33	±3.04	±3.45	±3.72	±2.67	±2.62
Pooled SE[a]	0.29	0.52	0.27	0.42	0.16	0.78	0.26	0.32

[a]Pooled standard error of measurement.

Table 3 The variance (%) in the pattern of n-alkane concentration and $\delta^{13}C$ values of n-alkanes explained by the first three principal component axes (PC1, PC2, and PC3) for each data set, and the residual variance (%) remaining after comparison by Orthogonal Procrustes Rotation (OPR) of the two principal component scores.

Marker	Variance explained (%)				Residual variance (%) remaining after OPR
	PC1	PC2	PC3	Total	
n-alkanes	54.6	22.1	14.1	90.8	
$\delta^{13}C$ of n-alkanes	35.7	22.3	16.0	74.0	84.7

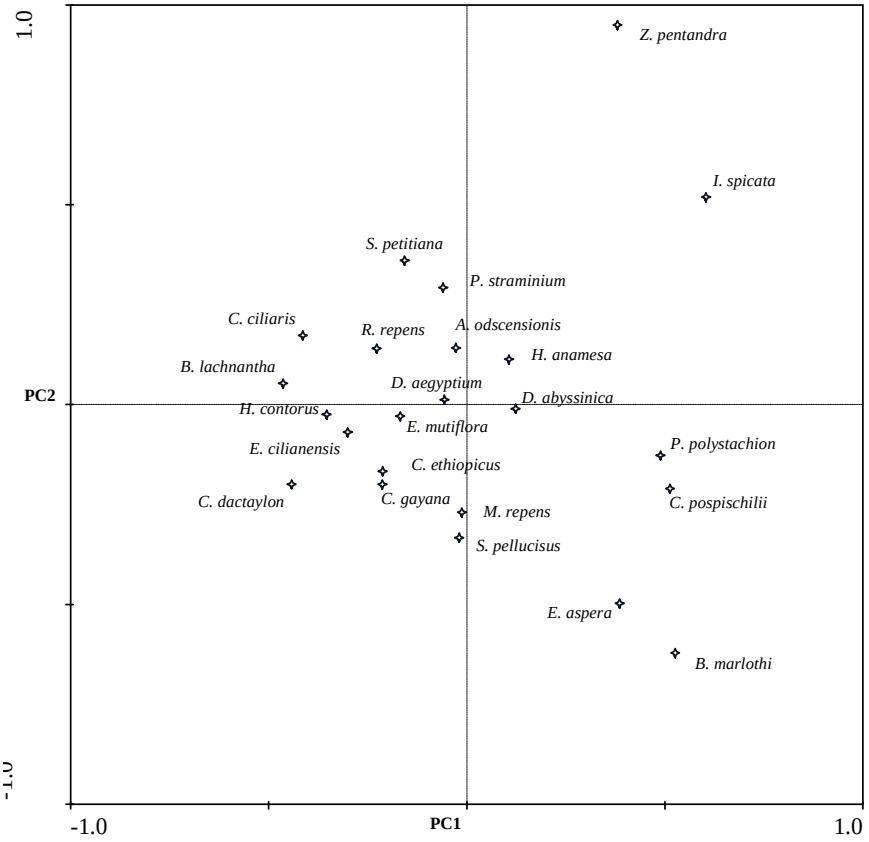

Figure 1 Scatter plot of pasture species on a two dimensional space using the first two principal components (PC1 and PC2) derived from PCA based on the n-alkane concentrations.

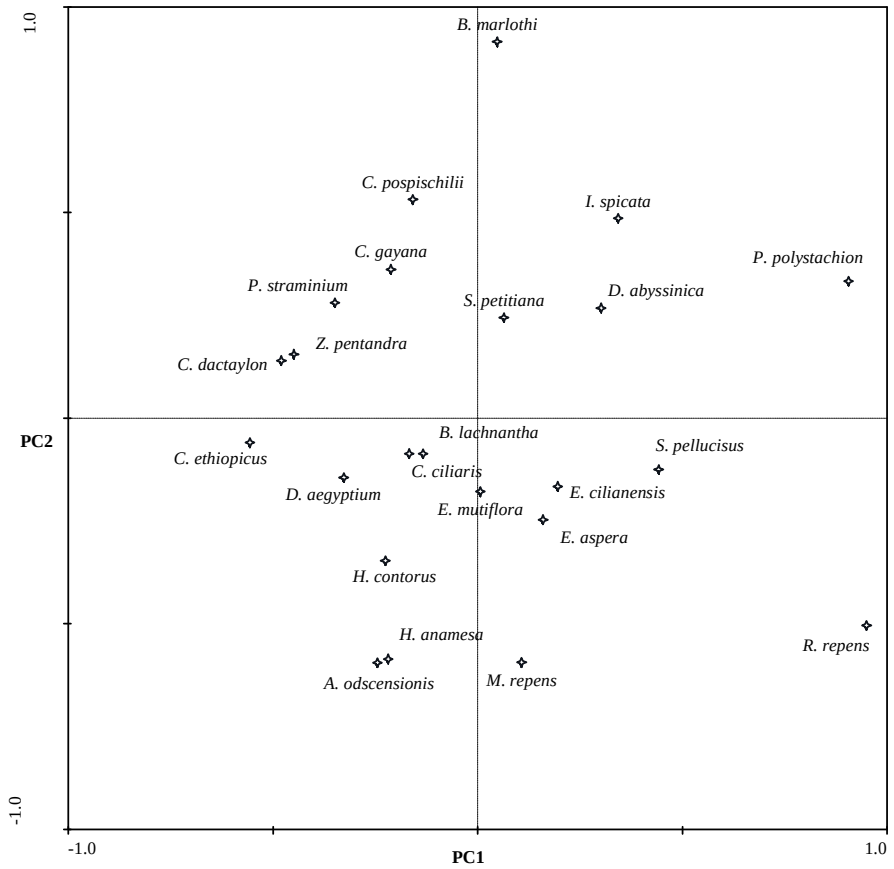

Figure 2 Scatter plot of pasture species on a two dimensional space using the first two principal components (PC1 and PC2) derived from PCA based on $\delta^{13}C$ values of n-alkanes.

The scatter plot based on n-alkanes (Figure 1) shows a good species separation. For example, *Brachiaria marlothi, I. spicata, Z. pentandra,* and *E. aspera* scattered widely from the rest of the species. *Brachiaria lachnantha, Heteropogon contortus, P. polystachion, C. pospischilii, C. ciliaris* and *C. dactylon* were also separated along the two principal axes. On the other hand, clustering between some of the species, like *Chloris gayana*, and *Cynodon ethiopicus* was observed. The scatter plot based on the $\delta^{13}C$ values of n-alkanes (Figure 2) showed that the species were scattered along the two axes in a different way. On one hand, those species which clustered closer when the analysis was based on n-alkanes (Figure 1), showed wider separation when the analysis was based on $\delta^{13}C$ values of n-alkanes (Figure 2). On the other hand some of the species (e.g. *C. dactylon and Z. pentandra*; *C. ciliaris* and *B.*

lachnantha), which were better separated with the n-alkane data set, showed aggregation with the isotope data set.

Comparison of the species ordinations by OPR revealed that the residual variance remaining after fitting the two PCA scores was 84.7% (Table 3). This indicated little similarity between the two PCA scores and that the majority of the variance explained by $\delta^{13}C$ values of n-alkanes was additional to that explained by the n-alkane profile of species.

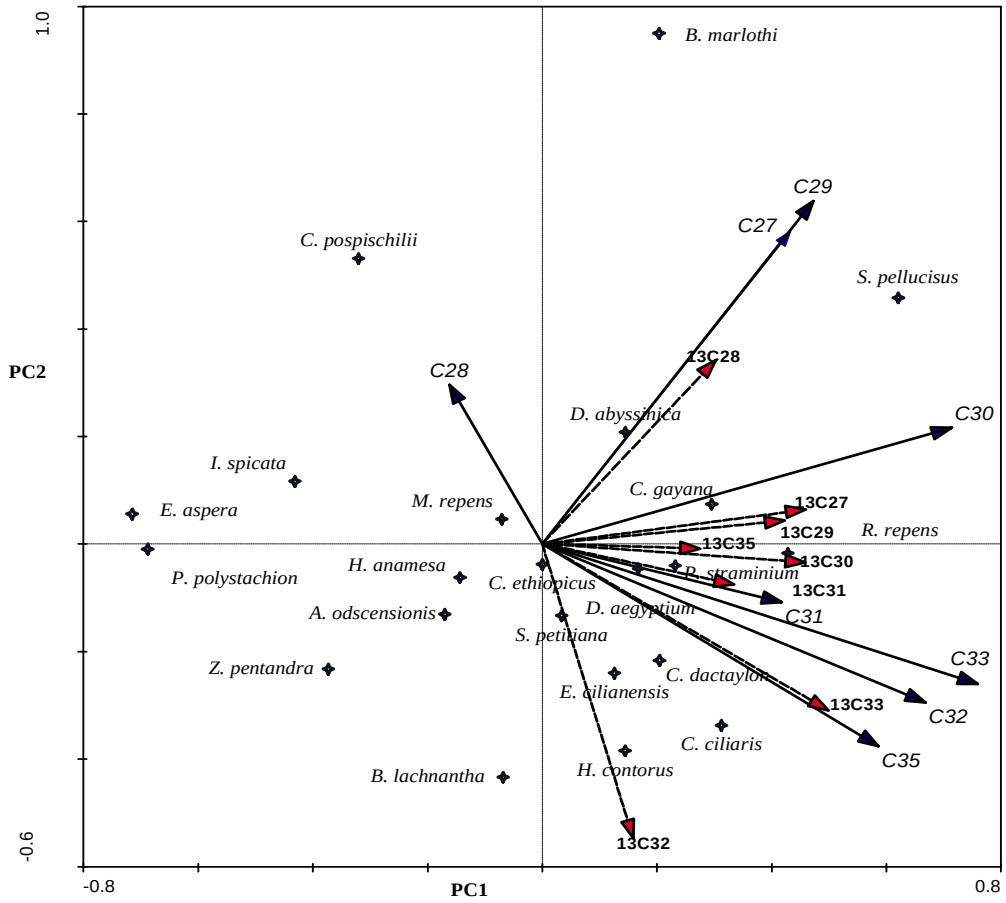

Figure 3 A tri-plot in the space of the first two principal axes derived from RDA of n-alkane concentration of species (solid arrows) as constrained by $\delta^{13}C$ values of n-alkanes (dashed arrows). The direction of the arrow points towards the steepest increase of the marker it represents.

Figure 3 shows a tri-plot of species points and the direction of the steepest increase for n-alkanes profile and $\delta^{13}C$ values of n-alkanes in the space of the first two principal axes derived from RDA. As shown in the graph, overlapping or clustering of species points was not

observed. Those species that were clustered together in Figure1 (PCA based on n-alkane) and Figure 2 (PCA based on $\delta^{13}C$ values of n-alkanes) appeared separated in Figure 3. On the other hand those species that had distinct coordinate points in either Figure 1 or Figure 2 remained distinct in Figure 3.

Discussion

n-Alkane concentrations

The presence of significant variability in the n-alkane profile between plant species has long been documented and this variability is increasingly being used for the indirect estimation of the diet composition of free-ranging herbivores (Dove and Mayes, 2005; Ferreira *et al.*, 2007b). To make effective use of these markers in nutritional studies, however, it is important to document location specific information on the n-alkane profiles of available herbage species (Ali *et al.*, 2005a). This is because environmental conditions and geographical locations could influence the pattern of cuticular wax profile of plant species growing in different places (Samuels *et al.*, 2008).

In the present report, the general trend that odd-chain alkanes exist in higher concentration than even-chain alkanes conforms to previous findings (Dove and Mayes, 1996). The dominance of C_{31} in the pasture species was also consistent with previous reports (Ali *et al.*, 2005a).This makes it easier to quantify odd-chain herbage alkanes more accurately than even-chain alkanes, and hence their role as a diet composition marker appears indispensable. Although found in small quantities some of the even-chain alkanes like C_{30} and C_{32} have shown high discriminatory potentials (Pueyo *et al.*, 2005).

Feed intake estimation using the ratio of a natural odd-chain to dosed even-chain n-alkane in the faeces is another important advantage of the n-alkane method (Mayes *et al.*, 1986). One of the requirements to estimate intake accurately using this approach is that the faecal recovery rates of the dosed and herbage n-alkanes should be similar (Dove and Mayes, 1991). Generally, pairs of n-alkanes with consecutive carbon chain lengths are reported to have similar recovery rates (Mayes and Dove, 2000). As a result a combination of either C_{31}/C_{32} or C_{33}/C_{32} has been used for this purpose. The present analysis also confirms herbage C_{31} and C_{33} alkanes as priority choices for intake estimation together with dosed C_{32}. The alkane C_{35} could also be used in combination with dosed $C_{36,}$ as it was found in considerable

amounts in many of the species (Table 1), and is known to have high faecal recovery rates (Ferreira *et al.*, 2009).

The $\delta^{13}C$ values of n-alkanes

It is known that all photosynthetic plants discriminate against the natural stable isotope ^{13}C during their CO_2 absorption and utilization. This results in the depletion of ^{13}C in organic tissues, as well as in specific compounds like n-alkanes of plants in comparison with the natural abundance (Bendle *et al.*, 2006). Plants that follow the C_3 photosynthetic pathway exhibit a higher level of carbon isotope fractionation than C_4 plants (Marshall and Zhang, 1994). The resulting difference in carbon isotope composition of the organic matter has been exploited to estimate the gross diet composition of herbivores in terms of the two plant groups (Coates *et al.*, 1987; Norman *et al.*, 2009).

Differences in carbon isotope fractionation between species that follow the same photosynthetic pathway have also been documented (Ehleringer, 1991). However, these differences have not been evaluated as an additional source of plant marker. The main reason for that may be the general assumption that the within photosynthetic group variations in carbon isotopic ratio are too small to be used as an additional marker (Osmond *et al.*, 1973).

From previous reports, the $\delta^{13}C$ values of n-alkanes range between -30 to -40‰ for C_3 plants and -17 to -24‰ for C_4 plants (Reddy *et al.*, 2000). The results of the present analyses largely agree with these ranges of values. *Indigofera spicata* was the only legume species analyzed in the present study, and the $\delta^{13}C$ values obtained for this species (-32.27 to-37.40‰) fall within the range of values observed for C_3 plants. This conforms to the established knowledge that in tropical grasslands, legumes are represented by C_3 plants (Dove and Mayes, 2005). The other species analyzed exhibited a range of carbon isotope enrichment which is typical of C_4 plants (Bendle *et al.*, 2006), although some of the species like *Z. pentandra* showed a lower level of enrichment. In the current study, the general isotope enrichment level of even- and odd-chain alkanes agrees with the finding of Reddy *et al.* (2000), who reported that even-chain alkanes were depleted by about –1‰ compared to the neighbouring odd-chain alkanes. This may suggest that during the biosynthesis of n-alkanes (elongation of carbon skeletons) there is a differential carbon isotopic fractionation.

Multivariate analysis

A variety of multivariate statistical procedures are available to study the patterns of interspecies variability in n-alkanes and other plant markers (Dove *et al.*, 1996; Dove *et al.*, 1999). Principal component analysis was chosen here as the n-alkane and ^{13}C analyses were based on bulked samples and the data do not provide within species variability in the two markers. The PCA carried out showed that most of the variance between species was explained by the first three principal components. This indicated the presence of a high variability among the plants studied, which can be ascribed to the patterns of the two markers. The results obtained regarding the n-alkanes is similar to several other investigations over the past decades for pasture and browse species (Ferreira *et al.*, 2007b). However, to our knowledge, there is no previous published report regarding the interspecies variability in the ^{13}C enrichment of n-alkanes.

One of the constraints in using n-alkanes is that the concentrations of many of the lower-chain n-alkanes and the even-chain n-alkanes are too low for accurate measurement. As a result the number of n-alkanes that can be used as markers is limited. This in turn may limit the number of diet components that can be effectively estimated. There are, however, circumstances that the diet of animals grazing on botanically diverse vegetation could contain more diet components than the number of effective n-alkane markers available for diet composition estimation. Taking this limitation into account, research in the area has focused on evaluating other plant wax components like long chain fatty alcohols and fatty acids for use as additional markers (Mayes, 1998). This has been supported by the development of exhaustive analytical laboratory protocols (Dove and Mayes, 2006).

Generally the use of a combination of plant wax component n-alkanes, long-chain fatty alcohols, and acids has provided increased accuracy and power in the estimation of diet composition (Bugalho *et al.*, 2004; Fraser *et al.*, 2006; Kelman *et al.*, 2003). The present analysis also showed that the interspecies variability in $\delta^{13}C$ values of n-alkanes could be used as an additional source of information to estimate diet components of herbivores. The scatter plot derived from RDA, by constraining the species dispersion based on the n-alkane profile by the isotope composition of n-alkanes, showed that those species that tended to cluster when the analysis was based on either n-alkanes or $\delta^{13}C$ values of n-alkanes appeared to be

separated. This graphical presentation supports the OPR result that the isotopic composition of the n-alkanes provides additional discriminatory power to the species separation.

The increased analytical capacity to separate specific compounds prior to isotope composition analysis (Muccio and Jackson, 2009) provides enormous potential to study the isotopic ratio of not only n-alkanes but also long-chain fatty alcohols and fatty acids. The possibility of generating two different types of internal markers from a single set of compounds such as n-alkanes would be a desirable feature in terms of increasing the discriminatory power of wax components.

Estimation of diet composition

Estimation of diet composition using plant wax n-alkanes as markers is achieved by relating the marker patterns found in faeces (corrected for incomplete recovery) to that calculated from the marker patterns of individual diet components (plant species or plant parts). A number of mathematical algorithms and approaches including least squares optimization procedures (Dove and Mayes, 2005) and linear programming (Barcia *et. al.*, 2007) have been developed and used for this purpose. Regarding the application of isotopic enrichment, Bugalho *et al.* (2008) demonstrated that the linear programming model of Barcia *et al.* (2007) can be effectively used to estimate the contribution of different sources to a mixture by relating the isotopic composition of carbon ($\delta^{13}C$) and sulphur ($\delta^{34}S$) in the sources and mixture. In relation to the present findings, it should also be possible to adopt a suitable mathematical model to use both the n-alkane profiles and their carbon isotopic compositions ($\delta^{13}C$) as input in the calculation of diet compositions.

It is now well established that the recovery of n-alkanes in faeces is incomplete with the recovery rate generally increasing in a curvilinear fashion with increasing carbon number. A suitable faecal recovery correction factor is therefore required to increase the accuracy of the calculations (Ferreira *et al.*, 2009). However, little is known about the relative recoveries of ^{12}C and ^{13}C isotopes for a particular n-alkane, which could potentially alter the carbon isotope enrichment values of the alkane in feed and faeces and influence diet composition estimation. Controlled *in vivo* experiments may be required to document the relative fates of the two carbon isotopes in the gut. Gut microorganisms are unable to synthesize long-chain n-

alkanes (Keli *et al*., 2008a) indicating that there would be no bias in the estimation of diet composition due to endogenous n-alkane excretion into faeces.

Due to the difficult nature of the measurement, the presence of differential recoveries (if any) of the same n-alkane originating from different plants has not yet been established. In view of the present study that the same n-alkane originating from different plants could have different isotopic composition and that this could be used as an additional marker, it would be interesting to investigate the issue of differential recoveries in relation to the molecular weight of the alkanes.

Conclusion

The n-alkane profile as well as the isotopic enrichment of n-alkanes showed considerable variability between the species studied. The majority of the interspecies variances explained by the two types of data sets are independent of each other. Therefore, generating information on the n-alkane concentration of plant species in combination with their isotopic enrichment could be a valuable tool to improve the accuracy of estimating diet composition and quality of free-ranging animals. However, further validations needs to be conducted with actual feeding experiments. Within species variations in the $\delta^{13}C$ values of n-alkanes, as well as changes with physiological maturity are also topics that need to be addressed.

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CHAPTER 3

Using n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) to estimate the botanical composition of pasture mixes from the Mid Rift Valley grasslands of Ethiopia

M. Bezabih[1,2], W. F. Pellikaan[1], W. H. Hendriks[1,3]

[1]Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH, Wageningen, the Netherlands

[2]Department of Animal and Range Sciences, Hawassa University, P.O. Box 5, Hawassa, Ethiopia

[3] Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.151, 3508 TD, Utrecht, the Netherlands

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Abstract

The present study was conducted to assess the accuracy of n-alkanes and their carbon isotope enrichment to estimate the diet composition of herbivores in the Mid Rift Valley rangelands of Ethiopia. Five common grass species which are abundantly available in the area were selected, from which several composite grass mixtures were prepared containing all the five species in varying proportions (from 0.05 to 0.35). The n-alkane profile and n-alkane isotopic ratio ($^{13}C/^{12}C$) of each grass species and composite grass mixtures were determined by gas chromatography/combustion isotope ratio mass spectrometry. The botanical composition of the composite mixtures was estimated using the n-alkane profile and n-alkane$^{13}C/^{12}C$-ratio of individual species using least squares optimization and linear programming procedures and compared to the actual botanical mixture composition. Three alternative scenarios (inclusion of an additional 0, 5, or 10 of species in addition to those that made up the mixes) and two options where additional botanical species were included were simulated. There was close alignment between estimated and measured botanical compositions with significant relationships ($P<0.001$).The percentage contribution of the species in the simulated pasture mixtures were accurately estimated when the five species making up the grass mixes were used as the only inputs in the calculation. However, when additional botanical species were introduced, the accuracy declined with a significant increase ($P=0.003$) in the mean square error of the prediction. The type of species in the extra inputs did not influence the results. In all scenarios, the combined use of n-alkanes and their $\delta^{13}C$ values improved the linear relationship and reduced ($P=0.002$) the mean square error between estimated and measured botanical compositions. The best fit equation ($R^2=0.996$, $P<0.001$) was obtained when n-alkanes and their $\delta^{13}C$ values were used together and no extra species was included as input in the calculation. The present study shows that a high degree of accuracy can be obtained in estimating the botanical composition of grass mixtures using n-alkanes composition and n-alkanes $^{13}C/^{12}C$-ratio. It is important to increase the number of markers used or limit the number of potential diet components to improve the quality of predictions.

Introduction

Free-ranging herbivores are the largest contributors to livestock output in tropical Africa. Animals in these areas consume a complex mixture of vegetation, typical of rangeland systems. This system is generally characterized by high temporal and spatial variations in herbage quality and availability (Corona *et al*., 1998; Hiernaux and Turner, 1996; Schlecht *et al*., 2006), which in turn results in fluctuation in nutrient and energy supply for livestock production along the grazing seasons (Georgiadis and McNaughton, 1990). The level of production obtained from animals grazing such vegetation depends on their ability to ingest a diet in excess of their maintenance nutrient requirements (Ash and McIvor, 1998, Celaya *et al*., 2007). The productivity and biodiversity of the vegetation is influenced, amongst others by the short and long-term grazing management practices put in place (Tefera *et al*., 2007a,b). Such knowledge of the plant-animal interactions is important for a sustainable ecosystem management and optimization of both animal and land productivity.

Plant cuticular n-alkanes are useful markers to estimate diet selection and nutrient intake of free-ranging herbivores (Dove and Mayes, 1996, Dove and Mayes, 2005). The application of cuticular n-alkanes for this purpose relies on the facts that plant species, and to some extent plant parts, show differences in n-alkane profiles. As n-alkanes are mainly indigestible and have high faecal recovery rates (Ferreira *et al*., 2009), they are suitable markers to estimate diet selection and nutrient intake. A major advantage of n-alkanes as markers is that diet composition of animals can be assessed with little interference to their normal foraging behaviour, which is particularly important in the case of semi-wild and wild herbivores. However, in botanically diverse vegetation where the number of plant species (diet components) is higher than the number of effective n-alkanes, it is necessary to either group plant species with similar marker profiles (Ferreira *et al.*, 2007c), or use additional markers to increase the discriminatory power. In the latter case, cuticular long chain fatty alcohols and fatty acids have been evaluated as important additional markers (Ali *et al*., 2005b; Bugalho *et al.*, 2004; Kelman *et al*., 2003). In addition, the carbon isotopic composition of the diet organic matter (Garcia *et al*., 2000), and more recently n-alkanes (Bezabih *et al*., 2011b) have shown promises to provide additional discriminatory information to estimate diet composition of herbivores.

Although the n-alkane method is widely applied in other parts of the world, there is limited information on its applications in tropical African ecosystems. Recently, pasture species commonly available in the Mid Rift Valley rangelands of Ethiopia were assessed for their n-alkane profiles and carbon isotopic composition of the alkanes (Bezabih *et al*., 2011b) and the result showed the presence of large between species variations. The present study aimed to further validate the suitability of n-alkanes alone or in combination with their $\delta^{13}C$ values to estimate the botanical composition of simulated pasture mixes prepared from selected grass species.

Materials and Methods

Preparation of simulated pasture mixtures and experimental design

Pasture species commonly available in the rangelands of the Mid Rift Valley areas of Ethiopia were collected. Sample collection and processing are described in detail in Bezabih *et al*. (2011b). For the present investigation, the pasture species were ranked according to their frequency of occurrence along the sampling transects in the field. The following five pasture species, namely *Chloris gayana, Cynodon dactylon*, *Pennisetum straminium, Cenchrus ciliaris*, and *Eragrostis aspera* were selected. The sample of each species was dried at 60°C for 48 h, ground to pass a 1-mm sieve, and pulverized in a ball grinder (Retsch MM 2000).

Table 1. Proportion of grass species in the composite grass mixtures.

Grass species	Composite mixtures				
	1	2	3	4	5
Cynodon dactylon	0.20	0.30	0.15	0.10	0.35
Pennisetum straminium	0.30	0.20	0.25	0.10	0.25
Cenchrus ciliaris	0.20	0.15	0.10	0.30	0.05
Chloris gayana	0.15	0.25	0.30	0.20	0.10
Eragrostis aspera	0.15	0.10	0.20	0.30	0.25

Five composite grass mixtures were prepared using the five pulverized species samples with each mixture containing all the five species but in different proportions (range 0.05 to 0.35) (Table 1). The n-alkane concentrations as well as the carbon isotopic ratio of the n-alkanes for each grass species and that of the five mixtures were analysed from n-alkane composition and n-alkane $^{13}C/^{12}C$-ratio in the laboratory. These data were used as input to

estimate the botanical composition of the mixtures by relating the marker profile of the individual species and the mixtures.

Chemical analysis

The chemical analysis was conducted at the Laboratory of Animal Nutrition Group of Wageningen University, the Netherlands. Extraction and analysis of n-alkanes was carried out as described by Mayes *et al.* (1986) with modifications by Salt *et al.* (1992) using tetratriacontane (C_{34}) as an internal standard. The extracted samples were analysed for n-alkanes (C_{27} to C_{36}) using a gas chromatograph (GC:Carlo Erba HRGC Mega 2 series) fitted to a flame ionizing detector (FID), using helium as the carrier gas. The column was a 40 m × 0.32 mm (i.d.) fused silica capillary (SPB-1) with 0.10 µm film thickness. A split type injector was used, with a split ratio of 1:10. The temperature for both the detector and injector was 340^{o}C.The starting temperature of the oven was set at 210^{o}C for 1 min followed by a 7.2^{o}C /min increase to 300^{o}C that was maintained for 6 min. Chrom Card Data System 2.2 (Thermo Finnigan, Waltham, MA) software was used to calculate peak areas. The alkane concentration was calculated according to the following formula:

$$\text{Alkane}_i\,(\text{mg/kg DM}) = [10 \times \text{area \% alkane}_i \times \text{IS wt (mg)}] / \text{SDW} \times \text{SRF}_i$$

where IS wt is the weight of the internal standard, SDW is sample dry weight, and SRF_i is the standard response factor for $alkane_i$, calculated as area % $alkane_i$ in the mixed standard divided by weight % $alkane_i$ in the mixed standard.

With the same extract, the carbon isotope composition of the alkanes was determined by fitting a GC (Finnigan_MAT, TraceGC Ultra), with a split/ splitless injector operated in split mode (split ratio 1:10) to a combustion interface (Finnigan_MAT Combustion interface III), which was connected to an elemental analyser isotope ratio mass spectrometer (Finnigan_MAT CN). Full base line separation of all individual alkanes was achieved by fitting the TraceGC with a capillary column as described earlier and using helium as carrier gas. The temperature setting of the column was identical to that described earlier. The isotope ratio of the alkanes was calculated in terms of conventional delta notation ($\delta^{13}C$).

Estimation of botanical composition of composite grass mixtures

The botanical composition of the composite grass mixtures was estimated by relating the marker profile of individual grass species to that of the mixtures using the following calculation programs: 1) the 'EatWhat' software program (Dove and Moore, 1995), 2) the linear programming model of Barcia *et al*. (2007) (LP_Tracer) and 3) the least-square optimization procedure (Hameleers and Mayes, 1998) using the Solver routine in Microsoft Excel. In the latter case, the Solver routine was programmed to solve the following function:

$$\text{Minimize} \sum [(\text{actual} - \text{calculated})^2] \text{ marker}_i \ldots n$$

where actual = measured concentration of marker_i in the mix; calculated = calculated concentration of marker *i* using the following formula

$$\text{calculated} = \sum[(X_j Y_{ij})] \ plants_j \ldots n$$

where X_j is the estimated proportion of plant species *j* in the mix; Y_{ij} is the concentration of marker *i* in plant species *j*; and $\sum X_j = 1$.

In each case, estimation of composite diet mixture composition was done using either n-alkane or a combination of n-alkane and their carbon isotope enrichment data as markers, under three scenarios: i) grass species that made up the mixture as input, and ii) including five additional species to those making up the mixtures, and iii) including ten additional species to those making up the mixtures. In scenario ii and iii, two options were used to include the additional species, following purposive selection based on their abundance or random selection from the available pool of species. This was done to examine the influence of the type of extra species included on the botanical composition estimations. Thus, a combination of the three calculation methods, two marker types, three scenarios and two options of extra species inclusion (3×2×(1+2×2)) resulted in a total of 30 validation tests.

Data analyses

The interspecies variability in n-alkane profile and carbon isotopic composition of the five species was analysed by principal component analysis (PCA) with Canoco for Windows 4.5. Estimated species proportions of pasture mixes were regressed against the measured values for each of the validation tests to determine estimation accuracy and differences from actual proportions using the slope of the regression lines and the intercepts, respectively. Mean square errors (MSE) were computed for estimated versus measured species compositions in

each of the validation tests and analysis of variance was conducted to examine the presence of significant differences. The regression and ANOVA were done in SAS® version 9.1.

Results

n-Alkane and carbon isotope enrichments ($\delta^{13}C$) of grass species and mixtures

The concentration of individual n-alkanes ($C_{27} - C_{35}$) and the $\delta^{13}C$ values of each hydrocarbon for the grass species and composite grass mixtures are shown in Table 2. The predominant alkanes in the grass species were C_{31} and C_{33}, the sum of which accounted for 73 and 64% of the total hydrocarbons ($C_{27} - C_{35}$) in *P. straminium* and *C. ciliaris*, respectively. *Eragrostis aspera* had an exceptionally low concentration of most of the n-alkanes analysed. As expected, the n-alkane profile of the composite grass mixtures followed the same trend as individual species, C_{31} and C_{33} being the dominant alkanes followed by C_{35}. The variation in n-alkane profile between mixtures was much smaller than that observed between species. The $\delta^{13}C$ values of the grass species varied between -20.10 and -27.84‰**.** Figure 1 shows the dispersion of the grass species along the first two principal components derived from PCA of the n-alkane profiles (panel A) and based on the $\delta^{13}C$ values of the alkanes (panel B). The first two principal components extracted about 95% of the interspecies variability when the analysis was based on n-alkanes. The species generally showed distinct positions along the two principal component axes with. *E. aspera* widely separated along the first principal component owing to its unique n-alkane profile. The first two principal components explained about 86% of the variances when the analysis was based on the $\delta^{13}C$ values of the alkanes. The species *E. aspera* was widely separated along the first principal component.

Table 2 Concentration (mg/kg DM) and carbon isotope enrichment ($\delta^{13}C$, ‰) of individual n-alkanes (C_{27} – C_{35}) for the five grass species and composite grass mixtures.

Samples	C_{27}		C_{28}		C_{29}		C_{30}		C_{31}		C_{32}		C_{33}		C_{35}	
	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$	conc	$\delta^{13}C$
Cynodon dactylon	64	-22.93	11	-27.36	67	-23.9	13	-25.79	153	-22.69	13	-27.84	186	-23.43	198	-23.66
Pennisetum straminium	40	-20.69	7	-23.09	130	-20.59	15	-22.26	596	-20.10	9	-24.50	126	-21.42	64	-21.71
Cenchru sciliaris	39	-20.85	8	-23.41	88	-21.13	13	-23.01	391	-21.80	14	-23.65	282	-21.94	210	-22.87
Chloris gayana	116	-21.37	18	-22.83	125	-21.55	11	-23.07	318	-21.08	11	-24.75	258	-22.26	165	-22.91
Eragrostis aspera	37	-21.09	19	-23.46	38	-22.13	5	-25.73	33	-21.95	2	-23.15	17	-24.52	18	-23.15
Mixture 1*	56	-21.33	11	-24.02	94	-22.50	12	-23.76	338	-21.38	10	-24.83	173	-22.52	128	-22.73
Mixture 2	66	-21.60	12	-24.39	94	-22.06	12	-23.98	307	-21.56	11	-25.30	190	-22.62	147	-22.91
Mixture 3	66	-21.33	13	-23.76	96	-21.74	11	-23.80	313	-21.32	9	-24.72	167	-22.65	120	-22.77
Mixture 4	55	-21.22	14	-23.67	83	-21.74	10	-24.04	265	-21.62	9	-24.22	173	-22.88	128	-22.93
Mixture 5	55	-21.68	13	-24.73	74	-22.36	11	-24.63	221	-21.99	10	-25.14	172	-23.11	144	-23.16

*For ingredients of composite grass mixtures see Table 1.

Table 3 Linear regression equations between predicted (Y) and measured (X) botanical composition of five mixtures using three calculation methods with n-alkanes or a combination of n-alkanes and their $\delta^{13}C$ values as markers.

Method[1]	Scenario[2]	Marker set					
		n-alkanes (C_{27}-C_{35})			n-alkanes and their $\delta^{13}C$ values		
		Linear equation	R^2	MSE	Linear equation	R^2	MSE
1	I	1.001x - 0.224	0.966	3.81[a]	1.006x - 0.081	0.997	0.20[b]
	II	0.957x - 0.751	0.708	27.6[c]	0.887x + 1.922	0.866	8.96[d]
	III	-	-	-	1.045x - 4.829	0.477	67.52[e]
2	I	1.027x - 0.004	0.920	6.38[a]	1.006x - 0.138	0.985	0.97[b]
	II	0.984x - 1.146	0.661	34.87[c]	1.029x - 1.987	0.819	13.40[d]
	III	0.893x - 0.754	0.460	61.84[e]	1.023x - 3.615	0.489	78.62[e]
3	I	0.956x + 0.611	0.913	5.42[a]	0.976x + 0.488	0.991	0.56[b]
	II	0.876x + 1.348	0.609	34.72[c]	0.918x + 0.352	0.816	12.95[d]
	III	0.640x + 6.235	0.329	64.58[e]	0.702x + 3.014	0.378	68.01[e]

[a,b,c]Values with different letters in the superscript are significantly different ($P<0.05$).

[1]Method: 1, EatWhat software; 2, LP_TRACER; 3, Solver routine in MS Excel.

[2] Scenario: I, five botanical species; II, I plus five additional species; III, I plus ten additional species.

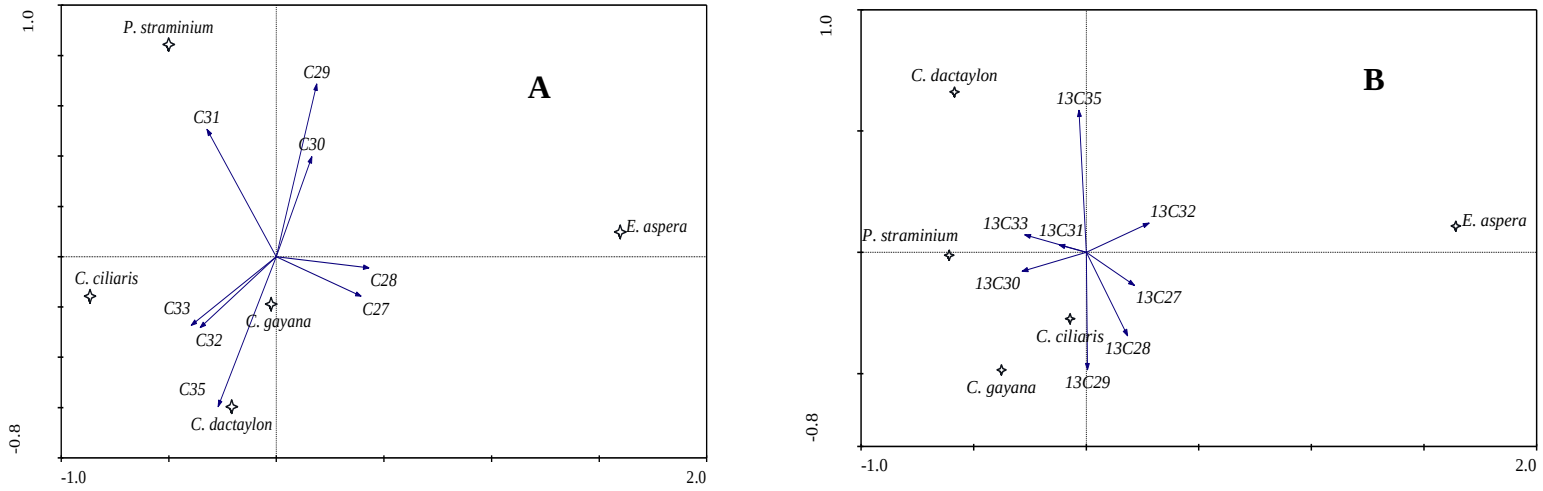

Figure 1 Bi-plot showing species positions on a two dimensional space derived from principal component analysis of n-alkane profile (Panel A) and $\delta^{13}C$ values of n-alkanes (Panel B) of 5 grass species from the Mid Rift Valley grasslands of Ethiopia.

Relationship between estimated versus measured botanical compositions

Table 3 shows the linear regression models explaining the relationship between predicted versus measured composite grass mixtures when the n-alkanes and a combination of n-alkanes and their $\delta^{13}C$ values were used as markers. n-Alkanes alone in the third scenario of calculation was not possible due to limited number of markers. In all other cases there was a linear relationship ($P<0.001$) between the estimated and measured compositions. The 95% confidence interval for the slope and intercept of the regression equations indicated that the regression lines of all the models were not statistically different from the line of equality. However, the coefficients of determination (R^2) varied widely ranging from 0.33 to 0.99. Best fit equations were obtained when the number of inputs was limited to those species that made up the grass mixtures (the first scenario). The third scenario, when 10 additional species were included as input, produced the lowest R^2. The two different options of additional species selection produced similar results and only the result with the first option is presented here. Using a combination of n-alkanes and their $\delta^{13}C$ values improved the R^2 compared to using n-alkanes alone (Table 3).

The mean square errors (MSE) calculated for predicted versus measured values increased from the first to the third scenario. The lowest MSE values (0.20-0.97) were obtained with the first scenario, in which a combination of n-alkanes and their $\delta^{13}C$ values were used as markers, whereas the largest values (68.01-78.62) were obtained with the third scenario, in which n-alkanes were used alone as markers. Analysis of variance and mean comparisons revealed differences ($P<0.001$) in the MSE between scenarios. Moreover, in the first and second scenarios, the MSE was reduced ($P=0.002$) when n-alkanes and $\delta^{13}C$ values were used together compared with the n-alkanes used alone. However, there was no statistically detectable difference among the calculation methods (Table 3) although the EatWhat program appeared to result in better predictions. Figure 2 shows the plots of the predicted composite grass mixture compositions derived using the EatWhat software.

Discussion

Plant cuticular n-alkanes have been used as useful markers to estimate the diet composition of free ranging herbivores (Dove and Mayes, 1996, Dove and Mayes, 2005). The first

requirement for the use of these hydrocarbons as diet composition markers is that the potential diet components (species) should have distinct n-alkane profiles to differentiate one diet component from the other (Dove and Mayes, 1991). A recent study regarding the plant cuticular n-alkanes and their carbon isotopic composition of pasture species commonly available in the Mid Rift Valley region of Ethiopia has shown promise for using these hydrocarbons as diet composition markers in this study area (Bezabih *et al*., 2011b).

The results of the separate principal component analyses conducted using n-alkanes and their $\delta^{13}C$ values show the presence of sufficient variability to discriminate between the species. A closer look at the orientation of the species in Figure 1 shows differences with regard to the loadings of the two principal component analyses. With n-alkanes, strong correlation was observed between C_{27} and C_{28}, C_{33} and C_{32}, and C_{29} and C_{30}. This appears desirable as the discriminatory power carried by the odd-chain alkanes could contain much of the needed information in case the even-chain alkanes are excluded due to low concentrations.

The significant linear relationships observed between predicted and measured botanical composition of pasture mixes (Table 3) further showed that the discriminatory information carried by the n-alkanes is suitable for use as diet composition markers. In each calculation method and scenario, the relationship was improved when n-alkane profiles were used in combination with their $\delta^{13}C$ values (Table 3). This confirmed the previous report that the carbon isotope enrichment of the n-alkanes can provide additional discriminatory information to estimate diet compositions (Bezabih *et al*., 2011b). A considerable decline in the R^2 values together with a significant increase in the MSE from the first to the third scenario indicates that the accuracy with which the percentage composition of grass species is predicted is dependent on the number of diet components used as input in the calculation. On the other hand, in the present trial, the prediction (scenario ii and iii) appeared to be insensitive to the combination of additional species included as input, indicating similar levels of interference of the additional species in the optimization procedures. This is generally in agreement with our previous observations (Bezabih *et al.*, 2011b) that showed most of the species under consideration were fairly evenly scattered along the first two principal component axes with no major clustering observed.

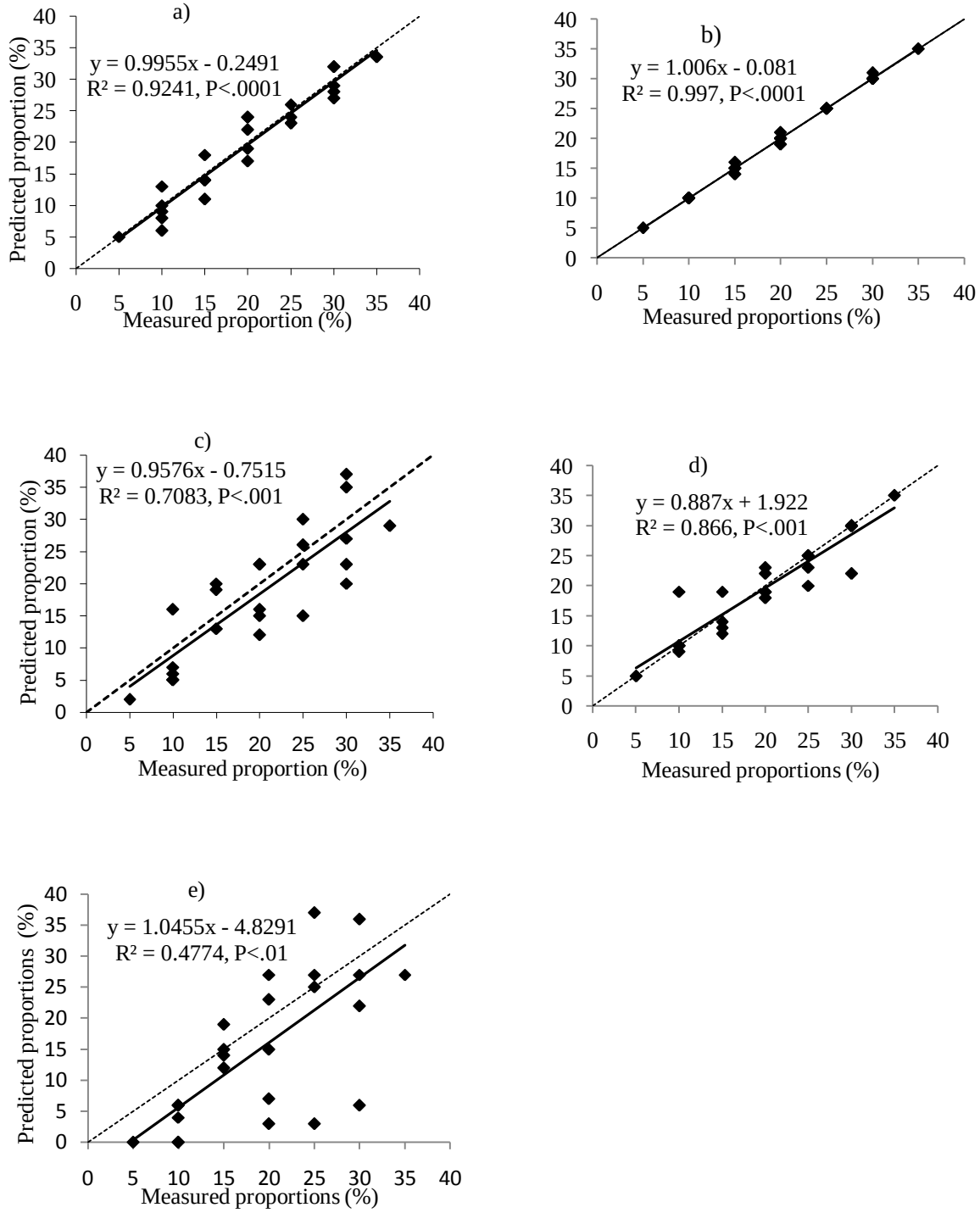

Figure 2 Plot of predicted versus measured composite diet mixture compositions derived from the EatWhat software; a) n-alkanes used as markers and no additional species used as input; b) n-alkanes and their $\delta^{13}C$ used as markers and no additional species used

as input; c) n-alkanes used as markers and five additional species added as input; d) n-alkanes and their $\delta^{13}C$ values used as markers and five additional species used as input; e) n-alkanes and their $\delta^{13}C$ values used as markers and ten additional species used as input. The dashed lines in the graphs indicate the line of equality.

Estimation of diet composition of free-ranging herbivores is predominantly concerned with generating two types of data. The first is the diet components (species) selected by the animal, and the second is the relative proportion of each component in the diet consumed. The former is particularly important if animals graze/browse botanically diverse vegetation and there is high degree of selection, where some plant species are preferentially consumed to others. The present results clearly indicated that in order to obtain a reliable estimation of the type of diet components consumed and the percentage contribution of each to the total, it is important to either increase the type of marker used or to restrict the inputs to those diet components which are most likely to be eaten by the animal. While the $\delta^{13}C$ values of n-alkanes demonstrated potential to increase the discriminatory power of n-alkanes in the present study, other research results are supportive of inclusion of other plant wax components such as long chain fatty acids and fatty alcohols (Ali *et al.,* 2005b; Dove and Mayes, 2005; Fraser *et al*., 2006). The carbon isotopic composition of long chain fatty alcohols and fatty acids has not yet been evaluated as additional internal markers. If beside inclusion of these other plant wax components also their $\delta^{13}C$ values are sufficiently discriminatory, there will be sufficient fingerprint information for a large number of diet components (species) in complex vegetation.

Animal experiments have confirmed that the recovery of n-alkanes and other wax components in the faeces is not complete (Dove and Mayes, 1996; Elwert *et al*., 2006), with the recovery rate generally increasing in a curvilinear fashion with increasing carbon number in ruminant animals. Hence, in order to relate the faecal patterns of the markers with that found in potential diet components, it is important to apply a suitable faecal recovery correction factor prior to diet composition estimation (Ferreira *et al*., 2009). When it comes to carbon isotope enrichments, faecal correction factors will not be required as $\delta^{13}C$ is a relative value.

As discussed elsewhere (Bezabih *et al*., 2011b), however, there is a lack of information as to whether there is a preferential degradation of ^{12}C and ^{13}C isotopes for a particular n-alkane in the gut and further investigation into this area will increase the accuracy of diet estimation using $^{13}C/^{12}C$ isotope ratios in n-alkanes.

Apart from increasing the type of markers used, the present study also indicates that restricting the inputs of diet components to those plant species that are actually eaten by the animal increases the accuracy with which the percentage of each component in the diet is predicted. Limiting the number of diet components (species) may be achieved by using qualitative methods such as visual observations or faecal microhistology (Dove and Mayes, 2006, Miller and Thompson, 2007). The use of such qualitative methods would enable identification of the plant species which are not ingested by the animal and thus help restrict the number of diet components included as input in the estimation of diet compositions. The microhistology method may also be used to set a maximum and minimum range for a particular diet component when diet composition is estimated with least-squares optimization (Miller and Thompson, 2007).

Generally, the three calculation methods used in the present evaluation produced similar results. It may, however, be important to consider developing an advanced mathematical algorithm, which takes into account the within and between species variability in the marker profiles and hence sets statistical confidence interval for the estimates of diet composition (Dove and Mayes, 2006). When different types of markers are used in combination, it may also be necessary to take into account the variation in measurement units and magnitudes of the different markers.

Conclusion

The n-alkane profile of five of the major botanical species found in the Mid Rift Valley rangelands contained sufficient discriminatory information to estimate their proportions in composite diet mixtures. The $\delta^{13}C$ values of n-alkanes provided additional discriminatory information and improved the linear relationship between the predicted versus measured composite diet mixtures. The accuracy of the estimate was affected by the number of species included as input, while the three calculation methods evaluated produced similar results. The results indicate that in botanically complex vegetation, combining n-alkanes with their $\delta^{13}C$

values can improve the accuracy of diet composition estimation. In addition, restricting the number of diet components that are used as input to those which are most likely be eaten by the animal increases the reliability of the prediction.

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CHAPTER 4

Estimation of feed intake and digestibility in cattle consuming low-quality tropical roughage diets using molasses-based n-alkane boluses

M. Bezabih[1,2], W. F. Pellikaan[1], A. Tolera[2], W. H. Hendriks[1,3]

[1]Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands

[2]Department of Animal and Range Sciences, Hawassa University, P.O. Box 5, Hawassa, Ethiopia

[3] Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80151, 3508 TD Utrecht, the Netherlands

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Abstract

A feeding experiment was conducted to measure the faecal recovery rates of n-alkanes and to evaluate molasses-based alkane boluses for feed intake and digestibility estimations in cattle consuming low-quality tropical roughages. The experiment was performed in a cross-over design with four experimental diets, four 21-day feeding runs and eight bulls. The animals received a measured amount of the experimental diets that resulted in little refusal throughout the experiment. After seven days of adaptation, the animals were dosed with molasses-based alkane boluses (each containing 200 mg C_{32} and 150 mg C_{36}) twice daily at 07:00 and 18:00 h. Concurrent with the alkane dosing, faecal spot samples were taken twice daily until the end of each run. In addition, total faecal collections were performed over the last 5 days of each run. The mean faecal recovery rate of both natural and dosed n-alkanes ranged between 0.61 and 0.86, with the recovery showing an upward trend with increasing carbon-chain length. The recovery rate of dosed alkanes was considerably higher than that of adjacent odd-chain alkanes. While diets did not differ ($P \geq 0.23$) in the recovery of even-chain n-alkanes, an effect of diet ($P \leq 0.01$) was observed in the recovery of odd-chain n-alkanes. The faecal concentration of dosed alkanes reached equilibrium 3.30 days into the alkane dosing. On the assumption of similar faecal recovery of adjacent n-alkanes, intake was underestimated by 12% ($P < 0.001$) when C_{31}/C_{33} and C_{33}/C_{32} alkane pairs were used and by only 1.5% ($P \geq 0.42$) when C_{35}/C_{36} was used. Correction for differences in the faecal recovery of adjacent n-alkanes considerably improved the intake prediction when C_{31}/C_{32} and C_{33}/C_{32} pairs were used. Digestibility of diets was accurately predicted using either C_{36} as external marker or C_{35} as internal marker corrected for incomplete recovery. The results showed that molasses-based boluses administered twice daily are suitable, and that knowledge of the faecal recovery rates of adjacent n-alkanes improves the reliability of the predictions.

Introduction

The production performance of farm animals within their genetic limits depends on the level of feed intake and the quality of the diet ingested (Coleman and Moore, 2003). Accurate measurement of feed intake and digestibility are, in this respect, important to meet nutritional requirements of the animal and optimize production (Mayes and Dove, 2000). Feed intake, diet composition and dietary nutrient digestibility are however difficult to measure accurately in free-ranging animals, and often indirect methods have to be used. Over the past two decades, the indirect method of estimation where n-alkanes are used as digesta markers, has received increasing acceptance (Keli *et al*., 2008b). The advantages of the n-alkane method over other approaches include low invasiveness, accuracy and the possibility of taking into account diet-animal interactions (Dove and Mayes, 1991; Mayes and Dove, 2000; Dove and Mayes, 2005). In addition, n-alkanes are chemically discrete components which can be easily analysed by gas chromatography.

Early studies showed that the recovery of n-alkanes in faeces was incomplete (Mayes and Lamb, 1984), with adjacent carbon chain alkanes showing similar recovery rates (Dove and Mayes, 1991). The concurrent use of adjacent natural odd- and dosed even-chain n-alkanes to estimate intake was developed (Mayes *et al*., 1986) based on the premise that estimation of intake from the simultaneous computation of digestibility (using natural odd-chain n-alkane) and faecal output (using dosed even-chain n-alkane) will cancel out the errors arising from the incomplete recovery of the alkanes and provides unbiased intake estimation. The accuracy of the intake estimation using this method is thus related to the similarity of faecal recovery rates of the dosed and herbage odd-chain alkanes (Dove and Mayes, 1991). Indoor validation experiments conducted under temperate climatic conditions with sheep and cattle have also confirmed that whenever the faecal recovery rates of adjacent alkanes were similar, the alkane method predicted intake accurately (Hameleers and Mayes, 1998; Estermann *et al.*, 2001). On the other hand, when there was a considerable difference in recovery rates between adjacent alkanes, the prediction was less accurate (Berry *et al*., 2000; Ferreira *et al.*, 2007a; Oliván *et al*., 2007b; Keli *et al*., 2008b).

Previous studies showed that the faecal recovery of alkanes is affected by animal species (Ferreira *et al*., 2009), physiological status and diet type (Lin *et al*., 2007; Elwert *et al*., 2008). Comparison between sheep, goats, and cattle consuming similar types of diet

indicated that cattle showed the highest and goats the lowest faecal recovery rates (Ferreira *et al*., 2009). In addition, the faecal recovery rate of alkanes seemed to be more variable in cattle than in sheep (Dove and Mayes, 1991).

The effect of diet on the recovery of n-alkanes appears to be variable among previous studies. For instance, Brosh *et al*. (2003) reported no effect of diet in a digestibility trial with three different diets fed to goats, cows and calves, but suggested more research to a wider range of diets, animals, environmental conditions and physiological and reproductive state. Elwert *et al.* (2006) reported only numerical differences in sheep fed different roughage diets, with high between-animal variability in faecal alkane recovery. On the other hand, the reports of Lin *et al.* (2007) and Ferreira *et al.* (2010) showed effect ($P<0.05$) of diet on the alkane recovery in sheep. The presence of such contrasting reports may be due to confounding effects of both diet compositions (ingredients with different cuticular wax characteristics) and physical and chemical properties of the diet. In this regard, Ferreira *et al*. (2005) reported that the diet composition of goats affected ($P<0.05$) the faecal recovery of alkanes when there were considerable differences in *in vivo* digestibility, with a negative correlation between alkane recovery and diet digestibility. Similar negative correlations were also observed by Elwert *et al.* (2008).

In the light of the importance of a reliable faecal recovery data to accurately estimate feed intake, diet composition and digestibility, researchers have recommended that the application of the n-alkane method for grazing animals should be preceded by calculation of the actual alkane faecal recoveries for different diet types and experimental conditions (Brosh *et al*., 2003; Valiente *et al*., 2003; Ferreira *et al*., 2005; Ferreira *et al*., 2010). Only few reports are available on the alkane faecal recovery of tropical forages and the applicability of the n-alkane method in cattle under tropical conditions (Hendricksen *et al*., 2002; Molina *et al*., 2004).

The present experiment was conducted with the following two objectives: 1) to measure the faecal recovery rate of n-alkanes in indigenous zebu cattle fed different types of tropical roughages in Ethiopia and, 2) to evaluate the suitability of molasses-based boluses to administer synthetic n-alkanes for intake and digestibility estimations.

Materials and Methods

Experimental animals and housing

Eight local Borana growing bulls with an average weight of 160±8 kg, which were raised on natural pasture in the Borana rangeland, were purchased from the local market and housed at Hawassa University (7°04´N and 38°29´E, Hawassa, Ethiopia) research farm. Animal care, handling and maintenance throughout the experiment were in accordance with the animal welfare regulations of the University. Upon arrival, the bulls were dewormed with Albendazole and group-fed for one week on a *Rhodes* grass and *Desmodium* hay-based uniformity diet provided *ad libitum*, with free access to water. After one week, the animals were transferred to individual concrete pens (2.5 m × 1.5 m) and fitted with faecal collection bags until the end of the experiment. Each pen contained a feeding and watering trough allowing individual feed intake measurement. The same *Rhodes* grass and *Desmodium* hay diet was offered twice daily in equal portions with the amount adjusted to ensure minimal to no refusals, while water was provided freely. Faecal collection bags were checked every hour 24 h a day and emptied of their contents when faeces were present. The animals were adapted to these conditions for 14 days before the start of the experiment.

Experimental diet

Four experimental diets (Table 1) were prepared from different roughage ingredients. All ingredients were sun-dried and chopped to a size of 3-4 cm. Diet 1 and 2 contained five species, whereas diet 3 and 4 contained ten species. The species and composition of the diets were chosen to simulate the type of diet cattle would consume during the dry and rainy seasons in Ethiopia. Each day, the amount of each dietary ingredient was precisely weighed out and uniformly spread over one another on a plastic sheet. Once all the species making up a diet were weighed out, the diet was thoroughly mixed by hand and stored in a bag until fed to the animal. Samples of individual ingredients were taken daily and stored for chemical analysis.

Experimental design and sampling

Following the 14 day adaptation period, the bulls were randomly assigned to one of the four treatment diets in a 4 × 4 double Latin square with each period lasting 21 days. The animals

received an accurately measured amount of the experimental diet twice a day (at 8:00 and 17:00 h) that resulted in minimal to no refusal to avoid selection. After a week of feeding, each animal received a molasses-based bolus twice daily (each bolus containing 200 mg C_{32} and 150 mg C_{36}) at 07:00 and 18:00 h with the aid of a balling gun. The bolus was made from alkanes/sucrose fatty acid ester tablets, each containing 0.20 g C_{32} and 0.15 g C_{36} per gram of a tablet (Argenta Manufacturing, New Zealand). The tablets were originally made to be used with controlled release devices (CRD). However, the company ceased production of alkane CRD, and in the present study these tablets were used to produce molasses-based alkane boluses. The tablets were crushed into a powder using mortar and pestle, and divided into 1.00 g portions. Eighteen grams of a carrier mixture (55% molasses, 20% hydrated calcium sulphate and 25% solvent-extracted linseed meal) was weighed into a lubricated (olive oil) glass beaker and carefully mixed with 1.00 g of the crushed alkane tablet to form a single dose bolus. With the addition of calcium sulphate powder, the boluses were shaped to fit into a balling gun whereafter they were dried in a forced-air oven at 60°C for 12 h. After restraining the animal, the bolus was placed over the tongue at the back of the throat, and a small amount of water (150-200 ml) was given to facilitate swallowing. Two days before the start of marker dosing until the end of each period (day 6 to 21), faecal spot samples were taken twice daily at the time of dosing. During the last five days within each period, every faecal defecation was quantitatively collected, pooled per day while placed in a fridge, and after thoroughly mixing, 10% of the daily fresh faeces was sampled and stored at -20°C.

Sample preparation

Samples of each feed ingredient collected over the experimental periods were pooled into one sample. Background faecal samples collected prior to marker dosing were pooled per diet while faecal spot samples collected from the beginning of dosing until the start of the total collection period were bulked per animal per day. The faecal spot samples obtained during the total collection period were pooled into morning and afternoon spot samples per animal. All the samples were dried at 60°C in a forced-air oven for 48 h, ground to pass a 1-mm sieve, and stored at 5°C in plastic bottles pending chemical analysis.

Chemical analysis

All chemical analyses were conducted at the Laboratory of the Animal Nutrition Group of Wageningen University (Wageningen, the Netherlands). For n-alkane analysis, ground samples were pulverised using a bullet mill (MM 2000; 4 min at 80 Hz; Retsch Technology GmbH, Haan, Germany) before extraction and analysis of n-alkanes as described by Mayes *et al.* (1986) with modifications by Salt *et al.* (1992) using tetratriacontane (C_{34}) as an internal standard. The extracted samples were analysed for n-alkanes (C_{27} to C_{36}) using a gas chromatograph (GC:Carlo Erba HRGC Mega 2 series) fitted to a flame ionizing detector (FID), using helium as the carrier gas. The setting of the gas chromatograph was as described by Bezabih *et al.* (2011b).

The samples were also analysed for dry matter (DM), ash, crude protein (CP), NDF and ADF. The content of DM was determined by oven drying at 103°C (ISO 6496; ISO, 1999), ash after incineration at 550°C (ISO 5984; ISO, 2002) and CP (6.25 × N) by using the Kjeldahl method (ISO 5983; ISO, 2005). Neutral detergent fibre (aNDF) was determined according to Van Soest *et al.* (1991) and acid detergent fibre (ADF) according to Van Soest (1973). The content of aNDF was determined with the use of sodium sulphite and alpha amylase. Both aNDF and ADF were expressed inclusive of residual ash.

Calculations

Faecal recovery of individual n-alkanes was calculated as the ratio of the n-alkane excreted in the faeces to that consumed from the diet as follows:

$$R_i = \frac{(FO \times F_i)}{(DMI \times H_i)}$$

where R_i is the faecal recovery rate of alkane *i*, FO is the daily faecal output (kg DM), F_i is the concentration of alkane *i* in faeces (mg/kg DM), DMI is the daily dry matter intake (kg), and H_i is the concentration of alkane *i* in the diet consumed (mg/kg DM)

Feed intake was estimated using the double n-alkane method according to Mayes *et al.* (1986) using the following formula:

$$\text{Daily diet intake (kg DM)} = \frac{((F_i/F_j) \times D_j)}{(H_i - F_i/F_j \times H_j)}$$

where F_i represents the feacal and H_i the herbage odd-chain alkane *i* concentrations (mg/kg DM), F_j resembles the fecal and H_j the herbage even-chain alkane *j* concentrations (mg/kg DM), and D_j equals the daily dose of even-chain alkane *j*. Three types of intake estimates were generated using C_{31}/C_{32}, C_{33}/C_{32} or C_{35}/C_{36} alkane pairs.

Faecal output was estimated from C_{36} concentration in the faeces as follows:

$$\text{Daily faecal output (kg DM)} = D_{C36}/(F_{C36} - B_{C36}),$$

where D_{C36} is the daily dose of C_{36}, F_{C36} is the faecal concentration of C_{36} (mg/kg DM) corrected for incomplete recovery, and B_{C36} is the background faecal concentration of C_{36} (mg/kg DM).

Apparent DM and organic matter (OM) digestibility estimates were calculated as:

1 – indigestibility, where indigestibility is the ratio of estimated daily faecal output to estimated intake.

Digestibility was also predicted using natural C_{35} alkane as an internal marker:

$$\text{DM digestibility} = (F_{C35} - D_{C35})/F_{C35},$$

where F_{C35} is the faecal C_{35} concentration (corrected for incomplete recover) and D_{C35} is the dietary C_{35} concentration.

Statistical analysis

The data on the faecal recovery of dosed and natural n-alkanes were analysed using the following general model:

$$Y_{ijkm} = \mu + D_i + P_j + A_k + e_{ijkm}$$

where, Y_{ijkm} is the dependent variable, μ is the overall mean; D_i is the fixed effect of diet *i* (i=1,...4), P_j the random effect of period *j* (*j*=1,...4), A_k the random effect of animal (*k*=1,…8), and e_{ijkm} is the error term.

The PROC MIXED procedure of the SAS statistical package (version 9.1) was used for the analysis. Paired *t*-test comparison between actual and predicted values of intake and digestibility was performed to examine the accuracy of the estimations. Analysis of variance was employed to investigate differences between faecal sampling methods in the estimation of intake and digestibility. Linear regression was conducted to examine the relation between the n-alkane profiles of total faecal collection samples and faecal spot samples, as well as the diet digestibility values predicted using the alkanes C_{36} and C_{35} as markers. In addition, a broken

stick regression was employed using the PROC NLIN procedure of SAS to determine the day at which a plateau of dosed alkane concentration was achieved in faeces after the first day of dosing.

Results

Chemical composition of diets and faecal alkane concentrations

The chemical composition of the four experimental diets is shown in Table 1. The CP concentration ranged from 57.7 g/kg DM in diet 1 to 74.8 g/kg DM in diet 4, whereas the aNDF concentration varied between 653 and 626 g/kg DM. The pattern of dosed C_{32} and C_{36}-alkane concentrations in faeces over time is shown in Figure 1. The concentration of dosed n-alkane increased steadily in the first three days of dosing before reaching equilibrium. The nonlinear regression revealed that a plateau faecal concentration of the dosed alkanes was achieved 3.30±0.20 (mean±s.e.) days into the marker dosing. Figure 2 shows the linear relationship ($P<0.001$, $R^2=0.99$) in the pattern of n-alkane concentration between the total faecal collection and morning faecal spot samples. In all the diets, the slope and the intercept of the regression lines were not different from 1 and 0, respectively.

Faecal recovery of n-alkanes

The mean faecal recoveries of individual n-alkanes during the measurement period are shown in Table 2. The actual alkane contents of the molasses-based boluses determined in the laboratory were used to calculate the faecal recovery of dosed alkanes. The mean recovery rates ranged from 0.61 to 0.86 with dosed alkanes showing higher recovery than the natural alkanes. The recovery rate increased with increasing carbon number among the natural alkanes. An effect of diet ($P\leq0.01$) on the recovery of the odd-chain alkanes was observed, with diet 1 having the lowest and diet 4 the highest recovery rates. The even-chain alkanes showed similar recovery values among the different diets. The average ratio between the faecal recovery of dosed even-chain and adjacent odd-chain alkanes was 0.90 for C_{35}/C_{36}, 0.84 for C_{31}/C_{32} and 0.82 for C_{33}/C_{32} (Table 2). These ratios varied ($P\leq0.03$) between diets, following the same pattern as the odd-chain alkanes.

Table 1 Ingredients and chemical composition of the four experimental diets.

Ingredients/chemical composition	Diet 1	Diet 2	Diet 3	Diet 4
Ingredients (proportion)				
Rhodes grass (*Chloris gayana*) hay	0.45	0.40	0.25	0.20
Lablab (*Lablab purpureus*) hay	0.05	0.10	0.05	0.07
Haricot bean (*Phaseolus vulgaris*) hay	0.15	0.25	0.13	0.15
Maize (*Zea mays*) stover	0.10	0.05	0.05	0.05
Teff (*Eragrostis tef)* straw	0.25	0.20	0.20	0.15
Desmodium (*Desmodium intortum*) hay	-	-	0.12	0.12
Wheat (*Triticum aestivum*) straw	-	-	0.07	0.10
Oat (*Avena sativa*) hay	-	-	0.08	0.10
Napier grass (*Pennisetum purpureum*) hay	-	-	0.03	0.05
Cow pea (*Vigna unguiculata*) hay	-	-	0.02	0.01
Chemical composition (g/kg DM)				
Dry matter (DM)	936	934	933	908
Organic matter (OM)	846	846	843	818
Crude protein (CP)	58	64	73	75
Neutral detergent fibre (aNDF)	653	632	630	626
Acid detergent fibre (ADF)	397	407	402	470

Intake and digestibility estimates

Table 3 presents actual and predicted mean dry matter intakes according to diet, sampling method and pair of n-alkanes used in the calculation. The result shows that the assumption of similar faecal recovery underestimated ($P<0.001$) intake by 11.8% with C_{31}/C_{32} and 12.6% with the C_{33}/C_{32} pairs of n-alkanes. On the other hand, with the C_{35}/C_{36} pair, the difference between actual and predicted intakes was less than 1% ($P\geq0.42$) (Table 3).

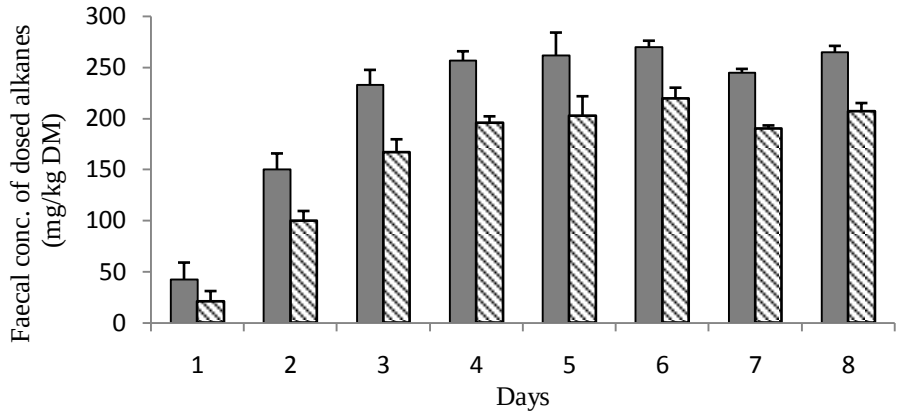

Figure 1 C_{32} and C_{36} alkane concentration in the faeces after dosing bulls 200 mg C_{32} and 150 mg C_{36} twice daily (07:00 and 18:00h).

Table 2 Mean faecal recovery of n-alkanes and the ratio of dosed and adjacent odd-chain alkanes in cattle fed four different tropical roughage diets.

Alkane	Diet 1	Diet 2	Diet 3	Diet 4	Pooled SEM	P-value
C_{27}	0.56^{a}	0.61^{ab}	0.62^{b}	0.64^{b}	1.80	0.005
C_{28}	0.69	0.67	0.64	0.68	6.71	0.260
C_{29}	0.68^{a}	0.73^{ab}	0.73^{b}	0.74^{b}	2.13	0.010
C_{30}	0.74	0.72	0.71	0.78	5.67	0.236
C_{31}	0.67^{a}	0.73^{ab}	0.74^{b}	0.75^{b}	1.97	<0.001
C_{32}	0.85	0.86	0.86	0.86	2.28	0.751
C_{33}	0.65^{a}	0.71^{ab}	0.72^{b}	0.73^{b}	1.99	0.002
C_{35}	0.67^{a}	0.74^{b}	0.74^{b}	0.76^{b}	2.44	0.002
C_{36}	0.81	0.82	0.81	0.80	2.07	0.481
C_{31}/C_{32}	0.794^{a}	0.850^{b}	0.861^{b}	0.876^{b}	0.017	0.032
C_{33}/C_{32}	0.769^{a}	0.831^{b}	0.836^{b}	0.846^{b}	0.016	<0.001
C_{35}/C_{36}	0.821^{a}	0.894^{b}	0.921^{b}	0.956^{b}	0.031	0.028

^{a,b,c} Means not sharing a common superscript within a row differ by P<0.05.
For diet ingredients see Table 1.

Correcting for faecal recovery considerably improved the prediction with the first two pairs of n-alkanes ($C_{31}/C_{32;}$ C_{33}/C_{32}), but not with the third pair (C_{35}/C_{36}). Using such correction overestimated intake on average by 4.1% with C_{31}/C_{32}, 4.9% with C_{33}/C_{32} and 8.4% with C_{35}/C_{36}. In this case, predicted intakes using total collection and morning spot samples did not differ from the actual values, except with C_{35}/C_{36} where predictions from both morning and afternoon spot sample were different ($P \leq 0.02$) from the actual value.

In all three cases, the predictions using the afternoon spot sample was higher ($P \leq 0.002$) than the actual value. Generally, comparison among the three faecal sampling methods in the predicted intake resulted in similar values, except in diet 4, where there was a difference ($P<0.05$) between the sampling methods when C_{31}/C_{32} was used (Table 3). Figure 3 presents plot of the observed error in individual intake estimation versus the difference in faecal recovery rates of the alkane pairs across diets. With all three options, a linear relationship (R^2=0.75-0.82; $P<0.001$) was observed (Figure 3).

Dry matter and organic matter digestibility (DMD and OMD) values predicted using C_{36} as an external marker are presented in Table 4. Comparison between actual and predicted digestibility showed a high level of similarity with no difference in all the three sampling procedures. The actual DMD was overestimated by only 0.50% with the total faecal collection sample and from 1.13 to 2.40% with morning and afternoon spot samples.

Use of C_{35} as an internal marker predicted the mean DMD of the diets as 48.1±7.7%, resulting in underestimation by 4.19% ($P=0.105$). Figure 4 shows a strong linear relationship ($P<0.001$, R^2=0.99) between dosed C_{36} and herbage C_{35}-predicted (corrected for incomplete recovery) DMD values, with the slope of the regression not different from 1.

Discussion

Chemical composition of diets and dosing of synthetic alkanes

Tropical forages are generally characterized by a low CP and high NDF content (Preston, 1982; Wassmann and Velk, 2003) with season of sampling (dry or rainy) having an important effect on the chemical composition (Machado *et al*., 2007; Miller and Thompson, 2007). In the long dry season, the CP content of forages may fall to less than 70 g/kg DM (Tefera *et al*., 2009), which is considered to be limiting for an optimum rumen microbial growth and fermentation (Van Soest, 1982; Leng, 1993). The chemical compositions and the species

richness of forage diets used in the present experiment (Table 1) resemble the type of diet animals would consume in the dry and rainy seasons.

Table 3. Actual and predicted dry matter intake (kg/day) using three different odd- to even-chain n-alkane ratios and three faecal sampling procedures in cattle fed four different tropical roughage diets.

Dry matter intake	Diet 1	Diet 2	Diet 3	Diet 4	Mean	P-value[†]
Actual intake	2.78	2.69	2.80	2.81	2.78	-
Predicted intake assuming similar faecal recovery of n-alkanes						
C_{31}/C_{32}						
TC	2.27	2.38	2.53	2.59[a]	2.47	0.001
MS	2.31	2.37	2.29	2.35[b]	2.34	0.001
AS	2.35	2.54	2.61	2.70[a]	2.55	0.001
P-value[‡]	0.885	0.481	0.081	0.006		
C_{33}/C_{32}						
TC	2.31	2.34	2.46	2.50	2.41	0.001
MS	2.25	2.31	2.26	2.24	2.31	0.001
AS	2.32	2.54	2.58	2.64	2.57	0.001
P-value[‡]	0.922	0.586	0.069	0.059		
C_{35}/C_{36}						
TC	2.67	2.56	2.74	2.85	2.75	0.423
MS	2.59	2.74	2.74	2.76	2.77	0.561
AS	2.52	2.77	2.83	2.87	2.80	0.705
P-value[‡]	0.879	0.898	0.961	0.920		
Predicted intake corrected for differences in faecal recovery of alkanes						
C_{31}/C_{32}						
TC	2.86	2.80	2.94	2.95[ab]	2.89	0.101
MS	2.74	2.81	2.72	2.79[a]	2.77	0.421
AS	2.79	3.00	3.10	3.20[b]	3.02	0.002
P-value[‡]	0.763	0.543	0.421	0.032		
C_{33}/C_{32}						
TC	2.88	2.82	2.95	2.96[ab]	2.90	0.135
MS	2.76	2.82	2.75	2.73[a]	2.77	0.451
AS	2.85	3.09	3.15	3.22[b]	3.08	0.001
P-value[‡]	0.621	0.302	0.081	0.021		
C_{35}/C_{36}						
TC	2.94	2.86	2.98	2.98	2.94	0.115
MS	2.91	3.06	3.03	3.06	3.02	0.021
AS	2.84	3.10	3.17	3.23	3.08	0.001
P value[‡]	0.672	0.251	0.402	0.153		

[a,b,c] Means not sharing a common letter in the superscript within a column differ by $P<0.05$. TC=total faecal collection samples; MS=morning faecal spot samples; AS=afternoon faecal spot samples. [†]Comparison between actual and predicted dry matter intakes across diets; and [‡] comparison between sampling methods; For diet ingredients see Table 1.

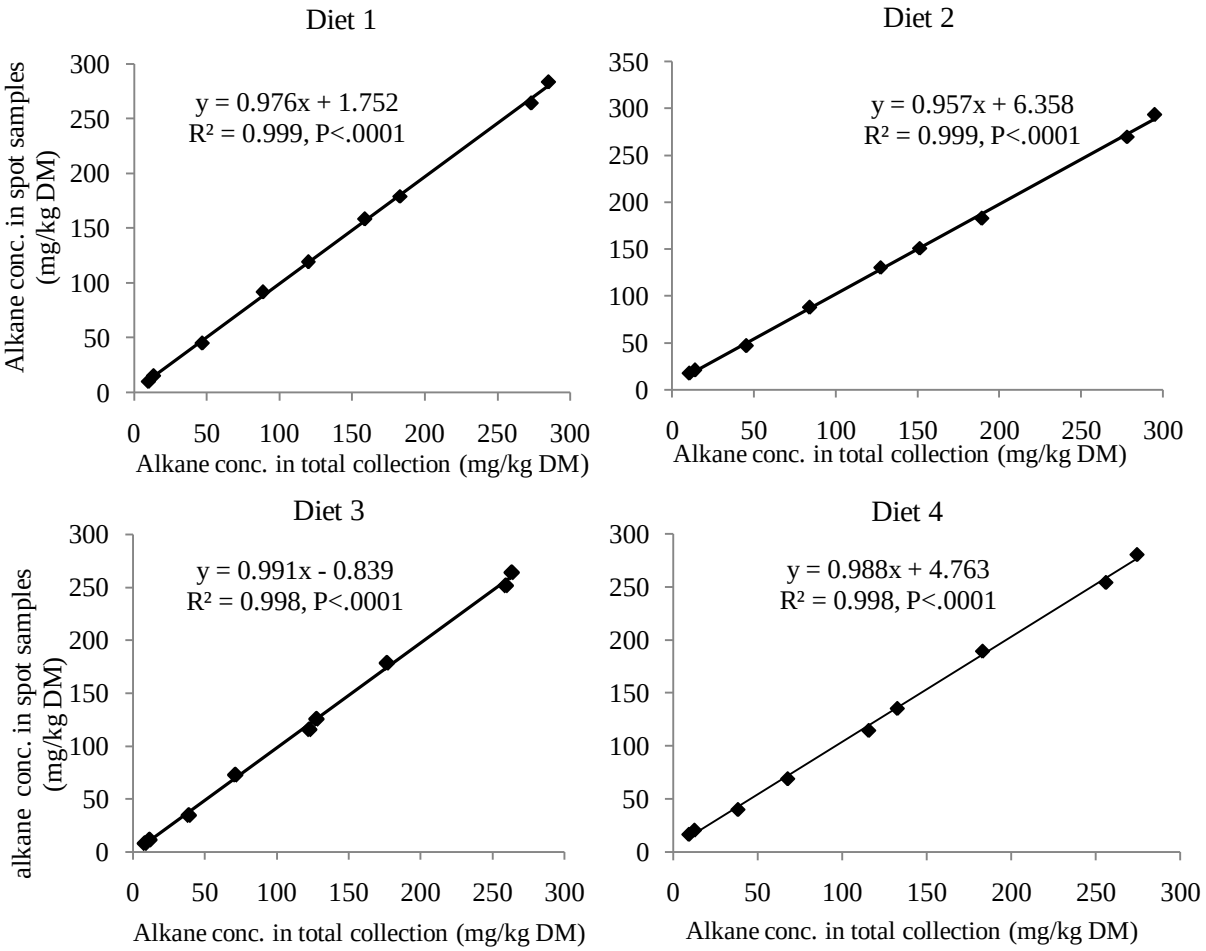

Figure 2 Linear relationships between n-alkane (C_{27}-C_{36}) concentrations (mg/kg DM) in total faecal collection and morning spot samples in cattle fed different tropical roughage diets.

Estimation of feed intake and faecal output using the n-alkane technique requires dosing of the animal with one or more synthetic n-alkanes (Mayes *et al.*, 1986). The carrier material used, the frequency of dosing, and faecal sampling schedules are factors that may have an influence on the pattern of faecal concentration of dosed n-alkanes (Dove *et al.*, 2002; Giráldez *et al.*, 2004; Smith *et al.*, 2007). In previous studies involving paper pellets and controlled-release devises for alkane dosing, a 5-day equilibrium period has been recommended (Berry *et al.*, 2000; Hendricksen *et al.*, 2003; Mayer *et al.*, 2003; Ferreira *et al.*, 2007a; Oliván *et al.*, 2007a). In the present study, the concentration of dosed n-alkanes

reached a plateau between the third and fourth day of dosing. This result indicates that with the molasses-based boluses and twice administration regime, faecal sampling for intake and digestibility estimation can be conducted from the fourth day onwards.

The strong relationship observed (Figure 2) between total faecal collection and spot samples in the concentration of dosed and natural n-alkane shows a desirable feature in the pattern of recovery of the alkanes in faeces. As commercial alkane CRD are currently not available on the market, the molasses-based alkane boluses tested in the present study appear to be a suitable alternative for alkane dosing. Although the sources of the alkanes in the present experiment were tablets (originally made for the manufacture of CRD), whose production also ceased, the molasses-based boluses can be produced from other sources such as pure alkanes pre-absorbed in cellulose fibre.

Hendricksen *et al*. (2003) compared molasses as a carrier for C_{32} and C_{36} with a commercially manufactured CRD and concluded that molasses containing C_{32} and C_{36} markers given three or more times daily to steers was as accurate as the commercial CRD. The two times daily dosing of the molasses boluses in the present study appears to have been an equally adequate frequency of dosing, as faecal spot and total collection samples produced similar alkane profiles (Figure 2) and intake and digestibility estimations (Table 3 and 4). This would be convenient in grazing experiments, as animals are usually corralled in sheds overnight and dosing can be done before animals are turned into pasture in the morning and after their return from pasture in the afternoon, eliminating interference while they are grazing.

Recovery of n-alkanes

The incomplete recovery of n-alkanes in faeces in the present study is in line with earlier studies when these hydrocarbons were first considered as nutritional markers (Mayes and Lamb, 1984). Further studies have shown effects of animal species (Ferreira *et al*., 2009) and diets on the recovery of n-alkanes. Although the effects of diet are variable, differences in n-alkane recoveries between diet types have been observed (Hendricksen *et al*., 2002; Monks *et al*., 2005; Elwert *et al.*, 2006).

In the present experiment, the recovery values of both natural and synthetic n-alkanes were lower than that reported for temperate regions (Ferreira *et al*., 2009), but the increase in

recovery rate with increasing carbon chain length (Table 2) is in general agreement with previous reports (Dove and Mayes, 2005). Previous experiments on tropical forages also showed lower levels of n-alkane recovery compared with temperate conditions (Hendricksen *et al*., 2002). This difference in faecal recovery rate between temperate and tropical forages strengthens the recommendation that the alkane method needs diet and species-specific trials to increase the accuracy of its predictions.

The four experimental diets used in the present trial differed both in species richness and gross chemical composition to examine different diet scenarios on the faecal n-alkane recoveries and hence on the estimates of intake and digestibility in grazing cattle. The results showed that as the diets differed in composition, differences ($P<0.05$) in the faecal recovery rates of odd-chain n-alkanes were observed. This is evident when compositional and recovery values of diet 1 and diet 4 are compared (Table 1 and 2). While the two diets had the largest difference in their chemical composition, they showed differences ($P\leq0.05$) in the faecal recovery of the odd-chain n-alkanes. In contrast to the odd-chains, the recovery of even-chain n-alkanes did not differ due to the change in the diet composition. For the natural even-chain alkanes this lack of difference may be attributed to their low concentration in combination with a considerable between animal variability (Elwert *et al*., 2006). The observation that the dosed n-alkanes were superior in faecal recovery than the natural alkanes (Table 2) is consistent with previous studies (Berry *et al*., 2000; Hendricksen *et al*., 2002). Herbage n-alkanes are associated with the solid phase of the digesta, while the dosed n-alkanes are associated with the liquid phase (Dove and Mayes, 1991; Bulang *et al*., 2008). This may contribute to a higher passage rate in the gut of dosed n-alkanes and therefore a higher recovery.

Freeze-drying is generally the preferred method of sample preparation for alkane analysis. In the present study, oven-drying of feed and faecal samples at 60°C for 48 h was used. Although oven-drying at 100°C considerably affected n-alkane concentration in comparison to freeze-drying (Dove and Mayes, 1991; Elwert *et al*., 2006), drying at 60°C for 48 h produced similar results to freeze-drying (Elwert *et al*., 2006). It was therefore assumed that the drying procedure used in the present study had little effect on the alkane content of the samples analysed and on the faecal recovery rates calculated.

Table 4 Actual and predicted digestibility of dry matter and organic matter in cattle fed four different tropical roughage diets.

Digestibility	Diet 1	Diet 2	Diet 3	Diet 4	Mean	P value[†]
Dry matter digestibility						
Actual	0.50	0.50	0.49	0.52	0.52	
Predicted						
TC	0.48	0.49	0.51	0.53	0.53	0.479
MS	0.51	0.51	0.50	0.55	0.55	0.253
AS	0.52	0.51	0.49	0.52	0.52	0.547
P-value[‡]	0.454	0.716	0.653	0.608		
Organic matter digestibility						
Actual	0.55	0.55	0.55	0.57	0.57	
Predicted						
TC	0.51	0.52	0.54	0.56	0.56	0.084
MS	0.54	0.54	0.58	0.58	0.58	0.795
AS	0.54	0.54	0.57	0.55	0.55	0.575
P-value[‡]	0.512	0.753	0.409	0.820		

TC=total faecal collection samples; MS=morning faecal spot samples; AS=afternoon faecal spot samples.

[†]P-value for comparison between actual and predicted digestibility across diets; and [‡]for comparison between sampling methods; for diet ingredients composition see Table 1.

Intake and digestibility estimations

Estimation of intake with the double n-alkane method (Mayes *et al*., 1986) assumes that the faecal recovery rate of adjacent odd- and even-chain n-alkanes is similar. There are, however, observations in which adjacent natural and dosed n-alkanes showed considerable differences in their faecal recovery rates that could lead to biased intake estimations (Berry *et al*., 2000).

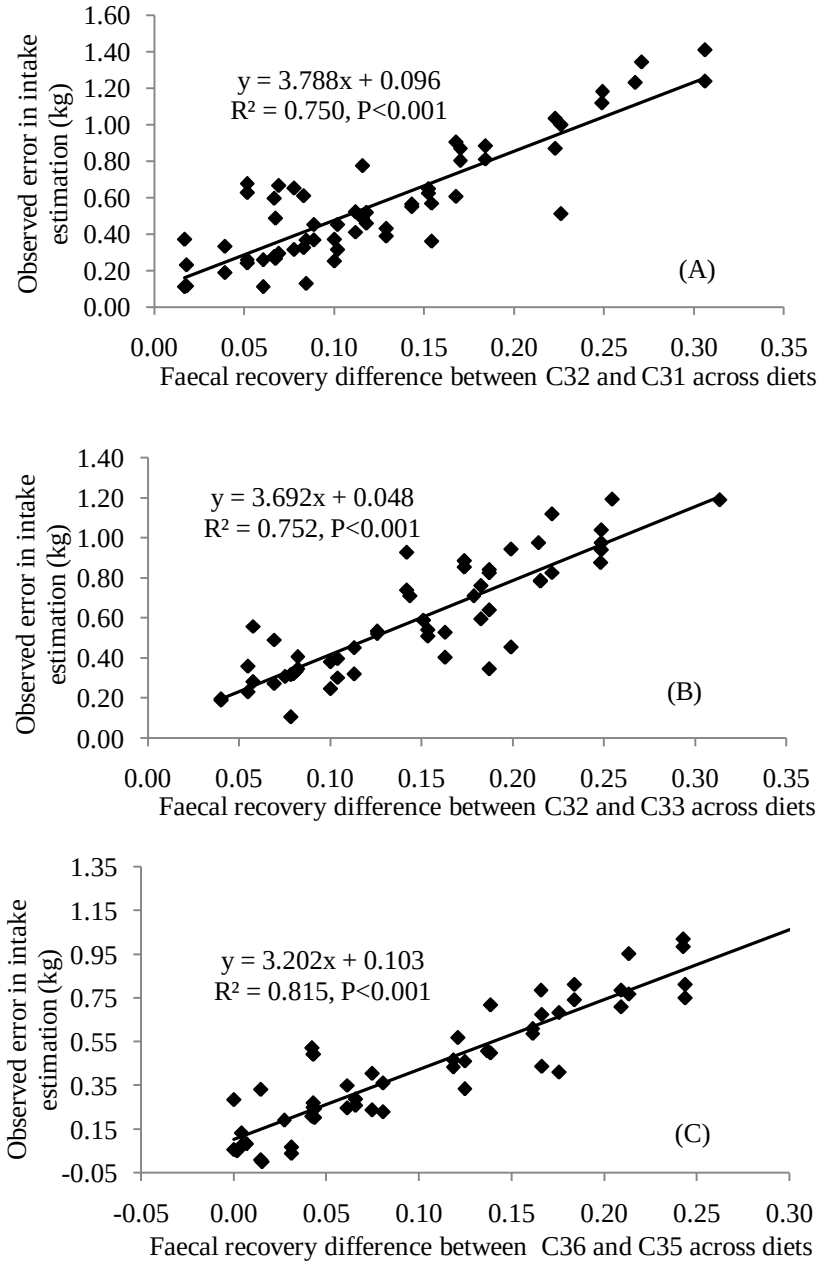

Figure 3 Plot of observed error in the individual intake estimation (measured - estimated intake, kg) versus differences in faecal recovery rates of alkane pairs across diets: Panel A, using C_{31}/C_{32}; Panel B, using C_{33}/C_{32}; and Panel C, using $C_{35}/C_{3}6$. The faecal recovery rate of the alkanes is calculated as the proportion of the alkane consumed that is excreted in faeces.

In the present study (Table 2), the ratios of the faecal recovery rate of the three alternative combinations of odd- to even-chain alkanes were less than unity on average by 10 to 18%, which indicates that if similar faecal recovery is assumed, an underestimation of the actual intake by the same magnitude would be expected. This is evidenced by the strong linear relationship observed (Figure 3) between the error in intake prediction and the difference in faecal recovery rates of the alkane pairs used.

The presence of effect of diet on the ratio of recovery of adjacent n-alkanes (Table 2) implies that, if corrections are made for differences in the faecal recovery, diet-specific correction factors may provide better results than average values. As shown in Table 3, the comparison between actual and predicted intakes largely agrees with the above explanation. Without correction for differences in recovery of n-alkane pairs, an underestimation of the actual intake by about 12% was observed with C_{31}/C_{32} and C_{33}/C_{32} pairs. On the other hand, the predictions using C_{35}/C_{36} were close to the actual values, mainly because the ratio of the faecal recovery of this pair of n-alkanes approaches unity better than the other two pairs of n-alkanes. The fact that correction for differences in recovery improved prediction with C_{31}/C_{32} and C_{33}/C_{32} alkane pairs and not with C_{35}/C_{36} indicates the importance of acquiring measured faecal recovery values to increase the accuracy of intake estimation.

Generally, the use of a correction factor and its magnitude should depend on the n-alkane pair and the type of diet consumed by the animal. In free-ranging animals, however, it is difficult to generate specific faecal n-alkane recovery data and the correction factor should depend on those values obtained from controlled experiments (Dove and Mayes 1991). In this respect, a wide range of balance studies involving different diet scenarios that simulate the diet of the grazing animal appears indispensable.

DMD and OMD were estimated with a high accuracy with the use of C_{36} as external marker to estimate faecal output (Table 4). Moreover, the digestibility estimated with C_{35} as an internal marker was not different from the actual values and that of C_{36} estimations (Figure 4). Thus, it appears that the natural C_{35} alkane may be used as an internal marker to conduct a quick diagnosis of digestibility in grazing animals without having to dose a synthetic n-alkane. However, for both digestibility and intake estimations, obtaining a representative sample of the diet consumed by the animal is essential.

There was a good agreement among total collection, morning spot and afternoon spot samples with regard to the prediction of intake and digestibility. For each of the sample types, faeces collected over five consecutive days were pooled into a sample, which may have contributed to the similarity among them. Samples pooled over several days are known to give more accurate results than single-picked samples (Berry *et al*., 2000; Smith *et al*., 2007). Generally, the observed similarity between total faecal collection and spot samples in the prediction of intake and digestibility has an important practical significance as the ultimate goal is to estimate diet intake and digestibility in free-ranging animals where total faecal collection is impractical.

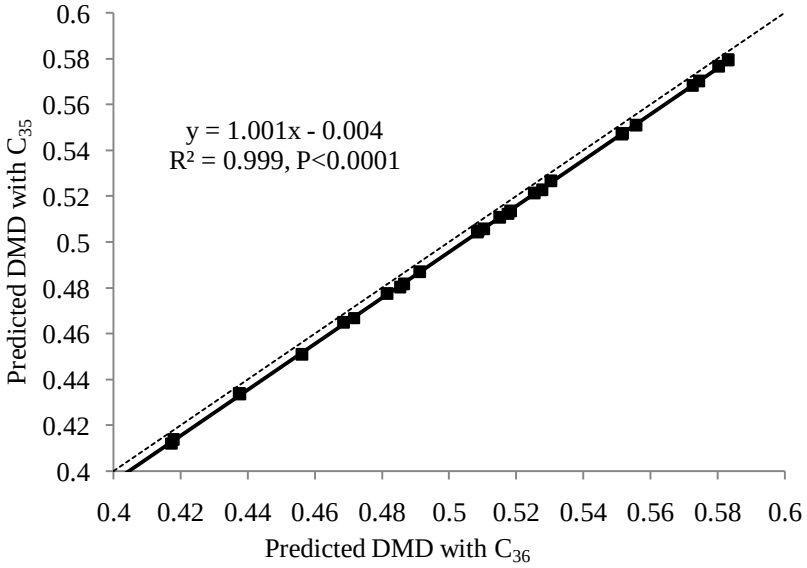

Figure 4 Linear relationship between C_{36} predicted and C_{35} predicted (corrected for incomplete recovery) dry matter digestibility values in cattle fed low-quality tropical roughage diets. The dotted line shows the line of equality (1:1).

Conclusion

Molasses-based boluses containing C_{32} and C_{36} alkanes administered twice daily are suitable to conduct intake and digestibility measurements in growing bulls. The type of diet consumed affected the faecal recovery rate of odd-chain n-alkanes with a positive relationship between diet digestibility and faecal recovery rate. The assumption of similar recovery of dosed even-

chain and adjacent odd-chain alkanes underestimated intake using C_{31}/C_{32} and C_{33}/C_{32} n-alkane pairs, while it enabled accurate intake prediction when the C_{35}/C_{36} pair was used. Digestibility of diets was accurately predicted using either C_{36} as an external marker or C_{35} as an internal marker corrected for incomplete recovery. Accurate measurement of the faecal recovery rates of dosed and natural alkanes appears to be essential to produce reliable estimates of intake and digestibility.

Acknowledgment

We thank Mr Michel Breuer and the laboratory staff of Animal Nutrition Group of Wageningen University for their assistance during the chemical analyses. This work was funded by the Netherlands Organisation for International Cooperation in Higher Education and the Ministry of Education of Ethiopia.

CHAPTER 5

Chemical composition, *in vitro* total gas and methane production of grass and browse species from the Mid Rift Valley grasslands of Ethiopia

M. Bezabih[1,2], W. F. Pellikaan[1], A. Tolera[2], N.A. Khan[1], W. H. Hendriks[1,3]

[1]Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

[2]Department of Animal and Range Sciences, Hawassa University, P.O. Box 5, Hawassa, Ethiopia

[3] Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80151, 3508 TD Utrecht, the Netherlands

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Abstract

The aim of this study was to assess the nutrient composition, feeding value and methane production potential of pasture and browse species from the Mid Rift Valley grasslands of Ethiopia. Samples of pasture and browse were collected during the main rainy season (July-August, 2009) from various locations in the region and sorted by species. Oven-dried and ground samples of these species were analyzed for nutrient composition, and *in vitro* total gas and methane production potentials. Organic matter digestibility (OMD) and metabolizable energy (ME) contents were predicted from the *in vitro* total gas and nutrient composition data. Large variability was observed among the forage species in all variables considered. The neutral detergent fibre (NDF) varied from 184 to 684 g/kg dry matter (DM), the acid detergent fibre from 85 to 385 g/kg DM, and crude protein (CP) from 54 to 438 g/kg DM. The mineral contents (g/kg DM) varied in the following ranges: Ca, 2.2-26.6; P, 1.4-3.8; K, 8-75; Na, 0.03-0.75; Mg, 2.1-16.5; Mn, 0.051-0.225; and Cu, 0.019-0.093. Among the minerals, Na was deficient in most (83%) of the species, whereas Ca was limiting in about 7% of the species. The other minerals were present in adequate amounts for ruminant production. The *in vitro* gas volume after 72 h of incubation ranged from 133 to 283 ml/g OM, while the methane volume ranged from 33 to 64 ml/g OM. The mean ratio of CH_4 to total gas increased linearly from 0.17±0.03 at 12 h to 0.24±0.04 at 72 h. The relative ranking of the species in terms of total gas volume and CH_4 to total gas ratio changed with incubation time. The estimated OMD ranged between 42 and 73%, and the ME content varied between 5.8 and 10.2 MJ/kg DM. While the NDF content of the samples was positively correlated with total gas ($r=0.41$; $P<0.05$) and CH_4 ($r=0.40$; $P<0.05$) production, the CP content was negatively correlated ($r=-0.39$; $P<0.05$) with total gas production. Generally, the pasture stand during the main growing season was evaluated as of moderate nutritional quality and that any decrease in animal productivity during this time is likely to primarily originate from biomass availability (DM intake) rather than feed quality.

Introduction

Natural pasture is the major feed resource for grazing ruminants in many developing countries such as Ethiopia. The performance of animals grazing tropical pastures is mainly influenced by availability and nutritional quality of the biomass on offer (Vazquez and Smith, 2000). While availability of biomass in the tropical grasslands depends on the rainfall pattern (Clary, 2008; Lenz and Facelli, 2006), the quality is mainly affected by the stage of maturity, the type forage species and the levels of soil fertilization (Aumont *et al*., 1995; Perez Corona *et al*., 1998). Tropical grasses generally grow and mature faster, and reach the age of senescence much quicker than temperate grasses, thereby become more fibrous and less digestible in a short duration (Hennessy *et al*., 2000; Leng, 1990). This creates a challenge to provide the ruminant with good quality forages over extended periods, and often animals have to cope with poor quality feeds (Miller and Thompson, 2007; Sampaio *et al.*, 2010).

To optimize the use of pasture resources in a sustainable manner, it is important to quantify the nutrient composition of the available forage species (Peacock *et al.*, 2005). Such information is vital to adequately assess the feeding values of available forages and identify possible limiting nutrients, based on which a suitable grazing management (Sternberg *et al*., 2000) and a feasible supplementation strategy (Huston *et al*., 1999) can be implemented. Nutritive evaluation of forages is also important for the selection and breeding of appropriate indigenous forage species to improve the carrying capacity and quality of the natural grasslands.

Because of the growing concern of global warming, CH_4 emission from ruminant livestock production has currently received the attention of nutritionists. CH_4 is a greenhouse gas, which is about 20 times more potent than CO_2. It is produced in the rumen as a by-product of microbial fermentation of forages, and is eructated as gas into the atmosphere, through which ruminants lose up to 11% of the gross energy they consume (Waghorn *et al*., 2002). CH_4 production in the rumen is thus both wastage of energy and a cause of damage to the environment. The current nutritive evaluation of forages, therefore, needs to take into account methanogenesis and antimethanogenic activities of various feeds. In this respect, analysis of chemical composition and *in vitro* assays are valuable proxies for nutritive evaluation of feedstuffs. The *in vitro* total gas production technique together with chemical composition data (Menke and Steinggass, 1988) is a widely accepted method to estimate the

degradability kinetics and energy values, and CH_4 outputs of a range of feed ingredients and feed additives (Getachew *et al*., 2005; Kumara Mahipala *et al*., 2009; Mc Geough *et al*., 2011). The total gas production technique has the advantage that it is cost effective as compared to *in vivo* assays (Blümmel and Becker, 1997) and that large number of plant species can be screened rapidly for further study in forage breeding programs (Blümmel *et al*., 2005; Bodas *et al*., 2008; Soliva *et al*., 2008). The accuracy of the method is evident from the high correlation between *in vitro* total gas production and apparent *in vivo* digestibility (Bhatta *et al*., 2006), as well as the similarity between *in vitro* CH_4 and *in vivo* respiration chamber CH_4 measurements (Blümmel *et al*., 2005).

Ethiopia has the highest ruminant livestock population in Africa (FAO, 2010), and the Mid Rift Valley region of the country supports the bulk of this livestock population (CSA, 2008). Improving the nutrition of the grazing ruminant in the region has thus wider implications in terms of supporting the national economy and mitigating methane emissions into the atmosphere. Therefore, this study was conducted to document the chemical composition, *in vitro* total gas and CH_4 production of grass and browse species collected from the Mid Rift Valley grasslands during the main pasture growth period.

Materials and Methods

Research site and forage sampling

The research site was in the Mid Rift Valley of Ethiopia (7°34´N to 7°35´N and 38°33´to 38°34´E, with an elevation of 1650 meters above sea level). The site has a semi-arid agro-ecology, and scarcity of water is the major constraint for crop and livestock productivity. Maize cultivation and cattle rearing on natural pasture are the main source of livelihood for the farming communities in the area. For the present study, feed samples consumed by cattle in the area including pasture species, maize stover and acacia fruit were collected from the grazing lands (enclosed ranch and open grasslands). The procedure of sample collection is described elsewhere (Bezabih *et al.*, 2011b). Briefly, several transect walks were conducted across the grazing sites, and forage samples were randomly collected from areas of 1 m^2 at various locations along transects. The forages were cut at a height of 5 cm from the ground, after which the samples were sorted and bulked by species. Freshly fallen *Acacia tortilis* fruit were collected from the area under several trees along the sampling lines.

Chemical analysis

Sample of each pasture species was dried in an air-draft oven at 60°C for 48 h, and ground to pass a 1mm sieve.The samples were then analysed for the contents of dry matter (DM), ash, crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and minerals. Dry matter was determined by oven drying at 103°C (ISO 6496; ISO, 1999) and ash after incineration at 550°C (ISO 5984; ISO, 2002). The contents of CP (6.25 × N) was determined by using the Kjeldahl method (ISO 5983; ISO, 2005), NDF according to Van Soest (1991) and ADF according to Van Soest (1973). The contents of Ca, P, K, Na, Mg, Mn, and Cu were determined by atomic absorption spectrophotometer (Buck Scientific 240VGP, Milan Italy) after digestion with tri-acid mixture of nitric, perchloric and sulphuric acids. CH_4 was determined using a gas chromatograph (GC8000 Top, CE Instruments, Milan, Italy) fitted to a flame ionization detector, using a packed column (Porapak, 6 m × 1/8 in., 50–80 mesh, Grace/Alltech, Lexington, Kentucky, USA) with nitrogen as carrier gas (100 kPa) and an oven temperature maintained at 60°C.

***In vitro* gas and methane production measurement**

A fully automated *in vitro* gas production apparatus (Cone *et al*., 1996), with modifications on the incubation bottles as described by Pellikaan *et al.* (2011), was used to measure cumulative gas, CH_4 and fermentation end-products at the laboratory of Animal Nutrition Group of Wageningen University, The Netherlands. Forage samples were incubated according to the procedure described by Cone *et al.* (1996). Rumen fluid was collected from two ruminally canulated Holstein Friesian dry cows (about 2 h after the morning feeding), pooled together, and stored in a pre-warmed insulated thermos prefilled with CO_2. The cows received standard dry cow ration containing silage (grass and maize) and concentrates. The rumen fluid was filtered through cheesecloth and mixed (1:2, v/v) with an anaerobic buffer/mineral solution, after which 500 mg of ground samples were incubated in duplicate with 60 ml of the buffered rumen fluid at 39°C. Cumulative gas production was recorded automatically during 72 h of incubation.

To determine CH_4 concentration in the cumulative gas produced, small aliquots of gas (10 µl) were sampled (at 0, 2, 4, 6, 8, 10, 12, 24, 30, 48, 56, and 72 h) from the headspace

using a gas tight syringe (Hamilton 1701N, point style 5 needles, 51 mm; Hamilton, Bonaduz, Switzerland) and were immediately analyzed by gas chromatography. The CH_4 concentrations of individual bottles were expressed relative to the maximum concentration to normalize the data, and were plotted against time. Finally, the data were fitted to a nonlinear monophasic equation (Eq.1: Groot *et al*. 1996), and the curve fit parameters were used to compute CH_4 concentrations at each individual valve opening. Cumulative CH_4 production was calculated as the sum of the increase in headspace CH_4 between successive valve openings and the amount of CH_4 vented. All measurements were corrected for blank (gas produced in buffered rumen fluid without sample).

Curve fitting and calculations

The cumulative gas and CH_4 production over time were fitted iteratively with a monophasic equation (Groot *et al*., 1996) of the following form using the NLIN procedure of Statistical Analysis System (SAS®; Version 9.2):

$$G= A/(1+(C/t)^B \quad (1)$$

where, G is total gas or CH_4 produced, A equals the asymptotic total gas or CH_4 production (ml/g organic matter (OM)), B is the switching characteristic of the curve, C is the time at which half of the asymptotic total gas or CH_4 production had been reached (half-time; T1/2h), and t is the time (h).

Maximum gas production rate (R_{max}, ml/h) was calculated according to Bauer *et al*. (2001) as follows:

$$R_{max} = [A\times C^B\times B\times (TR_{max}^{(-B-1)})]/[1+(C^B\times TR_{max}^{(-B)})^2] \quad (2)$$

where TR_{max} is the time at which R_{max} occurs; and $TR_{max}=C\times[[(B-1)/(B+1)]^{(1/B)}]$

The *in vitro* organic matter digestibility and metabolizable energy contents of the samples were estimated from the net 48 h gas volume, CP and ash contents (Menke and Steinggass, 1988) according to the following equations:

$$OMD = 14.88 + 0.889GV + 0.45CP + 0.0651XA \quad (3)$$

$$ME = 2.20 + 0.136GV + 0.057CP \quad (4)$$

where, OMD is organic matter digestibility (g/100 g); ME is metabolizable energy content (MJ/kg DM); GV is net gas volume at 48 h fermentation (ml/g DM); CP is CP content (g/100 g DM); XA is ash content (g/100 g DM).

Results

Chemical composition and mineral profiles

Table 1 show the chemical and mineral composition of pasture and browse species, while Figure 1 visualizes the range and distribution of the major chemical fractions. The result showed a large variation in the chemical composition among the botanical species analyzed. The NDF content, which represents the cell wall components, was the highest in *Hyparrhenia anamesa* (684 g/kg DM) and the lowest in the pods of *Acacia tortilis* (184 g/kg DM). In the same pattern, the ADF content ranged from 85-385 g/kg DM. The highest CP content was observed in the seeds (438 g/kg DM) and fruits (210 g/kg DM) of *A. tortilis*. Among the pasture species, *Indigofera spicata* had the highest CP content (228 g/kg DM), whereas *Sporobolus pellucisus* contained the lowest CP content (54 g/kg DM).

The mineral contents (g/kg DM) varied among the species with the following ranges: Ca, 2.2-26.6; P, 1.4-3.8; K, 8-75; Na, 0.03-0.75; Mg, 2.1-16.5; Mn, 0.051-0.225, and Cu, 0.019-0.093. Table 2 shows the percentage of grass species with mineral contents below, above or within the normal range of mineral requirements in reference to the National Research Council (1996) recommendations. Except Ca and Na, the analyzed minerals were available in adequate amounts for ruminant production. Na was deficient in most of the samples (83%) analysed, whereas Ca appeared to be limiting in about 7% of the species.

Table 1 Chemical composition and mineral profile of grass and browse species from the Mid Rift Valley grasslands of Ethiopia.

Species	DM	NDF	ADF	CP	Ash	Ca	P	K	Na	Mg	Mn	Cu
	g/kg DM										mg/kg DM	
Cynodon dactylon	180	525	242	171	119	12.6	2.95	68	0.67	9.1	68	37
Pennisetum straminium	250	536	258	167	96	6.0	2.60	58	0.45	5.9	110	30
Cenchrus ciliaris	260	563	302	96	111	12.8	3.40	50	0.42	12.0	145	83
Cymbopogon pospischilii	310	608	354	68	75	2.2	1.40	13	0.72	8.2	113	26
Indigofera spicata	210	363	226	228	92	6.5	2.30	48	0.40	15.5	63	43
Heteropogon contortus	321	615	338	56	82	4.4	3.00	56	0.41	16.5	187	59
Zaleya pentandra	165	523	309	83	95	4.0	2.15	73	0.38	3.4	101	22
Chloris gayana	212	627	335	98	83	4.2	2.75	62	0.45	5.7	110	28
Eragrostis aspera	174	566	292	137	113	2.4	2.35	37	0.36	5.5	108	28
Eragrostis cilianensis	205	578	315	93	92	4.0	2.65	57	0.29	6.5	85	32
Cynodon ethiopicus	155	535	252	134	97	12.6	2.90	41	0.61	8.9	60	32
Eleusine mutiflora	225	631	333	74	125	11.6	2.95	65	0.57	9.1	70	37
Brachiaria lachnantha	270	643	355	106	101	9.6	3.30	46	0.59	16.4	102	43
Aristida adscensionis	240	653	339	83	94	4.0	2.15	73	0.38	3.4	95	22
Bracheria marlothii	195	554	360	91	85	4.5	2.05	65	0.37	7.2	120	31
Sporobolus pellucisus	325	657	336	54	92	4.3	3.05	46	0.39	13.1	100	30
Dactyloctenium aegyptium	168	621	317	83	87	26.6	3.30	55	0.59	16.4	165	43
Digitaria abyssinica	210	570	355	77	86	6.7	3.12	34	0.43	7.2	51	42
Pennisetum polystachion	305	624	363	59	98	4.6	2.01	40	0.52	11.8	65	48
Hyparrhenia anamesa	324	684	385	84	115	12.4	3.45	54	0.39	11.0	120	54
Snowdenia petitiana	350	621	351	54	105	5.7	3.63	75	0.75	8.2	96	35
Rhynchelytrum repens	215	542	325	77	83	4.9	2.6	42	0.64	6.3	130	53
Melinis repens	260	564	345	83	90	5.2	2.01	53	0.32	10.3	117	28
Harpachne schimperi	340	674	358	55	55	11.8	2.40	47	0.285	7.4	68	37
Themeda triandra	245	649	333	92	80	15.7	2.05	35	0.525	14.6	165	93
Zea mays (stover)	852	663	336	39	75	4.3	3.05	37	0.385	13.1	99	30
Acacia tortilis (fruit)	540	230	129	210	66	6.5	2.85	9	0.06	2.24	206	32
Acacia tortilis (seed)	650	326	221	438	52	6.3	3.80	8	0.11	2.1	165	19
Acacia tortilis (pod hull)	475	184	85	103	69	6.6	2.40	9	0.03	2.3	225	38

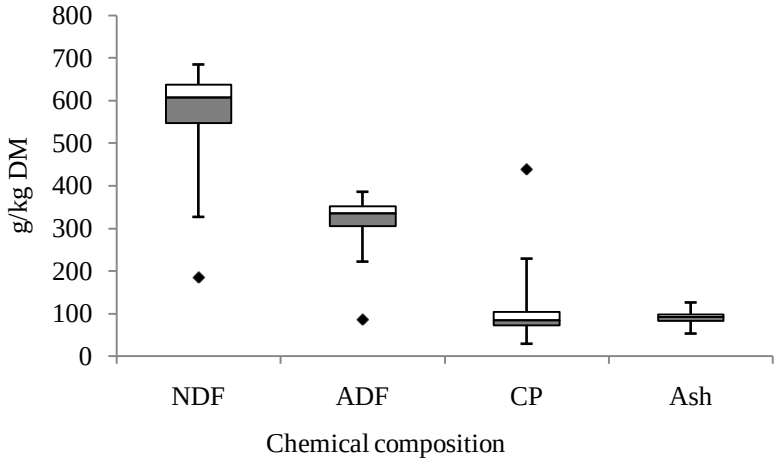

Figure 1 Chemical composition (NDF, ADF, CP, and ash) of grass and browse species from the Mid Rift Valley grassland of Ethiopia (a box-plot presentation showing the median, the middle 50% of the data (box), outliers (♦) and data ranges): NDF = Neutral Detergent fibre; ADF = acid detergent fibre, CP = crude protein, and Ash = crude ash.

Table 2 Percentage of grass species from the Mid Rift Valley grasslands of Ethiopia with mineral concentrations below, above or within the normal range of mineral requirements for ruminants.

Mineral	Normal requirements (g/kg DM)*	Below	Above	Within
Ca	3.4–7.0	7	31	62
P	1.2–2.1	-	93	7
K	6.0–7.0	-	100	-
Na	0.6 – 1.0	83	-	17
Mg	1.0–2.0	-	100	-
Mn	0.02 – 0.04	-	100	-
Cu	0.007–0.011	-	100	-

*Recommended requirements according to NRC (1996).

In vitro total gas and methane production

The *in vitro* total gas production (ml/g OM) after 12, 24, 48, and 72 h of incubation are shown in Table 3, while Figure 2 shows the box-plot presentation of the range and distribution of gas volumes produced at the selected incubation hours. The forage samples had large variation in

the total volume of gas produced. At 12 h of incubation the lowest gas volume (57 ml/g OM) was recorded in *Harpachne schimperi*, whereas the highest gas volume (180 ml/g OM) was observed in *Heteropogon contortus*. At 24 h the gas volume ranged from 94 ml/g OM (*H. schimperi*) to 232 ml/g OM (*Dactyloctenium aegyptium*), while it varied between 133 to 283 ml/g OM at the end of the incubation (72 h). In reference to the total volume of gas produced at the end of the incubation, 55±9% of the total gas was produced in the first 12 h, 82±6% after 24 h, and 97±1% after 48 h of incubation.

The parameters of total gas production also showed large variation between species (Table 3), with $T_{1/2}$ (time at which half of the asymptotic total gas is produced) ranging from 6.5 to 21.3 h, R_{max} (maximum rate of gas production) from 6.7 to 28.8 ml/h, and TR_{max} (time at which R_{max} occurs) from 1.1 to 7.9 h. Species such as *H. contortus, Zaleya pentandra,* and *Brachiaria lachnantha* had fast degradation with relatively short $T_{1/2}$ and TR_{max} and high R_{max}, whereas species such as *Sporobolus pellucisus, H. schimperi* and maize stover showed slow rates of degradation. The slow rate of degradation in the latter groups of species was also associated with low level of total gas production at the end of the incubation.

The *in vitro* CH_4 production, CH_4 to total gas ratio at the end of the incubation, and estimated nutritive values of the feed samples are presented in Table 4. The range of CH_4 production (ml/g OM) was 14-27; 24-43; 31-57 and 33-64 after 12, 24, 48 and 72 h of incubation, respectively, showing a linear increase in the volume of CH_4 produced over the incubation time. Distinct variability between species was observed in CH_4 production. In general, grasses such as *S. petitiana, M. repens, Z. pentandra, E. mutiflora,* and *A. tortilis* (fruit, seed and pod) produced the lowest CH_4 volumes (33-43 ml/g OM) after 72 h. On the other hand, species such as *B. lachnantha, C. ciliaris, P. straminium and D. aegyptium* produced the highest CH_4 volumes (61-64 ml/g OM). Compared to the percentage of total gas production much lower percentage of the total CH_4 gas were produced after 12 (55 *vs.* 40%) and 24 h (82 *vs.* 64%). As a result the ratio of methane to total gas increased steadily from 0.17±0.03 at 12 h to 0.24± 0.04 at 72 h (Figure 2).

Table 3 *In vitro* total gas (ml/g OM) production of grass and browse species from the Mid Rift Valley grasslands of Ethiopia, after incubation with rumen fluid for 72 h.

Species	Volume of gas after incubation time *t* (h)				Total gas kinetics*		
	12	24	48	72	$T_{1/2}$ (h)	R_M (ml/h)	T_{RM} (h)
Cynodon dactylon	107	163	192	197	13.1	13.6	1.5
Pennisetum straminium	139	208	236	239	11.1	18.4	2.7
Cenchrus ciliaris	130	202	235	241	12.3	16.8	3.1
Cymbopogon pospischilii	114	177	209	220	13.3	14.1	3.0
Indigofera spicata	122	179	201	206	10.7	16.6	3.2
Heteropogon contortus	180	224	237	240	6.7	28.8	1.5
Zaleya pentandra	140	174	180	183	6.5	26.1	1.8
Chloris gayana	118	189	228	236	13.4	15.4	4.1
Eragrostis aspera	132	200	231	236	11.5	18.4	3.9
Eragrostis cilianensis	154	218	242	246	9.8	20.7	2.4
Cynodon ethiopicus	134	201	230	235	11.4	17.3	2.2
Eleusine mutiflora	151	216	255	269	11.7	18.9	2.6
Brachiaria lachnantha	170	230	249	254	8.7	23.9	2.1
Aristida odscensionis	135	197	230	239	11.8	17.2	2.9
Bracheria marlothi	135	210	246	252	12.8	16.8	2.3
Sporobolus pellucisus	108	170	218	232	19.2	12.5	1.1
Dactyloctenium aegyptium	150	232	276	283	12.6	19.1	3.2
Digitaria abyssinica	161	219	249	255	9.8	21.3	2.6
Pennisetum polystachion	92	168	204	208	14.9	13.4	5.7
Hyparrhenia anamesa	124	197	233	241	12.7	17.8	5.3
Snowdenia petitiana	108	162	181	187	11.1	14.8	3.6
Rhynchelytrum repens	100	161	204	212	15.4	12.3	3.2
Melinis repens	94	156	199	212	15.5	11.9	4.9
Harpachne schimperi	57	94	127	133	17.7	6.7	4.4
Themeda triandra	91	154	201	209	14.0	18.4	3.8
Zea mays (stover)	79	136	187	198	21.3	9.1	3.3
Acacia tortilis (fruit)	96	146	172	177	12.2	13.5	4.7
Acacia tortilis (seed)	75	115	132	134	12.0	12.1	7.9
Acacia tortilis (pod hull)	106	160	191	198	12.3	14.2	3.3

*$T_{1/2}$ = time at which half of the asymptotic gas production has been reached; R_M = maximum rate of gas production; T_{RM} = time at which the maximum rate of gas production occurs.

Table 4 *In vitro* methane (ml/g OM) production, methane to total gas ratio (v/v), estimated *in vitro* organic matter digestibility (OMD) and metabolizable energy (ME) content of grass and browse species from the Mid Rift Valley grasslands of Ethiopia.

Species	ml CH_4 after incubation time *t* (h)				CH_4:Total	OMD	ME
	12	24	48	72	gas	(%)	(MJ/kg DM)
Cynodon dactylon	19	31	43	49	0.25	64	8.4
Pennisetum straminium	26	43	57	63	0.26	71	9.6
Cenchrus ciliaris	23	40	55	62	0.26	68	9.1
Cymbopogon pospischilii	21	34	48	55	0.25	60	8.3
Indigofera spicata	21	35	48	55	0.26	67	9.0
Heteropogon contortus	27	38	47	50	0.21	64	8.9
Zaleya pentandra	21	30	35	37	0.20	57	7.6
Chloris gayana	21	33	48	56	0.24	65	9.0
Eragrostis aspera	21	36	51	59	0.25	69	9.3
Eragrostis cilianensis	25	38	51	57	0.23	68	9.3
Cynodon ethiopicus	20	35	50	58	0.25	68	9.2
Eleusine mutiflora	20	27	31	33	0.12	72	9.6
Brachiaria lachnantha	25	39	53	61	0.24	70	9.6
Aristida odscensionis	22	34	48	55	0.23	66	8.9
Bracheria marlothi	24	38	52	59	0.23	68	9.4
Sporobolus pellucisus	19	31	47	56	0.24	62	8.4
Dactyloctenium aegyptium	25	41	56	64	0.22	73	10.2
Digitaria abyssinica	21	34	48	56	0.22	68	9.4
Pennisetum polystachion	18	31	46	54	0.26	60	8.1
Hyparrhenia anamesa	20	35	50	58	0.24	68	9.0
Snowdenia petitiana	14	29	39	43	0.23	56	7.4
Rhynchelytrum repens	17	27	40	47	0.22	60	8.2
Melinis repens	15	25	36	41	0.20	60	8.1
Harpachne schimperi	16	26	40	49	0.37	42	5.8
Themeda triandra	20	31	42	47	0.22	60	8.2
Zea mays (stover)	16	26	39	48	0.24	55	7.5
Acacia tortilis (fruit)	17	25	34	39	0.25	65	9.0
Acacia tortilis (seed)	17	24	31	35	0.26	52	7.0
Acacia tortilis (pod hull)	17	26	36	41	0.25	72	9.9

The relative ranking of species according to this ratio varied with the time of incubation. The species *E. mutiflora* had the lowest CH_4 to total gas ration (0.12) at the end of the incubation,

whereas *H. schimperi* had the highest ratio (0.37). The other species clustered with a CH_4 to total gas ratio of 0.19-0.26.

Organic matter digestibility and metabolizable energy contents

The estimated organic matter digestibility (OMD) varied between 42 and 73% (Table 4). The lowest digestibility was observed in the species such as *H. schimperi* (42%), *A. tortilis* seed (52%), maize stover (55%) and *Snowdenia petitiana* (56%), whereas the highest OMD was observed in *D. aegyptium* (73%), *A. tortilis* pod (72%), *E. mutiflora* (72%), *P. straminium* (71%) and *B. lachnantha* (71%). The estimated ME content varied from 5.8 to 10.2 MJ/kg DM, following the same trend as the OMD. The large variability in OMD and ME content appeared to reflect the considerable difference in the nutritive values of the feed samples. The correlation matrix of the chemical composition, *in vitro* gas production and estimated nutritive value of the feed samples is presented in Table 6. The NDF content was positively correlated with total gas ($R=0.41$; $P<0.05$) and CH_4 ($R=0.40$; $P<0.05$) production, while strongly negatively correlated with CP content ($R=-0.65$; $P<0.001$). The CP content was also negatively correlated ($R=-0.39$; $P<0.05$) with total gas production. The ash content was negatively correlated with total gas, OMD and ME contents. As expected strong positive correlations ($P<0.001$) were observed among *in vitro* total gas, CH_4, OMD and ME contents.

Discussion

Nutrient composition of pasture species

Nutrient composition data serve as a first-hand tool to evaluate the feeding value of forages. Several factors including forage genotype, stage of maturity, season of harvest, and management influence the nutrient composition of forages (Aumont *et al*., 1995). In the present analysis, forage samples were collected at their flowering stage, and samples of the same species collected from different quadrates were pooled together to account for spatial variability. Although this sampling method did not allow observation of intraspecies differences in nutrient concentration and feeding values, the bulked samples adequately represent individual species, and used for examining the interspecies variability.

The minimum CP level in the diet of ruminants required for adequate rumen function is about 70 g/kg DM (Van Soest, 1994) and the same nutrient required for optimum growth

and lactation in cattle is about 150 g/kg DM (Poppi and McLennan, 1995; Tessema and Baars, 2004). Among the pasture species, about 74% had CP content higher than the minimum requirements for normal rumen functioning and 24% had CP content higher than the requirements for optimum growth and lactation. The higher CP contents of *A. tortilis* fruit and pod is typical for tropical multipurpose trees (Soliva *et al*., 2008), and their potential as a protein supplement, particularly during the dry period has been documented (Anele *et al*., 2009; Berhane *et al*., 2006; Coppock and Reed, 1992). The level of cell wall fraction (NDF) in tropical grasses beyond which the dry matter intake of cattle would be negatively affected is considered around 600-650 g/kg DM (Van Soest *et al*., 1991), and about 81% of the forage samples analyzed contained NDF levels lower than this threshold. Considering the two important chemical fractions (CP and NDF) influencing ruminal degradability and hence the nutritive value feeds, the standing grass biomass in the natural grassland during the main growing season (sampling period) can be considered as of moderate quality.

Previous reports on the mineral status of forages and crop residues indicated marginal to deficient levels of Na, Cu, and P, and adequate supplies of K, Ca, Mg, Mn and Zn for ruminant animal production in the Rift Valley of Ethiopia (Kabaija and Little, 1988; Kabaija and Little, 1991). In the present result, except that of Na, the concentration of other minerals appeared to be adequate for ruminant production. This observation contrasts with previous findings that Cu deficiency is an important constraint (Faye *et al*., 1991; Faye *et al*., 1983) for grazing animals in the Rift Valley of Ethiopia. However, forage Cu concentration alone is of limited value in assessing Cu adequacy unless forage concentrations of Cu antagonists such as Mo, S, and Fe are also considered (NRC, 1996). Faye *et al.* (1991) reported that the soil formation in the Rift Valley is characterized by a high Mo, S, Fe, and a low Cu, Zn content, which leads to Mo-induced Cu deficiency in the grazing animal. These authors reviewed cases of *Enzootic ataxia* in new-born small ruminants as an evidence for the widespread Cu deficiency in the region. As Mo, S, and Fe contents of forages were not determined in the present study, the possible interference in Cu absorption of these minerals could not be inferred. Clinical signs of Cu deficiency were, however, not observed in cattle during the study period. In line with this, Kabaija and Little (1991) reported that supplementary P (in the form of bone meal) and injectable Cu had no remarkable effects on the serum mineral concentrations and body condition of 2-3 years old male cattle, suggesting that dietary

contents of these minerals may not be limiting for cattle production with the existing nutritional regime in the area. The current observation that Na was deficient in most of the samples analyzed (Table 2) is in line with previous observations. A routine supplementation of this mineral as common salt to the grazing animal is thus essential.

***In vitro* total gas and methane production**

The *in vitro* gas production method (Menke *et al*., 1979) has become a widely used technique to study the fermentation kinetics of feeds in rumen fluid (Blümmel and Becker, 1997; Blümmel and Ørskov, 1993; Sandoval-Castro *et al*., 2005). This method has been fully automated (Cone *et al*., 1996) to generate large time-series data points, allowing more accurate prediction of fermentation characteristics. Results of the total gas volume in the present experiment largely agree with those reported by others (Anele *et al*., 2009; Berhane *et al*., 2006; Soliva *et al*., 2008). The nature and amount of the cell wall fraction as well as the CP content of forages are known to influence the degradability and hence nutritive values (Van Soest *et al*., 1991). In the present experiment the species with optimum level of NDF and CP (e.g. *D. aegyptium*) produced the highest gas volume, whereas those with the highest NDF and lowest CP content (e.g. *H. schimperi*) produced the lowest gas volume. The browse *A. tortilis* fruit and seed produced low gas volumes despite the low NDF (230-326 g/kg DM) and high CP (210-438 g/kg DM) content in the samples, while the pod of the same species (NDF: 184 g/kg DM and CP: 103 g/kg DM) showed optimum level of degradability. The low gas volume in the fruit and seed may be due to the high content of phenolic compounds in the seeds (Kumara Mahipala *et al*., 2009), which could inactivate microbial enzymes and reduce protein degradation in the rumen (Kumar and Singh, 1984). The large differences observed in the gas production kinetics (Table 3) provides further evidence about the variation among the forage species in their potential feeding values. Low total gas production coupled with slow rate of fermentation indicates poor digestibility and feeding value, with species such as *S. pellucisus,* and *H. schimperi* included under this category. On the other hand, high total gas production associated with moderate to rapid rate of fermentation indicates better digestibility and utilization by ruminants, with species such as *C. ciliaris, D. aegyptium*, *B. marlothi* and *P. straminium* falling under this category. In addition to their value for screening forages, the gas production parameters provide valuable information in choosing mixtures of forage

species that optimizes microbial fermentation in the rumen when artificial pastures are established or a cut and carry system is used in feeding ruminants (Williams, 2000).

Microbial degradation of feeds in the rumen results in the production of short-chain fatty acids, gases (mainly CO_2 and CH_4), and synthesis of microbial biomass. The CH_4 gas produced is a potent greenhouse gas, through which a potentially productive feed energy is also lost (Getachew *et al.*, 2005; Moss *et al.*, 2000). In recent times, the concurrent measurement of CH_4 production during the *in vitro* incubation enabled to simultaneously screen animal diets both for their degradability and low CH_4 production potentials (Bodas *et al.*, 2008; Getachew *et al.*, 2005; Pellikaan *et al.*, 2011). In the present experiment, the observed variability in CH_4 production among species reflects the large scope for selecting forage species with lower CH_4 production potentials.

The range of CH_4 proportion (0.12-0.37) in the total gas in the present study was similar to that reported for tropical browses by Soliva *et al.* (2008), although appears to be higher than that reported for other tropical tannin containing plants (Hariadi and Santoso, 2010) and for cereal crop residues (Blümmel *et al.*, 2005), measured using the Menke and Steingass (1988) batch incubation system. The CH_4 to total gas ratio reported by Meale *et al.* (2012) for forage grasses and legumes is less than 0.08, which is much lower than that reported for tropical tanniniferous plants with a high anti-methanogenic activity (Bodas *et al.*, 2008).

The relative ranking of species according to CH_4 to total gas ratio changed with the incubation time, indicating that the time-series measurement method provides valuable insights into the kinetics of CH_4 production of substrates than the batch system. From the present observation species including *E. mutiflora, M. repens and Z. pentandra* showed consistently lower CH_4 to total gas ratio, which may be worth considering for further studies in an effort to find low CH_4 emission forage diets. It is important to note that the pod of *A. tortilis* is rich in protein, and is highly degradable (Table 1 and 3) with moderate CH_4 to total gas ratio, which make it an ideal feed supplement for ruminants.

As indicated in Figure 2, the volume of CH_4 produced increased linearly with the incubation time. This is in line with the observation that forage diets with a high *in vivo* passage rate (thus less resident time in the rumen) are likely to produce less CH_4 than those with a low passage rate (Waghorn *et al.*, 2002). Fibre quality and particle size of forage diets

are among the main factors that affect *in vivo* passage rate of digesta, and thus should be targeted in the manipulation of the nutrition of ruminants.

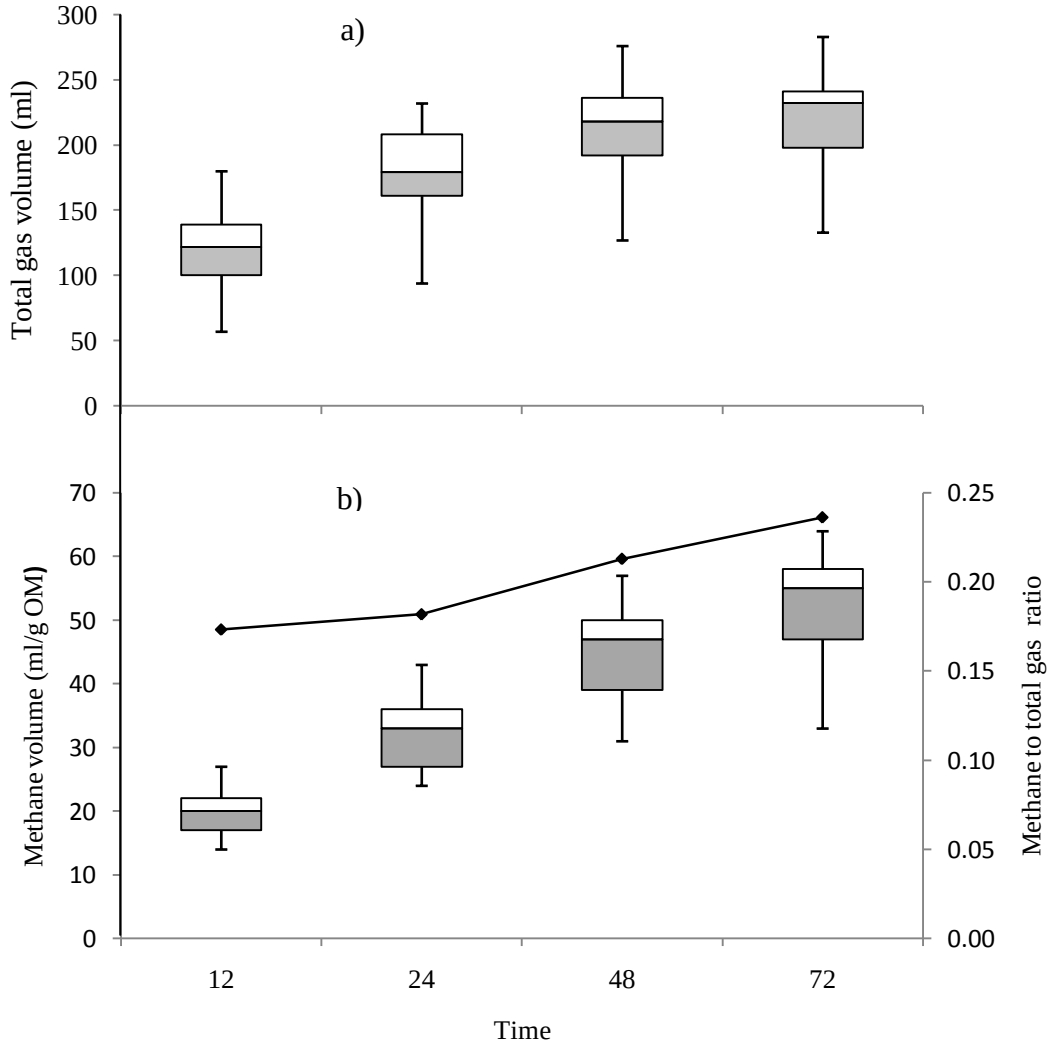

Figure 2 *In vitro* total gas (a), CH_4 (b), and CH_4 to total gas ratio (b: line-graph) for grass and browse species from the Mid Rift Valley grassland of Ethiopia after incubation with rumen fluid for 72 h (a box-plot presentation showing the median, the middle 50% of the data (box), and gas volume ranges).

Organic matter digestibility and energy contents

Organic matter digestibility is a valuable predictor of the nutritive value of feeds (Lukas *et al*., 2005). The predicted OMD of the forage samples (Table 4) reflects a large variation in the nutritive value of the feeds. Forages containing 700 g OMD/kg DM are considered to be of high quality (Meissner *et al*., 2000), and about 17% of the feed samples studied were predicted to have OMD above this threshold. The majority of the remaining species (62%) contained 610-690 g OMD/kg DM, which can be considered as of moderate quality under tropical conditions (Kumara Mahipala *et al*., 2009; Leng, 1990). The ME content of 52% of the species was above 9 MJ/kg DM, a level comparable to good quality forages; 31% of the species contained 8-9 MJ/kg DM, a level comparable to a low quality hay; and the other 14% contained 6.5-8.0 MJ/kg DM, a level comparable to ammoniated straws (Leng, 1990). The species *H. schimperi* contained the lowest ME content (5.8 MJ/kg DM), which is comparable to a straw diet. This species also showed the lowest OM content and the highest methane production, indicating that it has a low feeding value in all parameters considered.

Table 5 Pearson correlation coefficient (R) matrix of chemical composition, *in vitro* gas production and estimated nutritive value of grass and browse species from the Mid Rift Valley grasslands of Ethiopia.

	ADF	CP	Ash	Total gas	CH_4	OMD	ME
NDF	0.92***	-0.65***	0.38*	0.40*	0.40*	0.04	-0.15
ADF		-0.58***	0.27	0.33	0.31	-0.05	-0.23
CP			-0.24	-0.39*	-0.23	-0.09	-0.03
Ash				-0.57*	-0.26	-0.59**	-0.41*
Total gas					0.60***	0.86***	0.83***
CH_4						0.50**	0.45*
OMD							0.91***

*P<0.05; **P<0.01; ***P<0.001.

The observed positive correlation between NDF and *in vitro* gas production (Table 5) agrees with that reported for browse species by Kumara Mahipala *et al*. (2009). The observed significant negative correlation between NDF and CP is in line with the established knowledge that with advancing maturity the NDF content of forages increases while the CP

content declines (Machado *et al*., 2007). The significant negative effect of CP on total gas volume is in contrast with the expected positive association between CP and *in vitro* OM degradability in grasses and browses (Cilliers and van der Merwe, 1993; Datt *et al.*, 2008; Kaitho *et al*., 1998). Similar negative correlation between CP and *in vitro* total gas volume in browse was also reported by Mahipala *et al*. (2009), who discussed that the negative effect could be due to high soluble nitrogen content, which has been observed to reduce cumulative gas production at the early stages of incubation in rumen fluid (Cone and Van Gelder, 1999).

Generally, the predicted feeding values reflected the characteristics of the feeds. In the prediction of ME, however, it may be beneficial to include CH_4 to total gas ratio as a predictor variable in addition to total gas volume, because different substrates with similar levels of gas volume were observed to have different level of CH_4 to total gas ratio.

Conclusions

The nutrient composition and predicted feeding values of the forages studied showed large variability. Such information on the nutrient composition, feeding values and CH_4 production kinetics of the pasture species provides an opportunity for selection of desirable pasture species. Overall, the pasture stand in the Mid Rift Valley of Ethiopia has moderate quality to support ruminant production in the wet season, and that any possible limitation during this time is likely to originate from biomass availability (DM intake) rather than feed quality. The role of *A. tortilis* pods as a protein and energy supplement appears indispensable, and proper storage and utilization of this feed would help improve animal performance. Routine supplementation of Na to the grazing animals is essential as most species were deficient in this mineral in the study area.

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CHAPTER 6

Nutritional status of cattle grazing natural pasture in the Mid Rift Valley grasslands of Ethiopia measured using plant cuticular hydrocarbons and their isotope enrichment

M. Bezabih[1,2], W. F. Pellikaan[1], A. Tolera[2], W. H. Hendriks[1,3]

[1]Animal Nutrition Group, Department of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands

[2]Department of Animal and Range Sciences, Hawassa University, P.O. Box 5, Hawassa, Ethiopia

[3] Animal Nutrition Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80151, 3508 TD Utrecht, the Netherlands

Submitted

Abstract

The seasonal diet composition, digestibility and nutrient intake of cattle grazing on natural pasture in the Mid Rift valley region of Ethiopia was determined using an improved n-alkanes method. Sixteen local Borana and Arsi cattle (8 bulls and 8 heifers, 175±10 kg weight) were randomly selected from herds at two sites; a moderately grazed ranch and a heavily grazed, communal grassland area. Grazing behaviour was observed and herbage species consumed sampled during five periods (early-dry, dry, short-rainy, main-rainy and end-of-rainy seasons) throughout the year at the two grazing sites. During each period, animals were dosed twice daily with 152±4 mg of C_{32} and 150±3 mg C_{36} alkanes for 10 consecutive days, with faeces sample collected in the morning during the last five days to determine dry matter intake (DMI).The proportion of consumed herbage species in the diet was determined using n-alkanes and their carbon isotope enrichments as markers, while the energy and nutrient intakes were derived from the DMI, digestibility, and diet composition of the DM consumed. Marked seasonal variations ($P<0.05$) were observed in the species diversity of diets consumed as well as intake of DM (65-98 g/kg$^{0.75}$/d), crude protein (222-448 g/d), metabolizable energy (20-37 MJ/d) and minerals. Energy intake was more limiting than crude protein for weight gain during most of the seasons. During the dry period, animals were in negative energy and nutrient balance with a predicted body weight loss of approximately 110 g/d, whereas in the main rainy season the intakes supported 500-800 g daily weight gains. Predicted weight gains agreed well with the body condition recorded for the same period. The n-alkanes method coupled with isotope enrichment in n-alkanes and visual observations as used in the present study provided realistic nutritional data for free-ranging cattle which correlated well with changes in body conditions.

Introduction

Natural pastures are the major feed resource for livestock in the arid and semi-arid regions of the tropics and generally show high temporal and spatial variation in herbage quality and availability (Corona *et al*., 1998; Schlecht *et al*., 1999; Hiernaux and Turner, 1996). The primary factor affecting pasture availability and quality in these regions is the rainfall pattern. During the long dry season, pasture availability and quality generally declines, whereas pasture abundance increases with a concurrent improvement in quality in the rainy season (Bastin *et al*., 2003; McIvor, 2007). As a result, herbivores grazing such grasslands experience marked seasonal fluctuation in nutrient intake and production performance (Ash and McIvor, 1998).

Other factors that influence pasture availability and quality include longer-term grazing management practices by animal herders (Verlinden and Kruger, 2007). Over the past few decades, evidence has shown that high grazing pressure has resulted in replacement of highly palatable and productive perennial species with unpalatable annual species in several tropical pasture lands, with a concomitant loss of soil fertility (Asefa *et al.*, 2003; Semmartin *et al*., 2010; Tefera *et al*., 2007b). Unsustainable grazing management practices, which focused only on increasing immediate farm production with little consideration for ecological stability, have been the major cause for the loss of palatable species and soil fertility (Scott *et al*., 2000). However, the issue of sustainable use of pasture resources has received increasing attention, with emphasis given to improved management of existing grassland resources rather than focusing on immediate farm returns (Dumont *et al*., 2007; Kemp *et al*., 2000).

Development of effective and sustainable grazing systems requires knowledge of the seasonal intake, composition and nutrient digestibility of forages by grazing animals (Ngugi *et al*., 2004; Prache *et al*., 1998). Such knowledge forms the basis for improving the nutrition of the animal through optimal allocation of forage resources, increasing the carrying capacity of grasslands through reseeding with desirable species, and identifying supplementation strategies for a target production. However, unlike barn feeding where feed intake, diet composition and nutrient digestibility of animals can be directly and accurately measured, direct measurement of these variables in free ranging animals is difficult or impractical, and often indirect methods have to be used (Mayes and Dove, 2000). Inaccuracies inherent in

these indirect methods have remained major limitations in the study of the nutrition of free grazing animals.

Over the last two decades, the use of plant cuticular hydrocarbon (n-alkanes) markers to estimate feed intake, diet composition and digestibility has gained increasing acceptance (Dove and Mayes, 2006) due to its low invasiveness, accuracy and the possibility of taking into account diet-animal interactions (Dove and Mayes, 1991, 2005). Although the majority of the validation studies and application of the n-alkane method has been conducted under temperate conditions, the few validation studies conducted under tropical conditions showed the suitability of this method to estimate intake, diet composition and digestibility of grazing animals (Bezabih *et al.*, 2011a; Bezabih *et al.*, 2011b; Hendricksen *et al.*, 2002; Laredo *et al.*, 1991). Recently, the accuracy of diet composition estimation by the n-alkane method was significantly improved (Bezabih *et al.*, 2011a) by the inclusion of additional information of the carbon isotope enrichment in individual alkanes.

The Mid Rift Valley region supports large grazing livestock populations in Ethiopia (CSA, 2006). Currently, improving the management of the available natural grasslands in the region has a high priority, owing to its potential for commercial livestock production and the increasing human population pressure in the area. However, information is lacking on the nutritional status of the grazing animal, based on which various grassland management strategies and decisions can be made. The few studies available report information on feed and blood samples opportunistically collected (Kabaija and Little, 1991; Khalili *et al.*, 1993a, b).

The present study was designed to estimate the seasonal pattern of diet composition, feed intake and digestibility of grazing cattle in selected sites in the Mid Rift Valley of Ethiopia. For the study, a combination of the n-alkane method, isotope enrichment in n-alkanes and visual observations was used.

Material and methods

Study area

The research was conducted in the Mid Rift Valley region of Ethiopia called Abernosa (Figure 1), which is located approximately 176 km south of Addis Ababa (at 7°34´N to 7°35´N and 38°33´E to 38°34´E and with an elevation of 1650 meters above sea level). The

Abernosa ranch was established more than 50 years ago by the government with the aim of improving the genetic potential of Boran cattle and serving as a multiplication centre for crossbred heifers. With a total area of 23,000 hectares, the ranch has been privatized since 2008, and during the research period (Nov 2009 to Sep 2010), the herd size at the ranch was around 1500 heads of cattle. The pasture production on the ranch and adjacent communal grasslands is mainly organic without application of artificial fertilization. While cattle were the only livestock species grazed within the ranch, goats and cattle were reared together by local farmers on the adjacent communal grasslands.

The agro-ecology in the area is generally classified as semi-arid with an annual rainfall varying between 500 and 700 mm/annum, and mean maximum and minimum temperature of 14° and 28°C, respectively. The rainfall pattern is bimodal with short rains from April to May, followed by the main wet season from July to October (Figure 2).

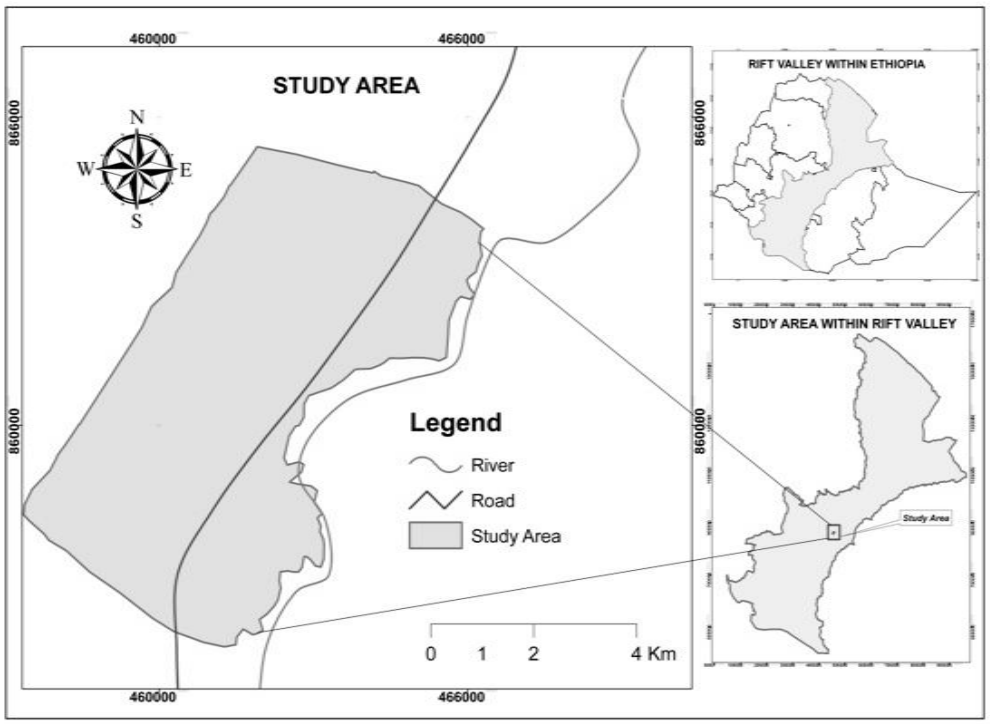

Figure 1 Location map of the study area in the Mid Rift Valley of Ethiopia.

The landscape exhibits typical savannah woodland with a scattered population of trees such as *Acacia tortilis, A. seyal, A. senegal* and *Balanites aegyptiaca* and some broadleaved shrubs. The undergrowth is dominated by grasses such as *Themeda triandra, Chloris gayana,*

Cenchrus ciliaris, Sporobolus pyramidalis and *Sporobolus pellucisus.* Farming communities in the study area practice settled mixed farming, mainly based on cattle rearing and maize cultivation. Due to an increasing population pressure, the woodlands are continuously converted into cropland, resulting in shrinkage of and high pressure on the communal grasslands.

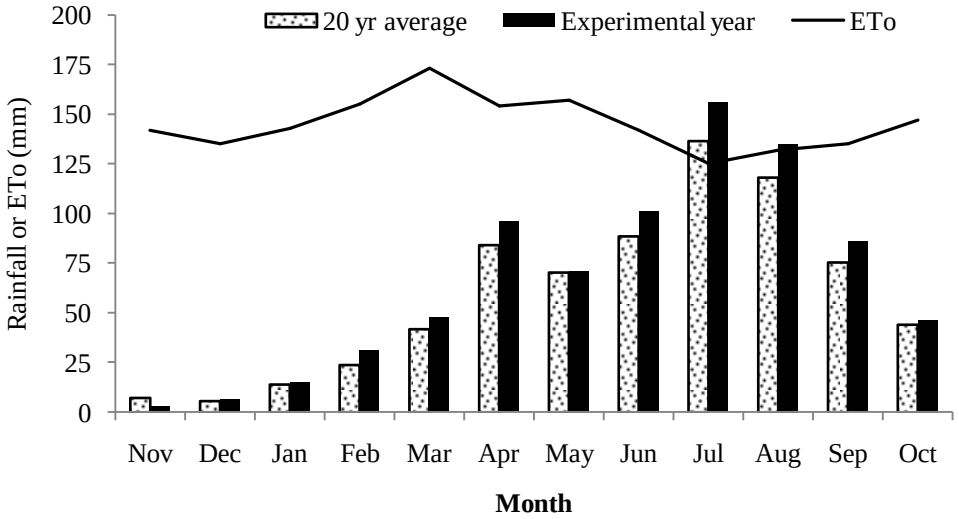

Figure 2 Monthly rainfall during the experimental year (Nov 2009 to Oct 2010), 20 year average (1990-2009) rainfall, and mean monthly reference evapotranspiration (ETo) at the research site.

Experimental design, animals and measurements

The experiment was designed to determine feed intake and diet quality of grazing cattle at two research sites. The first site was contained within the boundaries of the enclosed ranch (ER), Abernosa, which represented a moderately grazed pasture, and the second site was the adjacent communal grassland (CG), which represented a heavily grazed pasture due to high grazing pressure (Tessema *et al.*, 2011). Four bulls and 4 heifers of the local Borana and Arsi breed with a mean live weight of 175±10 kg were randomly selected from the herd grazing on each site. Feed intake and diet quality was measured during five periods (early-dry: November to December; dry: January to March; short-rainy: April to May; main-rainy: June to August; and end-of-rainy season: September to October) from November 2009 to October 2010. Data collection was conducted in the middle of each period for 15 days, during which time the

animals were housed in individual pens at night and allowed to graze within the herd during the day. All animals had access to water during the day with feed and water not provided overnight, which is a common cattle husbandry practice in the region.

At the start of each data collection period, a background faecal spot sample was collected from each animal once per day in the morning (while housed in individual pens) for two days. The spot samples were oven-dried (60°C for 48 h) and bulked per animal per period. The animals were provided a mixture of 2.0 g of alkane-labelled sawdust (containing 152±4 mg C_{32} and 150±3 mg C_{36} alkanes) mixed with 40 g wheat bran and 40 ml molasses in plastic feeders twice daily for 10 consecutive days. The daily alkane dose was given in the morning before the animals were released onto the pasture (6:00 h) and in the afternoon (19:00 h) when the animals returned from grazing. During the last five days of alkane dosing, daily faecal spot samples were collected from each animal in the morning, oven dried (60°C for 48 h) and bulked to one sample per animal per period. Concurrent with the faecal spot sampling, the grazing behaviour of the animals was observed from a close distance (5-6 m) by four trained staff, and samples of herbage species grazed by the animals collected taking into account grazing height. During this herbage sampling period, each animal was continuously observed for 30 min per day (2.5 h over 5 sampling days), during which, the time spent on a herbage patch before moving to the next was recorded using a time counter. The herbage samples collected were manually sorted by species and pooled per plant species per period. Herbage species were identified as described by Bezabih *et al.* (2011b). The faecal and forage samples were dried in a forced-air oven at 60°C for 48 h, ground to pass through a 1-mm sieve and stored in plastic bags pending chemical analysis.

In addition to the collection of herbage samples, the above-ground biomass cover was measured by total destruction of herbage from 50 quadrats (1×1 m^2) at each site. The contribution of major botanical species to the above-ground biomass cover was estimated by sorting the collected samples according to species and measuring the contribution of each to the total fresh and DM weight. Tree density was measured by counting all trees and shrubs (taller than 1.5 m) in 50×50 m^2 quadrats along diagonal transect lines. Body condition score of animals was determined using a five point scale (where 5 is over conditioned, and 1 is extremely emaciated) once during each period by four staff members (Ferguson *et al.*, 1994).

Alkane dose preparation

Equal amounts of synthetic crystals of C_{32} and C_{36}-alkanes (Argenta, New Zealand) were dissolved together in n-hexane, and the resulting alkane solution was absorbed in sawdust. The sawdust was washed with boiling water and liquid detergent (Tide-Febreze®) for 40 min after which it was oven dried (103°C for 12 h) and ground to a particle size of 4 mm. Next, 30.4 g of each alkane (60.8 g total) was weighed into a 5 L Erlenmeyer flask and dissolved in 4 L n-hexane, with the solution heated to 55°C in a water bath. Ground sawdust (300.0 g) was uniformly spread on a tray and dried in an oven at 70°C for 30 min. While warm, the alkane solution was added by continuous mixing ensuring the solution was uniformly distributed in the sawdust. The tray was left in the sun for several hours until the hexane completely evaporated, and was heated at 80°C for 30 min to facilitate the absorption of the alkanes into the sawdust. Finally, after thorough mixing, portions of 2.00 g alkane labelled sawdust were made and stored at room temperature until use. The above procedure was repeated to produce sufficient amounts of alkane labelled sawdust for the entire experiment. From each batch, five samples were randomly taken and analysed for C_{32} and C_{36} concentrations.

Diet botanical composition

The diet composition of the animals was estimated using a combination of the herbage n-alkanes and their carbon isotope enrichments appearing in faeces as markers and visual observations of plant species consumed. Faecal n-alkane concentrations were corrected for incomplete recovery (Bezabih *et al.*, 2012) before the diet composition calculations were made according to the least square optimization procedure using the Solver routine in Microsoft excel:

$$\text{Minimize} \sum [(\text{actual} - \text{calculated})^2] \; \text{marker}_i \ldots n$$

where actual = measured concentration of marker *i* in the diet; calculated = calculated concentration of marker *i* using the following formula:

$$\text{calculated} = \sum[(X_j \times Y_{ij})] \; \text{plant}_j \ldots n$$

where X_j is the estimated proportion of plant species *j* in the diet; Y_{ij} is the concentration of marker *i* in plant species *j*; and $\sum X_j = 1$. The solver routine was constrained to yield none zero values, as the input herbage species were those observed to be consumed by the animals.

Feed intake and digestibility estimations

The concentration of individual n-alkanes in the diet was calculated by summation of the proportion of individual plant species in the diet multiplied by the respective concentration of the particular n-alkane. Feed intake was calculated according to Mayes *et al.* (1986), with corrections for differences in the recovery of dosed and herbage n-alkanes (Bezabih *et al.*, 2012).

Faecal output was estimated from C_{36} concentration in the faeces as follows:

$$\text{Daily faecal output (DM kg)} = D_j/(F_j - B_j),$$

where D_j is the daily dose of C_{36}, F_j is the faecal concentration of C_{36} (mg/kg DM) corrected for incomplete recovery, and B_j is the background faecal concentration of C_{36} (mg/kg DM). Apparent digestibility of DM was calculated as: 1 – indigestibility, where indigestibility is the ratio of estimated daily faecal DM output to estimated DMI.

The chemical composition of the diets was calculated by summation of the proportion of individual plant species in the diet multiplied by the respective species chemical composition. Intakes of protein and minerals were then calculated from the estimated intake of DM and chemical composition of the diets. Metabolizable energy intake was derived from the estimated apparent DM digestibility and forage intake (Freer *et al.*, 1997).

Faecal recovery of *A. tortilis* seeds and estimation of the fruit intake

During two of the five measurement periods (early-dry and dry), fallen *A. tortilis* fruit was consumed in considerable amounts. During these two periods, faecal subsamples were taken from the bulked morning spot samples to determine the concentration of intact acacia seeds appearing in faeces. The DM contents of the subsamples were first determined, and then the samples were immersed in water for about 2 h, after which the seeds were collected using a 3 mm sieve and counted.

The faecal recovery rate of *A. tortilis* seeds was determined in a separate indoor trial. Four bulls (135±4 kg live-weight) fitted with faecal collection bags were housed in individual pens and provided with hay and freshly cut grass twice daily. After three days of adaptation, each animal received 250 g of dried *A. tortilis* fruit per day for five consecutive days. Total faecal collection was performed starting from the day of acacia fruit consumption until no acacia seed was observed in the faeces. The daily faecal collections were washed with water

on a 3 mm sieve and the seeds were recovered. The faecal seed recovery was calculated as: total seed count in faeces/total seed consumed with the pod. Samples of acacia pod were opened to determine the number of seeds per pod, seeds per gram of dried fruit and seed to pod ratio (w:w).

The acacia fruit consumption of the grazing animals was calculated from the faecal seed count as follows:

Daily acacia fruit consumed (g DM/d) = $[(S_{faeces}/R_s)/S_{fruit}]\times DM_{faeces}$

where S_{faeces} is acacia seed count per gram of faeces DM, R_s is the faecal recovery rate of acacia seeds (determined indoors), S_{fruit} is mean acacia seed count per gram of dried acacia fruit, and DM_{faeces} is the daily total faecal DM output (g).

Chemical analysis

The herbage species samples were analysed for DM, ash, crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and minerals, whereas the faecal samples were analysed for DM, ash and CP. Dry matter was determined by oven drying at 103°C (ISO 6496; ISO, 1999) and ash after incineration at 550°C (ISO 5984; ISO, 2002). Crude protein (6.25 × N) was determined using the Kjeldahl method (ISO 5983; ISO, 2005) with NDF determined according to Van Soest *et al.* (1991) and ADF according to Van Soest (1973). The contents of Ca, K, Na, Mg, Mn, and Cu in forage samples were determined by atomic absorption spectrophotometer (Buck Scientific 240VGP) after digestion with tri-acid mixture of nitric, per-chloric and sulphuric acids (Chitra *et al.*, 1996).

For n-alkane analysis, ground samples were pulverised using a bullet mill (MM 2000; 4 min at 80 Hz; Retsch Technology GmbH, Haan, Germany) prior to extraction and analysis of n-alkanes as described by Mayes *et al.* (1986) with modifications by Salt *et al.* (1992) using tetratriacontane (C_{34}) as an internal standard. The extracted samples were analysed for n-alkanes (C_{27} to C_{36}) using a gas chromatograph (CarloErba HRGC Mega 2 series) fitted to a flame ionizing detector (FID), using helium as the carrier gas.

The carbon isotope ratio of the alkanes was determined by fitting a GC (Finnigan_MAT, TraceGC Ultra) with a split/splitless injector operated in split mode (split ratio 1:10) to a combustion interface (Finnigan_MAT Combustion interface III), which was

connected to an elemental analyser isotope ratio mass spectrometer (FinniganMAT CN). The temperature settings of the GC/CIRMS were as described by Bezabih *et al.* (2011b).

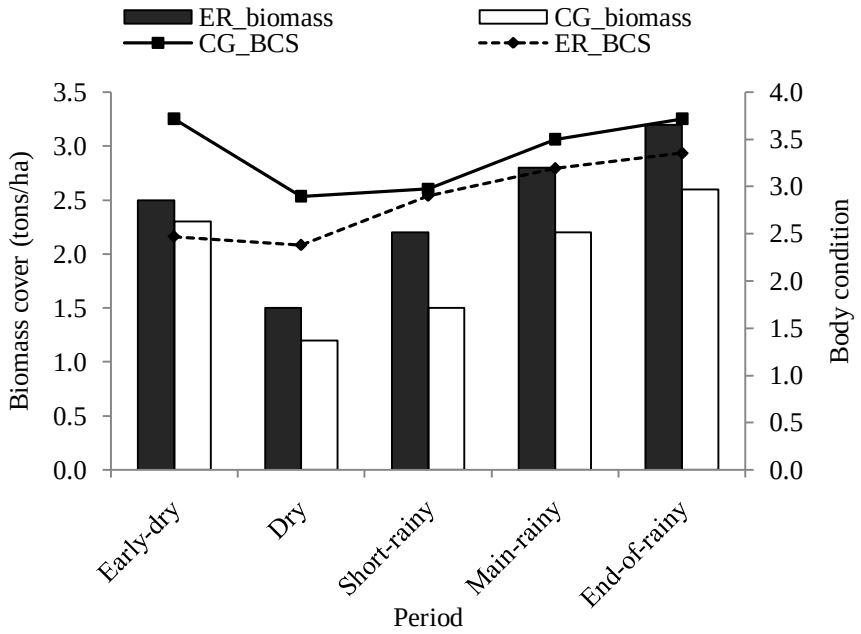

Figure 3 Grass biomass cover (bar-graph) of grassland at the enclosed ranch (ER) and communal grassland (CG) and the body condition score (BCS; in a five point scale) of the cattle grazing on the two sites measured during five seasons/periods in the Mid Rift Valley of Ethiopia.

Data analysis

Individual animals were considered as the experimental unit. The data on diet chemical composition, intake, DM digestibility, and OM digestibility were analyzed with the GLM of SAS (9.1) using the model:

$$Y_{ijm} = \mu + S_i + P_j + e_{ijm,}$$

Where Y is the dependent variable, μ is the overall mean; S_i is the effect of site, P_j is the effect of period, and e_{ijm} is the error term. The interaction effect was omitted from the model after observing that it was not significant. There was also no difference between the sexes (bull or heifer) on the diet composition and nutrient intakes and therefore the data from both sexes were pooled. Diversity indices of diets selected and similarity indices between non-

consecutive periods (Feinsinger *et al.*, 1981; Hill, 1973) were calculated to evaluate seasonal effects on diet composition of the grazing animals.

Results

Above-ground biomass cover and diet botanical composition

The estimated biomass yield ranged from 1.2 to 2.6 ton/ha in the CG and from 1.5 to 3.2 ton/ha in the ER (Figure 3). As expected, the abundance of the biomass cover was strongly related with the rainfall, with the lowest biomass recorded in the dry period and the highest biomass obtained towards the end-of-the rainy period. The body condition of the animals followed the same trend as the abundance of above-ground biomass (Figure 3). In the dry periods, the body condition of the animals in the CG was higher than those in the ER, although the biomass was higher in the latter.

Table 1 shows the diet composition of the grazing animals estimated using n-alkanes and their carbon isotope enrichments as markers in combination with visual observations. The diets of bulls and heifers did not differ significantly and the results were pooled. In total, 31 diet components/herbage species were identified to have been consumed in considerable quantities (≥3%) over the collection periods. In the CG, species such as *C. dactylon*, *D. aegyptium*, *S. spyramidalis, P. polystachion*, maize stover and haricot bean straw were each consumed in quantities more than 10% of the total DM consumed during one or more of the measurement periods. In the ER, the diets contained large proportions of *C. gayana, C. ciliaris*, *T. triandra, Pennisetum straminium*, *C. pychnotrix*, and *A. tortilis* fruit. Among the grass species *C. gayana* dominated the diet of animals in the ER ranging from 8-22%, whereas *A. tortilis* fruit constituted up to 32% of the diet in the early dry period. Maize stover constituted 23-31% of the diet of the animals in the CG during the early dry and dry periods. Some of the species such as *B. marlotii, Desmodium sericeum*, *Z. pentandra,* and *M. repens* were short lived and appeared in the diet of the animals only in one or two seasons.

As for the above-ground biomass, the species richness of the diets varied between the grazing locations and the measurement periods. The diets selected in the ER contained more species than those in the CG. Moreover, the species richness of the diets reached a maximum during the rainy season and a minimum during the dry season. The early-dry and dry period diets were less diverse ($P<0.05$) than the wet period diets in both grazing locations (Table 2).

Moreover, in the end-of-rainy period, diets were more diverse (P<0.05) than the short-rainy and main-rainy periods. The similarity indices of diets between non-consecutive measurement periods (values ranging from zero to one, with one being most similar and zero most dissimilar) were less than 0.50 (Table 2), except for the short-rainy *vs.* end-of- rainy period diets (0.56) in the ER. The lowest similarity index (0.18) was that of the diets of the dry period *vs*. the main rainy period in the CG.

Diet chemical compositions

The NDF content of the diets ranged from 585 g/kg DM (main-rainy season) to 657 g/kg DM (dry season) in the ER, whereas it ranged from 595 to 665 g/kg DM in the CG (Table 3). The CP content varied between 85-118 g/kg DM in the ER, and 71-112 g/kg DM in the CG. The major mineral profiles were in the following ranges (g/kg DM): Ca, 6.1-14.2; P, 1.5-3.0; K, 30-58; Na, 0.38-0.58; and Mg, 8.5-11.4 (Table 3). The concentration of Cu was in the range of 9-49 mg/kg DM, and that of Mn was 63-95 mg/kg DM. The effect of period (season) was significant (P<0.05) for all nutrients analysed, whereas the effect of site of grazing was significant (P<0.05) only for CP, Ca, K, Mn, and Cu. Generally the CP and mineral contents reached a maximum level in the rainy season and declined afterwards into the dry season.

Estimated intakes of dry matter, metabolizable energy and nutrients

The DMI of the cattle varied between 65 and 98 g/kg$^{0.75}$, with the lowest and highest intakes observed in the dry and main rainy periods, respectively (Table 4). The ME intake varied between 20 and 37 MJ/d, while the intake of other nutrients varied in the following ranges (g/d): CP, 222-556; Ca, 19-65; P, 5-14; K, 97-273; Na, 1.3-2.1; Mg, 30-54; Mn (mg/d), 246-406; and Cu (mg/d), 28-231. The effect of period on the intake of energy and nutrients was significant (P<0.05). In most measurement periods, the intakes were higher on the ER than the CG, with differences being significant (P<0.05) for CP, Mg, Mn, and Cu and trends observed for Ca. There was a trend (P=0.065) for the digestibility of the DM consumed (0.49-0.58) to be different between periods with estimates for the two sites being not different (Table 4).

Table 1 Dry matter fraction of plant species consumed by cattle grazed on grassland of an enclosed ranch (ER) or communal grassland (CG) in the Mid Rift Valley of Ethiopia during five seasons/periods estimated using a combination of plant n-alkanes and their carbon isotope enrichments and visual observations.

Diet components/species	Early-dry		Dry		Short-rainy		Main-rainy		End-of-rainy	
	ER	CG	ER	CG	ER	CG	ER	CG	ER	CG
Cynodon dactylon	0.06 (0.01)†	0.12 (0.03)	0.08 (0.02)	0.15 (0.04)	0.09 (0.03)	0.19 (0.08)	0.07 (0.02)	0.15 (0.06)	0.05 (0.01)	0.14 (0.06)
Chloris gayana	0.15 (0.05)	0.08 (0.02)	0.22 (0.06)	0.10 (0.03)	0.18 (0.07)	0	0.20 (0.08)	0.04 (0.01)	0.08 (0.02)	0.05 (0.01)
Brachiaria lachnantha	0	0	0	0	0	0.03 (0.01)	0.05 (0.01)	0.09 (0.03)	0	0.06 (0.02)
Chloris pychnotrix	0	0	0	0	0.04 (0.01)	0	0.08 (0.04)	0	0.11 (0.03)	0
Melinis repens	0	0	0	0	0.05 (0.02)	0	0.06 (0.02)	0	0	0
Desmodium sericeum	0	0	0	0	0	0	0.05 (0.01)	0	0.07 (0.02)	0
Acacia tortilis (fruit)	0.32 (0.16)	0.05 (0.03)	0.11 (0.01)	0.03 (0.01)	0	0	0	0	0	0
Cenchrus ciliaris	0.10 (0.03)	0	0.05 (0.01)	0	0.08 (0.03)	0	0.10 (0.04)	0	0.08 (0.03)	0
Dactylactium aegyptium	0.05 (0.01)	0	0	0	0.09 (0.02)	0.04 (0.01	0.10 (0.03)	0.08 (0.03)	0.07 (0.03)	0.06 (0.02)
Eragrostis aspera	0	0	0.03 (0.01)	0	0.07 (0.03)	0.04 (0.01)	0	0	0	0
Aristida adscensionis	0	0	0	0	0.02 (0.01)	0	0.03 (0.02)	0	0.05 (0.01)	0.04 (0.01)
Cymbopogon pospischilii	0	0.05 (0.02)	0.03 (0.01)	0	0	0	0	0	0.03 (0.01)	0
Cynodon ethiopicus	0	0	0	0	0	0.09 (0.03)	0	0.04 (0.02)	0.03 (0.01)	0
Eleusine mutiflora	0	0	0	0	0	0	0.03 (0.01	0	0	0.04 (0.01)
Maize stover	0	0.22 (0.05)	0	0.31 (0.08)	0	0.07 (0.01)	0	0	0	0
Panicum coloratum	0.06 (0.02)	0	0.05 (0.02)	0	0	0	0.07 (0.02)	0.05 (0.01)	0.06 (0.02)	0.09 (0.03)
Pennisetum straminium	0.08 (0.03)	0	0	0	0.10 (0.02)	0	0.05 (0.03)	0	0.07 (0.02	0
Phaseolus vulgaris (straw)	0	0.13 (0.04)	0	0.14 (0.02)	0	0	0	0	0	0
Digitaria abyssinica	0	0	0	0	0.07 (0.02)	0.06 (0.01)	0.04 (0.01)	0.03 (0.01)	0.07 (0.02)	0

Table 1 Continued.

Diet composition	Early-dry		Dry		Short-rainy		Main-rainy		End-of-rainy	
	ER	CG	ER	CG	ER	CG	ER	CG	ER	CG
Bracheria marlothii	0	0	0	0	0	0	0.04 (0.02)	0	0.07 (0.03)	0
Cynodon plectostachyus	0.04 (0.01)	0.03 (0.01)	0.05 (0.02)	0.03 (0.01)	0	0	0	0	0	0.07 (0.02)
Eragrostis cilianensis	0	0	0	0	0	0	0	0.08 (0.03)	0.03 (0.01)	0.07 (0.02)
Themeda triandra	0.07 (0.04)	0.07 (0.03)	0.20 (0.07)	0.09 (0.02)	0	0	0	0	0.05 (0.01)	0.10 (0.03)
Pennisetum polystachion	0	0	0	0	0	0.13 (0.05)	0	0.06 (0.02)	0	0.05 (0.01)
Zaleya pentandra	0	0	0	0	0	0	0.03 (0.01)	0.04 (0.01)	0	0.03 (0.01)
Harpachne schimperi	0	0	0	0.04 (0.01)	0	0.11 (0.04)	0	0.05 (0.02)	0	0
Heteropogon contortus	0.03 (0.01)	0	0.10 (0.03)	0	0.07 (0.02)	0	0	0.06 (0.02)	0.03 (0.01)	0.04 (0.02)
Hyparrhenia anamesa	0	0	0.03 (0.01)	0	0	0.12 (0.03)	0	0.08 (0.03)	0	0
Sporobolus pellucisus	0	0.09 (0.04)	0	0.11 (0.03)	0	0.12 (0.02)	0	0	0	0.07 (0.02)
Sporobulus pyramidalis	0	0.12 (0.03)	0.05 (0.02)	0	0.08 (0.03)	0	0	0.11 (0.03)	0	0.06 (0.01)
Indigofera spicata	0.04 (0.01)	0.04 (0.01)	0	0	0.06 (0.01)	0	0	0.04 (0.01)	0.05 (0.02)	0.03 (0.01)

†Values in parenthesis are standard deviations of the estimate.

Table 2 Diversity and similarity indices of the diet of cattle grazed on grassland of an enclosed ranch (ER) or communal grassland (CG) during five seasons/periods in the Mid Rift Valley of Ethiopia.

Season/Period	Index	
	ER	CG
Diversity of diets within period		
Early-dry	7.53[a]	7.95[a]
Dry	7.45[a]	6.02[b]
Short-rainy	10.69[b]	8.54[a]
Main-rainy	11.23[b]	12.11[c]
End-of-rainy	15.01[c]	13.37[d]
SEM	0.36	0.33
Similarity of diets between periods		
Early-dry *vs.* Short-rainy	0.48	0.34
Early-dry *vs.* End-of-rainy	0.49	0.39
Dry *vs.* Main-rainy	0.38	0.18
Dry *vs.* End-of-rainy	0.37	0.34
Short-rainy *vs.* End-of-rainy	0.56	0.37

[a,b,c]Diversity indices within a column with different superscripts are significantly different ($P<0.05$).

Table 3 Dietary compositions of cattle grazed on grassland of an enclosed ranch (ER) or communal grassland (CG) in the Mid Rift Valley of Ethiopia during five seasons/periods.

Season/Period	Site	DM	CP	NDF	ADF	Ca	P	K	Na	Mg	Mn	Cu
		g/kg	g/kg DM			g/kg DM					mg/kg DM	
Early-dry	ER	326	101	633	318	7.3	2.3	35	0.38	11.3	85	21
	CG	333	83	620	341	6.4	2.1	37	0.41	10.6	91	24
Dry	ER	373	85	657	322	8.5	1.7	30	0.42	9.2	95	11
	CG	362	71	665	338	6.1	1.5	31	0.41	10.1	94	9
Short-rainy	ER	232	107	596	316	7.8	2.2	45	0.50	9.1	63	23
	CG	245	89	610	320	9.3	1.9	42	0.45	8.5	78	24
Main-rainy	ER	174	118	585	314	13.7	3.0	58	0.58	11.4	85	49
	CG	195	112	595	317	14.2	2.7	53	0.49	10.4	83	45
End-of-rainy	ER	237	98	605	315	10.1	2.5	45	0.51	10.1	80	30
	CG	235	96	615	325	10.5	2.2	44	0.47	10.3	87	32
Pooled SEM		9.5	3.3	21.6	11.3	0.33	0.08	1.5	0.02	0.36	2.9	1.3
Statistical significance												
Period		0.041	0.002	0.003	0.007	0.001	0.001	0.047	0.006	0.005	0.004	0.002
Site		0.621	0.037	0.203	0.321	0.004	0.172	0.021	0.304	0.291	0.032	0.042

DM=dry matter, CP= Crude protein, NDF=Neutral detergent fibre, ADF=Acid detergent fibre.

Table 4 Estimated intake of dry matter (DM), metabolizable energy (ME), crude protein (CP), minerals and digestibility of DM and organic matter (OM) for cattle grazed on grassland of an enclosed ranch (ER) or communal grassland (CG) in the Mid Rift Valley region of Ethiopia over five seasons/periods.

Estimated variable	Season/Period										Pooled	Significance	
	Early-dry		Dry		Short-rainy		Main-rainy		End-of-rainy				
	ER	CG	ER	CG	ER	CG	ER	CG	ER	CG	SEM.	Site	Period
Intake of													
DM ($g/kg^{0.75}\ d^{-1}$)	76	75	68	65	81	78	98	94	90	97	3.0	0.851	0.001
ME (MJ/d)	26	24	21	20	28	27	37	32	32	32	1.3	0.870	0.031
CP (g/d)	369	300	278	222	417	334	556	507	424	448	7.9	0.042	0.001
Ca (g/d)	27	23	28	19	30	35	65	64	44	49	0.8	0.056	0.001
P (g/d)	8	8	6	5	9	7	14	12	11	10	0.2	0.231	0.051
K (g/d)	128	134	98	97	175	158	273	240	195	205	3.6	0.750	0.001
Na (g/d)	1.4	1.5	1.4	1.3	2.0	1.7	2.7	2.2	2.2	2.2	0.08	0.158	0.042
Mg (g/d)	41	38	30	32	35	32	54	47	44	48	0.9	0.033	0.055
Mn (mg/d)	311	328	311	294	246	293	401	375	346	406	6.9	0.020	0.001
Cu (mg/d)	77	87	36	28	90	90	231	204	130	149	3.1	0.048	0.001
Apparent total tract digestibility													
DM	0.53	0.51	0.50	0.49	0.54	0.54	0.58	0.53	0.55	0.52	0.025	0.75	0.065
OM	0.59	0.56	0.56	0.54	0.60	0.59	0.64	0.59	0.60	0.58	0.032	0.65	0.210

Table 5 *Acacia tortilis* seed count, seed to pod ratio and the faecal recovery of seeds in cattle (n=50).

Observation	Minimum	Maximum	Mean	SEM
Seed count per g dried fruit	6	9	7	0.16
Seed to pod ratio (w:w, DM basis)	0.47	0.51	0.48	0.004
Seed faecal recovery (%)	51.7	59.6	55.6	0.31
Tree density per hectare				
Enclosed ranch	66	80	72	0.71
Communal grassland	15	31	21	0.71

Estimated intake of *Acacia tortilis* fruit by the grazing cattle

The mean faecal seed recovery of *A. tortilis* seeds was 55.6±2.2%, seed count per gram dried fruit 7±1.1, and seed to pod ratio 0.48±0.03 (Table 5). The density of *A. tortilis* tree per hectare varied from 66 to 80 in the ER and from 15 to 31 in the CG. The trees commenced fruit production towards the end of the rainy season and continued into the middle of the dry period. The grazing cattle were observed to consume fallen *A. tortilis* fruits in the early-dry and dry periods. During the early-dry period, the estimated fruit consumption in the ER and CG was 324±68 and 53±14 g/kg DM intake, respectively. In the dry period, the estimated acacia fruit intake was 105±10 g/kg DM intake in the ER and 32±6 g/kg DMI in the CG.

Discussion

Biomass abundance and diet botanical diversity

Studies on the ecology of pasturelands have shown that pasture species diversity is highly dependent on the level of grazing pressure (Asefa *et al.*, 2003; Mwendera *et al.*, 1997; Tefera *et al.*, 2007). At high grazing pressure, pasture biomass cover and species diversity decline (Golodets *et al.*, 2010). In the present study, the difference observed in biomass abundance and diet species diversity consumed by the animals on the two grazing sites during the five periods show the spatial and temporal variations in the level of stress on the pasturelands. For example, the lowest biomass cover and diet species diversity was observed for the CG during the dry period, whereas the highest species diversity and biomass cover was recorded for the ER during the main rainy period.

In addition to a decline in species diversity and biomass cover, several studies have shown that high grazing pressure results in a shift from a perennial grass to an annual and less palatable grass dominated pasture (Loeser *et al*., 2007; Oba *et al.*, 2000). In line with this, the proportion of unpalatable grasses in the present experiment such as *Sporobulus* species was higher on the CG than the ER. Moreover, some of the highly palatable perennial species such as *C. ciliaris* and *P. straminium* were completely absent in the diet of animals grazing on the CG. On the other hand, other studies have shown that at a low level of grazing pressure (high biomass cover), the pasture composition of grasslands is gradually dominated by few competitive species that vigorously use the favourable environment and suppress the growth of others (McIvor, 1998). This phenomenon has led to the hypothesis that maximum species diversity is obtained at an intermediate level of pasture stress or grazing pressure (Loeser *et al*., 2007). In the present study, the increased species diversity in the ER indicates that the pastures responded positively to a decreasing grazing pressure. However, because only two grazing pressures were studied, it is not possible to infer if the grazing pressure in the ER can be considered as optimal.

Estimated chemical composition of diets

Grazing animals often exploit the heterogeneity of forage resources through selective grazing, choosing a diet which is of better quality than the average vegetation on offer (Prache *et al*., 1998). As a result, measuring or estimating the chemical composition and other functional properties (digestibility and intake) of the diets of grazing animals on heterogeneous vegetation is always difficult (Boval *et al*., 2004). The major advantage of the method used in the current study was that the estimation was done with little interference to the normal grazing behaviour of the animals, thus simulating the natural condition.

Except in the dry season, the fibre content of the diets was in the range that does not limit the dry matter intake of the animals (Van Soest *et al*., 1991). Nitrogen is considered as a limiting nutrient for animals grazing tropical grasses during the dry periods (Boval *et al*., 2002; Coppock *et al.*, 1986). The CP content of the diets in the present study was lowest in the dry period, but remained above the minimum CP concentration (70 g/kg DM) required for normal rumen microbial fermentation (Van Soest, 1994). In the dry periods, *A. tortilis* fruit appears to have had an important role in increasing the CP content of the diets, as it was

consumed in large quantities (especially in the ER) and this fruit is known to contain a high CP content (129 g/kg DM) (Bezabih *et al*. 2012). Earlier studies have also shown the role of this tree fruit as an alternative low cost protein source to optimize animal performance during dry periods (Coppock and Reed, 1992). Acacia trees in these grasslands also provide shade for the grazing animals and minimize evapo-transpiration, with palatable grass species growing underneath the tree canopy.

The significant difference observed in fibre, CP and mineral content of diets between measurement periods and grazing sites indicate that the energy and nutrient supply of the grazing animals fluctuated. While the differences observed between the periods can be explained by the amount of rainfall available for plant growth, the difference observed between the two sites mainly originates from the grazing pressure/land use management of the grasslands. Stage of growth is the main factor affecting the nutritive value of individual pasture species (Aumont *et al*., 1995), and as the growth of pastures is dependent on the rainfall pattern, there is a distinct seasonality in the nutritive quality of the diet consumed by the grazing animals. The mineral content of diets was significantly higher in the rainy season compared with the dry seasons, mainly due to the higher leaf to stem ratio of pasture stands in the rainy season than the dry seasons. As the cell content of pastures decline with advancing maturity, so does the soluble mineral content of the pasture.

Estimated intake of dry matter, nutrients, and animal response

The nutrient intakes beyond maintenance requirements are important nutritional variables to predict production performance of animals. In the present study, the fluctuation in the estimated DMI corresponded to the seasonal changes in biomass abundance and quality in the grazing lands. However, despite the variation in biomass abundance and pasture quality between the ER and the CG, the estimated DM intakes between the two grazing sites were not different. It appears that the low biomass cover in the CG was compensated by extended grazing duration and timing of grazing that the farmers practiced in the CG. Several reports have shown that extended duration of grazing and timing of grazing significantly increases the DM intake and animal performance, especially during the dry season when herbage abundance declines (Ayantunde *et al*., 2000, 2001). In the CG, cattle had access to pasture on average for 13 h (6:00-19:00) per day, whereas in the ER the average grazing time was 8 h

(9:00-17:00) per day. As foraging activity in grazing animals is known to peak during the hours just after sunrise and during the hours before sunset (Hodgson, 1990), it appears that the animals in the ER have missed the opportunity to graze during these periods and were forced to graze during periods of the day when they would normally seek shade for thermoregulation. This difference in the grazing management mainly originates from the fact that in the ER animals are herded by formal employees who go to work during standard working hours whereas the CG cattle are herded by family members who spend most of their time with their animals.

Moreover, while animals at both sites grazed in groups, the size of the groups differed considerably, being about 250 animals per group in the ER and about 40 animals per group in the CG. Social facilitation is known to affect grazing behaviour and herbage intakes, with animals consuming more when they are in a group than when isolated (Forbes, 1995). However, the large group size in the ER seemed to have induced more grazing competition and mobility than that in the CG. This might have negatively affected the DM intake of the animals in the ER especially in the dry periods. Previous reports have also documented the negative effects of social facilitation particularly in low forage resource conditions (Vallentine, 1990). In this respect, introducing improved management practices such grazing paddocks, rotational grazing, grouping of animals into a manageable size according to age/physiological state and timing of grazing are important intervention measures to optimize productivity in the ER. Although the farmers optimized the DM intake of their animals in the CG, the high grazing pressure appears to be unsustainable in the long-term as evidenced by the dominance of less palatable annual grasses and disappearance of desirable perennial species from the CG (Table 1). Collective measures are therefore required to reduce the grazing pressure on CG by means of improving availability of alternative feed resources, allowing regeneration of grasslands through enclosures and improving the utilization of crop residues.

The estimated ME and nutrient intakes were highest during the main rainy season, following the increased biomass abundance and improved forage quality. This increase in the ME and nutrient intake coincided with an improvement in the body condition score, indicating that the animals were consuming energy and nutrients above their maintenance requirements. The ME required for maintenance functions represents approximately 70% of the total ME

required by mature cows and more than 90% of the energy required by breeding bulls (NRC, 1996). The maintenance energy requirement is higher in free-ranging animals than in penned animals due to the additional energy required for walking. The extra cost of grazing depends on the herbage quality and type of terrain, being about 10–20% of the basic maintenance requirement for cattle grazing on plain grounds, and about 50% for cattle on extensive and hilly pasture where animals walk considerable distances (NRC, 1996). Considering similar basal ME requirement for maintenance (450 kJ/kg$^{0.75}$/d) and an extra allowance (15%) for grazing (NRC, 1996), the energy intake of the animals in the present study appeared to marginally cover maintenance requirements during the early-dry period. The energy balance was negative in the dry period, followed by a return to a positive energy balance in the subsequent wet periods. In the main-rainy season, the estimated ME intake exceeded the maintenance requirements by up to 43%.

Assuming that the excess ME is used for weight gain and that the ME requirement for weight gain is about 27 MJ/kg (NRC, 1996), the performance of the animals in terms of weight gain was predicted to reach 160 g/d during the short-rainy, 500 g/d in the main-rainy season, and 300 g/d towards the end of the rainy period. The energy content per unit of live weight gain or loss in cattle is considered similar (NRC, 1996), and based on this assumption the body weight loss in the dry period was on average predicted to be around 110 g/d.

A shift from a period of energy restriction to a period of energy supply well beyond maintenance requirements may result in compensatory growth (Tolla *et al*., 2003; Warren *et al*., 1998). The extent to which animals compensate the period of energy restriction depends on the severity and duration of the restriction and the nutritional regime during the re-aliment period (Hornick *et al*., 2000; Sainz *et al*., 1995). The predicted energy restriction in this study appeared to be less severe and the body condition of the animals at both grazing sites rarely fell below 2.0 in the dry period, with a return to the initial body condition observed in the rainy season. However, it should be noted that during the study year, the research area received above average rainfall (Figure 2), which might have reduced the degree of energy restriction in the dry period.

The calculated CP requirement for maintenance for the animals in the present study ranged from 228-285 g/d (NRC, 1996). In the dry period, the estimated mean CP intake (Table 4) appeared to marginally fulfil maintenance requirements in the ER, while it was

below maintenance in the CG. In the remaining measurement periods, the CP intakes were above maintenance requirements. In the main-rainy season, the CP intake reached about twice the maintenance requirements. Assuming a CP requirement for growth of 340 g/kg weight gain (NRC, 1996), the excess CP supply appeared to support a weight gain of up to 350 g/d in the short-rainy period, 800 g/d in the main-rainy season, and 520 g/d at the end-of-rainy period. The above performance predictions seem to indicate that energy intake is more limiting than protein intake for the productive performance of cattle grazing in the study area. While energy and protein intakes are interrelated, the amount of ME intake is directly influenced by the level of DM intake and its digestibility (Estermann *et al*., 2001; Freer *et al*., 1997).

Macro- and micro-minerals play essential roles in the metabolism of animals and, while marginal deficiencies in the diet may impair animal performance, severe restrictions will result in clinical signs of disorders. When the estimated daily intake of macro- and micro minerals in the five measurement periods were compared with requirements for maintenance and growth (NRC, 1996), Ca, Mg, K, and Mn appeared to be consumed in sufficient amounts. The intake of P was above maintenance in all measurement periods. However, in the dry period, the amount of P consumed was marginal in excess of maintenance requirements and might not support a daily weight gain of more than 76 g, provided that energy and all other nutrients are not limiting. The grazing cattle were in a negative energy balance during the dry period and thus the marginal P supply may not be of concern. However, if the animals are supplemented with energy and protein rich concentrates to maintain uninterrupted growth/weight gain during this period, the P nutrition should be given due attention. In the short-rainy period, the P intake could support up to 425 g/d weight gain, while the same prediction for the main-rainy season and end-of-rainy periods were, respectively, 800 and 600 g/d. The intake of Na was below maintenance in all measurement periods, and the balance shows that there is a need to supplement the grazing cattle with about 80 g of Na daily in the form of common salt. The intake of Cu appeared to be marginally below maintenance during the dry period, but in the remaining periods the intakes were according to the recommendation. It should, however, be noted that the requirements for Cu can vary widely depending on the concentration of dietary Mo, S and Fe (NRC, 1996). Because these Cu antagonists were not determined in the present study, the result should be considered

cautiously, particularly in light of previous reports that the soil formation in the Rift Valley of Ethiopia contained high Mo-S complex resulting in Mo-induced Cu deficiency in small ruminants (Faye *et al*., 1991; Kabaija and Little, 1991).

Although allowances should be made for underestimation of energy and nutrient intakes due to the inherent errors of the method used in this study (particularly in relation to the use of hand-plucked samples to mimic herbage selected by the grazing animal), the estimated energy and nutrient intakes were confirmed by the pattern of body condition score of the animals over the measurement periods. With the present method, the visual observation of the grazing behaviour helped to restrict the herbage species used as input for the estimation of diet composition, while the combined use of the n-alkanes and their $\delta^{13}C$ values increased the number of markers available for the estimation, both of which having an important role in improving the accuracy of diet composition estimation (Bezabih *et al.*, 2011a) and hence energy and nutrient intake predictions.

In the present study, the use of *A. tortilis* seed count in faeces to estimate the intake of the fruit seemed to be a suitable approach. Previous reports have shown that the recovery of acacia seeds varies according to the animal species, with cattle showing higher faecal recovery of acacia seeds than sheep and goats (Razanamandranto *et al*., 2004; Shayo and Udén, 1998). The observed recovery rate of *A. tortilis* seed (Table 5) agrees well with that of Shayo and Udén (1998), who reported a mean seed recovery rate of 58% for heifers, 10% for mature sheep and 24% for mature goats. The estimated intake of *A. tortilis* fruit calculated from the faecal seed count was considerably higher in the ER than in the CG animals owing to the large difference in the tree density and hence availability of the fruit (Table 5). The grazing animals showed a high preference for the acacia fruit, as evidenced by the frequent movement of the animals to grazing underneath the trees. Incidences of bloat have been reported by farmers particularly when animals had access to large amounts of acacia fruit before they consume roughage diets in the morning (personal communication). Overall, the *A. tortilis* trees play an important role in maintaining the ecology of the area. Despite its ecological importance, however, the tree has been excessively cut for illegal charcoal making, decreasing the tree density at alarming rates in recent years. For a sustainable farming system in the Mid Rift Valley of Ethiopia, it is essential to protect the existing acacia trees and emerging new seedlings.

Conclusion

The diet composition of cattle grazing on the CG were less diverse (dominated by unpalatable annual grasses) compared with that of the ER. Although the DM intake of the animals grazing on the two sites was not different, the grazing pressure on the CG appeared to be unsustainable in the long-term. There was marked seasonal variation in the estimated diet composition and nutrient intakes. The estimated intakes showed that in the dry period the animals were in negative energy and nutrient balance, while in the main rainy season the intakes were sufficient for optimum daily weight gains. The method used in the present study appeared to provide accurate data on the nutrient intake of the grazing cattle when compared to the changes in the body condition score of the animals. The seasonal diet composition and nutrient balance data generated from this study can be used as valuable inputs to design a sustainable cattle production system in the regions.

Acknowledgments

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CHAPTER 7

General discussion

Introduction

The aim of the research described in this thesis was to: 1. determine the potential of plant cuticular n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as markers to study the nutritional ecology of grazing animals under tropical conditions and, 2. use this method to estimate the seasonal pattern of nutrient intake and diet composition of grazing cattle in the Mid Rift Valley grasslands of Ethiopia.

For the first aim, the variability in the n-alkane profile and $\delta^{13}C$ of the n-alkanes among commonly available pasture species in the Mid Rift Valley of Ethiopia was researched. The multivariate analysis in Chapter 2 indicated that the pasture species show large variability, and that the interspecies variability explained by the n-alkanes differs from that explained by the $\delta^{13}C$ values of the alkanes. In a further validation study, the botanical composition of composite pasture mixes were accurately predicted using n-alkanes and their $\delta^{13}C$ values as markers (Chapter 3). In freely ranging animals, diet composition is estimated by relating the marker profile found in faeces with that of potential dietary components. Correction for incomplete recovery of faecal n-alkane concentrations is necessary before diet composition is estimated using this approach. In Chapter 4, the faecal recovery rate of natural and dosed n-alkanes in cattle fed tropical roughage diets were measured. In estimating forage intake using the alkane method, the alkane pairs used (natural and dosed alkanes) should have similar faecal recovery or the difference in faecal recovery should be established to apply a suitable faecal correction factor. The faecal recovery of dosed alkanes administered in the form of molasses-based bolus in the present study showed a higher faecal recovery than adjacent odd-chain alkanes, and use of a correction factor appears necessary to improve the accuracy of intake predictions (Chapter 4). For a sustainable management of natural grasslands it is important to acquire information on the nutritive value of available pasture species as well as the possible environmental impacts of their utilizations. With this objective in mind, the nutritive value and greenhouse gas production potential of various pasture species collected from the Mid Rift Valley region during the main pasture growth season were explored by carrying out chemical analysis, *in vitro* gas and methane production measurements (Chapter 5). The results showed large variability in nutritive value among the pasture species, with scope for selection of species with high nutritive value and low methane production to improve grassland productivity and mitigate methane emission from ruminants.

For the second aim, the nutritional status of grazing cattle in the Mid Rift Valley region in terms of diet composition and nutrient intake was estimated during five measurement periods/seasons over one year using the n-alkanes and their $\delta^{13}C$ values as markers (Chapter 6). Comparisons between estimated nutrient intakes and requirements revealed nutritional limitation in different grazing seasons. Management options to optimize both animal and pastureland productivity have been discussed based on the results generated.

Variation in alkane profile and carbon isotope enrichment of alkanes between species

The important attributes of markers in nutritional studies include high faecal recovery and accurate quantitative measurement both in faeces and in the plant (Kotb and Luckey, 1972). Apart from this, sufficient variability between dietary components/species in the marker profile is an essential requirement for the use of one or more plant chemical compounds as diet composition markers (Dove and Mayes, 1996). Numerous research reports conducted mainly on temperate pasture species confirmed the presence of distinct variability in the n-alkane profiles between species and, to some extent, between plant parts, providing fingerprint information to estimate diet composition of grazing herbivores (Mayes and Dove, 2000).

With this background, a research interest arose to establish whether the cuticular n-alkane profile of pasture species commonly available in the rangelands of Ethiopia will show a sufficiently wide variability in their n-alkane profile to be used as nutritional markers. Individual pasture species were collected from the Mid Rift Valley grasslands of Ethiopia during the main pasture growth period, and the n-alkane concentrations were determined (Chapter 2). The n-alkanes with carbon chain lengths ranging from C_{27} to C_{35} were found in adequate concentrations, with C_{31} and C_{33} being the dominant alkanes measured. In addition, the δ^{13} C values of individual n-alkanes were determined for each species. The multivariate analysis employed to examine the interspecies variability revealed that most of the pasture species vary widely in their n-alkane profiles and, in addition, carbon isotope enrichments, and that this variability can be exploited to predict the diet composition of grazing animals (Chapter 2).The results show that the interspecies variability explained by the $\delta^{13}C$ values of the alkanes provides additional information, and that use of a combination of the two data sets considerably increases the discriminatory potential of the alkanes (Chapter 2 and 3).

The $\delta^{13}C$ values of forages have long been used to estimate the proportion of C_3 and C_4 plants in the diet of herbivores (Coates *et al.*, 1987). García *et al*. (2000) reported an increase in the accuracy of diet composition estimation of cows when a combination of the n-alkanes and $\delta^{13}C$ values of the organic matter consumed was used. To the author's knowledge, the work presented here is first to use compound specific (alkane) carbon isotopic enrichments to discriminate plants at a species level. The use of n-alkanes, rather than the whole organic matter for carbon isotope analysis, is more advantageous, because the former is relatively stable both in feed and faeces, whereas much of the latter is digested and absorbed in the gut, with highly digestible dietary components represented less in the faeces organic matter compared to poorly digestible components. Moreover, research reports have shown that rumen microbes are unable to synthesize or degrade n-alkanes (Mayes *et al.*, 1988; Keli *et al.*, 2008a). Although trace levels of alkanes have been detected in ruminant tissues (Di Muccio *et al.*, 1984), the possible secretion of alkanes into the gut is also negligible in comparison to the high concentration of alkanes present in the digesta (Dove and Mayes, 1991). Evidence therefore provides a strong argument that the carbon isotope enrichment of alkanes would remain undiluted in the faeces. Unlike n-alkanes, for which correction is required for incomplete faecal recovery during diet composition estimation, $\delta^{13}C$ values of n-alkanes do not require corrections as they are relative values (ratio of ^{13}C and ^{12}C isotopes in relation to the natural abundance), allowing their use in diet composition estimation without the need to conduct a specific *in vivo* balance study. This approach, however, assumes that there are no differential recoveries of individual n-alkanes due to differences in their molecular weight or source of plant.

n-Alkane profile and carbon isotope enrichment of alkanes over the plant growth period

The cuticular wax, in addition to its role as a protective cover, plays an essential function in limiting non-stomatal water loss from plants, and as a result it is one of the key adaptations in the evolution of terrestrial plants (Samuels *et al.*, 2008). When stressed with moisture scarcity, plants tend to respond by accumulating more wax on the outer surface of their leaves, with the level of response varying according to the genotype of the plant (García *et al.*, 2002). Because n-alkanes are an important component of the cuticular wax, such environmental factors may also influence the alkane profile of the plants. This signifies the importance of documenting

location specific information on the alkane profile of available herbage species for use as nutritional markers (Ali *et al.,* 2005a).

An effect of age or stage of plant maturity on the alkane profile of herbage species has been documented (Dove *et al.,* 1996). The effect of stage of growth/age can be looked from two angles. On one hand, young herbage seedlings have less wax sealing on their leaves and stems, but as the leaves develop the epidermal cells increasingly synthesize wax and seal the cuticular surface (Van Maarseveen *et al.,* 2009). On the other hand, the different morphological fractions (stem, leaf sheath, and leaf blade) of a plant differ in their n-alkane profile, with stems generally containing low and leaf blades high concentrations of alkanes (Dove *et al.,* 1996). As the plant mature, the proportion of these morphological fractions changes, which in turn affects the n-alkane profile of the plant species.

In this thesis the herbage species sampled during five measurement periods (Chapter 6) were not separated into stem and leaves and the change in the alkane profile within the morphological fractions over the different seasons could not be examined. Figure 1 shows the concentration of the major odd chain alkanes for selected perennial grass species (hand-plucked by simulating the grazing height of cattle) over the five measurement periods. The concentration of the alkanes in each species changed over time, with the pattern tending to be different from species to species and from alkane to alkane. The $\delta^{13}C$ values of individual alkanes of the grass species were also not constant during the five measurement seasons, with a tendency for the enrichment to be higher (0.5 to 1.5 delta units) during the rainy season than the dry period in some of the species. Over the measurement periods, however, there was consistently large variability between the species, which appears important from the point of view of using these markers to discriminate the diet of grazing animals. This result indicates that concurrent sampling of faeces and herbage consumed by the grazing animal is essential, and that the presence of a time gap between faeces and herbage sampling could introduce a considerable error in the estimation of diet composition and intake of the grazing animal. This feature appears as a weakness of the n-alkane method because herbage samples analysed at one time may not be suitable for estimating the diet composition of the grazing animal at the other time.

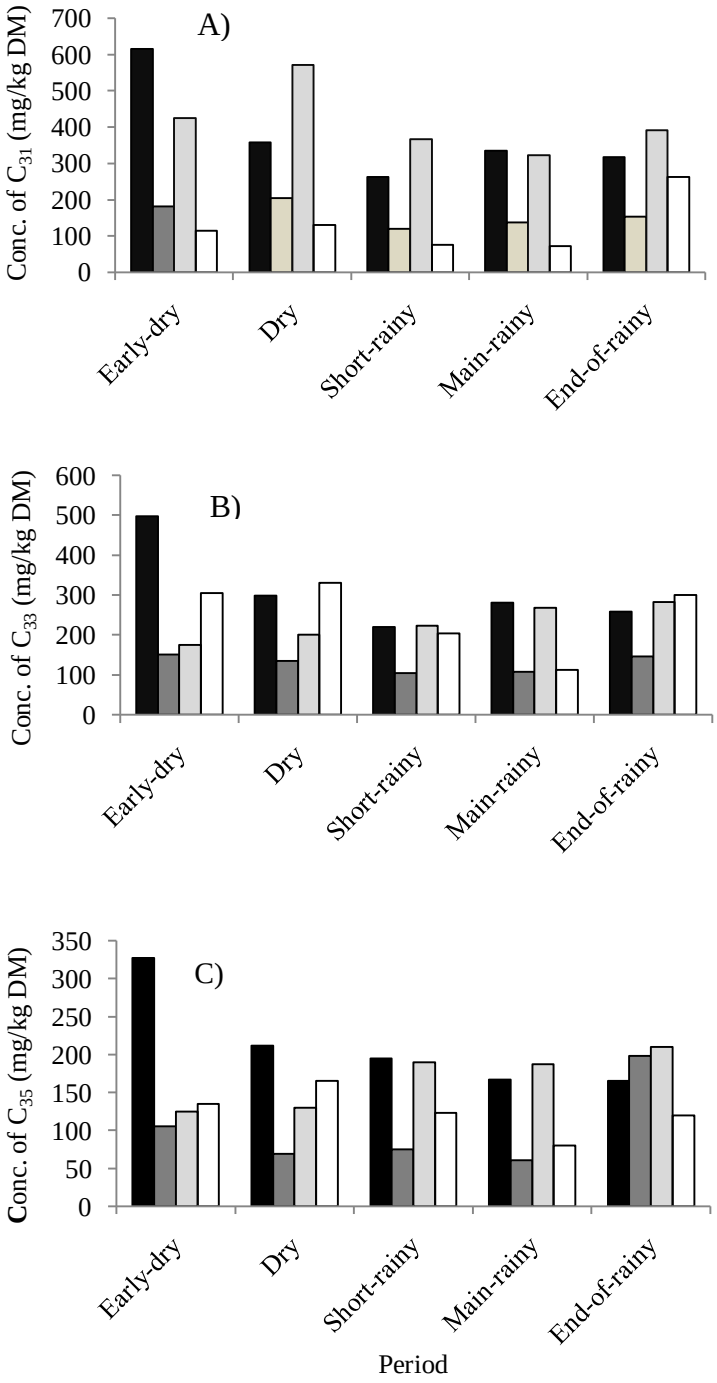

Figure 1 The concentration of C_{31} (panel A), C_{33} (panel B) and C_{35} (panel C) in grass species (■*C. gayana*; ■*C. dactylon*; □*C. ciliaris;* □*S. pyramidialis*) sampled by mimicking grazing height of cattle during five measurement periods over one year.

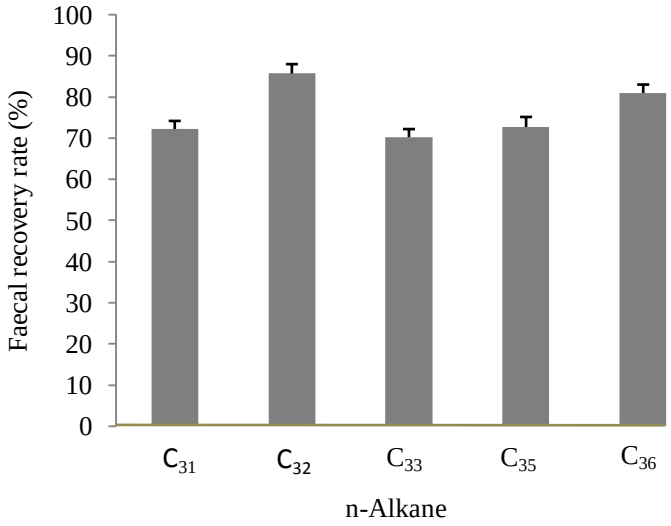

Figure 2 Mean faecal recovery rate of dosed synthetic even-chain (C_{32} and C_{36}) and adjacent odd-chain alkanes in zebu cattle fed low quality tropical roughage diets.

Importance of faecal recovery of alkanes

The recovery of n-alkanes in faeces is incomplete (Mayes and Lamb, 1984), with the recovery rate showing an upward trend with increasing carbon-chain length (Ferreira *et al.*, 2009). A reliable faecal recovery data is required for two important purposes. The first is to correct faecal n-alkane concentrations for the incomplete recovery before estimating diet composition using either least squares optimization procedures or linear programming (Dove and Moore, 1995; Barcia *et al.*, 2007). The second is to select alkane pairs (dosed even-chain and natural odd-chain) with similar faecal recovery rates for forage intake estimation using the double n-alkane method (Mayes *et al.*, 1986). In the latter case, it is necessary to ensure that the assumption of similar faecal recovery of alkane pairs holds true, or that the difference in the faecal recovery is quantified to apply an appropriate correction during forage intake estimations.

Differences exist among animal species in the faecal recovery of alkanes (Mayes *et al.*, 1986). Studies have shown that the effect of diet on the faecal recovery of alkanes is variable (Brosh *et al.*, 2003; Elwert *et al.*, 2008), with data for tropical forages being scarce. A balance study was thus conducted to determine the faecal recovery of natural and dosed alkanes in zebu cattle consuming tropical roughage diets (Chapter 4). The results show that the faecal

recovery of n-alkanes was lower than that reported for temperate forages (Ferreira *et al.*, 2009). The low faecal recovery may be a characteristic of tropical forages, as the few previously published reports generally agree with the results presented in this thesis (Table 1). The synthetic alkanes (C_{32} and C_{36}) dosed in the form of molasses-based boluses (Chapter 4) showed a higher faecal recovery than adjacent odd-chain alkanes (Figure 2).The tendency for the dosed alkanes to have higher faecal recovery rates than that expected from the carbon-chain length has been known from earlier studies when the alkane method was evaluated for intake measurement (Dove and Mayes, 1991). In the present work (Figure 1), the faecal recovery of dosed synthetic alkanes and natural odd-chain alkanes were considerably different making the assumption of similar recovery not hold true.

Nutrient intake estimation in grazing cattle

Nutrient intake is an important factor determining the production performance of domestic animals. Quantifying nutrient intake in grazing animals is complex, as it is dependent on the amount and the nutrient content of the plant species (plant parts) consumed, and direct measurement of these variables is difficult (Mayes and Dove, 2000). In complex vegetation, free ranging animals exert a selection pressure by consuming the available plant species. As the different species or plant parts may vary considerably in their nutrient concentrations, estimation of daily nutrient intake of animals requires measuring both the daily forage intake and the diet composition of the forage consumed.

Although several indirect approaches are available to measure nutrient intakes of grazing animals, most of them suffer from low accuracy, invasiveness or an inability to measure intakes on individual animals (Dove and Mayes, 1991). The use of the n-alkane method has emerged as an approach to estimate nutrient intake with increased accuracy and little interference to the normal grazing behaviour. With the double n-alkane method, herbage intake is estimated using the ratio in faeces of dosed even-chain and adjacent natural odd-chain alkanes (Mayes *et al*. 1986), on the assumption that the subsequent odd- and even-chain alkanes have similar faecal recovery. In most of the pasture species studied (Chapter 2), the odd-chain n-alkanes, mainly C_{31}, C_{33}, and C_{35}, were found in sufficient concentrations to be used for intake estimations. Following this, the accuracy of intake estimation with the alkane method was evaluated by conducting a feeding experiment with growing local Borana bulls in

Ethiopia (Chapter 4). The animals were dosed daily with C_{32} and C_{36} synthetic alkanes in the form of molasses-based boluses, and thereafter the pattern of faecal concentrations of dosed alkanes with time was studied. The analysis showed that a steady-state of dosed alkane concentration in faeces was achieved at 3.3 days into the marker dosing. Thus with molasses-based alkane boluses, faecal collection to estimate nutrient intake and digestibility can be made from the fourth day onwards. Comparison between actual and predicted intakes showed that with the C_{35}/C_{36} alkane ratio, intake was accurately estimated (with less than 1.5% difference between actual and predicted intakes), whereas with the C_{31}/C_{32} and C_{33}/C_{32} alkane ratios, the assumption of similar faecal recovery of adjacent n-alkanes underestimated the intake by about 12% (Chapter 4). The result further revealed that correction for the difference in the faecal recovery of the dosed C_{32} and natural C_{31} and C_{33} alkanes considerably improved the intake estimation.

Table 1 Faecal recovery rate of n-alkanes summarized from different published references.

Source	Faecal recovery of alkanes (%)								
	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{35}	C_{36}
Tropical forages									
This thesis	61	67	72	74	72	86	70	72	81
Hendrichsen *et al.* (2002)	-	-	-	56	76	87	80	74	86
Molina *et al.* (2004)	-	-	-	-	79	99	87	-	-
Morais *et al.* (2011)	42	94	56	82	71	96	75	77	86
Mediterranean/temperate forages									
Dillon (1993)	68	-	77	-	81	86	85	90	87
Ferreira *et al.* (2009)	71	55	86	86	88	103	103	-	-
Herd *et al.* (2003)	55	-	53	-	78	87	68	-	63
Dove and Mayes (1991)*	50-58	58-67	63-70	57-68	60-86	70-90	71-90	78-100	78-94

*A review.

As presented in Chapter 4, three faecal sampling methods, i.e., total faecal collection, morning faecal spot, and afternoon faecal spot sampling were compared to evaluate whether faecal spot samples can yield the same result as total faecal collection. This similarity has practical significance, as total faecal collection of grazing animals is difficult to achieve under field conditions. The alkane profile of the three fractions (Figure 3), and the subsequent feed intake estimations using these samples were not statistically different (Chapter 4), showing that the use of faecal spot samples can yield the same result as that of total faecal collection.

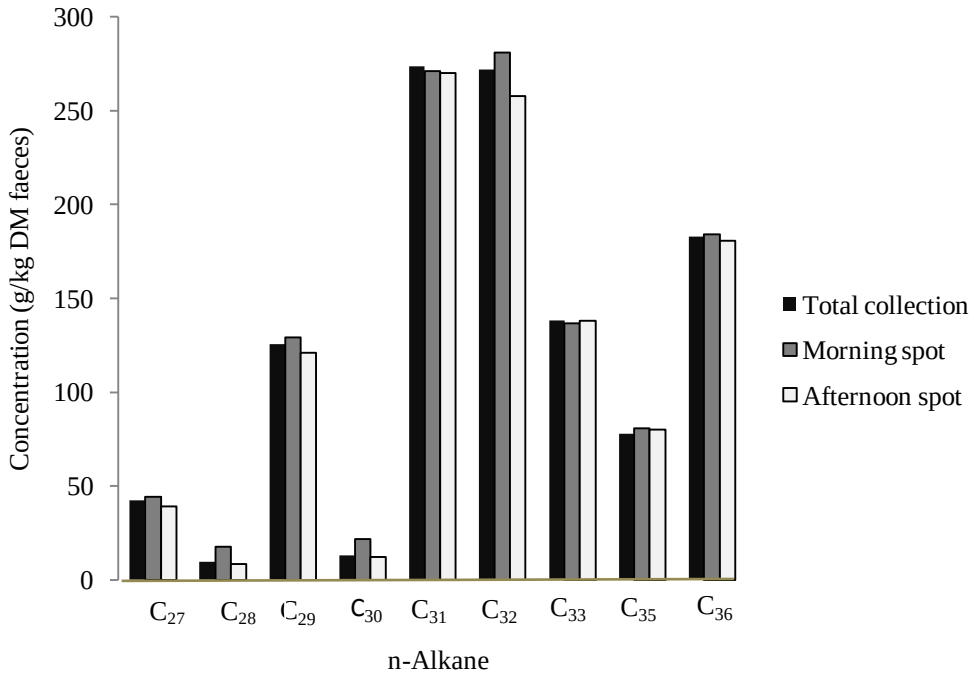

Figure 3 Faecal n-alkane concentrations (mg/kg DM faeces) determined from total collection, morning spot and afternoon spot samples for cattle fed low-quality tropical feeds.

Table 2 Range of chemical composition and predicted nutritive value of feed resources analyzed during the main pasture growth period.

Parameter	NDF	ADF	CP	OMD	ME	*In vitro* CH_4:total
	g/kg DM			%	MJ/kg DM	gas ratio (v/v)
Minimum	184	85	39	42	5.8	0.12
Maximum	684	385	439	73	10.2	0.37
Mean	556	305	110	63	8.7	0.24
SD	126	70	78	7	0.96	0.04

NDF=neutral detergent fibre, ADF=acid detergent fibre, CP=crude protein, OMD=organic matter digestibility, ME=metabolizable energy.

Variation in the nutritive value of forages in the Mid Rift Valley of Ethiopia

The nutritive value of forages varies according to species, stage of maturity, soil fertilization and other climatic factors (Aumont *et al.*, 1995; Mislevy *et al.*, 2003; Machado *et al.*, 2007). Knowledge of the nutritive value of available forage species is important to predict the

nutritional status of animals and to screen desirable forage species for pasture improvement (Coleman and Moore, 2003; Meale *et al.*, 2012). The experiment described in Chapter 5 provides information on the nutritive value of available forages in the Mid Rift Valley of Ethiopia in terms of their chemical composition, mineral profile, feeding value as assessed by *in vitro* gas production and environmental impact in terms of methane production during the main pasture growth period. A large variability between pasture species was observed, providing scope for selection of species with a high nutritive value and low methane emission potential (Table 2). With predicted organic matter digestibility ranging from 42 to 73% and metabolizable energy content from 5.8 to 10.2 MJ/kg DM, the pasture stand during the main rainy season has been evaluated as moderate in quality. The pasture species reached their full vegetative stage in the middle of the rainy season, which corresponded with the pasture sampling for the experiment presented in Chapter 5. It can thus be hypothesized that any nutritional limitations during the main pasture growth period in the Mid Rift Valley of Ethiopia primarily originates from biomass availability rather than feed quality (Chapter 5).

Nutritional status of grazing cattle in the Mid Rift Valley of Ethiopia

Grazing cattle are the dominant farm animals reared in the Rift Valley regions of Ethiopia, and they serve as an important source of livelihood for the farming community (CSA, 2008). Improved nutritional management of the grazing cattle is, therefore, one of the mechanisms by which the livelihood of the farming community can be improved in a sustainable manner. Towards this goal, the seasonal patterns of the nutrient intake and diet composition of the grazing cattle were measured using n-alkanes and their $\delta^{13}C$ values as markers in combination with visual observations (Chapter 6). The approach used in this study minimized interference to the normal grazing behaviour, although there remains an observer bias in mimicking the grazing height and sampling of forage species for n-alkane and nutrient composition analysis. In view of the grazing animal's ability to select highly palatable part of the forage on offer (Dumont *et al.*, 2007a,b), the hand-plucked samples may lead to an underestimation of energy and nutrient intakes of the animals. Moreover, the n-alkane profile of such samples may not exactly represent that of the diet selected because of the presence of variation between plant parts of the same species (Dove *et al.*, 1996), which may in turn introduce an error in the estimation of intake and diet composition.

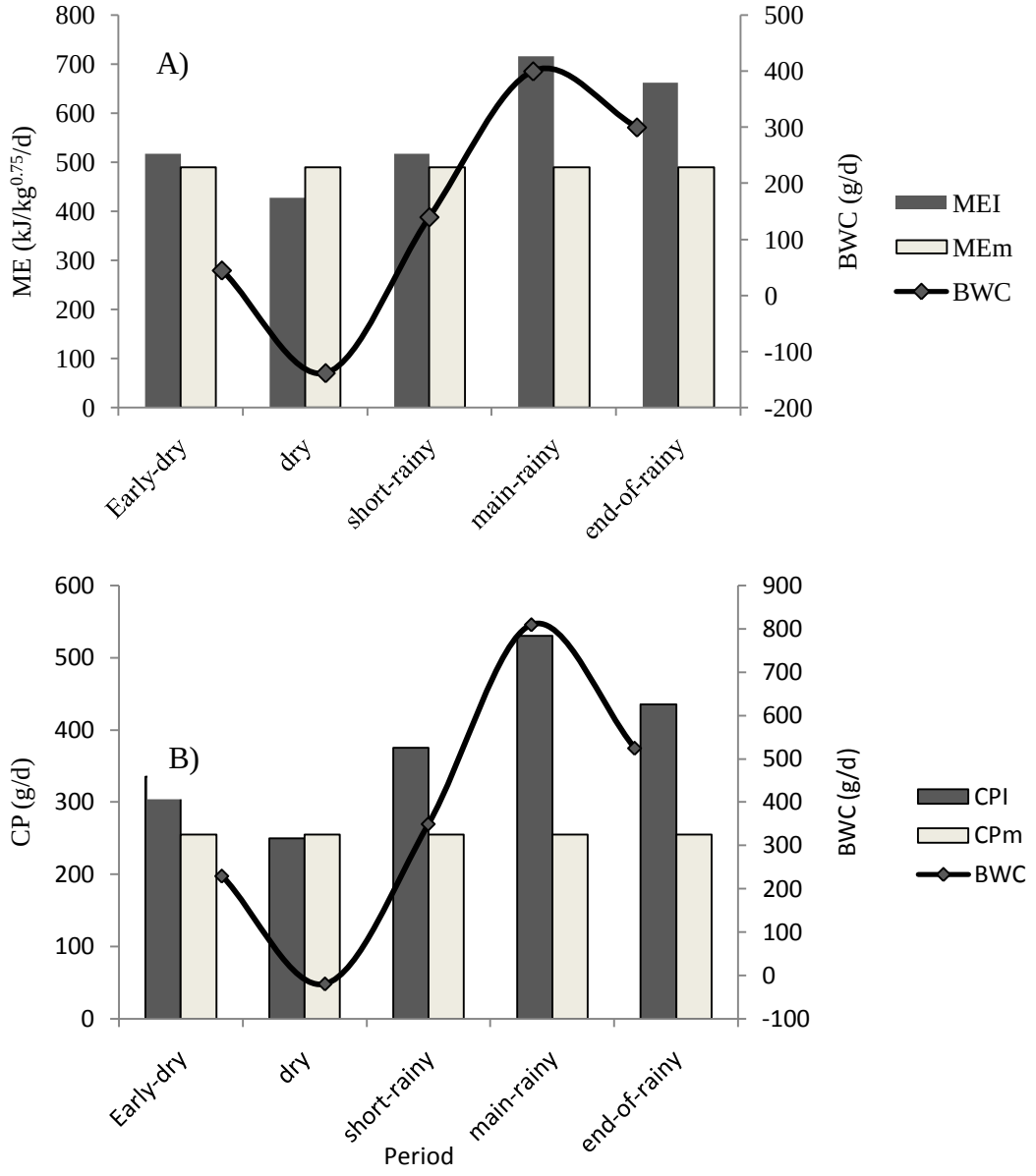

Figure 4 Predicted body weight change (BWC) of cattle based on intake of metabolizable energy (MEI; panel A), or crude protein (CPI; panel B) in reference to maintenance requirement for energy (MEm) or crude protein (CPm).

The quality of diet selected and the level of energy and nutrient intake determined with this method depended greatly on the rainfall pattern, reaching a maximum during the main rainy season and minimum during the dry period (Chapter 5 and 6). As presented in Figure 4,

positive energy and nutrient balances in the wet periods and negative balances in the dry period are evident, implying that acquiring periodic nutrient balance data is important for routine grassland management decisions.

A synchronized supply of energy and nutrients is necessary for efficient utilization of the available nutrients that animals consume. In this respect, an important observation in this thesis is that the pasture quality and availability reached a maximum level at the same time, allowing the increased intake of energy to coincide with increased intake of other nutrients (Chapter 5 and 6). It can be hypothesized that this phenomenon helps the grazing animal to use the energy and nutrient intakes consumed in excess of maintenance optimally. However, the extent to which the excess intakes support animal production varies according to nutrients. For example, energy is more limiting than crude protein for body weight gain during most of the grazing seasons (Chapter 6, Figure 3). In the rainy season, this limitation in energy intake most likely originates from suboptimal organic matter intake (Chapter 5), whereas during subsequent grazing seasons, a combination of reduced intake of organic matter and low quality of pasture contributes for the low energy intake (Chapter 6).

Supplementation of the grazing animal is one of the feeding management options to correct energy and nutrient deficiencies. Because of the cyclic positive (wet periods) and negative (dry period) energy and nutrient balances experienced by grazing animals, the amount, composition and timing of the supplementation should be planned to maximize returns in terms of animal performance. The nutrient balance data presented in Chapter 6 allows for a strategy to be developed where the grazing cattle can be divided into three groups for strategic supplementation; matured non-producing animals, recently weaned/young animals before the age of puberty, and producing animals (lactating cows and draft oxen). Under normal rainfall distribution, the first group of animals (mature non-producing animals) are able to tolerate the nutritional stresses during the dry period and can regain their lost weight during the following wet periods (Chapter 6). If finishing is planned for this group of animals by the use of supplementary feeding, it is advantageous to supply this with the onset of the wet period to maximize the efficiency of feed utilization from compensatory growth. For the second group of animals (recently weaned/young animals), the weight loss sustained during the dry periods may result in stunted growth, and such growth checks before the age of puberty are known to impair the production performance of animals in later life (Everitta and

Jurya, 1977; Coppock and Sovani, 1999; Jelantik *et al.*, 2008). Thus, for this group of animals, supplementation during the dry period appears necessary to avoid severe growth checks, while in the rainy season they can perform satisfactorily on pasture alone. Lactating cows and draft oxen need supplementary feeding during the dry period to maintain expected performances, but supplementary feeding during the rainy season should depend on the level of performance.

The pattern of energy and nutrient intake predicted in Chapter 6 also shows that energy concentrates should form the main components of supplementary feeding during the rainy season, whereas both energy and protein concentrates together with mineral supplements (P, Cu and Na) are necessary in the dry periods. Sodium intake is insufficient in all seasons and routine supplementation at a rate of about 80 g/day/head is required throughout the year. Molasses is a potential energy supplement in the area, while wheat bran and oil seed cakes can be used as sources of energy and protein. The pod of *Acacia tortilis* has a very good potential as a protein and energy supplement (Chapter 5), and proper use of this pod will minimize the cost of supplementary feeding. Moreover, plantation of adapted fodder trees along roadsides and within grazing lands will increase both feed biomass availability and dry season supplementary feeding (Sisay and Baars, 2002; Desta and Oba, 2004) .

Regulating the grazing pressure of the grasslands is another important management intervention to optimize the utilization of the available pasture resource in a sustainable manner (Kemp *et al.*, 2000). High grazing pressure leads to the disappearance of palatable/productive perennial species and low herbaceous basal cover, with a gradual loss of soil fertility and land degradation (Asefa *et al.*, 2003). In the result described in Chapter 6, although nutrient intakes of cattle grazing on the protected ranch and open grasslands were comparable, the absence of desirable species on the open grasslands coupled with very low basal coverage (mainly during the dry period) are indicators of an unsustainable grazing pressure in the communal grasslands. Community-based grassland management practices are, therefore, important to avoid further degradation and promote regeneration of bare lands. Over-sowing with improved forage seeds, planting adapted fodder trees, area enclosures and soil protection terraces are among the feasible intervention measures that can be implemented at a community level. On the other hand, the performance of animals on the protected ranch seems to have been limited by the grazing management practiced, particularly with regard to

the grazing time, grouping of animals, and grazing paddocks. In this respect it may be important to reschedule the grazing time (to include early morning and late afternoon grazing), divide the pasture into smaller paddocks and group animals into manageable sizes, whereby the animals can be allocated to new paddocks in a rotation schedule to efficiently utilize the available pasture resources. These measures will help to increase the performance of the animals reared on commercial ranches in the region in a sustainable manner.

As the use of hand-plucked samples to mimic the herbage selected by the grazing animal remains one of the weak sides of the method used in this study, the sampling method for herbage consumed should be a subject for further improvement. Moreover, with our current level of knowledge, a given n-alkane originating from different plant species, plant parts or different stages of growth/maturity is assumed to have a similar faecal recovery. However, further research is needed to establish if the physical and chemical properties or type of species of herbage consumed have an influence on the faecal recovery of n-alkanes. Such work would enable to examine if dietary indices are available that can be used to predict the faecal recovery of cuticular n-alkanes for a given forage. Another topic worth considering in this respect would be to study if the same n-alkane (carbon chain length) having different molecular weights (due to different level of isotope enrichment) shows similar recovery in the gut.

Conclusions

The use of plant cuticular n-alkanes as markers in nutritional studies has received increasing acceptance over the last two decades due to their wide distribution in the cuticular wax, large interspecies variability, high faecal recovery and ease of laboratory analysis. The evaluation conducted with pasture species in the Mid Rift Valley region in Ethiopia showed that the pasture species have large interspecies variability in their n-alkane profile, which can be used to estimate the diet composition of grazing animals. The variability in the carbon isotope enrichment of the hydrocarbons also provides additional information to increase the accuracy of diet composition estimation using the n-alkane method.

The faecal recovery of synthetic dosed even-chain alkanes (C_{32} and C_{36}) were found to be considerably higher than adjacent natural odd-chain alkanes, implying that correction for

the difference in the faecal alkane recovery rate of dosed and natural alkanes may be required to improve the accuracy of intake estimation using the double n-alkane method.

The available pasture species in the Mid Rift Valley of Ethiopia showed a large variability in their nutritive value, with scope for selection of desirable species for grassland improvement and environmental protection. The quality of the pasture stand in the main pasture growth period was generally of moderate quality, implying that constraints of nutrient intake of grazing cattle during the main rainy season are not likely to occur due to forage quality. *A. tortilis* trees play important ecological functions in the grasslands, and their proper management and utilization will help to optimize cattle production in the region.

The nutritional status of the grazing cattle in the Rift Valley Grasslands as measured using the n-alkane technique and visual observation has a cyclic negative (dry period) and positive (wet period) energy and nutrient balance. Energy intake appears to be more limiting than crude protein for weight gain. While mature animals can cope with the negative energy and nutrient balance during the dry period, young animals before the age of puberty may require supplementary feeding to avoid stunted growth. Supplementary feeding of finishing animals should coincide with the beginning of the rainy period to exploit the advantages of compensatory growth, and energy concentrates should form the major component of such supplementary feeding. Improvement in the management of grasslands and control of illegal cutting of acacia trees is required to optimize the productivity of grazing cattle in a sustainable manner.

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SUMMARY

-

SAMENVATTING

Summary

Grazing ruminants are the largest contributors to livestock output in tropical regions. The production performance of grazing ruminants, within their genetic boundaries, depends on the level of nutrient intake. Acquiring reliable nutrient intake data is, therefore, an important pre-requisite to predict production performance of grazing animals on pasture, and design appropriate feeding and management strategy to achieve target productions. Direct measurement of nutrient intake in grazing animals is difficult and often a variety of indirect methods, including observation of grazing behaviour and application of markers, are used. Over the past two decades, the use of n-alkanes as markers for the measurement of nutrient intake in grazing animals has gained increasing acceptance. Information is however scarce on the suitability of this method to measure intake and diet composition of grazing animals under tropical conditions. Ethiopia has the largest livestock population in Africa and the Mid Rift Valley region in the country supports large grazing livestock populations. Improving the nutrition of the grazing cattle in the region has wider implications in terms of improving the livelihood of the farming communities and supporting the national economy. The first aim of this thesis was to determine the potential of using cuticular n-alkanes and their carbon isotope enrichment ($\delta^{13}C$) as markers to study the nutritional ecology of grazing animals under tropical conditions. In addition, seasonal patterns of nutrient intake and diet composition of grazing cattle in the Mid Rift Valley grasslands of Ethiopia were assessed using this indirect method.

In Chapter 2, commonly available pasture species in the Mid Rift Valley grasslands of Ethiopia were collected during the main pasture growth season and analyzed for n-alkane concentrations and their $\delta^{13}C$ values. The n-alkanes C_{27} to C_{35} were found in adequate concentrations in the species with C_{31} (283±246 mg/kg DM) and C_{33} (149±98 mg/kg DM) being the dominant alkanes. The $\delta^{13}C$ values of the alkanes ranged from -19.37 to -37.40‰. Principal component analysis (PCA) revealed the presence of large variability among the pasture species. Orthogonal procrustes rotation analysis indicated that the majority of the interspecies variability explained by the two data sets was independent of each other. In general, n-alkane profiles of the pasture species in the study region can be used as markers for diet composition estimation and $\delta^{13}C$ values in n-alkane can provide additional information to discriminate diets of grazing animals.

In Chapter 3, the accuracy with which the botanical composition of composite pasture mixtures can be estimated using n-alkanes and their $\delta^{13}C$ values as markers was investigated. Several composite pasture mixtures containing different proportions of five dominant grass species from the Mid Rift Valley rangelands were used. The n-alkane profile and their $\delta^{13}C$ values were determined for individual species and composite pasture mixtures. The botanical composition of composite mixtures was then estimated using least squares optimization procedures and linear programming. Three alternative scenarios were evaluated in the calculation, where 0, 5, or 10 species in addition to those that made up the composite mixtures were included. There was close alignment between estimated and actual botanical compositions. Introduction of additional botanical species reduced the accuracy of the prediction. The best fit equation (R^2=0.996) was obtained when n-alkanes and their $\delta^{13}C$ values were used together and no additional species were included. Increasing the number of markers used or limiting the number of potential diet components (using qualitative methods) is important to improve the quality of diet composition predictions.

Reliable data on faecal recovery of alkanes is required to apply corrections to calculations of diet compositions. In Chapter 4, the faecal recovery rates of n-alkanes and the accuracy of molasses-based alkane boluses for feed intake and digestibility estimations were measured in eight growing bulls consuming low-quality tropical roughages. The experiment was performed in a 4 × 4 double Latin square with each period lasting 21 days (2 wks adaptation and 1 wk measurement period) and animals were assigned to one of four diets. During the last 2 wks of each period animals received 200 mg C_{32} and 150 mg C_{36} twice dailyin the form of molasses-based alkane bolus. The mean faecal recovery of natural and dosed n-alkanes ranged between 0.61 and 0.86, with the recovery showing an upward trend with increasing carbon-chain length. Dosed alkanes had considerably higher recovery than adjacent odd-chain alkanes and correction for the differences in the faecal recovery appeared necessary when intake is predicted using C_{31}/C_{32} and C_{33}/C_{32} ratios. The results showed that molasses-based boluses administered twice daily are suitable, and that knowledge of the faecal recovery rates of adjacent n-alkanes improves the reliability of the predictions.

In Chapter 5, the nutritive value of forage species and environmental impact of their utilizations were evaluated by conducting chemical analysis, *in vitro* total gas and methane production measurements. The forage samples were collected during the main pasture growth

season at approximately similar stage of growth. Large variability was observed among the species in their nutritive value and *in vitro* methane production potentials, with an opportunity for selecting species with high nutritive value and low methane production potential for a sustainable grassland improvement. The pasture quality during the main growth season was evaluated as optimum, and any nutritional limitation in the Mid Rift Valley region during this period is likely to originate primarily due to the quantity rather than the quality of the forage consumed.

Chapter 6 aimed to determine the nutritional status of cattle grazing in the Mid Rift Valley region using a combination of n-alkanes and their $\delta^{13}C$ values as markers and visual observations. For this study, two research sites (a moderately-grazed ranch and heavily-grazed communal grassland) and 16 experimental animals (8 on each site) were used. Measurements of above ground herbaceous biomass, diet composition, nutrient intake, and body condition of grazing cattle were conducted during five periods for one year. The estimated biomass cover, the species diversity of the diets, the dry matter and nutrient intakes of the animals showed high seasonal variation following the rainfall pattern, reaching a maximum during the rainy season and minimum during the dry period. In the dry period the animals were in a negative energy and nutrient balance (with a predicted weight loss of about 110 g/d), whereas in the main rainy season, energy and nutrient intakes were in excess of maintenance (with a predicted daily weight gains of 500-800 g/d). Energy intake was more limiting than crude protein for body weight gain in most of the seasons. Na, P and Cu were limiting for one or more of the grazing seasons. It appears that the nutritional restriction experienced during the dry period was tolerable by mature and non-producing animals but may not be so for young animals before the age of puberty, for which supplementary feeding may be required. The body condition of the animals recorded over the measurement periods agreed well with the predicted performances.

Alkane profiles of pasture species in the Mid Rift Valley of Ethiopia are suitable for use as nutritional markers. Accuracy of diet composition can be improved when used in combination with their $\delta^{13}C$ values. The faecal n-alkane recovery of the tropical roughage feeds studied in this thesis tended to be lower than temperate forages; further investigation into the underlying factors affecting the faecal recovery rate of alkanes will improve the accuracy with which nutrient intake is predicted

Samenvatting

Grazende runderen geven de grootste bijdrage aan het totaal van de dierlijke productie in de tropische gebieden. Het productieniveau van dit vee wordt, binnen het genetisch potentieel van de dieren, bepaald door het niveau van nutriëntopname. Het verkrijgen van betrouwbare informatie van de nutriëntenopname vormt dan ook een belangrijke randvoorwaarde voor het correct schatten van het productieniveau van weidend vee. Tevens is het van belang voor het ontwerpen van een doelmatig voer- en managementsysteem waarmee de gestelde productiedoelen behaald kunnen worden. Het direct meten van nutriëntopname door grazend vee is lastig en veelal worden verschillende indirecte methoden gebruikt zoals het observeren van het graasgedrag en het gebruikmaken van markeerstoffen. Gedurende de laatste twee decennia is het gebruik van n-alkanen als markeerstof voor het schatten van de gewasopname en nutriëntsamenstelling bij grazend vee als methode ontwikkeld en heeft een geaccepteerde status verworven. Er is echter slechts beperkte informatie beschikbaar over de geschiktheid van deze methode bij grazend vee welke onder tropische omstandigheden worden gehouden. Ethiopië is in Afrika het land met de grootste veepopulatieen hiervan wordt een grote populatie van het grazend vee gehouden in de Mid Rift Valley regio. Het verbeteren van de nutritionele status van het grazende vee in deze regio zal het levensonderhoud van de lokale boerengemeenschappen verbeteren evenals de nationale economie. Het eerste doel van het promotieonderzoek was om vast te stellen of het gebruik van cuticulairen-alkanen in combinatie met hun verrijkingsniveau van het stabiele isotoop van koolstof ($\delta^{13}C$) als markeerstoffen gebruikt kunnen worden om de nutritionele status van grazende dieren gehouden onder tropische omstandigheden te bestuderen. Daarnaast is de rantsoensamenstelling en nutriëntopname bij grazend vee in de Mid Rift Valley regio van Ethiopië in kaart gebracht met deze methode. Hierbij is eveneens gekeken naar de invloed van seizoen op de samenstelling en opname.

Voor Hoofdstuk 2 zijn de meest voorkomende grassoorten in het graasland van de Mid Rift Valley regio verzameld gedurende het hoofd-groeiseizoenen vervolgens geanalyseerd op n-alkaan samenstelling en de $\delta^{13}C$-verrijking van de alkanen. De n-alkanen C_{27} tot en met C_{35} werden in adequate hoeveelheden teruggevonden voor alle plantsoorten, waarbij C_{31} (283±246 mg/kg DM) en C_{33} (149±98 mg/kg DM) het meest duidelijk aanwezig waren. De $\delta^{13}C$-verrijking van alkanen varieerde van -19.37‰ tot -37.40‰. Data-analyse van n-alkaan

en δ^{13}C-waarden met behulp van een principale-componentenanalyse (PCA) liet een grote variatie zien tussen plantsoorten. Een orthogonal procrustus rotatie-analyse gaf aan dat de het grootste aandeel van de verklaarde tussensoorten-variabiliteit op basis van de twee datasets onafhankelijk van elkaar waren. Er werd dan ook geconcludeerd dat de n-alkaan profielen van plantsoorten in het studiegebied gebruikt kunnen worden als markeerstof om rantsoensamenstelling the schatten, en dat de δ^{13}C-verrijking in n-alkanen additionele informatie geeft om rantsoensamenstelling van grazende dieren te onderscheiden.

In Hoofdstuk 3 is onderzocht met welke nauwkeurigheid de botanische compositie van handmatig samengestelde graslandmengsels kan worden geschat aan de hand van de n-alkaan concentraties en hun δ^{13}C-verrijkingswaarden. Hiervoor werden een aantal graslandmengsels handmatig samengesteld uit de vijf meest voorkomende grassoorten in de Mid Rift Valley regio waarbij de grassen per mengsel in verschillende proporties werden toegevoegd. De n-alkaan samenstelling en de δ^{13}C-alkaanverrijking werden bepaald voor de individuele grassoorten en de samengestelde mengsels. De botanische samenstelling van de mengsels werd vervolgens geschat met behulp van een kleinste kwadraten optimalisatie procedure en lineaire programmering. Vervolgens werden drie alternatieve scenario's geëvalueerd met deze procedure. Hiervoor werden 0, 5 of 10 plantsoorten toegevoegd aan de 5 soorten waaruit de mengsels waren samengesteld.

Er werd een sterke overeenkomst gevonden tussen de geschatte en de feitelijke botanische samenstellingen. Wel bleek dat toevoeging van additionele grassoorten resulteerde in een verminderde nauwkeurigheid van de schattingen. De best schattende vergelijking (R^2=0.996) werd verkregen als n-alkanen gebruikt werden in combinatie met de δ^{13}C-verrijkingswaarden en zonder toevoeging van additionele plantsoorten. De conclusie uit dit onderzoek was dat het verhogen van het aantal markeerstoffen of het beperken van het aantal betrokken plantsoorten (door kwalitatieve methoden) belangrijk is in het verhogen van de nauwkeurigheid van het schatten van de rantsoensamenstelling.

Betrouwbare informatie over de faecale terugwinning van alkanen is benodigd om correcties door te voeren bij de berekening van de rantsoensamenstelling. Daarnaast is het belangrijk om even en oneven alkaan-paren te selecteren die een vergelijkbaar faecaal terugwinningspercentage hebben, ofwel een bekend verschil in terugwinning hebben, zodat correcties kunnen worden gemaakt. In Hoofdstuk 4 is een *in vivo* studie uitgevoerd waarbij de

faecale terugwinning van n-alkanen is vastgesteld bij acht stieren gehouden op rantsoenen van laagwaardige tropische grassen. Daarnaast werd de nauwkeurigheid van het schatten van de opname en rantsoensamenstelling met behulp van alkaan-houdende molasse bolussen vastgesteld. De dierproef werd uitgevoerd als een herhaalde 4 × 4 Latijnsvierkant waarbij iedere periode 21 dagen duurde (twee week adaptatie gevolgd door een meetweek). Dieren werden aan één van de vier proefrantsoenen toegekend. Vanaf de tweede week van adaptatie ontvingen de dieren tweemaal daags een alkaan-houdende melasse bolus (elk 200 mg C_{32} en 150 mg C_{36}) tot aan het einde van de meetweek.
De gemiddelde faecale terugwinning van de natuurlijke en de gedoseerde synthetische alkanen varieerde tussen 0.61 to 0.86, waarbij de terugwinning een opgaande tendens liet zien met een toenemende koolstof ketenlengte. De gedoseerde alkanen hadden een beduidend hogere terugwinning dan de naastliggende oneven alkanen. Dit betekent dat een correctie voor het verschil in faecale terugwinning aan de hand van de C_{31}/C_{32} en C_{33}/C_{32} ratio's noodzaak is voor het correct schatten van de opname. De resultaten lieten zien dat het tweemaal daags verstrekken van alkaan-houdende melasse bolussen een geschikte methode is om opname te schatten, en dat het corrigeren voor verschil in faecale terugwinning de betrouwbaarheid van de opnameschattingen verhoogt.

In Hoofdstuk 5 is de nutritionele waarde van plantsoorten en de effecten op het milieu onderzocht aan de hand van chemische analyses in combinatie met een *in vitro* gas en methaan productie studie. De plantmonsters werden verzameld tijdens de hoofd-groeiperiode bij vergelijkbare groeistadia. Er was een grote variatie in de nutritionele waarde en *in vitro* methaanproductie tussen planten. Dit biedt perspectief om te selecteren op plantensoorten met een hoge nutritionele waarde en een laag methaanproductie potentieel, waarmee de duurzaamheid van graslandaanwending verbeterd kan worden. De kwaliteit van de grassen tijdens het hoofd-groeiseizoen werd als matig bevonden. De nutritionele limitaties in de Mid Rift Valley regio tijdens deze periode kunnen dan ook voornamelijk toegeschreven worden aan kwantitatieve beperkingen en niet zozeer aan de kwaliteit van het opgenomen ruwvoer.

Hoofdstuk 6 beschrijft een studie waar de nutritionele status van grazend vee in de Mid Rift Valley regio gedurende het jaar is bepaald door middel van de nieuw ontwikkelde n-alkanen en de δ^{13}C-verrijking methode in combinatie met visuele waarnemingen. De studie werd uitgevoerd op twee onderzoek locaties (gematigd begraasd land *vs.* intensief begraasd

gemeenschappelijk land) met op elke locatie acht dieren. De bovengrondse biomassa, rantsoensamenstelling, nutriëntopname en conditiescore van dieren werd, verdeeld over vijf perioden, gedurende een jaar gemonitord.

De geschatte bovengrondse biomassa, de soorten diversiteit in het rantsoen, de droge stof en nutriëntenopname van dieren liet een grote seizoenvariatie zien gerelateerd aan het regenvalpatroon, waarbij het maximum bereikt werd tijdens het regenseizoen en het minimum tijdens de droge periode. Tijdens de droge periode waren de dieren in negatieve energie- en nutriëntenbalans (met een geschat gewichtsverlies van ~110 g/d). Gedurende de belangrijkste regenperiode echter lag de energie- en nutriëntenopname boven onderhoud (met een geschatte dagelijkse groei van 500-800 g/d). De energieopname bleek meer limiterend te zijn dan de eiwitopname voor groei gedurende het grootste deel van het seizoen. Na, P, en Cu waren één of meerdere keren limiterend tijdens het graasseizoen. De nutritionele beperkingen die ondervonden werden tijdens de droge periode werden verdragen door de volwassen niet-producerende dieren. Echter, het kan zijn dat jonge onvolwassen dieren wel gevoelig zijn voor deze beperkingen en waarvoor mogelijk aanvullende voedingmiddelen nodig is. De conditie score van de dieren liet een goed verband zien met de geschatte producties.

Alkaanprofielen van plantensoorten in grasland in de Mid Rift Valley regio in Ethiopië kunnen gebruikt worden als markeerstof voor rantsoenbepaling. De nauwkeurigheid van rantsoensamenstelling kan worden verbeterd als de n-alkaan methode gecombineerd wordt met δ^{13}C-verrijkingdata. De faecale terugwinningcoëfficiënten van de tropische grassen opgenomen in het huidige onderzoek neigden naar lagere waarden ten opzichte van ruwvoeders uit gematigde klimaatstreken. Verder onderzoek naar de achterliggende factoren die van invloed zijn op de faecale terugwinning van alkanen zal de nauwkeurigheid van nutriëntopnameschatting verbeteren.

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CURRICULUM VITAE

About the Author

Melkamu Bezabih Derseh was born on November 24, 1973 in Gojjam, Ethiopia. He attended elementary and high school education at his birthplace. From 1991 to 1995 he studied at Addis Ababa University-Awassa College of Agriculture and obtained a BSc degree in Animal Production and Rangeland Management with great distinction. Afterwards, he worked as a graduate assistant at Awassa College of Agriculture for two years. In 1998 he was awarded a two-year scholarship by the German Catholic Academic Exchange Service to pursue a graduate study at Bonn University, where he obtained MSc degree specializing in Animal Nutrition. His MSc thesis was about the efficiency of energy and nutrient utilizations in pre-ruminant goat kids. He returned back to Ethiopia and worked as a lecturer at Hawassa University, College of Agriculture, during which he was involved in teaching, research and outreach activities. He also handled extra assignments as associate dean of the School of Graduate Studies, and research coordinator at Hawassa University. In 2008, he joined Wageningen University to conduct a PhD study on a sandwich basis with financial support from Wageningen Institute of Animal Sciences, the Netherlands Organization for International Cooperation in Higher Education, and the Ministry of Education of Ethiopia. The results of his study are described in this thesis. During his PhD study he investigated the potential of cuticular n-alkanes and their carbon isotope enrichments as markers to estimate diet composition and nutrient intake of grazing cattle in tropical grasslands.

Publications

Refereed scientific publications

Bezabih, M. and Pfeffer, E., 2003. Body chemical composition and efficiency of energy and nutrient utilization by growing pre-ruminant Saanen goat kids. *Animal Science, 77:155-163.*

Bezabih, M., Pellikaan, W.F., Tolera, A. and Hendriks, W.H., 2011. Evaluation of n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as diet composition markers. *Animal, 5:57-66.*

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Bezabih, M., Pellikaan, W.F., Tolera, A. and Hendriks, W.H., 2012. Estimation of feed intake and digestibility in cattle consuming low-quality tropical roughage diets using molasses-based n-alkane boluses. *Animal Feed Science and Technology 177:161-171.*

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Khan, M.T, Khan, N.A., Bezabih, M., Qureshi, M.S., and Rahman, A., 2012. The nutritional value of peanut hay (*Arachis hypogaea L.*) as an alternate forage source for sheep. *Tropical Animal Health and Production, DOI 10.1007/s11250-012-0297-8.*

Habib, G., Khan, N.A., Ali, M. and Bezabih, M., 2012. *In situ* ruminal crude protein degradability of cereal grains, oilseeds and animal by-product protein feedstuffs *Livestock Science (Accepted)*

Bezabih, M., Pellikaan, W.F., Tolera, A. and Hendriks, W.H., 2012. Nutritional status of grazing cattle in the Mid Rift Valley grasslands of Ethiopia measured by use of cuticular hydrocarbons and their isotope enrichments (submitted)

Contributions to conferences and symposia

Bezabih, M., Pellikaan, W.F., and Hendriks, W.H., 2010. Use of a combination of n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as diet composition markers. In: proceedings of 61st Annual Meetings of the European Association for Animal Production, Crete, Greece, pp 29

Bezabih, M., Pellikaan, W.F., Tolera, A. and Hendriks, W.H., 2011. Evaluation of n-alkanes and their carbon isotope enrichments as diet composition markers. In: proceedings of 37th Animal Nutrition Research Forum, pp 43

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Bezabih, M., Pellikaan, W.F., Tolera, A. and Hendriks, W.H., 2011. Estimation of intake and digestibility in cattle consuming low quality roughage diets using molasses-based alkane boluses. In: proceedings of 8th International Symposium on the Nutrition of Herbivores, Aberystwyth, Wales, pp 458

Training and supervision plan			
Name	Melkamu Bezabih Derseh		
Group	Animal Nutrition Group		
Daily supervisors	Dr. W.F. Pellikaan and Prof. A. Tolera		
Supervisor	Prof. dr.ir. W.H. Hendriks		
The Basic Package		year	credits *
WIAS Introduction Course		2008	1.5
Philosophy of Science and Ethics		2008	1.5
International conferences			
Annual Nutrition Research (ANR) Forum, Leuven, Belgium		2011	0.3
Annual meetings of the European Association for Animal Production, Crete, Greece		2010	1.5
International Conference on the Nutrition of Herbivors, Aberystwyth, Wales, UK		2011	1.2
Seminars and workshops			
WIAS Seminar "Use of biomass: food, feed or fuel: Stakeholder Vision"		2007	0.2
WIAS Science day		2008	0.3
International summar school on Environment and Resource Protection		2008	1.5
Annual Research Review Workshop, Hawassa Univerisity, Ethiopia		2009	0.6
Presentations			
Poster EAAP conference, Crete, Greece		2010	1.0
Oral Anual Nutrition Research Forum, Leuven, Belgium		2011	1.0
Poster Anual Nutrition Research Forum, Leuven, Belgium		2011	1.0
Poster ISNH8, Aberystwyth, Wales, UK		2011	1.0
Disciplinary and interdisciplinary courses			
Interdisiplinary research: a crucial knowledge gap		2011	0.9
Modelling course		2008	2.0
Advanced statistics courses			
Design of Animal Experiments		2008	1.0
Statistics for Life Sciences		2009	2.0
Multivariate Analysis		2009	1.5
MSc level courses			
Animal Nutrition and Physiology (period 3)		2008	6.0
Professional Skills Support Courses			
Techniques for writing and presenting a scientific paper		2008	1.2
PhD Competence assessment		2008	0.3
Information literacy, including introduction Endnote		2009	1.0
Scientific Writing		2011	1.8
Wiriting proposition and the general section of thesis		2011	0.5
Research Skills Training			
Preparing own PhD research proposal		2007	6.0
Didactic Skills Training			
Handling undergraduate courses, Hawassa University		2010	6.0
Review of Research Master Cluster proposals		2011	0.5
Supervising BSc thesis work, Hawassa University (3 theses)		2009/10	3.0
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