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Carbon flow from pasture to milk – Carbon isotope analysis as a tool of authenticity testing and proof of origin for pasture-based milk production

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Contents

Abstract

Evermore consumers are interested in the authenticity and origin of foodstuff, in particular of meat and milk. Stable isotope analysis is a valuable tool for origin assignment. There is a measurable offset (discrimination) between diet and product in stable isotope composition due to physical and biochemical processes. The application of stable isotope analysis to infer the diet from the stable isotope composition of an animal's product relies on knowledge of isotopic discrimination during digestion and metabolism. The objective of this research was to quantify the discrimination of ^{13}C ($^{13}\Delta$) between diet, faeces, milk and milk components of dairy cows in stall and on pasture. In so doing, three prerequisites are necessary: (1) the composition of the feeding ration used to determine discrimination should not have a negative impact on the animal performance and health because both could affect discrimination; (2) the digestibility of diet organic matter must be known to calculate the carbon isotopic composition of the resorbed nutrients of the diet, and (3) the elution kinetic must be known to exclude that old body carbon with differing isotopic composition influences the apparent discrimination.

To determine the isotope composition of diet, faeces, milk and milk components (fat, casein, lactose), four experiments were conducted during two years. In all experiments, lactating dairy cows grazing an all-day pasture with continuous stocking were supplemented with grain maize as isotopic marker. Different amounts of grain maize were fed to grazing dairy cows and the influence on animal performance and health was examined. In a second experiment the digestibility of organic matter of pasture herbage was estimated using eight different methods. The third experiment examined the elution kinetic of grain maize in faeces, milk and milk components after cessation of grain maize supplementation to grazing. Finally, in the fourth experiment the isotopic discrimination of ^{13}C between diet, faeces, milk and milk components was determined from 232 measurements of these materials during a 61 day long period, during which the cows were kept close to isotopic and metabolic flow equilibrium.

It was found that (1) different amounts of supplemented grain maize (1 kg vs. 3 kg per cow per day) had no influence on animal performance and health; (2) the digestibility of organic matter of pasture herbage was similar to that of grain maize, and (3) grain maize feeding could be detected in faeces, milk and milk components only for three days or less when changing to pure grazing.

Overall, all three prerequisites were fulfilled. Concerning these results, discrimination as determined in this study should be near to true discrimination. Compared to the diet, there was a depletion of 0.4 ‰ in whole milk caused by the strong depletion in milk fat ($^{13}\Delta$ = 2.2 ‰), which was not fully compensated by the enrichment in casein ($^{13}\Delta$ = −1.1 ‰) and lactose ($^{13}\Delta$ = −0.7 ‰). Faeces also were depleted ($^{13}\Delta$ = 1.7 ‰).

The present work contributes to the use of stable isotope analysis as a tool of authenticity testing and proof of origin, which must be based on a profound understanding of isotope metabolism. Carbon isotope analyses can only falsify a predicated feeding regime but it cannot verify it in the case of agreement. Thus, stable carbon isotope analysis delivers valuable information that complements other investigations for authenticity testing and proof of origin of milk.

Zusammenfassung

Eine zunehmende Anzahl von Verbrauchern interessiert sich für die Herkunft von Lebensmitteln und die Korrektheit der Produktionsangaben, insbesondere von Fleisch- und Milchprodukten. Die Analyse der stabilen Isotopen kann zur Authentizitäts- und Herkunftssicherung eingesetzt werden, da die isotopische Zusammensetzung der tierischen Produkte streng von der des Futters abhängt. Allerdings besteht zwischen beiden aufgrund physikalischer und biochemischer Prozesse bei der Verdauung ein messbarer Unterschied (Diskriminierung). Um ausgehend von der isotopischen Zusammensetzung tierischer Produkte die Isotopie des Futters mithilfe der Stabilisotopenanalyse abschätzen zu können, muss die Diskriminierung während der Verdauung und Verstoffwechselung der Nährstoffe des Futters bekannt sein. Ziel dieser Arbeit war es, die Diskriminierung von ^{13}C ($^{13}\Delta$) zwischen Futter, Kot, Milch und den einzelnen Milchbestandteilen von weidenden und im Stall gehaltenen Milchkühen zu bestimmen. Zur Bestimmung der Diskriminierung müssen drei Grundvoraussetzungen erfüllt sein: (1) Die Rationszusammensetzung darf keinen negativen Einfluss auf die Leistung und Tiergesundheit haben, da beides die Diskriminierung beeinflussen könnte; (2) die Verdaulichkeit der organischen Masse der Ration muss bekannt sein, um die isotopische Zusammensetzung der resorbierten Nährstoffe ermitteln zu können und (3) die Auswaschkinetik muss bekannt sein, um auszuschließen, dass im Körper gebundener (alter) Kohlenstoff die scheinbare Diskriminierung beeinflusst.

Mit Versuchen, die sich über zwei Jahre erstreckten, wurden die Voraussetzungen überprüft und anschließend die isotopische Zusammensetzung des Futters, des Kotes, der Milch und der Milchbestandteile (Milchfett, Kasein und Laktose) bestimmt. Dazu wurden laktierende Milchkühe auf einer Kurzrasenweide gehalten und geschroteter Körnermais als isotopischer Marker zugefüttert. Es wurden unterschiedliche Mengen an Körnermais zugefüttert, um den Einfluss auf die Leistung, Tiergesundheit und die Mobilisierung von Körperreserven zu überprüfen. In einem zweiten Versuch wurde die Verdaulichkeit der organischen Masse des Kurzrasenweidegrases mit acht verschiedenen Methoden ermittelt. Im dritten Versuch wurde die Auswaschkinetik Körnermais-bürtigen Kohlenstoffs im Kot, der Milch und den Milchbestandteilen nach Beendigung der Körnermaiszufütterung bestimmt. In einem vierten Versuch wurde schließlich die Diskriminierung von ^{13}C zwischen Futter, Kot, Milch und Milchbestandteilen anhand von 232 Messungen während einer Versuchsdauer von 61 Tagen bestimmt. Während dieses Versuches wurden die Kühe nahe am isotopischen Gleichgewicht und in einer ausgeglichenen Stoffwechsellage gehalten.

Es zeigte sich: (1) Unterschiedliche Tagesmengen an Körnermais (1 kg vs. 3 kg) zeigten keinen Einfluss auf die tierischen Leistungen und die Tiergesundheit; (2) die Verdaulichkeit der organischen Masse des Kurzrasenweidegrases war ähnlich der Verdaulichkeit des Körnermaises und (3) Körnermais-bürtiger Kohlenstoff kann in Kot, Milch und den Milchbestandteilen nur drei Tage oder weniger nachgewiesen werden, wenn die Maisfütterung eingestellt wird. Da die drei oben genannten Voraussetzungen erfüllt waren, sollten die Ergebnisse der Diskriminierung nahe an der wahren Diskriminierung liegen. Verglichen mit dem Futter, war die Milch um 0.4 ‰ abgereichert, was sich durch die starke Abreicherung des Milchfettes ($^{13}\Delta$ = 2.2 ‰) erklärte, welche nicht vollständig durch die Anreicherung von Kasein ($^{13}\Delta$ = −1.1 ‰) und der Laktose ($^{13}\Delta$ = −0.7 ‰) kompensiert wurde. Auch der Kot war an ^{13}C abgereichert ($^{13}\Delta$ = 1.7 ‰).

Die vorliegende Arbeit leistet einen Beitrag zur Authentizitätsprüfung und Herkunftssicherung des Lebensmittels Milch mittels der Stabilisotopenanalyse, da die hierfür benötigten Diskriminierungen bestimmt wurden. Allerdings kann ein vorgegebenes Fütterungsregime durch die Analyse der Kohlenstoffisotopie nur falsifiziert, aber nicht eindeutig verifiziert werden. Die Analyse der Kohlenstoffisotopie liefert aber eine wertvolle Information, welche andere Methoden zur Authentizitäts- und Herkunftssicherung von Milch ergänzt.

Abbreviations

ADF	acid detergent fiber
ASAT	aspartate-aminotransferase
BCS	body condition score
BFT	back fat thickness
CP	crude protein
DM	dry matter
DMI	dry matter intake
ECM	energy-corrected milk
GLDH	glutamate-dehydrogenase
ME	metabolizable energy
OMD	organic matter digestibility
RNB	ruminal nitrogen balance

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Chapter I: General Introduction

The interest of consumers in authenticity testing and proof of origin of food is increasing (Rossmann *et al.,* 2000; Rossmann, 2001; Bahar *et al.,* 2005; Pillonel *et al.,* 2005; Reid *et al.,* 2006; Luykx and Van Ruth*,* 2008; Monahan *et al.,* 2010). It is particularly high for animal products like meat or milk (Baumont *et al.,* 2000; Piasentier *et al.,* 2003; Boner and Förstel, 2004; Karoui and De Baerdemaeker, 2007). In addition to the quality, the origin and authenticity of food define the price of a product. Milk and especially cheese of certain regions like "Allgäuer Bergkäse" in Bavaria, for example, advertise their products with the origin and advanced production regulations. The trade name "Allgäuer Bergkäse" is associated with grazing cows in the Alps. This husbandry condition is looked upon as the natural and most appropriate to the cows. Some of the consumers are willing to pay higher prices for the origin and this husbandry (Pillonel *et al.,* 2005; Lobsiger *et al.,* 2010). The trade name is protected and certificated ("protected designation of origin") (Council Regulation (EC) 510/2006). This protection is important for the producers and consumers, but the higher price for this product is seducing for fraud (Rossmann *et al.,* 2000; Camin *et al.,* 2004). In this context, stable isotope analysis is gaining more importance in official food control to proof origin, to verify authenticity and to avoid mislabelling of animal products (Pillonel *et al.,* 2005).

Most of human food, as well as milk and cheese, consist of complex mixtures of organic substances, minerals and water. Biomass of food is built of following elements: hydrogen, nitrogen, carbon, oxygen and sulphur. Stable isotopes occur naturally in all these bioelements. These elements have one overwhelmingly abundant isotope and one or more isotopes of low abundance (Crittenden *et al.*, 2007). Their concentrations are given as relative differences to international standards as δ-values (Brand and Coplen, 2012).

In Europe, diet of dairy cows (*Bos taurus*) normally mainly contains fresh herbage, grass silage, hay, maize silage and concentrates (Givens and Rulquin, 2004). Herbage and products of herbage (hay, grass silage) consist of C_3 plants. In contrast, maize is a C_4 plant. C_3 and C_4 plants use different metabolic pathways of photosynthesis. As a consequence of these different pathways, C_3 plants discriminate more against the heavy stable isotope (^{13}C) than C_4 plants (Craig, 1954; Smith and Epstein, 1971). This effect of photosynthesis is imprinted in plant carbon and causes characteristic $\delta^{13}C$ values for C_3 and C_4 biomass. These differences are forwarded along the trophic chain (Hesslein *et al.,* 1991; Codron *et al.,* 2007; De Visser *et al.,* 2008). In case that agricultural production systems differ in their use of C_3 and C_4 derived biomass, analysis of $\delta^{13}C$ can be used to identify the production system from the product. For example, Boner and Förstel (2004) found that beef produced in organic farming often has more negative $\delta^{13}C$ values than conventional produced beef. The reason is

that organic and conventional farms typically use different feed rations. Conventional farms often use maize silage (C_4) as the main fodder component for bulls, whereas on organic cattle farms the bulls mostly are fed on herbage or herbage products (C_3) as roughage (Boner and Förstel, 2004; Bahar *et al.,* 2008) because the ban of herbicides aggravates the organic cultivation of maize. Thus, the carbon stable isotope composition of beef can be used to distinguish between maize and herbage fed bulls, and – with some restrictions – even between conventionally and organically produced beef (Bahar *et al.,* 2005; Bahar *et al.,* 2008).

Along the same line stable isotope composition of animal products can also be used to proof the authenticity and origin of animal products given that the production areas differ significantly in the isotopic composition of the bioelements (Boutton *et al.*, 1988; Metges *et al.*, 1990; Kornexl *et al.*, 1997). This is based on the famous paradigm "You are what you eat (plus a few per ‰)" (DeNiro and Epstein, 1976). This "plus a few per ‰" describes that there is a measurable offset between the $\delta^{13}C$ of the diet and that of the product due to physical and biochemical processes in the animal. The product-specific discrimination must be known when using stable isotope analysis to infer the diet $\delta^{13}C$ from that of an animal's product (McCutchan *et al.*, 2003; Wittmer *et al.*, 2010; Martínez del Rio and Carleton, 2012). The smaller the differences in isotopic composition between products of different origin need to be detected and the higher the legal requirements for the detection of fraud are, the more precise the "few per mill" need to be known. These "few per mill" differ among different products like milk, meat or visceral organs. In addition, the discrimination of commercially irrelevant animal products like teeth, hair, breath, urine or faeces needs to be known (Ayliffe *et al.,* 2004; Sponheimer *et al.,* 2006; Appenzeller *et al.,* 2007) because some of these products can be obtained from living animals and as these products differ in their turnover times, they provide an isotope clock (Guelinckx *et al.,* 2008; Woodland *et al.,* 2012), that allows to gain information on the diet during different time spans.

There is always a construction and depletion of body material, however, each tissue has a different turnover rate (Gannes *et al.,* 1998; Ayliffe *et al.,* 2004). When the diet of a dairy cow changes the new diet is gradually incorporated in the animal tissues. However, true isotopic discrimination requires isotopic and energetic flow equilibrium conditions. Otherwise old body pools that reflect a previous, different diet still contribute significantly to the product and cause the apparent discrimination to vary over time until a constant equilibrium is reached (McCutchan *et al.,* 2003; Auerswald *et al.,* 2010). Hence, it is important to achieve such equilibrium conditions and to know the time span after a diet change that is necessary for equilibration (De Smet *et al.,* 2004). So the elution kinetic of maize derived ^{13}C is necessary

to determine $\delta^{13}C$ discrimination between diet and animal products like faeces, milk and milk components. For example, body proteins in heifers required more than 167 respectively 230 days to achieve a new isotopic equilibrium (Bahar *et al.*, 2005; Gebbing *et al.,* 2004) while one week was apparently sufficient to reach a new equilibrium in milk carbon and nitrogen isotope composition of dairy cows following a change of diet isotope composition (Wilson *et al.*, 1988; Knobbe *et al.*, 2006).

In literature, several studies with dairy cows can be found from which discriminations can be derived (Minson *et al.*, 1975; Tyrrell *et al*., 1984; Boutton *et al.*, 1988; Wilson *et al.,* 1988; Metges *et al.*, 1990; Schulze *et al.,* 1992; Masud *et al.,* 1999; Knobbe *et al.,* 2006; Camin *et al*., 2008), but only few of them specifically aimed to determine discrimination between diet and product. However, these experiments either used short equilibrium periods (Metges *et al.*, 1990; Schulze *et al.*, 1992; Masud *et al.*, 1999), only one or few cows (Minson *et al.*, 1975; Masud *et al.*, 1999) or bulk milk samples without knowing exactly the carbon isotope composition of the ingested feed (Camin *et al.*, 2008). Furthermore, no study delivered a consistent set of discrimination among diet, faeces, milk and milk components, and only one of these studies applies for grazing cows (Minson *et al.*, 1975).

The objective of this research was to describe the isotopic discrimination of $\delta^{13}C$ between diet, faeces, milk and milk components of cows in stall and on pasture. The present work contributes to the use of stable isotope analysis to proof of origin and authenticity testing which must be based on a profound understanding of isotope metabolism.

Four experiments were conducted over two years. In all experiments lactating dairy cows grazing an all-day pasture with continuous stocking were supplemented with grain maize. The grain maize served on the one hand as natural isotopic marker (maize as C_4 plant) and as energetic supplement to the protein-rich pasture herbage. In the first experiment the influence of different amounts of grain maize supplementation (1 kg vs. 3 kg fresh matter per day) to the animal performance and animal health of dairy cows grazing an all-day pasture with continuous stocking was tested. It has to be excluded that a change in maize supplementation causes a major change in the metabolic status or in health of the cows (Bowtell *et al.,* 1998; Carleton and Martínez del Rio, 2005; Fuller *et al.,* 2005) because both could affect the discrimination. The experimental breadboard and the results can be seen in Chapter II.

In the second experiment the digestibility of organic matter (OMD) of pasture herbage was estimated using eight different methods. The OMD is important for the interpretation of the

results of stable isotope analysis because it determines proportion of the different components in the diet that are digested and can be found in milk and milk components, whereas the indigestible proportions will emerge in faeces (Jones *et al.,* 1979). From milk and milk components the proportion of digested diet components can be calculated. To determine discrimination between diet and animal products, the digestibility of marker and pasture herbage must be known. The results of this experiment are already published (Schneider *et al.,* 2011, inclusion in a thesis or dissertation as full text allowed) and can be found in the Appendix.

In this experiment additionally the elution kinetic of grain maize derived ^{13}C was examined. Knowing the elution kinetic is a prerequisite for using discriminations to proof the origin and authenticity of a product. The time span of elution kinetic of grain maize in faeces, milk and milk components was established after cessation of grain maize supplementation to grazing. The experimental breadboard and the results are shown in Chapter III.

Furthermore, the stable isotope composition of carbon in grain maize, pasture herbage, milk, milk components and faeces of grazing dairy cows was examined and the isotopic discrimination between the diet and the products was determined. The experimental breadboard and the results are shown in Chapter IV.

Hence, the particular chapters answer questions than can be regarded independent and that require different methodological approaches, even though all are based on grazing experiments. Thus, the chapters are organized separately each with own introduction, material and methods, results and discussion.

Chapter II: Influence of different amounts of grain maize on animal performance and health

ABSTRACT

High intensity all-day pasture provides a diet rich in protein. Supplementation by energy concentrates may have beneficial effects on animal performance and health. A grazing experiment was undertaken to examine the influence of different quantities of daily grain maize supplementation (1 kg vs. 3 kg per cow per day) on the performance and animal health of lactating dairy cows. For the comparison, an all-day pasture with continuous stocking of 2.9 cows/ha was used. The experiment lasted eight weeks during the main vegetation period in 2009. Sixteen grazing Simmental dairy cows, averaging 31 kg/d of milk at the start of the experiment, were allocated to two groups of comparable age and milk yield. Group 1 was supplemented with 1 kg/d of grain maize (GM1) and group 2 with 3 kg/d (GM3). Grain maize was offered twice a day immediately after milking in the stall to ensure a 21 hour long grazing period per day.

Quantity of grain maize had no significant effect on milk yield (26.6 kg vs. 26.8 kg/day energy-corrected milk) or any other examined property (milk fat, milk protein, milk urea, back fat thickness and body condition score). The high digestibility of the pasture herbage and the substitution rate of 1.01 are the reasons for the small difference between the two groups. In conclusion, an enhancement of grain maize supplementation to grazing from 1 kg to 3 kg/day did not improve animal performance and health.

INTRODUCTION

Cow milk is produced by different feeding systems. There are high-input and low-cost strategies. The all-day pasture as a variety of the low-cost strategy is used for milk production in several countries like New Zealand, Ireland, and more recently in Switzerland and Germany (Thomet *et al*., 2002; Dillon, 2006; Steinwidder *et al.,* 2010). The all-day pasture strategy, which aims at reducing labor and feed costs of milk production by increasing the amount of pasture herbage in the ration, is based on the reasoning that pasture herbage delivers a low-cost feed (Gehman *et al*., 2006; Thomet *et al.,* 2004). Hence, the input of maize silage and concentrate feed is tremendously reduced. The genetically possible maximal milk yield of a single cow will not be achieved in an all-day pasture system. Therefore, the aim of this feeding system is not the maximal milk yield per cow, but the maximal milk production per hectare (Thomet *et al.,* 2002; Bargo *et al.,* 2003; Dillon, 2006). In particular, the productivity per area and per labor within an optimized grazing system is remarkable (Thomet *et al*., 2004).

Farmers in Germany, who begin or return to a grazing-based milk production system, often choose the system of all-day pasture with continuous stocking (in German: ”Kurzrasenweide”). The main difference of this grazing system to extensive continuous grazing systems, which were used over several hundred years, is the use of slurry and mineral fertilizer and the exact adaption of the stocking rate to the growth of herbage. The particular feature of the all-day pasture is the low average sward height of the herbage, which should be 5 to 7 cm (Thomet *et al.,* 2004). Thus, a high stocking rate becomes possible with intensive all-day pastures, and feed quality remains on a relatively high level during the main vegetation period (Holden *et al*., 1995; Schneider *et al*., 2011). Further characteristics of this system are the seasonal calving, an intensive early stocking before the main grazing season, and a high grazing pressure during the effective grazing season (Thomet *et al.,* 2002).

The energy uptake of grazing dairy cows depends on dry matter intake (DMI) and the energy concentration of the feed (Van Vuuren and Van den Pol-van Dasselaar, 2006). There is a lower DMI on pasture in comparison to conventional total mixed ration feeding (Kolver and Muller, 1998; Bargo *et al*., 2003; Van Vuuren and Van den Pol-van Dasselaar, 2006). Furthermore, grazing cows have a 10 to 20% higher energy requirement for their maintenance due to increased locomotion (Muller and Fales, 1998). Thus, the metabolizable energy (ME) uptake during pure pasture feeding is often not sufficient to meet the demands of cows with high milk yields (Van Vuuren and Van den Pol-van Dasselaar, 2006) although young grass normally has high energy contents. This circumstance is aggravated by the fact

that the proportion from protein to ME in herbage from all-day pasture is considerably above the accepted requirements of dairy cows (DLG, 1997). Van Vuuren and Van den Pol-van Dasselaar (2006) reported that a daily milk yield of 17 to 25 kg/d is possible under pure grazing, whereas the amount of protein would be sufficient for 26 to 28 kg potential milk production per day. Others have reported a maximum possible daily milk production of 20 kg/d (Fulkerson *et al*., 1998) and 30 kg/d (Kolver and Muller, 1998), but the protein surplus would be similar as expected by Van Vuuren and Van den Pol-van Dasselaar (2006).

Beever *et al.* (1986) have shown that, when dietary nitrogen exceeds 25 g of N/kg of organic matter preduodenal loss of nitrogen occurs. The excretion of surplus nitrogen is an energy-intensive process because a lot of energy is required to convert ammonia to non-toxic urea in the liver and excrete it in the urine (Fulkerson *et al.,* 1998; Gehman *et al.,* 2006). This enhances the energy shortage of the grazing dairy cow (Kolver *et al.,* 1998). A supplementation of concentrate feed only once or twice a day can cause temporary imbalances between protein and energy in the rumen (Morrison and Patterson, 2007). Supplementary feed with high-energy content increases the energy concentration of the total diet and so the feed intake (Bargo *et al*., 2003). However, there is a replacement of pasture herbage by the concentrate feed supplementation. The reduction in pasture DMI per kilogram of concentrate is termed the substitution rate (Meijs, 1986; Bargo *et al*., 2003; Dillon, 2006; Van Vuuren and Van den Pol-van Dasselaar, 2006). The substitution rate ranges between 0 and 0.9 (Meijs and Hoekstra, 1984; Meijs, 1986; Muller and Fales, 1998; Bargo *et al.,* 2003; Dillon, 2006).

Feedstuffs which feature high amounts of carbohydrates are sugar beet, citrus pulp, coconut expeller, rice, and potato starch (Van Vuuren and Van den Pol-van Dasselaar, 2006). Less suitable energy sources are wheat, barley, and tapioca. These latter mentioned feedstuffs also have high carbohydrate contents, but they are very quickly degraded and increase the risk of rumen acidosis (Van Vuuren and Van den Pol-van Dasselaar, 2006). Granzin (2004) found that pasture supplemented with corn led to higher milk protein concentrations and in one of two experiments higher milk fat concentrations and milk protein yields in comparison to barley-based supplementation. The explanation for this could be the slower and more incomplete degradation of corn starch in the rumen compared to barley starch (Owens *et al.,* 1986). Thus, a lower energy loss in rumen methane production and fermentation heat can be expected (Owens *et al.,* 1986), and the assumption that starch digested postruminally is used more efficiently for milk synthesis than starch digested in the rumen (Nocek and Tamminga, 1991; Knowlton *et al.,* 1998) can be confirmed.

In the present work the influence of different amounts of grain maize supplementation (1 kg vs. 3 kg per day) to dairy cows grazing an all-day pasture with continuous stocking is examined in regards to animal performance and health.

MATERIAL AND METHODS

Experimental design

The experiment was carried out on the agricultural research station of the University of Applied Sciences Weihenstephan, Freising, South Germany (latitude 48°26'N; longitude 11°46'E). It was initiated on 21 April 2009 and ended on 25 June 2009. Sixteen lactating Simmental cows grazing a 7.0 ha all-day pasture with continuous stocking were used for the experiment. More details about the experimental pasture, the mean annual temperature, the annual precipitation and the soil texture are given by Schneider *et al.* (2011). The botanical composition of the pasture was nearly the same as in the experiment of 2008 (Schneider *et al*., 2011). The sixteen cows were selected from a herd of 26 cows and matched for age, body weight, and milk production according to the current lactation and then allocated to one of two groups of eight cows. In each group one cow was in the first, two cows were in the second and five cows were in the third lactation. The cows in the first group were 72 days in milk whereas cows of the second group were 65 days in milk. The first group (GM1) received 1 kg/d grain maize and the second group received 3 kg/d (GM3).

Milk yield, milk components and the body condition score (BCS) were determined during the 3 weeks long transition period after the stall feeding (maize silage, grass silage, hay and concentrate feed) when the animals were already moved to the pasture before the experiment started. Back fat thickness (BFT) was measured before and during the experiment. Cows in group GM3 had an approximately 50 kg higher body weight than the cows of group GM1, whereas the BCS was nearly equal (3.7 vs. 3.6). The inflated milk urea values in both groups represent the high crude protein content of the herbage on the all-day pasture.

The animals had been acclimated to grazing by keeping them on an all-day pasture during the whole vegetation period of the previous year. The cows were supplemented with grain maize and 0.1 kg/d of a mineral supplement. The mineral supplement consisted of (g/kg): Ca 180; P 50; Mg 45; Na 90 and the following trace elements (mg/kg) Cu 8.000; Zn 12.000; Mn 5.000; Se 50; Co 50; J 100, according to the manufacturer. Concentrates were weighed daily and dry matter (DM) content was determined twice a week. After each milking, the cows from both groups were confined for 30 min and half of the daily allotment of grain maize was fed in individual troughs. Feed remains of grain maize were collected and weighed after each

feeding and DM content was estimated by drying to calculate the effective grain maize intake. The cows of both groups spent about 3 hours per day in the stall for the milking and the supplement intake. Hence, the pure grazing time was about 21 hours per day. All cows were inseminated during the experiment. The experiment was carried out without disturbance and no animal disorders occurred.

Data collection, sampling procedures and analytical methods

The milking was done in the same line as in the experiment of Schneider *et al*. (2011). Milk samples were taken four times during the experimental period (8 weeks). Body weight (weekly), BCS according to the method of Edmonson *et al*. (1989) and BFT (once before the experiment and twice during the experiment) were determined for each individual cow. BFT was measured sonographically (Esaote, Tringa Linear 50, Oberhausen, Germany) at the measuring point defined by Schröder and Staufenbiel (2006).

Blood samples were taken three times after morning milking (twice during the experiment and once four days after the experiment). Blood samples were analysed for aspartate-aminotransferase (ASAT) and glutamate-dehydrogenase (GLDH). ASAT was formerly called glutamate-oxaloacetate-transaminase.

Herbage from the pasture was sampled and analysed once a week. The gross composition (DM, crude protein, ether extract, and crude ash) of herbage samples was determined as described by Naumann and Bassler (2007). The calculation of the energy content of the herbage samples was based on the wether experiments of Schneider and Bellof (2009) and Schneider *et al*. (2011), which were calculated with the estimation formula of the GfE (1995).

Statistical analysis

Linear regressions were used to evaluate the datasets. Hypothesis testing on equal means of groups or on parity of the mean of the population and a specific value was carried out using Student`s t-test (two-sided). This was performed against a 95% confidence interval, preceded by a test for normal distribution. All procedures followed standard protocols (Sachs, 1984).

RESULTS

Feedstuff

Herbage from all-day pasture is denoted by high crude protein (CP) contents with moderate crude fiber contents, which results in high energy contents (Table II.1). The high nitrogen

surplus of the herbage is remarkable, which results in an inflated ruminal nitrogen balance (RNB).

Table II.1: Composition, nutrient and energy content of feedstuff fresh matter and dry matter.

Item	Feed component	
	Herbage (n = 4)	Grain maize (n = 1)
Dry matter content of fresh matter (g/kg)	175	885
Dry matter composition (g/kg)		
Crude ash	112	17
Crude protein	289	83
Ether extract	26	45
Crude fibre	224	37
NfE	346	801
nXP	162	154
RNB (g/kg)	20	–11
NEL (MJ/kg DM)	6.51	8.03

NfE, nitrogen free extract; nXP, usable crude protein; RNB, ruminal nitrogen balance; NEL, net energy content for lactation; DM, dry matter.

Feed intake and milk yield

Feed intake of the supplemented grain maize in the stall was almost complete. On average, there were only 1.8% orts of the grain maize. Milk yield was insignificantly higher in GM3, but this was compensated for by the insignificantly lower milk fat content and as such energy-corrected milk (ECM) yield was nearly identical for both groups (Figure II.1).

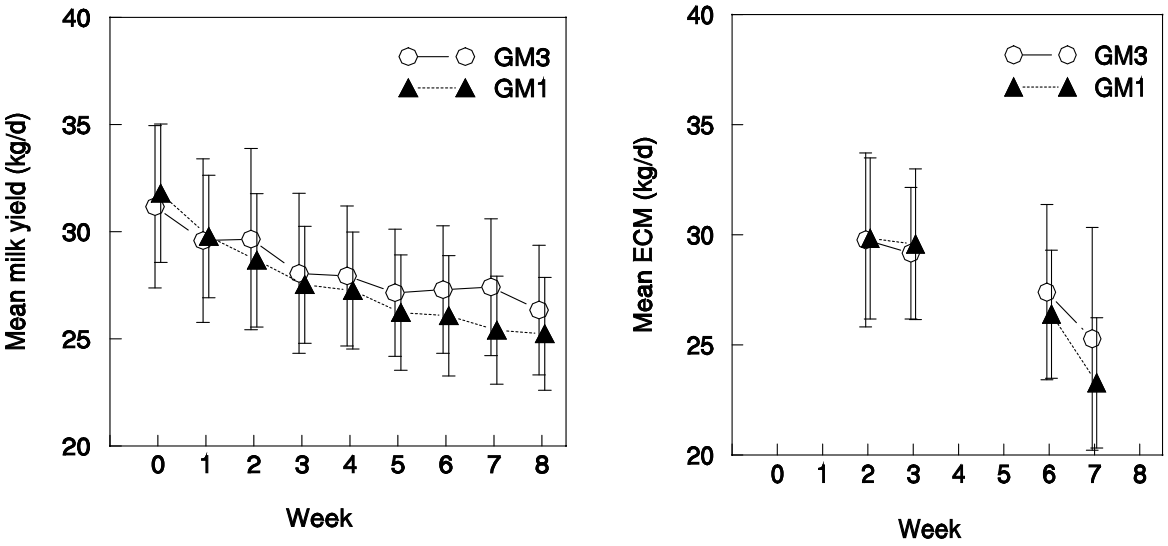

Figure II.1: Mean daily milk yield from GM1 and GM3 before and during the experiment and mean ECM from GM1 and GM3 during the experiment. Error bars denote the 95% confidence intervals of the mean (n = 8 for each data point).

The daily milk yield and ECM decreased during the experiment. This development was related to the progress in lactation (Figure II.1). Also milk protein content and milk urea content were not affected by different grain maize supplementation (Table II.2).

Table II.2: Average daily milk quality of the two groups (GM1 and GM3) before (16 measurements) and during the feeding experiment (64 measurements). *P* gives the probability of GM1 and GM3 not being different.

Item	Before experiment Feeding group			During experiment Feeding group		
	GM1	GM3	P value[1]	GM1	GM3	P value[1]
Milk quality						
Milk fat (g/kg)	45.1	48.9	0.496	38.3	35.2	0.108
Milk protein (g/kg)	32.8	33.2	0.771	34.3	34.5	0.846
Urea (mg/dl)	48.3	41.3	0.119	42.9	37.4	0.082

[1] Significance level.

BCS and BFT decreased in both groups (Figure II.2). In GM1 BFT diminished from 1.51 cm to 1.13 cm, while the decline in group GM3 was lower (1.60 cm to 1.34 cm). The difference in body weight between the two groups remained nearly constant during the experiment. The insemination index was 2.1 for GM1 and 1.5 for GM3. This difference was not statistically significant (P = 0.139).

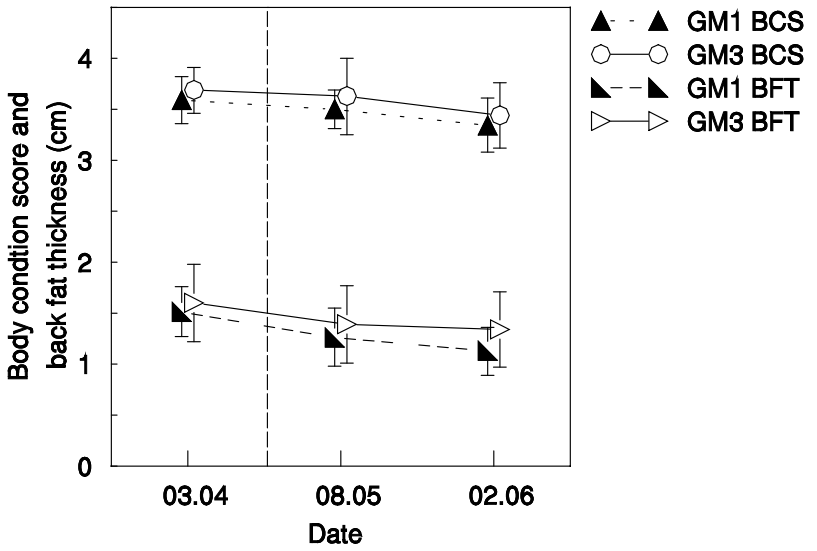

Figure II.2: Body condition score and back fat thickness from GM1 and GM3 before (03.04) and during the experiment (08.05/02.06). Error bars denote the 95% confidence intervals of the mean (n = 8 for each data point). The dashed vertical line denotes the start of the experiment.

Blood analysis

The blood enzyme levels did not differ significantly between both groups (Figure II.3) at all dates although there was some remarkable but inconsistent fluctuation over time. At the beginning of the experiment (24 April 2009), both groups already showed mean values close

to the critical values (according to Tiergesundheitsdienst Bayern) for both enzymes even when a few outliers to the top were not considered. For ASAT one outlier occurred at the second date in group GM3 and there was one extreme high GLDH value at the first date in GM1 and two in GM3 at the second date. In the final blood analysis, which was realized four days after the experiment when all cows were supplemented with 1 kg grain maize and 0.1 kg minerals per day, no outliers occurred.

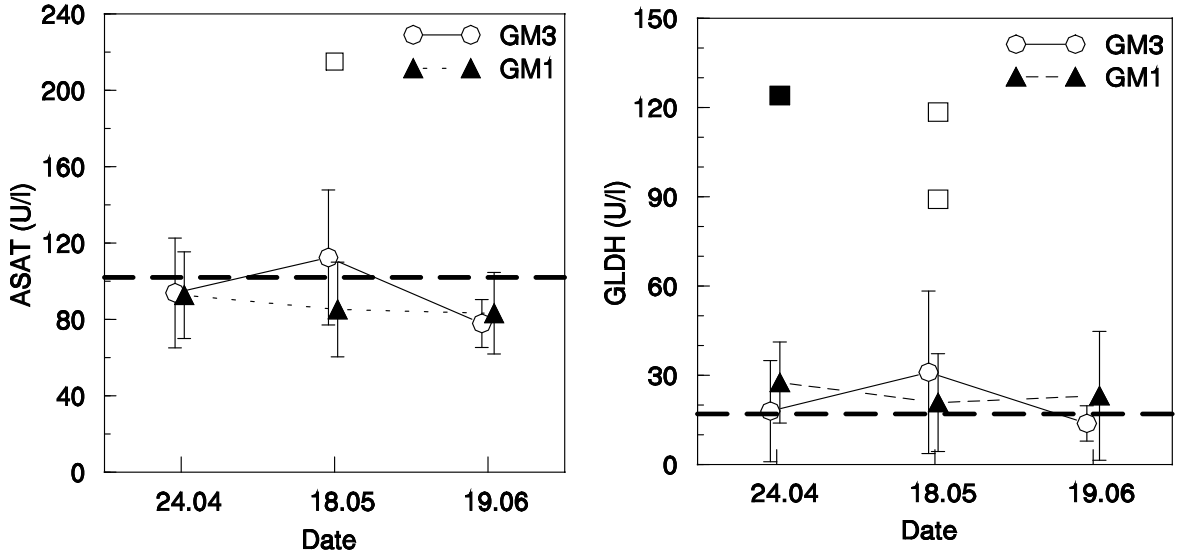

Figure II.3: ASAT and GLDH levels of the cows at three dates (start, mid and after the experiment). ASAT, aspartate-aminotransferase; GLDH, glutamate-dehydrogenase. Error bars denote the 95% confidence intervals of the mean (n = 8 except outliers). The critical values (Tiergesundheitsdienst Bayern) for ASAT max. 102 U/l and GLDH max. 17 U/l are shown as dashed horizontal lines. □ denotes one outlier for ASAT (GM3) and ■/□ denote three outliers for GLDH (GM1 + GM3).

The intervals of confidence were rather large despite the removal of outliers. This implies that even individual animals that were not removed as an outlier were considerably above the critical values. However, negative effects that could additionally indicate metabolic disorders were not evident for these cows and even not for the outliers.

DISCUSSION

The experiment clearly showed that the all-day pasture with continuous stocking provided a high-quality feed. Supplementing with 3 kg/d instead of 1 kg/d grain maize did not improve animal performance and health. The applied grain maize had some lower nutrient and energy content than is noted in DLG (1997). Schneider and Bellof (2009) and Schneider *et al*. (2011) noted that herbage from all-day pasture has comparatively high crude fiber (235 g/kg DM) and acid-detergent fiber (ADF) contents (296 g/kg DM) simultaneously coupled with high digestibility of organic matter (777 g/kg). Analysing the herbage in a wether test

resulted in an energy content of 6.53 MJ NEL/kg DM. Calculating the energy content from the gross composition of the herbage resulted in an energy content of 6.51 MJ NEL/kg DM.

Due to the high energy content of the herbage from the all-day pasture (Table II.1), there is only an energetic difference of 1.52 MJ NEL/kg DM between the supplemented grain maize and the pasture herbage. Additionally, the amount of grain maize (1 kg and 3 kg fresh matter, respectively), in comparison to the total DMI (18.4 kg/d, Table II.3), is of minor importance. Assuming an identical total dry matter intake for GM1 and GM3 (Holden *et al.,* 1995), the higher energy content of grain maize compared to pasture herbage would lead to a 2.69 MJ NEL higher energy supply for GM3 per day, which is less than is required for the production of 0.9 kg of milk. In conclusion, a much better animal performance could not have been expected.

To our knowledge, there are only few experiments about the feed value of herbage from all-day pasture under Central European conditions. Steinwidder *et al*. (2010) quoted the average CP content for herbage from all-day pasture in Austria during the main vegetation period to be 210 g/kg in DM, while Schneider *et al*. (2011) measured a slightly lower content of 205 g/kg in the previous year (May till July 2008). Muller and Fales (1998) described a comparable level (210 to 250 g/kg CP in DM) for herbage, which was harvested in spring, but CP contents in May and June were nearly 290 g/kg. This result agrees well with the result in this experiment (289 g/kg) and the results from Holden *et al*. (1995), which determined 270 to 330 g/kg for young pasture herbage. These high values were confirmed by Van Vuuren *et al*. (1991), which analysed maximum values of 300 g/kg for young pasture herbage. The high nitrogen surplus of an all-day pasture can normally be diminished by an energy rich supplementation, but in this study this was not the case. There was only a slightly lower milk urea content in GM3.

Concentrate feeding is associated with a replacement of herbage. The substitution rate depends on the herbage allowance and increases with higher pasture availability. With higher grazing pressure (= lower herbage allowance), concentrate feed will lead to higher substitution rates. Substitution rates described in literature range from 0 to 0.9 (Meijs and Hoekstra, 1984; Meijs, 1986; Muller and Fales, 1998; Bargo *et al*., 2003). Dillon (2006) calculated an average substitution rate of 0.6 from different literature data. At a value of approximately 1.0 a supplementation with concentrate feed only allows to increase the number of animals on pasture, but not to increase the feed intake per animal. The calculation with our data shows that for GM3 a decline of 1.7 kg of herbage intake can be assumed (Table II.3) corresponding to a substitution rate of 1.01. In comparison to the values in the

literature (Meijs, 1986; Holdon *et al.,* 1995), this result is high and indicates that a supplementation of 3 kg grain maize had no advantages in this experiment.

Table II.3: Calculation of energy supply and energy intake for estimating intake of pasture herbage.

Item	Feeding group	
	GM1	GM3
ECM (kg)	26.6	26.8
Required energy for performance (MJ NEL)	85.1	85.6
+ Required energy for maintenance (MJ NEL)	41.5	41.5
= Total energy demand (MJ NEL)	126.6	127.1
– Energy supply of fat mobilization (MJ NEL)	6.1	3.7
− Energy supply from grain maize (MJ NEL)	6.4	20.6
= Required energy uptake from pasture (MJ NEL)	114.1	102.8
Estimated dry matter intake with herbage (kg)	17.5	15.8
Estimated total dry matter intake (kg)	18.4	18.4
Estimated substitution rate		1.01

ECM, energy-corrected milk, ECM = (0.3246 x kilograms of milk) + (12.86 x kilograms of milk fat) + (7.04 x kilograms of milk protein).

The following assumptions were used for the calculation of Table II.3:

- The energy demand on pasture feeding systems compared to stall-feeding is increased by 10% (37.7 MJ NEL/day x 1.1 = 41.5 MJ NEL/cow per day) (Van Vuuren and Van den Pol-van Dasselaar, 2006).
- The energy supply from fat mobilization is estimated from the alteration of BFT (Figure II.2) with a decline of 1 mm of BFT corresponding to a fat mobilization of 5 kg (Klawuhn and Staufenbiel, 1998).
- The mobilization of 1 kg of body fat provides about 20 to 21 MJ NEL for the synthesis of milk (Kirchgessner *et al*., 2008).

The milk fat and protein content in this experiment showed a low milk fat: milk protein ratio of 1.13 for GM1, whereas this ratio was significantly ($P < 0.05$) lower at 1.01 for GM3. Ratios of ≤ 1.0 are known to indicate a ruminal acidosis (Mertens, 1997; Kolver and de Veth, 2002), which then cannot be excluded for all animals of the latter mentioned group. This threshold value is yet appropriate for stall-fed dairy cows. Whether it also applies for dairy cows on all-day pasture has to be validated.

High herbage CP contents connected with high intra-ruminal degradation rates (about 84%, Kolver and Muller, 1998; 70% to 80%, Van Vuuren and Van den Pol-van Dasselaar, 2006) and low energy supply result in high CP surplus. This caused the high milk urea contents in both experimental groups. When calculating the daily feed intake of both groups (Table II.1, Table II.3) the RNB was + 341 g for GM1 and + 287 g for GM3. Considering this high

nitrogen surplus, milk urea contents are relatively moderate. The slight decrease in milk urea content with increasing supplementation agrees with literature, where decreasing milk urea contents are described with increasing concentrate supplementation with grazing (Bargo *et al*., 2003). Gehman *et al*. (2006) described a negative correlation to the pregnancy rate even at milk urea contents up to 20 mg/dl. The insemination index in this experiment was yet not negatively affected by the high urea contents (1.5 and 2.1, respectively). This is in accordance to Fulkerson *et al.* (1998) and Thomet *et al.* (2004), who described that these negative effects may not be present in dairy cows grazing pasture.

Blood enzyme analysis showed critical values for both groups and individuals exceeded the critical values by as much as 700%. This would indicate severe metabolic stress. According to Owens *et al*. (1998) a ruminal acidosis is accompanied by low pH-values in blood, damage of the rumen wall and the intestine, and impair of the liver. However, no animal disorders occurred in this grazing system at these enzyme levels. More importantly, a higher supply with grain maize did not reduce the high enzyme levels, which implies that a feeding of 3 kg/d is not creating an advantage regarding animal health.

CONCLUSIONS

A supplementation of 3 kg grain maize per cow and day alongside all-day pasture with continuous stocking during the main vegetation period had no influence on the energy-corrected milk yield, milk ingredients, animal condition parameters and animal health when compared to a supplementation with 1 kg. Supplementation above 1 kg/d does not provide any advantage under conditions of all-day pasture with continuous stocking.

Chapter III: Elution kinetic of maize derived ^{13}C

ABSTRACT

Food safety and proof of origin are important in a world with global flows of foodstuff. Consumer interest in food origin and authenticity is particularly high for animal products like meat or milk. Stable isotope analysis is a valuable tool for origin assignment. But its application relies on knowledge of isotopic discrimination (Δ) during digestion and metabolism and the elution kinetic of the specific product. We assessed $^{13}\Delta$ for milk, milk components (fat, casein, lactose) and faeces in eight lactating dairy cows which grazed pasture or were fed fresh pasture herbage in the stall. All cows were supplemented daily with 1.72 kg dry matter of grain maize for eight weeks. Feed, faeces, milk fat, casein, lactose and whole milk were analysed for carbon isotope composition ($^{13}C/^{12}C$) four times a week during this 8 week period. When supplementation with grain maize ended after eight weeks and pasture herbage became the sole feed source, the fate of grain maize derived ^{13}C was traced in faeces, milk and milk components over a 10 day long period.

Grain maize feeding could be detected in faeces, milk and milk components only for three days or less when changing to pure grazing. Elution kinetic was fastest for lactose and faeces (36 hours) and seemed to almost follow a piston-flow pattern. Carbon isotope composition of milk, milk components and faeces thus only reflects the very recent feed but not feeding history. This fast elution kinetics is negative in regard to proof of origin but it offers numerous opportunities for isotope researchers in other study areas.

INTRODUCTION

When using stable isotopes to estimate diet from analysis of an animal product, the isotopic offset between diet and tissue or product must be known (McCutchan *et al.*, 2003; Codron *et al.,* 2005; Wittmer *et al.*, 2010; Martínez del Rio and Carleton, 2012; see Chapter IV). Near constant discriminations require isotopic and energetic flow equilibrium. Hence, it is important to achieve such equilibrium when determining the discrimination and to know the time span after a diet change that is necessary for equilibration (De Smet *et al.,* 2004). The time to achieve a complete turnover depends on the animal itself and the respective tissue. Full equilibration is often achieved in the order of many months (West *et al.*, 2004). Body proteins in heifers required more than 167 respectively 230 days to achieve a complete carbon turnover (Bahar *et al.*, 2005; Gebbing *et al.,* 2004), while for dairy cows one week was enough to reach a new equilibrium of milk elements (carbon and nitrogen) following a change in the type of diet (Wilson *et al.*, 1988; Knobbe *et al.*, 2006). Boutton *et al.* (1988) found a new equilibrium in cow milk carbon isotopes after a diet change already after four days, whereas Camin *et al.* (2008) found that 2 weeks are not long enough to obtain a complete turnover of carbon in casein. Faeces of herbivores also have short turnover rates of several days (Codron *et al.*, 2005). Jones *et al.* (1979) investigated changes in the $\delta^{13}C$ composition of faeces of steers and cattle depending on different diets. After the abrupt feed change from a C_4 to C_3 based diet they observed a complete turnover of the $\delta^{13}C$ values in faeces of steers within 6 days. Hair growth of steers recorded equilibrium following a change in diet from a C_4 to C_3 feed and then back to C_4 feed after 74 days (Jones *et al.*, 1981).

For an analytical point of view, diet-switch experiments to prove the isotopic coupling of diet and product are best carried out with large isotopic spacing like switching from pure C_3 to pure C_4 diet. For the determination of discrimination this has two disadvantages. First, the shift often also involves a pronounced change in the dietary value of the feed that affects the metabolism of the animal. Second, the period of feeding a constant diet must be long enough to achieve a constant isotopic composition of the product. Even for products with fast turnover, body pools with slow turnover may deliver enough substrate to influence the apparent discrimination if there is a large isotopic spacing between the actual feed and the former feed, which contributed most to the formation of slow body pools. This calls for diet-switch experiments with mixed diets of small isotopic spacing especially for products of fast turnover. Still, a certain proportion of lipids and protein from body stores will be directly incorporated in new tissues and products (Phillips and Koch, 2002). Furthermore, the different components of a mixed diet may not be passed on in equal relative amounts to the product, e.g. due to differences in digestibility (Jones *et al.,* 1979). The problems associated with varying contributions of different feed components to specific tissues and products are

probably less in the case of ruminants (Sponheimer *et al.*, 2003; Gannes *et al.*, 1997; Gannes *et al.*, 1998) as their dietary protein greatly reflects the diet because gut symbionts produce most of ruminant´s protein from bulk diet and urea recycling, which homogenizes the carbon skeletons from different dietary sources in body protein (Gannes *et al.*, 1998). But even in ruminants dietary components escape from the fermentative chamber and are absorbed in the lower gut without prior isotopic scrambling.

There are several studies measuring carbon isotope composition in dairy cows (Minson *et al.*, 1975; Boutton *et al.*, 1988; Wilson *et al.*, 1988; Metges *et al.*, 1990), but only few of them specifically aimed to determine discrimination between diet and product. These experiments either used short equilibrium periods (Metges *et al.*, 1990; Schulze *et al.*, 1992; Masud *et al.*, 1999), only one or few cows (Minson *et al.*, 1975; Masud *et al.*, 1999) or bulk milk samples without knowing exactly the carbon isotope composition of the ingested feed (Camin *et al.*, 2008). Several authors call for experiments with longer adaptation and observation periods under controlled conditions to ascertain constant discriminations between diet and animal tissues and products (Gannes *et al.*, 1997; Sponheimer *et al.*, 2003; Camin *et al.*, 2008; Norman *et al.,* 2009).

To our knowledge, this is the first experiment continuously measuring stable carbon isotopes of dairy cows over eight weeks simultaneously in faeces, milk and milk components, and subsequently determining elution kinetics in the respective tissues. It is a prerequisite to know the elution kinetic of a specific tissue when the discrimination between diet and product or tissue shall be determined (Monahan *et al.,* 2010).

The objectives of this research was to analyse the elution kinetics of grain maize (C_4) stable isotopes after a change from an all-day pasture supplemented with grain maize to a pure pasture diet (C_3 plants).

MATERIAL AND METHODS

The experiment was carried out on the agricultural research station of the University of Applied Sciences Weihenstephan, Freising, South Germany (latitude 48°26'N; longitude 11°46'E). More details about the experimental pasture, the mean annual temperature, the annual precipitation, the soil texture, the botanical composition and the fertilisation of the pasture are given by Schneider *et al.* (2011).

At the start of the experiment, eight pregnant, lactating multiparous Simmental cows in their second or third lactation were between 80 and 133 days in lactation. Energy corrected milk

(ECM) was calculated by standardizing actual milk production of a cow to 35 g/kg milk fat and 32 g/kg milk protein following Bernard (1997). ECM production per cow averaged 28 kg/d during the seven days before the commencement of the experiment. The animals grazed for six weeks before the experiment started to accustom them to grazing and the feed of a pure pasture diet.

The experiment was initiated on 19 May 2008 and ended on 28 July 2008. It consisted of two subsequent 28-day trial periods (19 May to 18 July 2008) and a 10 day long elution kinetic period (19 July to 28 July 2008). The first two periods are described in detail in Chapter IV. The elution kinetic period started with the cessation of grain maize supplementation on the morning of 19 July 2008 and herbage from pasture became the sole feed source for the next 10 days. During the elution kinetic period, samples of the pasture herbage were taken daily and immediately frozen at -18 °C. Faeces and milk were sampled twice a day for 5 days (19 July to 23 July) and afterwards once a day in the morning from day 6 to 10 (24 July to 28 July). The samples of faeces and milk were frozen immediately at -18 °C. After the feeding experiment herbage, faeces samples, and milk were defreezed and analysed (see Chapter IV).

RESULTS

The $\delta^{13}C$ of milk, milk components, and faeces did not change for 12 hours when switching diet from grain maize supplemented grazing (mixed C_3/C_4 diet) to pure grazing (C_3 diet) (Figure III.1). There was a significant ($P < 0.001$) shift in $\delta^{13}C$ of faeces, whole milk and milk components after this delay. Elution of maize derived ^{13}C in lactose with only one intermediate value 24 hours after the diet switch was faster than in casein and milk fat where the gradual change lasted about three days (Figure III.1). 24 hours after the last grain maize supplementation about 20% of the new signal was detected in faeces, while 36 hours after the last C_4 feeding the new signal had completely arrived. Elution kinetic of faeces was similarly fast than the elution kinetic of lactose and seemed to follow a piston-flow pattern (Figure III.1). Grain maize derived ^{13}C in lactose and faeces was detectable for 36 hours after diet change. In whole milk and casein, the elution of maize derived ^{13}C takes about 60 hours and was slowest in milk fat (72 hours). From day 4 after the diet switch, $\delta^{13}C$ composition for milk, milk components, and faeces remained constant and achieved a level which seemed to be typical for a pure C_3 pasture herbage diet (Figure III.1).

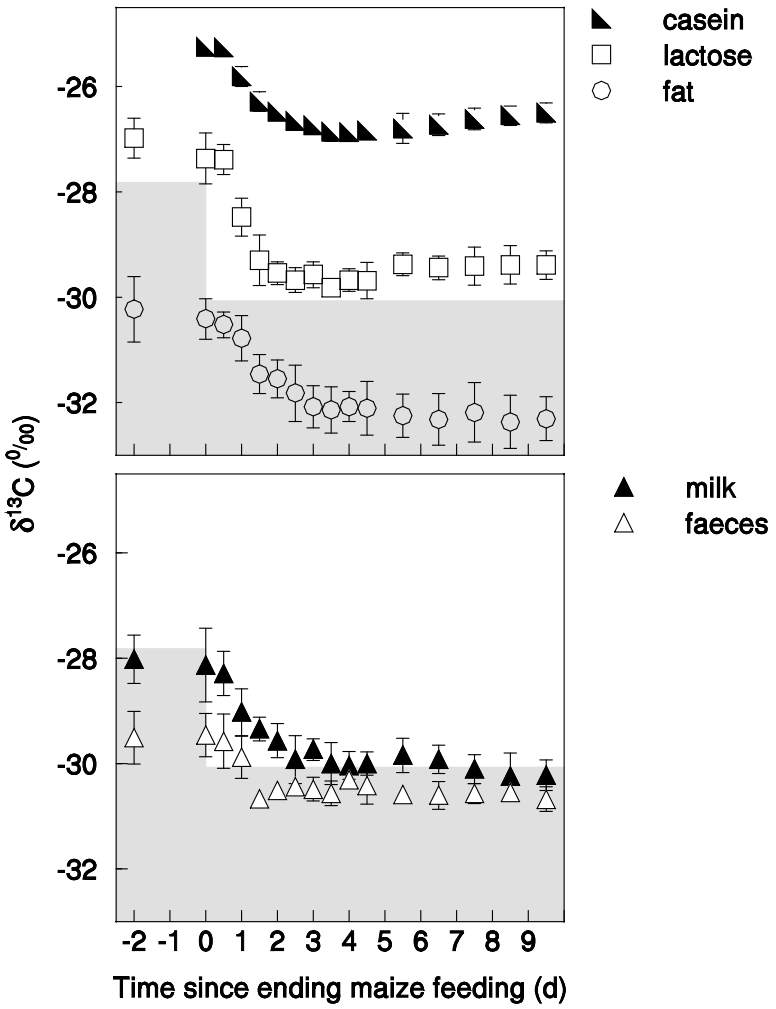

Figure III.1: Elution kinetics of maize derived ^{13}C in milk components (casein, lactose, fat), milk and faeces. The grey area shows the carbon isotope composition of the diet. Error bars denote the standard deviation (n = 8 for each data point).

After the delay $\delta^{13}C$ of casein of the eight cows scattered little, whereas scatter of milk fat was comparably large. The diet switch from grain maize supplemented grazing to pure grazing resulted a shift in the diet of 1.7 ‰ $\delta^{13}C$. In whole milk (1.8 ‰), milk fat (1.7 ‰) and casein (1.6 ‰) the shift in $\delta^{13}C$ was similar to the diet. The shift in $\delta^{13}C$ in lactose was larger (2.2 ‰) while the shift in faeces $\delta^{13}C$ was only 1.0 ‰.

DISCUSSION

Mixing of feed along the digestive tract was small. Only one faeces sample 24 hours after feed change indicated an intermediate isotopic composition, while 12 hours after the change still the previous feed arrived and 36 hours after the change the replacement seemed to be completed because no further change in isotopic composition occurred. This fast passage rate agrees with the high digestibility of organic matter of the diet (~ 78%, Schneider *et al.*, 2011) and is in the same range as found by Mambrini and Peyraud (1997), who described a

total digestive tract retention time for hay, ground hay and concentrates of 52, 46, and 41 hours for dairy cows. Nocek and Tamminga (1991) and Bayat *et al.* (2010) also have shown that coarse particles remained in the rumen for a considerably longer time until they entered the fraction of fines and passed on in the digestive tract. This implies that our results would have been slightly different if the C_4 component would have been offered as roughage and not as concentrate. The effect would not be visible during flow equilibrium when all material passes the digestive tract in proportional amounts even though retention times differ. The differences in passage rate only become isotopically effective at the diet change, but even there the effect would be small because the proportional amount being in the fines fraction would be identical and leave the digestive tract equally fast and thus produce the same initial pattern as that of the concentrates in our study. The proportional amount in the coarse fraction would pass the rumen slower, but the lower the passage rate becomes, the smaller the isotopic effect will be and thus its visibility decreases the more the passage rate differs leaving essentially the isotopic pattern caused by the fines.

The isotopic change in faeces was only 1.0 ‰ whereas diet changed by 1.7 ‰. This may indicate that the grain maize component that caused this shift had a higher digestibility than the herbage and thus was preferably removed from the faeces. However, the digestibility of herbage organic matter was already high (~ 78%) and there was only a slightly higher digestibility of the herbage-maize diet compared to the pure herbage diet as determined with various methods (Schneider *et al.*, 2011). Furthermore, a much higher digestibility of the grain maize should have increased the isotopic shift of milk compared to the shift in the diet, which was not the case. The shift in milk (1.8 ‰) was nearly identical to that in the diet (1.7 ‰). Hence, the smaller than expected shift in faeces must be caused by a high proportion of body material because the labelling period was too short to cause a (complete) turnover of body material (Ayliffe *et al.*, 2004; Bahar *et al*., 2005). The proportion of body material in faeces rises with increasing digestibility of the feed (Lukas *et al.,* 2005) and thus a high proportion can be expected with our highly digestible diet. This implies that the apparent diet-faeces discrimination can be highly variable in animals exposed to diet shifts were digestibility is high, while the shift should become more constant for diets of low digestibility. Whether the apparent diet-faeces discrimination before the diet shift reflects the true discrimination depends on whether the animal was in isotopic equilibrium with the first diet (Ayliffe *et al.,* 2004). The diet of our animals prior to the experimental period is not known with sufficient accuracy for the time of complete body turnover (> 167 days, Bahar *et al.,* 2005; > 230 days, Gebbing *et al.,* 2004) to answer this question without doubt. However, the grazing period without C_4 concentrates prior to the experimental period makes a bias in the

diet-faeces discrimination likely for both experimental periods with the true discrimination being between the apparent discriminations of both periods.

The elution kinetics of lactose followed a pattern similar to a piston flow. This indicates that lactose is directly derived from digested feed without intermediate storage that would cause a delay or buffering. This agrees well with the current understanding of lactose formation (Knowlton *et al.*, 1998).

The shift in lactose (2.2 ‰) was larger than the shift in diet (1.7 ‰) and thus caused a slightly higher shift in total milk (1.8 ‰). This indicates incomplete isotopic scrambling of the diet components during digestion. The structure of maize starch and the processing (drying after harvesting and grounding) cause about 30-40% of the starch to leave the rumen without degradation direct to the small intestine (Nocek and Tamminga, 1991). Starch via glucose is the main precursor for lactose synthesis (Bickerstaffe *et al.,* 1974; Waghorn and Baldwin, 1984) and thus appears predominantly in lactose without proper isotopic scrambling.

The elution kinetics of casein and milk fat were somewhat slower than that of faeces and lactose but even these two milk components completed the isotopic shift within 3 days after the diet shift and the same applied for the whole milk. Although in principle the body stores of protein and fat are large and could cause a slow elution, this seems to be not the case indicating that the body pools did not contribute significant amounts to milk production. Boutton *et al.* (1988) stated that for cows being near to energy balance, dietary carbon might be expected to contribute to nearly all milk carbon and the contribution of body carbon is small. The experimental cows in this study were in mid lactation. Milk yield was not very high and body weight, BFT and BCS did not change significantly during the experimental period (Schneider *et al.,* 2011). So no energy deficit of the cows can be expected, and due to the protein-rich herbage diet, contribution of body protein to milk synthesis can be expected to be small.

CONCLUSIONS

Grain maize feeding can be detected in faeces, milk and milk components only for three days when changing diet from grain maize supplemented grazing to pure grazing. Elution kinetics of lactose and faeces was even faster (36 hours) and seems to follow a piston-flow pattern. For proof of origin fast elution kinetic of cow milk is negative, but it offers numerous opportunities for isotope researchers in other study areas.

Chapter IV: ^{13}C discrimination between diet, faeces, milk and milk components

ABSTRACT

Food safety and proof of origin are important in a world with global flows of foodstuffs. Consumer interest in food origin and authenticity is particularly high for animal products like meat and milk. Stable isotope analysis is a valuable tool for origin assignment. But, its application relies on knowledge of isotope discrimination (Δ) during digestion and metabolism, which can modify the isotope signal in the product (e.g., $\delta^{13}C_{product}$) relative to that of the diet ($\delta^{13}C_{diet}$). For carbon, Δ is defined as $^{13}\Delta = (\delta^{13}C_{diet} - \delta^{13}C_{product}) / (1 + \delta^{13}C_{product})$. We assessed $^{13}\Delta$ for milk, milk components (fat, casein, lactose) and faeces in eight lactating dairy cows which grazed pasture or were fed the fresh pasture herbage in the stall. All cows were supplemented daily with 1.72 kg dry matter of grain maize for eight weeks. Feed components were collected daily, and faeces, milk fat, casein, lactose and whole milk samples four times per week during the 8 week long experiment. Carbon isotopic composition of each sample was analysed.

$\delta^{13}C$ was lowest in milk fat (−29.77 ‰) and highest for casein (−26.44 ‰). Compared to the diet, there was a depletion of 0.4 ‰ in whole milk caused by the strong depletion in milk fat ($^{13}\Delta$ = 2.2 ‰), which was not compensated by the enrichment in casein ($^{13}\Delta$ = −1.1 ‰) and lactose ($^{13}\Delta$ = −0.7 ‰). Faeces also were depleted ($^{13}\Delta$ = 1.7 ‰). Influences of feeding environment (stall vs. pasture) and herbage quality were minor (< 0.2 ‰). These discriminations between diet and milk are the first to cover simultaneously all main milk components and faeces. They cover both stall and pasture feeding under realistic conditions, and they are based on a much larger data set regarding number of cows and milkings than previous studies. Thus, they will improve the use of stable isotope analyses in regard to authenticity testing and proof of origin.

INTRODUCTION

Measurement of stable isotope composition has become an important tool in numerous fields of science like behavioural and nutritional ecology, physiology, archaeology, forensics, and food authenticity testing (Gannes *et al*., 1997; Kornexl *et al.*, 1997; Sponheimer *et al.*, 2003; De Smet *et al.*, 2004; Spangenberg *et al.*, 2006; Camin *et al.*, 2008). Stable isotopes naturally occur in all bioelements. These elements have one overwhelmingly abundant isotope and one or more isotopes of low abundance (Crittenden *et al.*, 2007). For carbon, the conventional definition of stable isotope composition is

$$\delta^{13}C = (R_{sample}/R_{standard}) - 1 \quad \textbf{(1)}$$

where R is the $^{13}C/^{12}C$ isotope-number ratio and standard is the Vienna Pee Dee Belemnite standard.

The $\delta^{13}C$ of plants varies between the metabolic pathways of photosynthesis. C_3 plants discriminate more against the heavy stable isotope (^{13}C) than C_4 plants (Craig, 1954; Smith and Epstein, 1971). This effect is imprinted in plant carbon and yields characteristic $\delta^{13}C$ values for C_3 and C_4 biomass. Maize is the most important C_4 crop in Europe and a frequent component of predominantly C_3-based cattle diets. Mixing models are usually used if two or more constituents contribute to the diet (Phillips and Koch, 2002). Simple linear mixing models contain a variety of unrealistic assumptions like the assumption that all dietary items are assimilated with the same efficiency (Jones *et al.*, 1979; Gannes *et al.,* 1998; Martínez del Rio *et al.*, 2009) or the assumption that the feed components are degraded and thus homogenized in the digestive tract and then used for tissue synthesis (Phillips and Koch, 2002) in equal relative amounts. The problem of incomplete homogenisation during digestion and resorption of undegraded, unhomogenized feed components is called “isotopic routing” (Gannes *et al.,* 1997; Martínez del Rio *et al.*, 2009). However, the animals that best fit this assumption are foregut fermenters like cows, where most of the carbon skeletons are actually degraded in the rumen to volatile fatty acids and bacterial protein (Van Soest, 1994) and thus largely meet the requirements of linear mixing models.

The $\delta^{13}C$ of the feed influences the $\delta^{13}C$ of cattle and their products (Minson *et al.*, 1975; DeNiro and Epstein, 1978; Tieszen *et al.*, 1983; Tyrrell *et al.*, 1984; Dawson and Brooks, 2001), including milk (Tyrrell *et al.*, 1984; Boutton *et al.*, 1988; Metges *et al.*, 1990; Kornexl *et al.*, 1997). However, physical and biochemical processes modify the partitioning of the different stable isotopes (isotopic discrimination), leading to a measurable offset between the $\delta^{13}C$ of the diet ($\delta^{13}C_{diet}$) and that of the product ($\delta^{13}C_{product}$) (Gannes *et al.,* 1998; Dawson and Brooks, 2001). There are several possibilities to describe this isotopic offset. Often, simply the absolute difference between the isotope composition of the substrate and product

($\Delta` = \delta_{tissue} - \delta_{diet}$) is used and called "trophic shift", "substrate-product shift" or "diet-tissue shift" (McCutchan *et al.,* 2003; Maennel *et al.,* 2007). Although this is convenient short-hand for discussing discriminations, it is not strictly correct (Sponheimer *et al.,* 2003; Auerswald *et al.,* 2010). The isotopic difference between two substrates is defined as $\alpha = R_{substrate\ 1}/R_{substrate\ 2}$ (Hobbie and Werner, 2004) where R is the $^{13}C/^{12}C$ ratio of the substrates. Discrimination Δ then is (Farquhar *et al.*, 1989; Hobbie and Werner, 2004)

$$\Delta_{substrate\ 1\ -\ substrate\ 2} = \alpha - 1 \quad \textbf{(2)}$$

when substrate 1 is the source and substrate 2 is the product.

An alternative would be to calculate the enrichment in which substrate 1 is the product and substrate 2 is the source. The discrimination can be calculated from stable isotope composition (Farquhar *et al*., 1989)

$$^{13}\Delta_{source\text{-}product} = (\delta^{13}C_{source} - \delta^{13}C_{product}) / (1 + \delta^{13}C_{product}) \quad \textbf{(3)}$$

It should be noted that Eq. (3) is formally equivalent to Eq. (1).

When using stable isotope analysis to infer the diet-$\delta^{13}C$ from that of animals' products (e.g. the $\delta^{13}C$ of whole milk) then the product-specific $^{13}\Delta$ (e.g. $^{13}\Delta_{diet\text{-}milk}$) must be known (McCutchan *et al.*, 2003; Wittmer *et al.*, 2010). The assessment of true $^{13}\Delta$-values requires isotopic and energetic flow equilibrium conditions. Otherwise old body pools that reflect a previous, different diet, still contribute significantly to the product and cause the apparent $^{13}\Delta$ to vary over time until a constant equilibrium $^{13}\Delta$ is reached (Auerswald *et al.,* 2010). Hence, it is important to achieve such equilibrium conditions when determining the $^{13}\Delta$ and to know the time span after a diet change that is necessary for equilibration. Complete equilibrium would need uniform feed from birth to death, similar to the classical experiments by DeNiro and Epstein (1978). Where maternal material constitutes a significant proportion of body mass, controlled and constant feeding must start already with the mothers as in the experiments by Hobson and Clark (1992). For specific animal parts or products with fast turnover near-equilibrium conditions may be obtained earlier. Body proteins in heifers required more than 167 respectively 230 days to achieve a new isotopic equilibrium (Bahar *et al.*, 2005; Gebbing *et al.,* 2004), while one week was apparently sufficient to reach a new equilibrium in milk carbon and nitrogen isotope composition of dairy cows following a change of diet isotope composition (Wilson *et al.*, 1988; Knobbe *et al.*, 2006). Boutton *et al.* (1988) reported a new equilibrium in cow milk $\delta^{13}C$ after a diet change already after four days, whereas Camin *et al.* (2008) found that 2 weeks were not long enough to obtain a new isotopic equilibrium of carbon in casein.

Faeces of herbivores also have short turnover rates of several days (Codron *et al.*, 2005). Jones *et al.* (1979) investigated changes in the $\delta^{13}C$ composition of faeces of steers and cattle depending on different diets. After an abrupt change from a C_4- to C_3-based diet they observed a complete equilibration of the $\delta^{13}C$ of the faeces within six days. Hairs of steers recorded equilibrium in the metabolic pools feeding hair growth following a change in diet after 74 days (Jones *et al.*, 1981).

In literature, studies can be found from which discriminations can be derived (Minson *et al.*, 1975; Tyrrell *et al*., 1984; Boutton *et al.*, 1988; Wilson *et al.,* 1988; Metges *et al.*, 1990; Schulze *et al.,* 1992; Masud *et al.,* 1999; Knobbe *et al.,* 2006; Camin *et al*., 2008), but only few of them specifically aimed to determine discrimination between diet and product. Hence, these experiments either used short equilibrium periods (Metges *et al.*, 1990; Schulze *et al.*, 1992; Masud *et al.*, 1999), only one or few cows (Minson *et al.*, 1975; Masud *et al.*, 1999) or bulk milk samples without knowing exactly the carbon isotope composition of the ingested feed (Camin *et al.*, 2008). Most of these studies (seven) treat diet-milk discrimination, whereas only one study was found for milk-casein discrimination and one for lactose-milk fat discrimination. A consistent set of discriminations among diet, faeces, milk and milk components does not exist. Furthermore, only one of these studies applies for grazing cows (Minson *et al.*, 1975).

The objective of this research was to describe the isotopic discrimination of $\delta^{13}C$ between diet, faeces, whole milk and milk components in stall and with grazing. To our knowledge, this is the first experiment continuously measuring stable carbon isotopes of dairy cows over eight weeks simultaneously in the diet, faeces, milk and milk components.

MATERIAL AND METHODS

Site conditions

The experiment was conducted at the agricultural research station of the University of Applied Sciences Weihenstephan, near Freising, southern Germany (48°26' N; 11°46' E), 493 m above sea level. The mean annual temperature and precipitation are 7.5 °C and 794 mm/yr, respectively. The experimental pasture was a 3.0 ha semi-natural grassland on silty loam soils. More details about the botanical composition and fertilisation of the pasture are given by Schneider *et al.* (2011).

Animals

At the start of the experiment, eight pregnant, lactating multiparous Simmental cows in their second or third lactation were between 80 and 133 days in lactation. ECM was calculated by standardizing actual milk production of a cow to 35 g/kg milk fat and 32 g/kg milk protein following Bernard (1997). ECM production per cow averaged 28 kg/d during the seven days before the commencement of the experiment. The animals grazed for six weeks before the experiment started to accustom them to grazing and the feed of a pure pasture diet.

Experimental setup

The experiment was started on 19 May 2008 and ended eight weeks later on 18 July 2008 (61 days). The cows were matched for age, body weight, calving date and milk production during the current lactation and then allocated to one of two groups of four cows. One group was kept on the experimental pasture, while the other four cows were penned individually indoors to measure their individual feed intake. The experiment was a crossover experiment to allow measuring feed intake for all cows. The two groups were switched after four weeks. Thus the eight weeks of the experiment consisted of two continuous 28 day long periods of constant feeding and keeping. We will report discriminations only for the data of the last three weeks of each period, while the first week of the first period is discarded because it may be influenced by the previous diet, and the first week of the second period is discarded to account for possible acclimatisation effects.

The cows in the stall were fed twice a day with clipped herbage, and feed intake was measured by weighing feed offered and refused at each meal. Feed on offer aimed to produce at least 10% feed remains. Dry matter content of offered and refused feed was determined by drying at 60 °C for 48 h. The herbage for the cows in stall was also collected on the experimental pasture. This was done twice a day by mowing between the grazing cows to obtain, as closely as possible, the same feed for the stall-fed and pasture groups. This procedure led to a total stocking density of 2.8 cows/ha. All cows had continuous access to fresh herbage and fresh water.

The cows on the pasture were allowed to graze from 07:00 to 16:00 and from 18:00 to 05:00, with two milkings per day between the grazing periods. After each milking, the cows of both groups were confined for 30 min and half of the daily allotment of grain maize (dry matter 1.72 kg/d) was fed in individual troughs. After this feeding, the cows were returned to the pasture or stall, as appropriate.

We chose this diet of isotopically distinct components for three reasons. First, this is a realistic scenario for the application because cows are usually fed heterogeneous diets

consisting of herbage and concentrates, which also implies isotopic heterogeneity. Second, by feeding the concentrate in constant amounts, while the herbage may to some degree differ in amount and isotopic composition, we assess the variability that results from herbage uptake that is unknown for grazing cows. Third, by labelling one compound (starch) fed in constant amounts, while the others differ, we will be able to see whether strong routing due to imperfect scrambling in the digestive tract affects discrimination to a degree that impedes the use of discriminations for authenticity testing.

All cows were milked in a 2 x 4 herringbone milking parlour. Milk production was recorded using flowmeters (Westfalia, Germany), and milk samples were taken four times a week twice a day in the morning and the afternoon for analysis of fat, protein and urea contents and pH value. Fat and protein contents were determined by infrared spectrophotometry (MilcoScan-FT-6000; Foss Electric, Hillerod, Denmark) by the Bavarian Association for Raw Milk Testing, Wolnzach, Germany.

During the entire experiment all cows were weighed twice a week on two consecutive days, and the average body weight for the two days was determined. BCS and BFT were determined at the beginning of the last week of each period by the same two independent evaluators. The reason for this lies in the fact that a strong decline or increase in body weight or BFT would influence the carbon isotopic composition of animal products by body carbon pools. The exact breadboard, e.g. BCS and BFT measurement or milking procedure, was described in more detail by Schneider *et al.* (2011). The animal performance study was carried out according to the standard of CCAC (1993).

Feed, faeces and milk sampling

Herbage was clipped on the experimental pasture with a green fodder harvester (Hege 212B, Waldenburg, Germany) to a stubble height of 30 mm. Samples of the harvested herbage and supplemented grain maize were taken daily and stored at -18 °C. About 50 g faeces per cow, obtained from all animals by rectal grab sampling in the parlour, were taken four times a week during the measurement periods at 06:30 and 17:30 after milking and frozen at -18 °C. Two aliquots of milk were taken four times a week at morning and evening milking and also stored at -18 °C. Altogether 61 grain maize, 61 herbage, 256 faeces and 256 milk samples were collected during the experimental period. The samples of the first week of the two periods were discarded because they may be influenced by the previous diet and possible acclimatisation effects.

Sample preparation and isotope analysis

All samples were analysed separately except for the grain maize samples, which were pooled for one experimental week. Herbage, grain maize and faeces samples were dried at 60 °C for 48 h and ball-milled to a homogenous fine powder. For carbon isotope analysis of herbage, grain maize and faeces, aliquots of 0.7 ± 0.05 mg were weighed into ultra-clean tin capsules (3.3 mm x 5.0 mm), which were also used for milk and milk components.

Thawed whole milk was homogenized and an aliquot of 5 µl transferred into tin capsules. It was then freeze-dried for 72 h, which does not influence the stable isotope composition (Knobbe *et al.*, 2006). The remaining milk was used for separation of milk fat, casein and the whey fraction. The milk fat was removed by centrifugation (12 min at 2 500 g). Casein was precipitated from the skim milk by acidification with 10% HCl to pH 4.3 and subsequent centrifugation (30 min at 2 500 g). The residue consisting of lactose and whey proteins was heated to 80 °C in a water bath and the flocculated whey proteins were filtered. The remaining filtrate contained mainly lactose. In spite of a small amount of nitrogenous compounds (C/N ratio 23) in the filtrate, this fraction is termed lactose in the following. The filtered whey proteins were not examined as they contributed little to total milk carbon. Lactose filtrate (5 µl) was pipetted into tin capsules and freeze-dried for 72 h. Milk fat and casein were freeze-dried for 72 h before weighing and then aliquots of 0.45 ± 0.05 mg of the fat and 0.35 ± 0.05 mg of the casein were weighed into tin capsules.

The tin capsules were combusted in an elemental analyser (NA 1110, Carlo Erba, Milan, Italy) interfaced (Conflo III, Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). Each sample was measured against a laboratory working standard CO_2 gas, which was previously calibrated against a secondary isotope standard (IAEA-CH_6). After every tenth sample an internal lab standard with similar carbon/nitrogen ratio as the respective sample material (horn or wheat flour) was run as a control. Standard deviation for repeated measurements of laboratory standards was 0.11 ‰ - 0.14 ‰.

Calculation of discrimination

Stable carbon composition of the diet was calculated with a linear mixing model from the two constituents with known isotopic composition (herbage and grain maize) and the contribution of the two constituents to the diet (Phillips and Koch, 2002). It was only calculated for the cows kept in the stall because direct feed intake measurements on the pasture were not possible. Discrimination was then calculated with Eq. (3).

Statistical analysis

Linear regressions were used to evaluate the datasets. Hypothesis testing on equal means of groups or on parity of the mean of the population and a specific value was carried out using Student`s t-test (two-sided). This was performed against a 95% confidence interval, preceded by a test for normal distribution. All procedures followed standard protocols (Sachs, 1984).

RESULTS

Of all diet-carbon intake 90.2% originated from herbage, the remainder from grain maize. The proportion of herbage slightly tended to increase with time (from 89.6% to 90.8%), but this effect was not significant (r^2 = 0.04). Additionally, some variation between animals occurred yielding a total range of 85 to 95% of herbage in diet-carbon ingestion. While $\delta^{13}C_{maize}$ was constant, $\delta^{13}C_{herbage}$ decreased by about 0.6 ‰ over the total duration of the experiment, but short-term fluctuation was much larger (range: 1.8 ‰; Figure IV.1), where the total range could be found on consecutive days. The range in $\delta^{13}C_{diet}$ was slightly smaller (1.37 ‰) due to the constant $\delta^{13}C_{maize}$, but the decrease over time (0.7 ‰) was slightly larger than the change in $\delta^{13}C_{herbage}$ because of the simultaneously increasing proportion of herbage in diet.

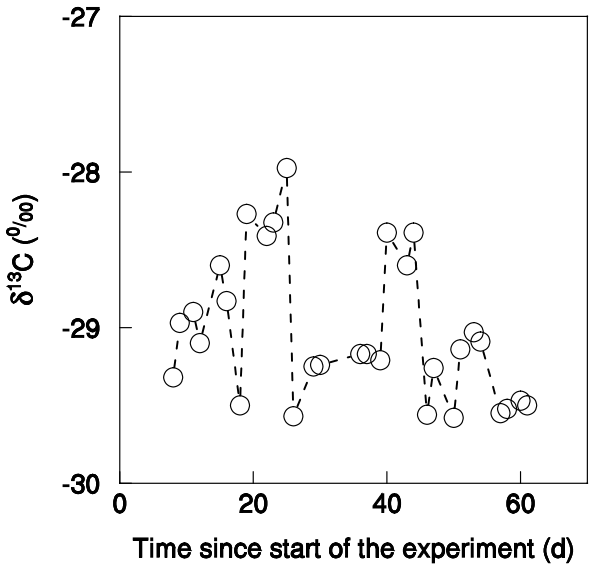

Figure IV.1: Relative carbon isotope ratio ($\delta^{13}C$) of herbage fed in stall.

The $\delta^{13}C$ differed significantly (P < 0.001) among diet, faeces, whole milk and milk components with milk fat having the lowest (–29.77 ‰) and casein the highest (–26.44 ‰) values (Figure IV.2; Table IV.1). The decrease in $\delta^{13}C_{diet}$ by 0.7 ‰ over time was slightly more put forward to faeces where it induced a change of 1.0 ‰, while the change in milk was only 0.6 ‰. Milk and faeces together again yielded a decrease 0.7 ‰ if we consider that

according to the digestibility of the diet (~ 0.78) only 22% of the diet is found in faeces, while 78% are metabolized. Due to this, discrimination did not change over time for faeces, milk and lactose, but it increased by 0.6 ‰ for milk fat (r^2= 0.185, P < 0.05) and decreased by 1.0 ‰ for casein (r^2 = 0.335; P < 0.001).

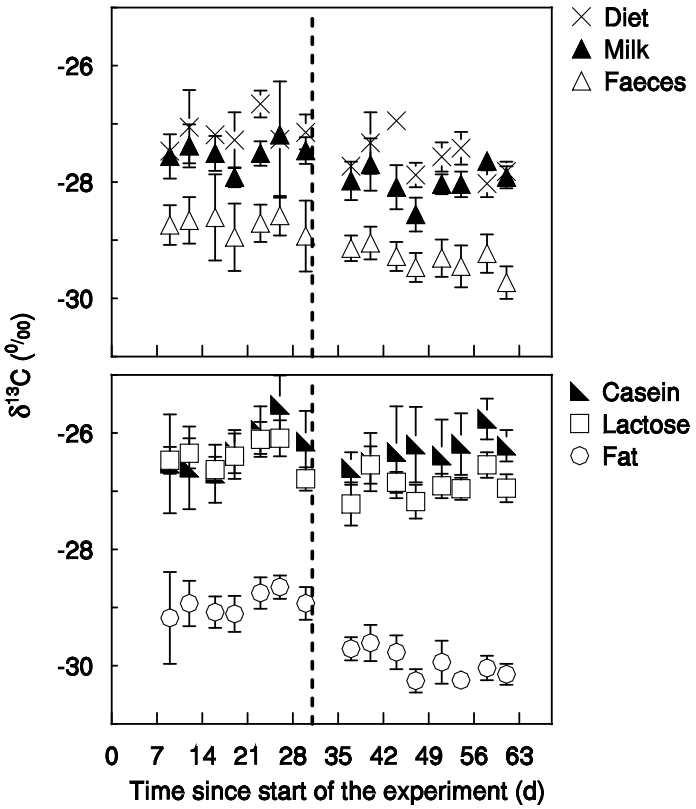

Figure IV.2: Relative carbon isotope ratio ($\delta^{13}C$) of diet organic matter, milk, faeces and milk components during the two experimental periods. The dashed vertical line denotes the change of the two experimental periods. Each data point comprises the average of four cows and two days (n = 8). Error bars denote the standard deviation.

The discrimination between diet, faeces, milk and milk components was only calculated for the cows in the stall where herbage intake had been measured and allowed calculating the $\delta^{13}C$ of the diet without the assumption regarding the herbage intake.

Table IV.1: $\delta^{13}C$ (‰) of herbage, grain maize, faeces, milk and milk components during the experimental period.

Material	Mean (‰)	Standard deviation (‰)	95% Confidence interval (‰)
Herbage	–29.03	0.46	0.05
Grain maize	–12.18	0.22	0.02
Faeces	–28.98	0.63	0.08
Milk	–27.92	0.46	0.06
Milk fat	–29.77	0.62	0.08
Casein	–26.44	0.64	0.08
Lactose	–26.71	0.36	0.05

Compared to the diet, whole milk, milk fat and faeces were depleted in ^{13}C with discrimination equal to 0.4 ‰, 2.2 ‰ and 1.7 ‰ respectively. In contrast, casein ($^{13}\Delta_{diet\text{-}casein}$ = –1.1 ‰) and lactose ($^{13}\Delta_{diet\text{-}lactose}$ = –0.7 ‰) were enriched in ^{13}C (Table IV.2). The range between individual cows in diet-product discrimination was largest for casein (1.3 ‰), while it was less than half of that for the other products (milk, milk components and faeces). Despite these variations within each material, the confidence intervals were even much smaller (between 0.05 ‰ and 0.16 ‰) due to the large number of samples (n = 232).

Table IV.2: ^{13}C discrimination ($^{13}\Delta$) between diet and milk and milk components and between diet and faeces in stall.

	Mean $^{13}\Delta$ (‰)	Standard deviation (‰)	95% Confidence interval (‰)
Diet – Milk	0.42	0.61	0.11
Diet – Milk fat	2.17	0.64	0.12
Diet – Casein	–1.14	0.87	0.16
Diet – Lactose	–0.73	0.58	0.11
Diet – Faeces	1.74	0.65	0.12

Milk fat was depleted in ^{13}C compared to whole milk ($^{13}\Delta_{milk–milk\ fat}$ = 1.9 ‰), while casein ($^{13}\Delta_{milk–casein}$ = –1.5 ‰) and lactose ($^{13}\Delta_{milk–lactose}$ = –1.2 ‰) had a less negative δ^{13}C value (Table IV.3). However, discrimination between milk and milk components differed slightly but significantly (P < 0.001) between stall- and pasture-fed groups (Table IV.3). Discrimination between milk and milk fat was 0.3 ‰ larger on pasture, while it was lower (by 0.2 ‰) between milk and lactose. In consequence, discrimination between fat and lactose averaged 3.2 ‰, but differed by 0.5 ‰ between stall and pasture.

Table IV.3: ^{13}C discrimination ($^{13}\Delta$) between milk and milk components in the stall and on pasture. P gives the probability of stall and pasture not being different.

	Stall	Pasture		Stall and pasture	
	Mean $^{13}\Delta$ (‰)	Mean $^{13}\Delta$ (‰)	P	Mean $^{13}\Delta$ (‰)	Standard deviation (‰)
Milk – Milk fat	1.78	2.04	< 0.001	1.91	0.51
Milk – Casein	–1.55	–1.48	0.43^{ns}	–1.51	0.68
Milk – Lactose	–1.14	–1.34	< 0.001	–1.24	0.42
Lactose – Milk fat	2.91	3.39	< 0.001	3.15	0.58
Casein – Milk fat	3.32	3.54	0.41^{ns}	3.43	0.83

DISCUSSION

Discriminations as determined in this study should be reliable, given I) the small isotopic change between the feed of the pre-experimental period (pasture herbage) and the experimental period (pasture herbage and about 10% of grain maize); II) the long equilibration time after shifting to the experimental feed, which was one week and about two

to eight times longer than the half-life times in the experiments by Boutton *et al*. (1988); there was no indication that turnover of body material influenced the data over the eight weeks of the experiment; III) the strictly controlled conditions especially with stall feeding, where feed and orts were determined daily; IV) the comparably small trend in feed isotopic composition (~ 0.7 ‰ for $\delta^{13}C$) during the experimental period and V) the large number of animals and milkings yielding a total of 232 sample pairs per discrimination. Altogether these favourable conditions led to 95% confidence intervals of far below 0.2 ‰ in the case of diet-product discrimination. Most of this small variation was caused by variation in the diet because variations in discriminations among milk components were only about half as large.

From the existing studies from which discriminations can be derived (Table IV.4, Table IV.5, Table IV.6) only one applies for grazing cows (Minson *et al.*, 1975). Also only one study was found for milk-casein discrimination (Camin *et al.,* 2008) and another one for lactose-milk fat discrimination (Schulze *et al.,* 1992). No study provided a complete overview among diet, faeces, milk and main milk components and no study used so many animals and samplings as our study. The variation in discrimination between the different studies was considerable. For instance, the diet-milk discrimination in the studies ranged from –2.9 ‰ to 2.2 ‰, whereas our study indicated a mean of 0.4 ‰ with an interval of confidence for the mean of only 0.1 ‰. The question arises what causes this large variation found in literature. Although we can only speculate because feeding conditions were often not described in sufficient detail, the following reasons can be identified:

1) The fast reaction of faeces, milk and milk components to the $\delta^{13}C$ variation of the diet (Boutton *et al.,* 1988; Knobbe *et al.,* 2006) requires that diet $\delta^{13}C$ is determined with high temporal resolution. Such variation over time is likely to occur. Even under our conditions which aimed to produce constant growing conditions by controlling pasture height, the herbage varied by about 2 ‰ between consecutive days. Also any selection of feed among individual animals can cause a scatter in estimated $^{13}\Delta$.
2) The temporal shift between intake and the effect in the product (1 d for milk and milk components) requires that constant feeding must be sufficiently long for a reliable calculation of discrimination. Only 29% of the discriminations from literature (Table IV.4, Table IV.5, Table IV.6) had experimental periods longer than 14 days, but some of these did not control the diet. In contrast we did not use the first 7 days after switching from pure herbage diet to the grain maize supplemented herbage diet and we maintained this diet for 61 days. The largest error in our discriminations would occur if the complete body carbon would have been exchanged during the experimental period. Excluding the digestive tract our cows contained approximately 110 kg carbon, while the total metabolized carbon intake (accumulated carbon intake multiplied by digestibility) during

61 days was 334 kg. Hence the body stores could on average contribute not more than 33%. A difference of 2 ‰ between body and diet would then lead to a bias of 0.7 ‰. The true bias of our discriminations must be smaller than that given that the turn-over time for body carbon is three times longer (Bahar *et al.*, 2005) and that the exchange will be largest during the first week, which was excluded from the analysis.

3) Large differences between feed components in digestibility can strongly affect discrimination (Jones *et al.,* 1979). Components with lower digestibility will be predominantly found in faeces and correspondingly will be underrepresented in milk (McCutchan *et al*., 2003; Hwang *et al*., 2007). Feed components, which predominantly are used for the synthesis of only one milk component like fatty acids supplying milk fat synthesis will also influence the discrimination if they are not uniformly distributed between feed components. In our study, digestibility of organic matter was high (~ 78%, Schneider *et al.,* 2011), and the C_4 component had practically the same digestibility than the C_3 component. This could cause only little deviation between the apparent and the true discrimination. Incomplete isotopic scrambling may further complicate the picture.
4) Under conditions of negative energy balance, where more energy is used in milk production than is assimilated from feed, body stores will play a larger role and can cause a bias in discrimination if the consumption of body mass is not accounted for.

All four mechanisms can cause a deviation between the apparent and the true discriminations. They can be expected to have a relatively small effect under the experimental conditions of this study. Hence, our discriminations should be close to the true discriminations.

Table IV.4: Carbon isotope discrimination ($^{13}\Delta_{diet-product}$) of faeces, milk and milk components relative to the diet of dairy cows from this study and from literature. Studies taken from literature are arranged according to decreasing percentage of C_3 plants in the diet.

Cows	Duration (d)	Sampling dates	C_3 in diet (%)	Mean $^{13}\Delta$ (‰)	Reference
			Diet – Milk		
8	61	16	90%	0.4	This study
3	42	42	100%	0.6	Boutton *et al*. 1988
3	49	7 (1. wk), 3 (2.-7. wk)	100%	0.2	Boutton *et al*. 1988
1	50	9	100%	–1.1	Knobbe *et al*. 2006
1	7	1	100% (2 diets)	–0.6,–0.6	Masud *et al*. 1999
6	14	2 diet/14 milk	100%	–1.8,–1.5,–0.7	Metges *et al*. 1990
2		1	100%	–2.9,–2.9	Minson *et al.* 1975
1	10	4	56%	2.1	Knobbe *et al*. 2006
1	7	1	50%	–1.0	Masud *et al*. 1999
6	14	2 diet/14 milk	50%	–1.3	Metges *et al*. 1990
3	42	42	0%	1.8	Boutton *et al*. 1988
3	49	7 (1. wk), 3 (2.-7. wk)	0%	2.2	Boutton *et al*. 1988
1	7	1	0%	3.0	Masud *et al*. 1999
6	14	2 diet/14 milk	0%	0.2, 0.3, 0.9	Metges *et al*. 1990
6		1	0%	1.5, 2.5	Minson *et al*. 1975
4	42	6	3 mixed C_3/C_4	–1.1, 1.0, 2.0	Tyrrell *et al*. 1984
bulk	>14	2-4	4 mixed (37-77%)	–0.9,–0.2, 0.6, 1.3	Camin *et al*. 2008
			Diet – Milk fat		
8	61	16	90%	2.2	This study
1	7	1	100% (2 diets)	–7.6, 1.6	Masud *et al*. 1999
4	10-14	2–3 (last 2-3 d)	100%	1.8	Wilson *et al*. 1988
1	7	1	50%	3.2	Masud *et al*. 1999
1	7	1	0%	7.9	Masud *et al*. 1999
4	8-9	2-3 (last 2-3 d)	0%	6.2	Wilson *et al*. 1988
4	12	11	mixed C_3/C_4	4.0	Schulze *et al*.1992
bulk	>14	2-4	8 mixed (37–77%)	–2.5,–1.6,–0.9,–0.7 –0.5,–0.3, 1.9, 2.4	Camin *et al*. 2008
			Diet – Casein		
8	61	16	90%	–1.1	This study
4	10-14	2-3 (last 2–3 d)	100%	–1.8	Wilson *et al*. 1988
4	8-9	2-3 (last 2–3 d)	0%	–3.2	Wilson *et al*. 1988
bulk	>14	2-4	8 mixed (37-77%)	–3.9,–3.7,–2.1,–1.4 –1.2,–1.1,–0.9,–0.5	Camin *et al*. 2008
			Diet – Lactose		
8	61	16	90%	–0.7	This study
1	7	1	100% (2 diets)	–0.4, 1.8	Masud *et al*. 1999
4	10-14	2-3 (last 2-3 d)	100%	0.0	Wilson *et al*. 1988
1	7	1	50%	–1.6	Masud *et al*. 1999
1	7	1	0%	0.9	Masud *et al*. 1999
4	8-9	2-3 (last 2-3 d)	0%	1.7	Wilson *et al*. 1988
4	12	11	mixed C_3/C_4	2.0	Schulze *et al*. 1992
			Diet – Faeces		
8	61	16	90%[1]	1.7	This study
4	10-14	2-3 (last 2-3 d)	100%	1.7	Wilson *et al*. 1988
4	8-9	2-3 (last 2-3 d)	0%	2.2	Wilson *et al*. 1988
4	42	6	3 mixed C_3/C_4	–1.1, 0.1, 0.5	Tyrrell *et al*. 1984

[1] Remainder is C_4.

Values are (re-)calculated as discrimination $^{13}\Delta = (\delta^{13}C_{diet} - \delta^{13}C_{product}) / (1 + \delta^{13}C_{product})$. All experiments were conducted in the stall except for Minson *et al*. (1975) who worked with grazing cows.

Table IV.5: Carbon isotope discrimination ($^{13}\Delta_{milk–milk\ component}$) between milk and milk components of dairy cows from this study and from literature. Literature studies are arranged according to decreasing percentage of C_3 plants in the diet.

Cows	Duration (d)	Sampling dates	C_3 in diet (%)	Mean $^{13}\Delta$ (‰)	Reference
			Milk – Milk fat		
8	61	32	90%	1.9	This study
1	7	1	100% (2 diets)	–7.0,–2.2	Masud *et al*. 1999
1	7	1	50%	–2.2	Masud *et al*. 1999
1	7	1	0%	4.6	Masud *et al*. 1999
bulk	>14	2-4	4 mixed (37-77%)	0.3, 0.6, 1.4, 1.9	Camin *et al*. 2008
			Milk – Casein		
8	61	32	90%[1]	–1.5	This study
bulk	>14	2-4	4 mixed (37-77%)	–0.8,–0.8,–0.9,–1.4	Camin *et al*. 2008
			Milk – Lactose		
8	61	32	90%	–1.2	This study
1	7	1	100% (2 diets)	0.2, 1.2	Masud *et al*. 1999
1	7	1	50%	–0.6	Masud *et al*. 1999
1	7	1	0%	–1.9	Masud *et al*. 1999

[1] Remainder is C_4.
Values are calculated as discrimination $^{13}\Delta = (\delta^{13}C_{milk} – \delta^{13}C_{milk\ component}) / (1 + \delta^{13}C_{milk\ component})$. All experiments were conducted in the stall except for this study.

Table IV.6: Carbon isotope discrimination ($^{13}\Delta_{component\ 1\ –\ component\ 2}$) between milk components of dairy cows from this study and from literature. Literature studies are arranged according to decreasing percentage of C_3 plants in the diet.

Cows	Duration (d)	Sampling dates	C_3 in diet (%)	Mean $^{13}\Delta$ (‰)	Reference
			Lactose – Milk fat		
8	56	32	90%	3.1	This study
4	12	11	mixed C_3/C_4	3.0	Schulze *et al*.1992
			Casein – Milk fat		
8	61	32	90%[1]	3.4	This study
4	10-14	2-3 (last 2-3 d)	100%	3.4, 4.0	Wilson *et al*. 1988
4	10-14	2-3 (last 2-3 d)	0%	4.9, 1.2	Wilson *et al*. 1988
bulk	> 14	2-4	8 mixed (37-77%)	0.7, 1.1, 1.4, 1.4 2.5, 2.8, 2.9, 3.0	Camin *et al*. 2008

[1] Remainder is C_4.
Values are calculated as discrimination $^{13}\Delta = (\delta^{13}C_{component\ 1} – \delta^{13}C_{component\ 2}) / (1 + \delta^{13}C_{component\ 2})$. All experiments were conducted in the stall except for this study.

CONCLUSIONS

Compared to previous studies this study provides the most complete set of discriminations between diet, milk, milk components and faeces that were determined over a long time. The effects of those parameters, which usually are not known in proof of origin and food safety analyses and hence cannot be predicted, namely the quality of the roughage and the keeping conditions, had only a small influence on the discriminations (usually < 0.2 ‰). The discriminations reported here should be superior to previously reported discriminations for

proof of origin and authenticity testing given the large number of samples, the well-controlled conditions and conditions that were close to practical agriculture including grazing. The largest problem in the interpretation of the isotopic composition of a mixed diet retrieved from the isotopic composition of the products and their discriminations results from the usually unknown variation in the C_3 herbage, which varied by almost 2 ‰ within consecutive days in our case.

Chapter V: General and Summarizing Discussion

In Europe, evermore consumers demand for natural and sustainable agriculture (Lobsiger *et al.,* 2010) and animal welfare (Dillon *et al.,* 2006). Milk production on pasture is associated with this request. Some of the consumers are willing to pay higher prices for organic origin or pasture husbandry (Pillonel *et al.,* 2005; Lobsiger *et al.,* 2010). Farmers can use this trend to realize advanced prices for their products, when they produce milk by pasture herbage or hay without silage (Lobsiger *et al.,* 2010). A variety of milk brands exist like milk from alpine regions that implicitly promise a certain quality mainly by defining the production system (Lobsiger *et al.,* 2010). Stable isotope analysis is used in authenticity testing, origin assignment, and food quality (Rossmann *et al.,* 2000; Rossmann, 2001; Kornexl *et al.,* 2007), and help to avoid mislabeling and fraud (Rossmann *et al.,* 2000). The objective of this research was to describe the isotopic discrimination of $\delta^{13}C$ between diet, faeces, milk and milk components of cows in stall and with grazing. Therefore four experiments were conducted over two years, and in all experiments lactating dairy cows grazing an all-day pasture with continuous stocking were supplemented with grain maize. A singularity of these experiments is the long experimental period.

To establish or use discrimination three prerequisites are necessary:
1) a negative impact of maize supplementation as natural isotopic marker must be excluded for grazing dairy cows, to avoid that a major change in the metabolic status or in health of the cows would affect discrimination; 2) a similar OMD allows using the amount of the sole components of the diet to calculate the carbon isotopic composition of the diet and 3) the elution kinetic, which describes the time span after a diet change until an isotopic equilibrium is achieved, must be known to ascertain that no or only little body material, which may have a different isotopic composition from an earlier feeding with differing isotopic composition, influences the discrimination. The present work contributes to the use of stable isotope analysis as a tool of authenticity testing and proof of origin, which must be based on a profound understanding of isotope metabolism.

In the first experiment different daily amounts of grain maize (1 kg vs. 3 kg/d) were supplemented to grazing dairy cows, and the influence on animal performance and health was examined (Chapter II). There was no beneficial effect of grain maize supplementation above 1 kg per day on the ECM, milk components, animal condition parameters and animal health under conditions of all-day pasture with continuous stocking. After a negative impact of grain maize supplementation can be excluded, grain maize of this amount can thus be used for grazing dairy cows as natural marker in stable isotope experiments.

Knowledge of digestibility is a key point for the determination of isotopic discrimination (Norman *et al.,* 2009) because of the fact that animals assimilate dietary components with varying efficiency (Gannes *et al.,* 1997). A mixed diet may hence not be passed on in equal relative amounts to the product if digestibility of the sole components differs (Jones *et al.,* 1979). The OMD is important for the interpretation of the results of stable isotope analysis because it determines the proportion of the different components in the diet that are digested and can be found in milk and milk components, whereas the indigestible proportions will emerge in faeces (McCutchan *et al*., 2003; Hwang *et al*., 2007). So OMD differing among feed components would lead to biased isotopic discrimination. It was expected that all-day pasture with continuous stocking (in German: "Kurzrasenweide") could provide C_3 roughage with similar OMD as C_4 concentrate (grain maize), and thus allow to vary the amount of C_4 component without a confounding change in OMD. To prove this expectation OMD of pasture herbage and grain maize were determined in the second experiment. The result showed that the pasture herbage diet had practically the same high OMD (~ 78%) as the grain maize supplemented pasture herbage diet (Appendix).

Further, the elution kinetic of grain maize derived ^{13}C was examined. In seeking to establish the isotopic discrimination, eight lactating dairy cows, which grazed an all-day pasture or were fed fresh pasture herbage in the stall, were supplemented with grain maize as isotopic marker. When supplementation with grain maize ended after eight weeks and pasture herbage became the sole feed source, the fate of grain maize derived ^{13}C was traced in faeces, milk and milk components over a 10 day long period (Chapter III). Due to the variation in equilibration time between tissues and differences between animal species, the tissue-specific elution kinetic of grain maize derived ^{13}C must be known when using the $\delta^{13}C$ technique to quantify short time dietary changes (McCutchan *et al.*, 2003; Norman *et al.,* 2009; Wittmer *et al.*, 2010). The experiment showed that grain maize feeding can be detected in faeces, milk and milk components only for three days after changing the diet from grain maize supplemented grazing to pure grazing. Elution kinetics of lactose and faeces was even faster (36 hours). Knowledge of this fast turnover is a prerequisite for the interpretation of stable isotope analysis by official food control. Camin *et al.* (2008) have shown that a mismatch between diet and animal products can be used for determining mislabelling of dairy products. The fast elution as shown in this experiment restricts the detection of such mislabelling to less than a week before sampling. A feed not in agreement with the labelling that was fed earlier could not be detected anymore in milk. In this case other body tissues reflecting longer time spans must be used. Hair is a prime tissue in this case (Schwertl *et al.,* 2005).

Isotopic discrimination can only be assumed to be constant when cows are in energetic and isotopic equilibrium (Ayliffe *et al.,* 2004). Even when cows are in energetic equilibrium, there is permanent renewal of body tissue (Gannes *et al.,* 1998; Ayliffe *et al.,* 2004), which also delivers old carbon to milk. Under Central European conditions, the seasonal cycle of feeding may result in isotopic variation because there are massive feed changes between winter stall feeding and pasture grazing during spring, summer and autumn (Kornexl *et al.,* 1997). This is especially true where all-day pasture with continuous stocking during the growing season is followed by conserved, maize dominated feed during the winter period when animals are housed (Bahar *et al.,* 2008). In the all-day pasture system with continuous stocking, which was used in our experiments, cows normally calve seasonally in late winter or early spring; so that the pasture herbage is the main and cheapest feed for milk production (Thomet *et al.,* 2002). During late lactation body reserves are rebuild for new lactation (Schröder and Staufenbiel, 2006) and dietary carbon might be expected to contribute nearly all milk carbon (Boutton *et al.,* 1988). In this special grazing system this takes place during winter stall feeding. Here, often grass and maize silage are fed as roughage. Cows are losing a large amount of body mass postpartum (50-60 kg, Baumann and Currie, 1980; Schröder and Staufenbiel, 2006), and the stable isotope composition of this different diet influences the stable isotope composition of the products. This negative energy balance in early-lactation was confirmed by Boutton *et al.* (1988), Phillips and Koch (2002) and Clark *et al. (*2005), who showed that body tissues are then a source of nutrients for milk synthesis, particularly during early lactation. During the first month of lactation the body reserves being utilized can be energetically equivalent to about 33% of the milk produced (Baumann and Currie, 1980). In an all-day pasture system with continuous stocking in spring all cows are simultaneously in negative energy balance during the time period after calving. This was one reason for the use of cows in mid lactation in an all-day pasture system with continuous stocking during the experiments.

During the lack of energy postpartum body fat is degraded and partially directly incorporated in milk fat (Baumann and Currie, 1980). This milk fat is then used to produce butter and cheese. Fat is depleted in $\delta^{13}C$ compared to other tissues (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987) and that is why there is a falsification of the isotope discrimination when body fat is mobilized for milk synthesis. Therefore in our experiments, the cows were in mid lactation, milk yield was not very high and body weight, BFT and BCS did not change significantly during the experimental period. So the cows were expected to be in energy balance. Hence, our apparent discrimination should be near to true discrimination.

The distinction between pasture-based milk production and conventional stall-based milk production systems, in which most cows are fed primarily with corn-based feed and concentrate supplements by carbon stable isotope analyses, is quite simple (Kornexl *et al.,* 2007; Lobsiger *et al.,* 2010). However, supplementation of herbage or grass silage with cereal-based concentrates may not be detected because stable carbon isotope composition of C_3 cereal-based concentrates is not greatly different of that of pasture herbage (Monahan *et al.,* 2010) due to the identical photosynthetic pathway (Gebbing *et al.,* 2004). Especially in this case it is difficult to deduce the feeding conditions only from the carbon isotope composition of milk and milk components. Isotope analysis can be used to verify whether a reported feeding regime fits to the measured isotopic composition, but in many cases it is impossible to predict the feeding regime from the isotopic composition because many differing feeding regimes could yield similar isotopic compositions. To verify pasture-based milk production, a wide range of chromatographic, spectroscopic, molecular and enzymatic methods are available to quantify for example fatty acids, volatile compounds, carotenoids and tocopherols (Monahan *et al.,* 2010). A multielement stable isotope analyses with carbon, sulphur, hydrogen, nitrogen and oxygen (Boner and Förstel, 2004) combined with measurements of other markers including trace elements (Camin *et al.,* 2004), fatty acids and carotenoids can help to trace back more exactly milk products (Rossmann *et al.,* 2000). Carbon stable isotope analysis can deliver one important facet, but additional methods are certainly necessary.

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Appendix

Suitability of eight techniques for estimating digestibility of herbage from continuously grazed all-day pasture

ABSTRACT

The objective of this study was to estimate digestibility of herbage using eight different methods. Organic matter digestibility (OMD) was estimated with titanium (Ti) dioxide and acid-insoluble ash (AIA) as indigestible markers, four faecal nitrogen (N) equations, which use the same raw data, the pepsin-cellulase method (*in vitro* OMD) and digestibility trials with wethers (*in vivo* OMD). An all-day pasture with continuous stocking at 2.8 cows/ha was chosen for the comparison because it restricted selection during grazing and thus allowed comparison of *in vitro*- and stall feeding-based methods with methods used for pastures. A crossover experiment with eight lactating Simmental cows was conducted from May until July 2008, with two consecutive experimental periods of 28 days. The cows were divided in two similar experimental groups. Four cows were put into individual stalls and fed herbage clipped from the experimental pasture and feed intake was measured. The other four cows were put onto the fenced pasture. All cows were supplemented with 2 kg/d fresh matter of grain maize. After four weeks, the treatment groups were switched.

OMD differed considerably between methods (by 20−110 g/kg). Applying the same method, OMD on pasture differed from OMD in-stall indicating that the grazing animal cannot be replaced by mowing even with high experimental effort and low opportunity for selection. It also differed over time. Only the faecal N methods were readily applicable on pasture at sufficiently high temporal resolution and – with one exception – produced similar results in-stall as the Ti method. They can in general be recommended for a large range of conditions including grazing studies but the variety of existing equations makes it difficult to select the appropriate one. This calls for the development of better defined and rigorously tested equations.

INTRODUCTION

The increasing costs of feed concentrates have led to increased interest in pasture-based milk production, particularly with respect to cost-effective pasture management systems. Feed intake and organic matter digestibility (OMD) ingested are the two most important components affecting the animal performance, but neither can be measured easily at pasture (Moore, 1996). Attempts to predict intake and OMD are quite numerous (Moore, 1994; Mayes and Dove, 2000), but all have their weaknesses (Moore, 1996; Delagarde and O´Donovan*,* 2005).

Feed intake at pasture can be modelled (Delagarde and O´Donovan*,* 2005) or it can be estimated from the excretion of indigestible markers, if the OMD of the ingested feed is known. Hence, knowledge of OMD is important in its own right and to estimate feed intake from the excretion of markers. The underlying concept is that when a known amount of an indigestible marker is fed and equilibrium is achieved, the same amount of that marker should then be voided with the faeces.

Several methods, each based on different principles, allow the determination of OMD. Each method is a simplification that reduces measurement effort and thus allows more repetitions, but it then introduces uncertainty regarding whether the obtained results can be generalized in a broader sense. The complexity of the examined situation increases from measuring *in vitro* OMD to *in vivo* techniques, the application of markers and the faecal N methods.

In vitro OMD methods are the least specific because they ignore the technical aspects of production and disregard the animal, except as a donor of rumen liquor. These techniques either simulate the rumen fermentation by enzymes (De Boever *et al.,* 1986) or the rumen liquor is used to measure OMD microbiologically (Tilley and Terry, 1963; Menke *et al.,* 1979). *In vivo* techniques, using sheep (wethers) or cattle, are more realistic since they are based on direct measurement of feed intake and faecal output in the stall under controlled conditions. These methods account for the animal digestive process, but wethers are often taken as the “standard ruminant” simply because of their ease of handling and management. The influence of species, lactation, breed, sex, age and level of nutrition are seldom considered. Furthermore, the standardized conditions for these *“in vivo”* tests may deviate considerably from real conditions that are encountered in normal animal keeping (Gabel *et al*., 2003).

Markers account for these influences. Internal markers, such as lignin, chromogen or acid-insoluble ash (AIA), are unavoidably taken up together with the feed while external markers,

such as metal oxides or rare-earth compounds are fed to the animals. N-alkanes are widely used markers, in which a combination of an internal and an external marker is used. Major limitations of such marker studies include non-representative sampling of the ingested feed, incomplete faecal recovery of the marker and differing recovery rates of the markers (Lancaster, 1949; Titgemeyer *et al.,* 2001; Mayes and Dove, 2000).

Recently, faecal-N methods gained interest as indirect methods (Schmidt *et al.,* 1999; Lukas *et al.,* 2005; Wang *et al.,* 2009) for allowing the calculation of OMD from faecal-N content using regression models. These faecal-N methods can be applied under a wide range of conditions, including grazing, given that fresh faeces can be sampled.

Stall experiments are comparatively easy to control through feed intake measurements and faecal sampling. However, the applicability of OMD obtained from stall-feeding studies to OMD under natural grazing has not yet been established. In many cases, the provision of cut forage samples to animals may not entirely supply the same feed quality as forage ingested during grazing. The same applies to other methods that employ forage samples, such as *in vitro* or *in sacco* digestibility tests. Cut or hand-plucked herbage may not simulate selection by the grazer (Baumont *et al.,* 2000; Schlegel *et al.,* 2000). Oesophageally fistulated animals avoid this source of error, but they require a surgical preparation of the animal and irritation of the animals may influence the results.

Comparisons of two or three methods for OMD estimation have already been done (Macoon *et al.,* 2003; Schiborra *et al.,* 2010). However, Schiborra *et al.* (2010) compared *in vivo* and *in vitro* OMD, but attributed the observed differences entirely to the grazing selection by the sheep. They assumed that both methods would lead to identical results if selection did not occur. Furthermore, studies that include a large range of methodologies are missing, to our knowledge. Thus, the question remains whether *in vivo* and *in vitro* OMD methods, and other OMD methods, yield similar results given similar conditions

Making this type of comparison would be relatively simple using confined animals but it is significantly more challenging under grazing conditions. In the present work, we introduce a new approach that allowed us to establish the same feed for animals both in-stall and at pasture, thus allowing comparison of several techniques for OMD estimation under grazing and stall-fed conditions.

The objectives of this research were: (1) to estimate OMD of herbage from all-day pasture using different techniques and (2) to compare the results of the different techniques.

MATERIALS AND METHODS

Experimental site

The experiment was conducted at the research station of the University of Applied Sciences Weihenstephan, near Freising, southern Germany (48°26'N; 11°46'E), 493 m above sea level. The experimental pasture was a 3.0 ha semi-natural grassland on silty loam soils. The mean annual temperature and precipitation are 7.5°C and 794 mm/yr, respectively. The pasture plot was fertilized with 54 kg/ha N (as calcium ammonium nitrate) before the experiment started and again with 54 kg/ha N after 28 days of grazing. The botanical composition of the pasture (Table 1), estimated by visual inspection by trained persons before and after the experiment, was dominated by grasses (0.82−0.86 in the harvested forage dry matter mass) with *Lolium perenne* (L.) contributing the most (0.60−0.75), while the legume *Trifolium repens* (L.) contributed 0.05−0.08.

Table 1: Botanical composition of the experimental pasture (fraction of standing dry matter).

Date	Start of experiment (28.04.2008)	End of experiment (18.07.2008)
Grasses		
Lolium perenne	0.750	0.600
Poa trivialis	0.032	0.086
Poa annua	0.016	0.019
Poa pratensis	0.000	0.036
Dactylis glomerata	0.062	0.079
Three other species	0.007	0.003
Sum grasses	0.867	0.823
Herbs		
Plantago major	0.020	0.060
Taraxacum officinale	0.003	0.017
Five other species	0.009	0.012
Sum herbs	0.032	0.089
Legumes		
Trifolium repens	0.084	0.054
Sum legumes	0.084	0.054
Miscellaneous	0.017	0.034
Total	1.000	1.000

Compressed sward height was measured daily before the evening milking with a rising-plate meter (Ashgrove, RD 10, New Zealand) applying a load of 4.8 kg/m^2. Approximately 150 measurements per day were taken, one at every ten steps along two diagonal lines across the experimental pasture. The target sward height was 6-7 cm.

Experimental animals

The experiment was initiated on 19 May 2008 and ended on 18 July 2008 and consisted of two continuous 28-day trial periods (experiment period 1 and 2) each consisting of one week of acclimatisation to the experimental conditions and three weeks of measurement (measuring period 1 and 2). Eight pregnant, lactating multiparous Simmental cows in their second or third lactation were combined in a total herd of 30 cows. At the start of the experiment, the cows were between 80 and 133 days in lactation. Energy corrected milk (ECM) was calculated by standardizing actual milk production of a cow to 35 g/kg milk fat and 32 g/kg milk protein following Bernard (1997). ECM averaged 28 kg/d during the seven days before the commencement of the experiment. The animals were accustomed to grazing and the feed by keeping them on a pure pasture diet for 6 weeks before the experiment started.

The cows were matched for age, body weight (BW), calving date and milk production during the current lactation and then allocated to one of the two groups of four cows. One group of four cows was put onto the fenced experimental pasture plot during period 1, while the other four cows were penned individually indoors to measure feed intake. The individual boxes in the stall had a floor area of 20 m^2 and the cows were allowed to move freely. The cows in the stall were fed with clipped herbage collected on the experimental pasture by mowing between the grazing cows. This was done to obtain, as closely as was possible, the same feed for the stall-fed and pasture groups. This strategy led to a total stocking density of 2.8 cows/ha. All cows had continuous access to fresh herbage and fresh water. After four weeks, the groups were switched, so that the four cows previously grazing on the pasture were moved to the stalls (P-S group), and the cows, that had been penned in the stall during period 1 were moved to the pasture (S-P group).

The dry matter (DM) intake in the stall was measured by weighing feed offered and feed refused after each meal before fresh herbage was fed. Feed on offer aimed to produce at least 10% feed remains. DM content was determined by drying at 60°C for 48 h.

The cows on the pasture were allowed to graze from 07:00 to 16:00 and from 18:00 to 05:00, with two milkings in between. The pasture had water tubs fitted with float-control devices to ensure permanent availability of fresh drinking water.

All cows were milked in a 2 x 4 herringbone milking parlour. Milk production was recorded using flowmeters (Westfalia, Germany) and milk samples were taken four times a week twice a day in the morning and afternoon, for analysis of fat, protein and urea contents and pH

value. Fat and protein contents were determined by infrared spectrophotometry (MilcoScan-FT-6000; Foss Electric, Hillerod, Denmark) by the Bavarian Association for Raw Milk Testing, Wolnzach, Germany, in order to convert milk production to ECM.

After each milking, the cows from both groups were confined for 30 min and half of the daily allotment of grain maize (1.72 kg/d DM) was fed in individual troughs. After this feeding, the cows were brought to their respective treatments. The grain maize served as the carrier for the Ti marker (twice daily dosing) and as an energy supplement to balance the high content of ruminally degradable protein in herbage. Weekly samples of maize were dried at 60°C for 48 h and the DM content was calculated. Feed remains of grain maize were collected and weighed and DM content was estimated by drying to calculate the effective maize and marker intake.

During the entire experimental periods all cows were weighed twice weekly on two consecutive days and the average BW for the two days was determined. Body condition score and back fat thickness were determined at the beginning of the last week of each period by the same two independent evaluators. Body condition score on a scale of 1−5 (1, very thin; 5, very fat) was defined by the method of Wildman *et al*. (1982) and back-fat thickness was measured sonographically (Esaote, Tringa Linear 50, Oberhausen, Germany) at the measuring point defined by Schröder and Staufenbiel (2006).

Experimental feed

Herbage was clipped on the experimental pasture with a green fodder harvester (Hege 212B, Waldenburg, Germany) to a stubble height of 30 mm. Excess feed on offer was reduced at the beginning of experimental week 4 by additionally harvesting 15% of the pasture. Feed properties are shown in Table 2.

Table 2: Feed properties of herbage harvested from all-day pasture above a stubble height of 3 cm and of grain maize.

Item	Herbage									Grain maize
Week:	1	2	3	4	5	6	7	8	Mean	
Period:	0	1	1	1	0	2	2	2		
Sward height (cm)	6.2	6.4	6.9	6.3	6.4	6.2	6.1	5.7	6.2	
Dry matter content of fresh matter (g/kg)	153	165	168	164	169	161	178	152	164	887
Dry matter composition (g/kg)										
Organic matter	902	903	905	896	899	901	893	895	899	982
Crude protein	216	188	190	189	201	211	213	230	205	94
Ether extract	39	36	42	37	41	43	38	46	40	46
eIOM	242	315	339	287	249	242	233	224	266	
eSOM	660	588	565	610	651	658	646	671	631	
NDF_{om}	533	554	501	506	499	549	517	522	523	336
NFC	114	125	172	165	159	97	125	96	131	506
ADF_{om}	307	297	310	311	284	304	271	283	296	38
Lignin(sa)	26	27	26	25	26	22	23	24	25	16

eIOM, enzymatically insoluble organic matter; eSOM, enzymatically soluble organic matter; NDF_{om}, neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive of residual ash; NFC, non fiber carbohydrates; ADF_{om}, acid detergent fiber not assayed with a heat stable amylase and expressed exclusive residual ash; Lignin(sa), Lignin determined by solubilization of cellulose with sulphuric acid. Period 0, adaptation; period 1 and 2, digestibility measuring periods.

The main part of the harvested herbage (fresh matter approximately 450 kg/d) was fed *ad libitum* to the four cows in the stall to measure feed intake. Representative samples of the harvested forage were taken daily to determine DM content by oven drying. For the digestion experiment with wethers, herbage (15 kg/d) was stored in plastic bags at −18°C.

Sample collection of herbage and faeces

A sample of the harvested herbage was immediately frozen and stored at −18°C for later chemical analyses. The frozen samples were thawed (36 h at room temperature), dried at 60°C for 48 h and then ball milled to a homogenous fine powder. Weekly samples were pooled and the gross composition (DM, crude protein, ether extract, ash) was measured. AIA contents (van Keulen and Young, 1977) and acid-detergent fiber (ADF_{om}), neutral detergent fiber (NDF_{om}) and lignin(sa) (Robertson and Van Soest, 1981; Van Soest *et al*., 1991) were measured. ADF_{om} and NDF_{om} were not assayed with a heat stable amylase and are expressed exclusive of residual ash. Lignin was determined by solubilization of cellulose with sulphuric acid. Non fiber carbohydrate (NFC) content was calculated as NFC = OM – CP – NDF_{om} – ether extract. The content of enzymatically insoluble organic matter (eIOM) was determined with the pepsin-cellulase method of De Boever *et al.* (1986).

About 50 g faeces per cow, obtained from all animals by rectal grab sampling in the parlour, were taken four times a week during the measuring periods, at 06:30 and 17:30, after milking. This grab sampling method avoided contamination of faeces by urine, insects or soil. All sampling was carried out by the same person in the same way and with the same tools. The samples were immediately stored in plastic containers and frozen at −18°C until analysis.

Daily excretion of faeces was measured by feeding 9 g Ti (as TiO_2) twice daily as external marker to every cow from the start of the experiment. The amount of an indigestible marker voided with the faeces, when fed continuously over time, should be constant after flow equilibrium is achieved (Rothfuss *et al.,* 1997; Myers *et al.,* 2004, Glindemann *et al.,* 2009). Thus, the concentration of the marker in faeces directly relates to faeces output (see Eq. (1)). The digestibility can be calculated when feed intake is measured simultaneously.

Faeces samples during the measuring periods were pooled by animal and by week. The samples of the acclimatization periods were not considered. This resulted in 48 pooled samples (8 cows x 3 weeks/period x 2 periods) that were analysed for N, AIA (van Keulen and Young, 1977) and the Ti content (absorption spectrophotometry).
Faecal DM output was calculated as follows (Lippke, 2002):

$$\text{faecal DM output (kg/d)} = \frac{[\text{Ti dosed per day (g/d)}]}{[\text{mean Ti content in faeces (g/kg)}]} \times \text{recover rate} \quad \textbf{(1)}$$

An average recovery of 960 g/kg was used in this study to calculate faecal DM output. The pasture DM intake was determined using the calculated faecal output and *in vitro* DM digestibility ($DDM_{in\ vitro}$) of pasture herbage according to the equation by Lippke (2002):

$$\text{DM intake (kg/d)} = \frac{[\text{faecal DM output (kg/d)}]}{[(1\text{-}DDM_{in\ vitro})]} \quad \textbf{(2)}$$

Determination of digestibility with wethers

Eight wethers, weighing 70−80 kg, were matched for age and BW and then divided into two similar groups of four wethers for *in vivo* digestibility determination. The animals were kept in single metabolic cages. Feed was given to the wethers twice a day in equal amounts at 07:00 and 18:00. Although other studies have often used dried herbage, we used thawed herbage because Beever *et al.* (1976) have shown that there is no influence of freezing and thawing on OMD. The frozen herbage, harvested as described above, was thawed 24 h in closed bags before feeding.
The first group of wethers received a pure herbage diet and 15 g/d mineral supplement consisting of (g/d): Ca 2.70; P 0.77; Mg 0.29; Na 1.50 and the following trace elements (mg/d): Zn 120; Mn 25.5; Co 0.4; I 0.9 and Se 0.9, as declared by the manufacturer

(Hoeveler Spezialfuttermittel GmbH und Co KG, Dormagen, Germany). The second group of wethers was fed grain maize in addition to the herbage and mineral supplement. To ensure a similar ration composition for both wethers and cows, the wethers of group 2 received grain maize to contribute, on average, a fraction of 0.124 (SD 0.006; n=4) to the total diet (DM basis). Fresh water was freely available to both groups.

The nutrition level during the experiments was adjusted to provide 1.1−1.2 times maintenance, according to Gabel *et al*. (2003) because the OMD is largest when energy level is near the maintenance requirement. Feeding times were 07:30 and 18:30 h and faecal collections were made at 07:00 and 18:00.

The experimental period for the wethers consisted of 14−day adaptation to the diet, followed by 8 days of intake measurements. The amounts of forage offered, the feed remains, and faeces excreted by each wether were weighed daily. DM of fresh herbage was determined twice daily by drying for 48 h at 60°C. The wethers were harnessed with faecal bags, which were tightened with three cordons at their body. The faecal bags were emptied twice daily and faeces were immediately frozen at −18°C. A single representative sample of faeces from each animal was obtained by pooling 20% of each defecation. Subsamples were stored at −18°C until DM determination. After 72 h freeze-drying, the faeces and the herbage samples were analysed for AIA as described for the cows' faeces.

The whole study was carried out according to the standards of CCAC (1993).

Calculation of digestibility

Digestibility of organic matter (OMD) was estimated with Ti and AIA as indigestible markers, four faecal nitrogen (N) equations, which use the same raw data, the pepsin-cellulase method (*in vitro* OMD) and the digestibility trials with wethers (*in vivo* OMD). All equations calculate digestibility as a fraction of OM. The equation to calculate OMD with the pepsin-cellulase method (Weissbach *et al.,* 1999) was:

$$OMD_{in\ vitro} = \frac{(940 - A - 0.62 \times eIOM - 0.000221 \times eIOM^2)}{(1000 - A)} \quad \textbf{(3)}$$

There are several equations to calculate OMD from the faecal N content. The equation by Schmidt *et al.* (1999) applies to fresh herbage:

$$OMD_{N1} = 0.8955 - \frac{4.6}{x}, \quad \textbf{(4)}$$

where x is N content in faeces OM (g/kg). Subsequently this method is termed 'faecal N method for fresh herbage'.

The equation after Schmidt and Jentsch (1994) applies for conserved forage-based diets:

$$OMD_{N2} = 0.9 - \frac{5.13}{x} \quad \textbf{(5)}$$

Subsequently, we term this method the 'faecal N method for forage-based diets'.

Although the following two methods are also based on the N content, we term them crude protein methods because the original equations were based on crude protein, which is obtained as faecal N content × 6.25.

The equation to calculate OMD with the faecal crude protein method by Lukas *et al.* (2005) is:

$$OMD_{N3} = a_i - 1.077 \exp^{(-0.01515p)} \quad \textbf{(6)}$$

where a_i is a location factor and p is crude protein content in faeces organic matter (g/kg). The location factor a_i is 0.7976 for Braunschweig and Hohenheim (Germany) and 0.7286 for Gumpenstein (Austria). The method is later termed OMD_{N3BH} when using a_i for Braunschweig and Hohenheim and OMD_{N3G} when using a_i for Gumpenstein. Both Braunschweig/Hohenheim and Gumpenstein share similarities with our experimental site. We found no criteria to decide beforehand which a_i was better suited in our case.

The equation to calculate *in vivo* OMD from the wether digestion test is

$$OMD_{\text{in vivo}} = 1 - \frac{[\text{faecal output OM (kg/d)}]}{[\text{feed intake OM (kg/d)}]} \quad \textbf{(7)}$$

Accordingly, as OM intake and faecal output from Eq. (1) for the cows in the stall was known, OMD_{Ti} was calculated as:

$$OMD_{Ti} = 1 - \frac{[\text{faecal output (kg/d)} - \text{faecal ash output (kg/d)}]}{[\text{dry matter intake (kg/d)} - \text{feed ash intake (kg/d)}]} \quad \textbf{(8)}$$

OMD_{AIA} was calculated as:

$$OMD_{AIA} = 1 - \left[\frac{\text{AIA content in feed OM (g/kg)}}{\text{AIA content in faeces OM (g/kg)}}\right] \quad \textbf{(9)}$$

Statistical methods

Linear regressions (weighted least-squares estimations) were used to evaluate the datasets. The coefficients of determination were tested with a two-sided test for significance of the regressions. Hypothesis testing on equal means of groups or on parity of the mean of the population and a specified value was carried out using Student's t-test (two-sided). This was performed against a 95% confidence interval, preceded by a test for normal distribution. All procedures followed standard protocols (Sachs, 1984).

RESULTS

Pasture and feed

The mean sward height during the experiment was approximately 6.2 cm (SD 0.9 cm, n = 8; Table 2). Sward height was closely correlated with forage composition (e.g., with crude protein content, r^2=0.74) even though variation in sward height was small (range 5.5−6.9 cm). AIA content of herbage was 13.4 g/kg (SD 1.7 g/kg; n=8). Due to beneficial conditions for herbage growth, sward height increased in weeks 2−3. Accordingly, the crude protein content decreased from 216 g/kg to 189 g/kg (Table 2). In the following period, protein content increased again, partially due to the excess harvesting of 15% of the pasture at the end of week 3 and the previous N fertilisation. After this intervention, the dry matter composition, eIOM and the mean sward height rapidly regained the levels seen in week 1.

Digestion experiment with wethers

The diet supplemented with grain maize had a significantly (P<0.05) higher $OMD_{in\ vivo}$ than the pure herbage diet (Table 3). The higher $OMD_{in\ vivo}$ of the supplemented diet was due to a significantly (P<0.001) higher digestibility of the NFC fraction, which is reasonable as supplemented grain maize mainly contributes to this fraction. The significantly (P<0.05) higher $OMD_{in\ vivo}$ in the ADF_{om} fraction in the grain-maize supplemented group could be caused by a better energy supply of cellulolytic bacteria in the rumen. AIA content in faeces of wethers was 59.5 g/kg for the grain-maize supplemented group and 57.0 g/kg for the pure herbage group.

Table 3: *In vivo* dry matter digestibility of two diets in the wether test (n = 4 for each diet)

Item	Herbage			Herbage and maize			P
	Content (g/kg)	Mean digestibility	SD	Content (g/kg)	Mean digestibility	SD	
Organic matter	904	0.777	0.008	913	0.794	0.013	0.0438
Crude protein	212	0.796	0.007	198	0.788	0.011	0.1477
NFC	131	0.684	0.025	175	0.794	0.004	0.0004
Ether extract	39	0.394	0.040	39	0.536 (0.473)	0.026 (0.128)	0.1428
NDF_{om}	522	0.821	0.011	501	0.817	0.021	0.3725
ADF_{om}	273	0.769	0.011	246	0.791	0.012	0.0235
Ash	96	0.467	0.033	87	0.465	0.026	0.4618

NFC, non fiber carbohydrates; NDF_{om}, neutral detergent fiber not assayed with a heat stable amylase and expressed exclusive residual ash; ADF_{om}, acid detergent fiber not assayed with a heat stable amylase and expressed exclusive residual ash. Values in parentheses are influenced by one outlier.

Cow performance

There were no clinical signs of diseases in either of the two groups during the entire experiment. The DM intake of herbage (15.5 kg/d vs. 15.8 kg/d) and grain maize (1.72 kg/d vs. 1.68 kg/d) did not differ significantly between the two groups in the stall. Milk production, BW, body condition score and back fat thickness throughout the experiment (Table 4) remained similar between both groups whether pasture- or stall-fed.

Table 4: Energy-corrected milk (ECM[1]), body weight, body condition score and back fat thickness during the two experimental periods. Each mean is calculated from 84 (ECM), 24 (body weight), 4 (body condition score) and 4 (back fat thickness) replicated measurements.

Item	Period 1				Period 2			
	Pasture group P-S		Stall group S-P		Pasture group S-P		Stall group P-S	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ECM (kg/d)	25.0	0.6	26.2	1.1	22.5	0.3	23.0	0.3
Body weight (kg)	649	56	623	52	619	52	663	58
Body condition score	3.31	0.24	3.31	0.29	3.13	0.48	3.31	0.13
Back fat thickness (cm)	1.02	0.26	0.95	0.18	0.91	0.17	1.11	0.30

[1] ECM, energy-corrected milk, ECM = (0.3246 × kilograms of milk) + (12.86 × kilograms of milk fat) + (7.04 × kilograms of milk protein).

The initial average ECM of 28 kg per day decreased slightly to 25 kg/d on the pasture and in the stall during the first three weeks of period 1 (Table 5), in agreement with other authors (Soriano *et al.,* 2000). This development related to changes in feed properties and the progress in lactation.

Table 5: Energy-corrected milk (ECM), feed intake and digestibility of organic matter (OMD) of ingested feed measured with the faecal N method for fresh herbage (OMD_{N1}) throughout the experimental period. Means are calculated from 28 milk and feed intake measurements or 4 digestibility measurements. Period '0' denotes the week of acclimatisation after an experimental change. SD is the standard deviation of replicates within each cell averaged over all weeks.

Week:	1	2	3	4	5	6	7	8	SD
Period:	0	1	1	1	0	2	2	2	
ECM in stall (kg/d)	28.9	27.2	26.4	25.1	22.5	22.8	22.5	22.2	2.6
ECM on pasture (kg/d)	27.2	25.6	24.8	24.5	25.3	23.1	22.7	23.3	1.5
Feed intake in stall (kg/d)		16.1	15.7	14.8		15.8	16.1	14.8	1.2
OMD in stall		0.767	0.752	0.734		0.749	0.742	0.755	0.007
OMD on pasture		0.782	0.775	0.771		0.761	0.763	0.763	0.005

Cow faeces

The crude protein content in faeces OM averaged 214 g/kg (SD 23 g/kg; n = 48) with significantly (P< 0.001) lower crude protein contents in the stall (199 g/kg; SD 17 g/kg; n = 24) than on pasture (229 g/kg; SD 17 g/kg; n = 24). In contrast, the difference between group S-P and group P-S was small and not significant (209 g/kg; SD 19 g/kg; n = 24 vs. 219 g/kg; SD 26 g/kg; n = 24). During stall feeding, the crude protein content decreased substantially from 224 to 178 g/kg during weeks 2 and 3 of period 1. This change probably reflected the change in sward properties (Table 2). In contrast, no strong decline was apparent during this time on the pasture, perhaps due to some selection by the cows, which compensated for changing sward properties. During the remaining period of the experiment, the N content in the faeces in the stall and on the pasture was more constant. The AIA content in faeces was 69 g/kg (SD 4 g/kg; n = 24) for grazing cows and 64 g/kg (SD 4 g/kg; n = 24) in the stall.

The Ti content in the faeces DM during the experiment varied little and was on average 3.78 g/kg (SD 0.5 g/kg; n = 48). A rather small difference was noted between the two experimental groups (3.82 g/kg for group S-P and 3.75 g/kg for group P-S). No significant difference was observed between the cows on the pasture and the cows in the stall. The lower N content in faeces during week 2 and 3 of period 1 was, therefore, not associated with a lower Ti content. This meant that ingestion on the pasture should have been greater than in the stall. However, no further evidence for this (e.g. in terms of higher milk yields) was noted, although the effect of higher intake should have been amplified by the higher digestibility of herbage on the pasture.

Comparison among the digestibility methods

OMD_{N1} (valid for fresh herbage) was 0.76 when averaged for all faeces samples, almost the same as that of OMD_{N2} (0.75) and OMD_{N3BH} (0.75) (Figure 1). This similarity was based on the corresponding equations, which predicted very similar OMD within the range of faecal N contents occurring in the experiment (Figure 2). However OMD_{N3G} (0.68) deviated considerably, without any obvious reason why the equation for OMD_{N3G} should have been less applicable than that for OMD_{N2} or OMD_{N3BH}. The N content of faeces, on which these calculations are based, differed significantly between stall- and pasture-fed animals (P<0.05). All three equations predicted that OMD on pasture was higher by about 0.02 (Figure 1). The difference between group S-P and P-S was small (about 0.005). The mean variation within each group was 0.015 (SD).

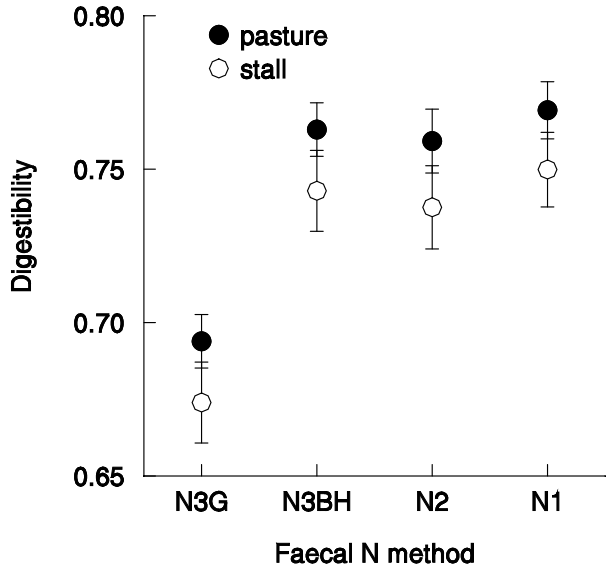

Figure 1: Digestibility of organic matter estimated by different faecal N methods. Error bars indicate standard deviation, which is caused by the variation in N content that is identical for all methods. N1, faecal N method for fresh herbage (OMD_{N1}); N2, faecal N method for conserved forage-based diets (OMD_{N2}); N3BH, faecal crude protein method for Braunschweig and Hohenheim (OMD_{N3BH}); N3G, faecal crude protein method for Gumpenstein (OMD_{N3G}).

OMD_{AIA} for stall-fed cows was significantly higher than estimated by the other methods (0.80). In the wether experiments $OMD_{in\ vivo}$ was, with the exception of the AIA method, consistently higher than estimated by the other methods (by 0.02−0.11; compare Figures 1 and 3). $OMD_{in\ vivo}$ was 0.79 for the herbage-maize diet and 0.78 for the pure herbage diet. $OMD_{in\ vitro}$ for pure herbage yielded 0.73 on average. The low level of $OMD_{in\ vitro}$ was due to the low values in weeks 2 and 3 (0.69 and 0.67).

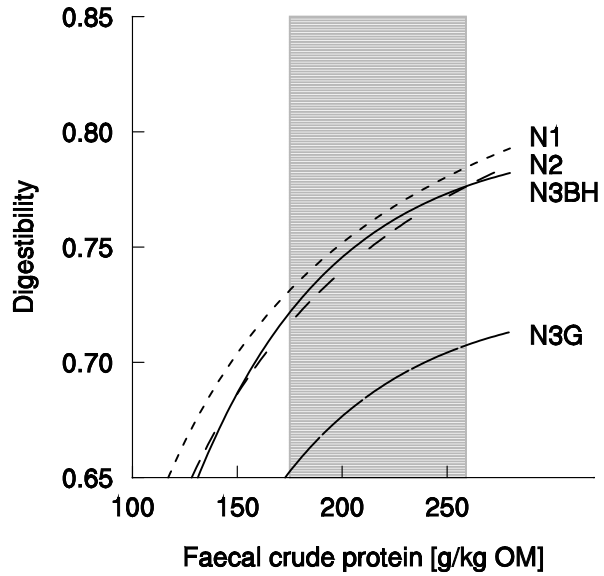

Figure 2: Calibration curves of different faecal N methods. N1, faecal N method for fresh herbage; N2, faecal N method for conserved forage-based diets; N3BH, faecal crude protein method for Braunschweig und Hohenheim; N3G, faecal crude protein method for Gumpenstein. The grey area shows the range of our data.

$OMD_{in\ vitro}$ was thus similar to the digestibilities based on faecal N, except for OMD_{N3G}, considering the slightly higher digestibility of the herbage-maize diet when compared to pure herbage (Table 3).

The AIA method and the Ti method were applicable only in the stall (group S-P in period 1 and group P-S in period 2), because of the need for measured feed intake. OMD_{Ti} was 0.75 and correlated significantly with OMD_{N1} (Figure 4) and the other methods based on faecal N. For OMD_{N1}, the regression did not differ from the 1/1 line and was significant, but weak (r^2 = 0.47). Excluding one animal, causing three outliers, from the calculation increased r^2 to 0.57. The weak correlation does not imply that both methods correlate weakly in general. In the present case, the correlation was weak due to the experimental setup, which aimed at a constant herbage quality during the whole experiment. If a constant digestibility had been fully achieved, a zero correlation would have resulted with all variation due to experimental error. An r^2 of about 0.5 indicates that inaccuracies of the methods contributed about half to the variation (0.02) while the other half (0.02) was attributable to true variation due to the lower herbage quality in weeks 2−4.

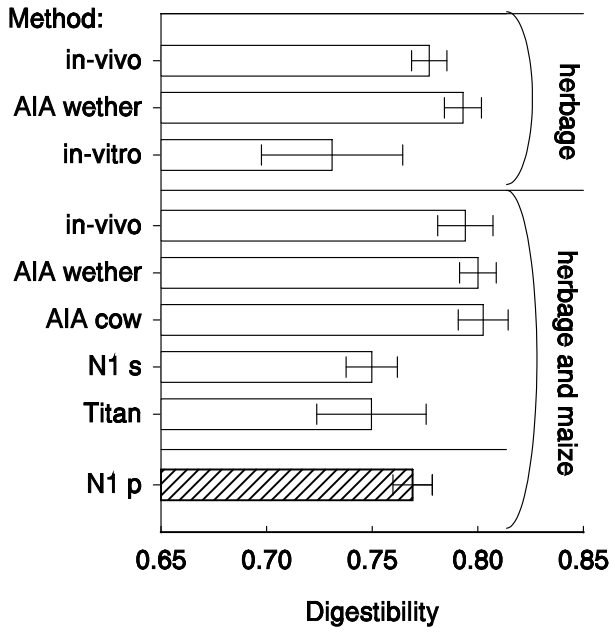

Figure 3: Organic matter digestibility estimated by the wether test (in vivo), the acid-insoluble ash method (AIA); the pepsin-cellulase method (*in vitro*), the Ti method (Titan) and the faecal N method depending on feed (herbage vs. herbage + maize) and location of measurement (stall: open bars, pasture: hatched bars). Error bars indicate standard deviation.

DISCUSSION

To our knowledge, this is the first study where herbage for stall-feeding was harvested twice daily between grazing animals on a pasture over a prolonged time. This method was developed to avoid problems of dissimilarities between grazed and mown swards and it

delivered a sound basis for estimating OMD of herbage from pasture using different methods. The mown herbage for evaluation in the stall has to be near-identical to the grazed herbage, which makes the selection of an appropriate mowing height critical. At high stocking densities the bite depth may vary between 45 mm (Meijs and Hoekstra, 1984; Mayne *et al.,* 1990), 44 mm (O´Donovan and Delaby, 2008) down to 15 mm (Illius and Gordon, 1987). We chose 30 mm mowing height, which is within these values and which agreed with observations in a previous experiment.

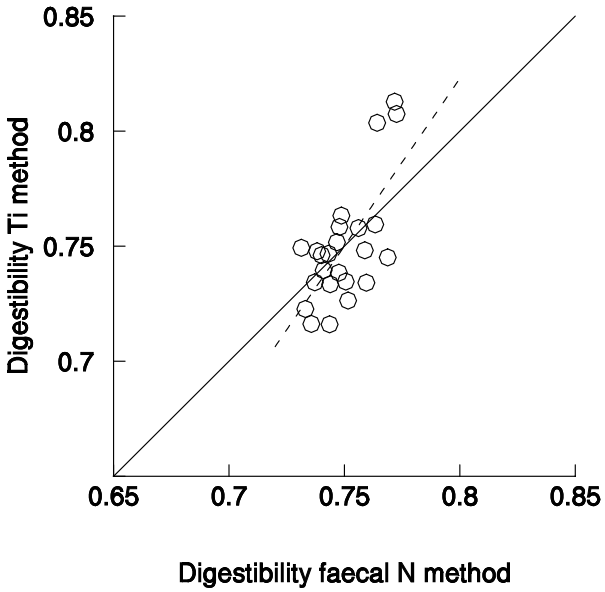

Figure 4: Relation between estimates of organic matter digestibility by the faecal N method (OMD_{N1}) and the Ti method (OMD_{Ti}) for individual animals. The solid line denotes unity. The regression (dashed line) is y= 1.455x – 0.342, r^2=0.47.

Only the faecal-N based methods were entirely applicable to grazing animals. The $OMD_{in\ vitro}$ method should theoretically also work on pasture, given that a representative sampling is possible, but it excludes animal-specific effects. Methods using artificially supplied tracers, like Ti in our case, require that some supplements must be fed, in order to apply the tracer, but this may help to access the animal. In the case of small ruminants, the tracer may be supplied directly, thereby avoiding supplementary feed (Glindemann *et al*., 2009, in the case of sheep), but this involves disturbance of the animals and a large work load. Alternatively, a bolus with the respective marker can be injected. However, a constant release of the marker over a prolonged period must then to be ensured.

Internal tracers, like AIA in our case, depend on the precondition that material of exactly the same composition as the feed grazed by the animals can be obtained as a reference, which becomes especially difficult where feed from different sources can be selected by the animal.

The small amounts of AIA, especially in herbage, where a relatively large variability can also be expected, requires sufficient material in order to control measuring errors.

N content in faeces of pasture- and stall-fed animals differed significantly in the present study, indicating a high sensitivity of the faecal-N methods to detect even small differences in OMD. Despite the higher OMD of grazed herbage, milk yield was slightly lower for grazing animals. This might be due to the energy demand of locomotion when grazing on pasture with low sward heights.
Both, the difference in milk yield and the difference in N content, indicate that even with a considerable effort to obtain the feed for the stall it was not fully possible to simulate a grazing cow. Even with grazing to a sward height of 6.2 cm and additional mowing every 10 days, some selection must still have occurred. The difference between grazed feed and sampled (mown) feed quite likely increases when the grazing systems allows for more selection or where the feed sample is obtained from outside the grazed area akin in the case of exclosure cages. Exclosures next to the experimental pasture are often used to harvest herbage and to calculate DM intake and digestibility (Polan *et al*., 1986; Holden *et al.,* 1994), especially when a control group is fed with herbage in the stall. The time of exclosure has to be sufficiently long to produce a clear difference between exclosure and the grazed area (e.g. one month in the experiments of Wittmer *et al.* (2010) and Schiborra *et al*. (2010)) as the DM intake is given by the difference between pre- and post-grazing biomass. The results of period 1 clearly show that, even under grazing, which should retard aging in the growing sward, the quality of feed can change within two weeks. This calls for a different approach.

Tracer methods require an assumption about the recovery rate. For Ti, Titgemeyer *et al.* (2001) measured a recovery rate of 900−950 g/kg and 930 g/kg in the case of cattle fed on forage-based diets. Hafez *et al.* (1988) observed 960−1.020 g/kg faecal recovery in dairy cows fed concentrate, grass-silage and corn-silage diets, although diurnal variation in excretion pattern was high. On average, for these studies recovery rate was 960 g/kg, which was assumed in this study. The recovery rate is especially critical for artificial tracers, which are applied for only a specific time period because full flow equilibrium may not have been achieved. In our case, the time course of Ti excretion provided no indication that flow equilibrium had not been achieved.

Faecal N methods are not affected by these uncertainties. These methods, however, suffer from other weaknesses. Their sensitivity is small when the digestibility is low, but with a grazing system providing feed of very high digestibility, as in our case, the sensitivity becomes high as indicated by the slope increasing with digestibility until the maximum

digestibility inherent in a certain equation is reached (Figure 2). $OMD_{in\ vivo}$, $OMD_{AIA\ wether}$ and $OMD_{AIA\ cow}$ all indicated that digestibility was 0.80 on average and higher in some cases (Figure 3). Even OMD_{Ti}, which presumably underrated digestibility due to overestimated recovery rate, partially produced digestibilities well above 0.80 (Figure 3). For OMD_{N3G} and OMD_{N3BH} digestibility already exceeded the maximum digestibilities (0.73 and 0.79) and thus must underrate digestibility in our case. Deviations in N content to lower values will lead to low digestibilities, while deviations to higher values cannot exceed the OM threshold in this case. Digestibilities over 0.80 can only be obtained with OMD_{N1} and OMD_{N2}. Calculating the digestibility from the average N content led to 1.5 g/kg higher digestibilities than did averaging the digestibilities as calculated from the individual N-faeces measurements. The faecal-N methods that are presently used suffer from a further and major disadvantage: the regression models to calculate OMD need a calibration and are only suitable for a particular measurement range.

OMD_{N1} and OMD_{N2} have no location factor and in our case delivered results that agreed with OMD_{Ti}. The main source of error arising during calculation of OMD with the faecal-N methods was the selection of an appropriate equation that included the location factor "a_i" in Eq. (6). For Braunschweig and Hohenheim (OMD_{N3BH}), the results agreed with the other faecal-N methods, whereas OMD_{N3G} significantly deviated. Lukas *et al.* (2005) favoured using OMD_{N3BH} for nearly all conserved foods and this was confirmed by studies of Schlecht *et al.* (2006), Schiborra *et al.* (2010) and Wang *et al.* (2009). The reason for the difference in location factors between Braunschweig/Hohenheim and Gumpenstein is unclear.

CONCLUSIONS

At present no unambiguously valid method exists for determining digestibility of feed grazed at pasture. The methods based on the N content in faeces have the largest potential because they are easily measured and the method does not rely on assumptions such as appropriate feed sampling or tracer recovery rate. The faecal-N methods (OMD_{N1} and OMD_{N2}) are suitable for highly digestible feed such as herbage from all-day pasture, where their sensitivity is good. The faecal-N methods also appear to be well suited for a wide range of experiments because of their low costs and methodological simplicity.

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