Passage of feed in dairy cows

Use of stable isotopes to estimate passage kinetics through the digestive tract of dairy cows

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Abstract

Dairy cows possess a unique digestive system to digest fibre-rich diets. Ingested feed is retained and degraded in the rumen by the enteric microbial population and is passed from the rumen to the following segments of the digestive tract. Passage of feed determines energy and protein supply to the animal and is a key parameter in several feed evaluation models for ruminants. Yet, quantitative data on passage of feed and particularly of single feed components are limited. Common techniques used to determine fractional passage rates of feed typically include indigestible markers that are not able to describe passage of distinct feed components. This thesis describes the use of stable isotope labelled feed components as a novel marker to determine feed type and feed component specific fractional passage rates. In a series of *in vivo* experiments, fractional passage rates of a typical dairy ration, including grass silage, maize silage and concentrates, were determined. The use of carbon (^{13}C) and nitrogen (^{15}N) stable isotopes as an internal marker inherent to the diet allowed to specifically determine fractional passage rates of plant cell walls such as structural fibre, fibre-bound nitrogen, *n*-alkanes, and intracellular components such as starch and total nitrogen. For grass silage and maize silage, stable isotopes gave slower fractional rumen passage rates compared to the commonly used external marker chromium mordanted fibre; for concentrates, stable isotopes gave faster rates than the external marker. Among isotopic labelled fractions, ^{13}C-labelled fibre and ^{15}N-labelled fibre-bound nitrogen gave the slowest rates. The isotopic signature of single feed components and further application of stable isotopes on a wider range of feeds and feed components offers scope for the future for a more detailed insight into nutrient-specific passage kinetics. This will ultimately allow to quantify nutrient supply in response to changes in diet composition and quality, and model animal response in relation to optimal animal performance, environmental and animal-health issues.

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Chapter 1

General introduction

The gastrointestinal tract of ruminants forms a unique digestive system, which is particularly adapted to digest low degradable feed rich in fibres. The time feed is retained in the various segments of the gastrointestinal tract (GIT) is essential for the digestion as it determines the time feed is exposed to fermentative degradation by the rumen microbial population. This is particularly important for herbivores which inhabit grassland areas but lack the enzymes to degrade plant fibres. The retention time in the rumen or any segment in the GIT can be expressed as a relative rate constant (the reciprocal of retention time) defining the proportion of a specific digesta pool passed out per unit of time (conventionally denoted as fractional passage rate).

Knowledge on fractional rates of rumen passage is essential to quantify the rumen escape part of nutrients as well as to quantify microbial growth efficiency. Fractional passage is, therefore, an important concept in feed evaluation systems for ruminants. Assessment of passage kinetics in ruminants has received much attention and the first attempts to measure fractional rumen passage were made with use of marker techniques as early as in the 1920s. Since then, considerable efforts have been made to develop markers to best estimate digesta passage. It is widely accepted that markers should be non-absorbable substances intimately associated with the digesta material of interest, should not disturb digestive actions in the GIT tract or be affected by them, and their analytical quantification should be sensitive and specific (after Faichney, 1975).

Modern feed evaluation systems often account for differences in rumen passage among feed components but still mainly rely on fixed fractional passage rates (e.g. the Dutch DVE/OEB_{2010} system) or incorporate some of the factors regulating passage to some extent (e.g. the British FiM system and the Scandinavian NorFor system). A more dynamic nutrient-specific representation of passage kinetics is mainly hampered by the current methodology used. Conventional marker techniques do not allow to quantify nutrient-specific passage kinetics.

Stable isotope labelled feed components may circumvent the limitations of conventional marker techniques. Stable isotopes are non-toxic, non-radioactive isotopes pertaining to various chemical elements and are thus inherent to the feed and distributed in the various feed components in small concentrations. Stable isotopes have been extensively used in nutritional studies (reviewed by Matthews and Bier, 1983), but were only recently applied in ruminant

digestion studies (Svejcar et al., 1993; Südekum et al., 1995; Huhtanen and Hristov, 2001; Pellikaan et al., 2013). Because stable isotope labelled feed components are subjected to the same digestive mechanisms as the ingested bulk feed, they appear to be a promising novel tool to measure digesta passage.

Aim and Outline of this Thesis

The main objective of the research described in this thesis is to assess passage kinetics of particle or solid phase associated feed fractions from feed types conventionally fed to dairy cows in intensive dairy systems. In order to assess passage kinetics of the major feed components, stable isotope labelled feed components were applied.

Chapter 2 is a review in which stable isotopes are compared with conventional marker techniques, and potential limitations and opportunities of each technique are discussed.

Chapter 3 describes passage kinetics of concentrates with special emphasis on fibres in concentrates by using the difference in the natural abundance of carbon stable isotopes between cool-season and warm-season concentrate ingredients.

Chapter 4 and 5 describe the results of two feeding experiments to assess passage kinetics of forages artificially enriched above natural abundance. **Chapter 4** describes the use of carbon stable isotope labelled maize silage to measure passage of fibres and starch. **Chapter 5** describes the use of carbon and nitrogen stable isotope dual labelled grass silage to measure passage of fibres and fibre-bound proteins.

Chapter 6 describes a study, which extends the use of stable isotope to minor plant compounds such as *n*-alkanes that typically do not undergo ruminal degradation in contrast to the dietary feed components tested in Chapter 3 through 5.

Chapter 7 discusses the major research findings from this thesis as well as implications of using stable isotopes for feed evaluation, offers recommendations for future research, and provides the main conclusions.

Chapter 2

Stable isotope labelled feed nutrients to assess nutrient-specific feed passage kinetics in ruminants. A review

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Abstract

Knowledge of digesta passage kinetics in ruminants is essential to predict nutrient supply to the animal in relation to optimal animal performance, environmental pollution and animal health. Fractional passage rates (FPR) of feed are widely used in modern feed evaluation models and mechanistic rumen systems but data on nutrient-specific FPR are scarce. Such models generally rely on conventional external marker techniques, which do not always describe digesta passage kinetics in a satisfactory manner. Here, we discuss the use of stable isotope labelled dietary nutrients as a promising novel tool to assess nutrient-specific passage kinetics. Some major limitations of this technique include a potential marker migration, a poor isotope distribution in the labelled feed, and a differential disappearance rate of isotopes upon microbial fermentation in non-steady state conditions. Such limitations can be often circumvented by using intrinsically labelled stable isotope plant material. Data are limited but indicate that external particulate markers overestimate rumen FPR of plant fibre compared to the internal stable isotope markers. Stable isotopes undergo the identical digestive mechanism as the labelled feed components and are thus of particular interest to specifically measure passage kinetics of digestible dietary nutrients.

Introduction

The digestive system of ruminants is the result of an evolutionary strategy of herbivores to digest low digestible fibre-rich diets. The ability to digest fibres is governed by the microbial degradation of feed particles in the rumen and their fractional passage rate (FPR; fraction per hour) from the rumen. The latter defines the time feed particles are retained in the rumen or any compartment in the gastrointestinal tract (GIT). It has been widely accepted that FPR is a key parameter in describing the extent of ruminal digestion (Waldo, 1972) and digestive mechanisms (Dijkstra et al., 2007) and, ultimately, in predicting nutrient supply to the animal. Accounting for FPR is, therefore, a prerequisite for many feed evaluation systems and mechanistic rumen models to quantify nutrient absorption and excretion with respect to environmental concerns and diet-related disorders (Kebreab et al., 2009).

Ruminal degradation has been studied for a wide array of feeds; yet, FPR is difficult to quantify and experimental data are relatively scarce (Dijkstra et al., 2007; Kebreab et al., 2009). Various techniques to measure FPR have been suggested but are often limited to a specific rumen pool of defined characteristics, such as the rumen liquid phase or solid phase of defined particle size. Extrapolation and a direct comparison among studies employing different techniques is, therefore, problematic. In principle, the most ideal technique to estimate passage kinetics of ingested dietary components is the use of such dietary components as internal markers. However, data based on such markers are scarce.

Isotopic labelled feed has been applied in early ruminant metabolic studies (Alexander et al., 1969a) but was only recently applied in ruminant digestion studies (Svejcar et al., 1993; Südekum et al., 1995) or specifically to quantify passage kinetics (Huhtanen and Hristov, 2001; Sponheimer et al., 2003; Pellikaan et al., 2013). Here, we provide an overview of recent digesta passage research and discuss opportunities and limitations of using the isotopic signature (δ) of dietary components as a potential novel marker to quantify FPR in ruminants.

Digesta Passage Kinetics to Assess Digestive Mechanisms

Passage Kinetics for Feed Evaluation, Environmental Pollution and Animal Health

An accurate description of digesta passage kinetics is essential to describe extent and mechanisms governing rumen fermentation and, more specifically, predict volatile fatty acid

production and efficiency of microbial protein synthesis (Dijkstra et al., 2007), as well as rumen fill and dry matter (DM) intake regulation (Jung and Allen, 1995). Several modern feed evaluation systems incorporate the concept of FPR to predict the amount of nutrients bypassing the reticulorumen and the amount of microbial protein produced in the rumen. Because of a lack of experimental data on FPR, early feed evaluation systems relied on fixed ruminal FPR (e.g. Madsen, 1985; Vérité et al., 1987; AFRC, 1992; Tamminga et al., 1994), ranging from values of 0.02 to 0.08/h. While it became increasingly apparent that FPR is influenced by a wide range of animal- and diet-related factors, some of these factors were included in several recently updated feed evaluations systems. Such adjustments to FPR include effects of feed intake, body weight and the roughage or concentrate proportion in the diet (NRC, 2001; Thomas, 2004; Volden, 2011), as well as feed component specific FPR (e.g., different FPR for starch and NDF; van Duinkerken et al., 2011; Volden, 2011). Attempts have been made to include some known passage determinants in empirical models to predict ruminal FPR in small and large ruminants (e.g. Owens and Goetsch, 1986; Sauvant et al., 1995; Chilibroste et al., 1997; Cannas and Van Soest, 2000; Seo et al., 2006a; Krizsan et al., 2010a), with some of these models forming the basis of current feed evaluation systems.

Knowledge on FPR has also been applied in mechanistic models describing nutrient supply and nutritional requirements of ruminants (Baldwin et al., 1987; Dijkstra et al., 1992; Lescoat and Sauvant, 1995; Fox et al., 2004; Danfaer et al., 2006). More specifically, FPRs were used to describe microbial population dynamics (Dijkstra et al., 2002) and their contribution to methane emissions from ruminants (Mills et al., 2001; Kebreab et al., 2004), as well as to predict nitrogen and phosphorus excretion to the environment and rumen health issues (e.g. sub-acute rumen acidosis; reviewed by Kebreab et al., 2009). Passage kinetics of forages were further used in comparative rumen physiology studies to identify evolutionary mechanisms between browsing and grazing ruminants (Clauss and Lechner-Doll, 2001; Clauss et al., 2003) and in non-ruminant herbivores (Sponheimer et al., 2003).

Factors Involved in Digesta Passage Kinetics

Several factors determining passage behaviour of feed in ruminants have been identified, which can be animal and diet related, or related to the physical appearance and properties of the ingested feed particles.

Physical Feed Characteristics. It has been widely accepted that the probability for feed particles to pass the reticulorumen via the reticulo-omasal orifice depends on their physical characteristics, that is, on the time required to reduce particle size (Welch, 1982) and increase

particle density (Ehle et al., 1984). This later has led to the notion for rumen passage being based on a specific particle size (Poppi et al., 1980), gravity (Hooper and Welch, 1985), and effective particle density (buoyancy; Sutherland, 1988). Fermentation gases upon microbial feed degradation can influence the specific gravity of feed particles, e.g. by increasing their buoyancy due to gas entrapment in feed particles (Sutherland, 1988) and, thereby, reduce their probability of rumen escape. In contrast, density of small particles is higher due to poor gas entrapment, which increases their probability of rumen escape. This correlation observed between feed particle size and density (Sutherland, 1988) suggests that neither particle size nor particle density alone can fully explain FPR. Furthermore, particle shape (Troelsen and Campbell, 1968) and the physical location of feed particles within the rumen (Welsh, 1982; Poppi et al., 2001) might be relevant as the probability of rumen escape was observed to be lower for less compact particles or particles entrapped in the rumen fibrous matt, although these mechanisms might be confounded with effects related to particle density.

Physical feed characteristics are not easily obtainable and include various separation techniques based on wet- and dry-sieving (for particle size determination) and particle immersion in a solution with known specific gravity (for particle density determination). These techniques are laborious and often not standardised and difficult to operate (Kennedy, 2005). Measurable parameters which are considerably easier to obtain are potential animal- and diet-related factors, although this approach is associated to a loss of degree of detail in studying the mechanisms involved in digesta passage.

Animal and Diet-Related Factors. A central issue to differential passage behaviour of feeds is the feed intake level by the animal. Fractional passage rates were observed to be higher with increased feed intake, which itself depends on stage of lactation (Robinson et al., 1987; Tamminga et al., 1989a; Colucci et al., 1990), reticular contractions (Kennedy, 1985), climatic factors (Kennedy et al., 1986) or characteristics of the diet. Potential diet-related factors can be attributed to the diet composition and quality, including forage type (Mulligan et al., 2002; Lund et al., 2006; Bayat et al., 2010), vegetative forage part (e.g. stem-leaf proportion; Poppi et al., 1981; Lamb et al., 2002), concentrate proportion in the diet (Colucci et al, 1990), and various forage and pasture management practices, such as stage of maturity (Bosch et al., 1992a; Rinne et al., 1997a), stage of regrowth (Bosch and Bruining, 1995; Bayat et al., 2011), harvesting and forage conservation practices (Beauchemin and Buchanan-Smith; 1989; Kokkonen et al., 2000), and grazing time (Gregorini et al., 2008). Common feed processing techniques (e.g. pelleting; Bernard et al., 1998; de Vega et al., 1998) generally increased FPR. Various feed additives and by-product feeds (Owens and Goetsch, 1986;

Firkins, 1997) have been reported to be associated with increased FPR through improved diet digestibility or through increased feed intake.

Stable Isotopes as Digesta Passage Markers

Stable Isotopes in Relation to Conventional Passage Markers

Passage kinetics have been routinely derived from marker studies, in which a digesta marker is administered at known concentrations and FPR is estimated based on the marker dilution in faeces or digesta collected at various segments in the GIT. Frequently, these markers were externally applied substances like various transition metals, mainly chromium and rare earth elements (e.g. ytterbium, lanthanum). These substances are not intrinsic to the feed and are, therefore, either administered separately or bonded to feed particles (e.g. mordants). Hence, it can be hypothesised that such markers do not reflect the passage behaviour of the feed particles or digesta phase they are associated with. In particular, they may reduce the digestibility of the feed particles they are associated with by increasing their density (Udén et al., 1980) and specific gravity. In addition, a preferential binding association of external markers was observed, resulting in marker migration to particles or rumen fluid not originally labelled (Mader et al., 1984; Bernard and Doreau, 2000); or, if sprayed on feed, resulting in an uneven marker distribution with a closer affinity to material of smaller particle size (Siddons et al., 1985).

Other techniques such as rumen evacuations or measurements on digesta contents after slaughter offer a direct measurement of FPR. Such techniques involve the use of a marker substance naturally present in the feed and the determination of the rumen pool size to be able to distinguish the marker from the bulk material. Fractional passage rates can then be calculated from the flow of the respective marker and its rumen pool size (Robinson et al., 1987) assumed to be constant. A constant rumen pool volume may be difficult to achieve, particularly when animals are not continuously fed. Alternatively, FPR may be estimated using consecutive rumen evacuations, separated by periods of feed deprivation, assuming a first order kinetics of particular markers (Taweel et al., 2005). Such markers, conventionally referred to as inert internal markers, are preferred over external markers because they are intrinsic to the feed and, hence, circumvent the inherent limitations of external markers. Various indigestible inert components present in feed in low (e.g. lignin, acid-insoluble ash) or in more abundant amounts (e.g. indigestible fibre) were commonly used. However, these

techniques cannot distinguish between feed types, are laborious, invasive and frequent sampling is difficult, prohibiting the assessment of particle dynamics through the GIT.

The techniques described above provide information on the specific particles of defined characteristics (external markers) or of the entire diet (inert markers upon rumen evacuation) but do not describe passage of specific feed nutrients. An alternative to such markers is the use of distinctive internal markers, e.g. by labelling feed with dyes or specific isotopes. Dyed feed did not gain much acceptance due to difficulties in quantitatively measuring the marker substance in faeces (Udén et al., 1980). In contrast, the analytical quantification of isotopes is specific and sensitive, and was applied in early ruminant metabolic studies, e.g. to measure the transfer of radioactive carbon isotopes from an isotopic labelled diet to volatile fatty acids and microbial protein in the rumen (Alexander et al., 1969b).

Restrictions on the use of radioactive isotopes can be avoided by using stable isotopes, which were applied to metabolic studies quantifying the transfer of stable carbon isotopes from isotopic labelled feed to milk (Boutton et al., 1988) and animal tissue (Richter et al., 2012) of ruminants. Because these isotopes are present in low quantities in feed, the isotopic ratio needs to be increased in favour of the stable (^{13}C) or radioactive carbon isotope (^{14}C) relative to the main carbon isotope (^{12}C) to distinguish the marker from the bulk material. Various procedures were described for ruminant nutrition studies and include either switching from a naturally low ^{13}C enriched diet based on C_3 plant species (e.g. ryegrass, lucerne, wheat) to a naturally higher ^{13}C enriched diet based on C_4 plant species like maize and some tropical grass species (Südekum et al., 1995; Sponheimer et al., 2003); or labelling the plant material with isotopes above their respective natural abundance. The latter procedure has been described for harvested plants soaked in a radioactive isotope solution (Mayes et al., 1997) as well as for growing plants labelled with radioactive (Smith et al., 1963; Keith et al., 1963) and stable isotopes (Svejcar et al., 1990). Applications to ruminant studies followed by labelling growing plants in a closed system under field conditions (Svejcar et al., 1993; Pellikaan et al., 2013) and under controlled conditions in a greenhouse (Alexander et al., 1969a; Huhtanen and Hristov, 2001). Only a few studies have so far reported FPR based on stable isotopes and reported values ranging from as low as 0.019/h for low-quality grass hay in small ruminants ($\delta^{13}C$; Sponheimer et al., 2003), 0.015–0.017/h and 0.023–0.035/h for grass silage in large ruminants ($\delta^{13}C$ in the NDF and DM fraction, respectively; Pellikaan et al., 2013), up to 0.036/h and 0.044/h for lucerne hay and silage in large ruminants ($\delta^{15}N$ in the NDF and ADF fraction, respectively; Huhtanen and Hristov, 2001).

Limitations and Opportunities of Stable Isotope Labelled Nutrients as Digesta Passage Markers

The extensive microbial fermentation of labelled feed components in the rumen was considered a major constraint for the use of stable isotopes as passage markers in earlier studies (Udén et al., 1980; Südekum et al., 1995; Huhtanen and Hristov, 2001) and it was suggested to isolate an isotopic labelled but indigestible feed fraction to abide to the ideal marker condition of non-absorbability (Faichney, 1975). However, the isotopic ratio (e.g. the ^{13}C:^{12}C ratio) may be constant between the moment of marker administration and moment of collection time in the undigested faecal matter. Recent studies have shown that microbial fermentation did not affect the ^{13}C:^{12}C ratio in apparent undigested material *in vitro* (Pellikaan et al., 2013) and *in vivo* (L.M.M. Ferreira et al., unpublished data), and that the ^{13}C:^{12}C ratio remained constant over time. Hence, digestion of the labelled nutrient did not alter the isotopic ratio and its FPR in those studies. Caution must, however, be exercised if the labelling procedure consists of pulse dosing or external application of the isotopes (e.g. by spraying techniques) as the isotope distribution will likely not be uniform and ^{13}C might disappear at a different rate than ^{12}C upon microbial fermentation. In contrast, intrinsically labelled material (e.g. by photosynthetic carbon isotope incorporation) circumvents the problem of inhomogeneous isotope distribution (Gorissen et al., 1996; Ippel et al., 2004).

A further potential limitation of stable isotope labelled nutrients is their potential incorporation into and passage with the microbial biomass (Firkins et al., 1998; Pellikaan et al., 2013). Microbial biomass in the faeces calculated from the number of intact faecal bacteria can account up to 8 g carbon per kg faecal DM (van Vliet et al., 2007) suggesting that a potential marker migration may bias the estimation of FPR to some extent although effects appear to be minor. However, when damaged microbial material (mainly originating from the rumen) is included, simulations indicate that 12–24% of faecal C is microbially derived (van Vliet et al., 2007) and this higher value is of significance in FPR estimation. Determination of $\delta^{13}C$ in an indigestible fraction will circumvent this problem as none or negligible amounts of microbial matter are analytically recovered.

Towards a Nutrient-Based Representation of Passage Kinetics with Stable Isotopes

Isotopic labelled nutrients as passage markers offer the opportunity to assess nutrient–specific passage, in contrast to external markers corresponding to a specific rumen pool of similar characteristics, or rumen evacuation studies limited to the use of strictly indigestible fractions.

In Figure 1, typical small particle external markers (chromium mordanted fibre; rare earth elements) yielded a FPR (mean ± SD) of 0.039 ± 0.0120/h and 0.053 ± 0.0200/h for roughage (n = 553) and concentrates (n = 195), respectively (Seo et al., 2006a). Indigestible NDF based on rumen evacuation studies yielded a mean FPR of 0.026 ± 0.0067/h (n = 172; Krizsan et al., 2010a). Fractional passage rates for isotopic labelled nutrients as internal markers in large ruminants were 0.034 ± 0.0080/h for DM (n = 7) and 0.017 ± 0.0052/h for NDF (n = 7) in roughage based on a limited dataset (Pellikaan et al., 2013; Pellikaan, 2004; B. M. Tas et al., unpublished data).

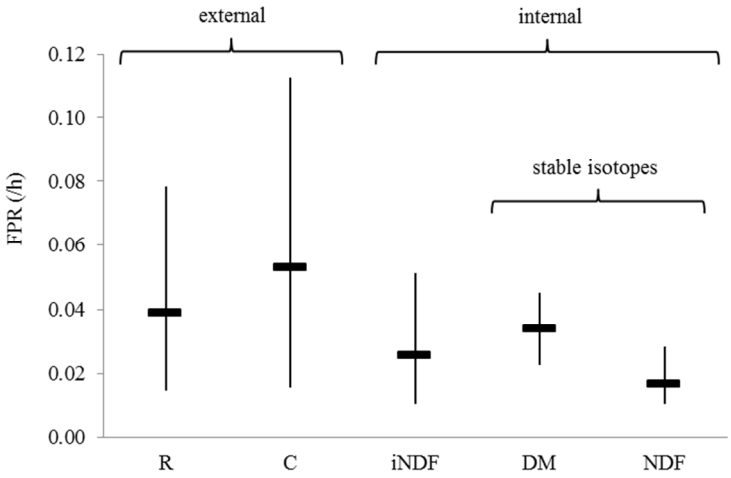

Figure 1. Mean ruminal fractional passage rates (FPR) estimated from faecal marker excretion patterns for cattle as reported in the literature. FPR and treatment means (n) are shown for external markers (Seo et al., 2006a) in roughage (R; n = 553) and concentrates (C; n = 195); for indigestible fibre (iNDF) from rumen evacuation studies (n = 172; Krizsan et al., 2010a); and for isotopes in the dry matter (DM; n = 7) and fibre fraction (NDF; n = 7) of roughage (Pellikaan, 2004; Pellikaan et al., 2013; B. M. Tas et al., unpublished). Bars represent means of FPR; whiskers represent respective minima and maxima.

Up to now, FPR predictions largely rely on empirical equations based on body weight and feed intake. Recently, fibre intake has been identified as the main explanatory parameter for rumen passage in two literature studies based on external markers (Cannas and Van Soest, 2000) or on internal marker following rumen evacuations (Krizsan et al., 2010a). A more mechanistic approach needs to account for biologically important variables determining FPR, i.e. the physical attributes of feed particles (Seo et al., 2006b) and their dynamic changes in the digestive tract (Dijkstra et al., 2007). Isotopic labelled nutrients have the advantage of

sharing the physical properties of the feed ingested by the animal, making them a valuable tool to describe dynamic changes of the physical feed properties.

The production of intrinsic labelled plant material for passage studies can be constrained by its costs and difficulties in producing uniformly labelled material of a comparable nutritional composition to that of the bulk diet. Costs of production of material are negligible when the natural difference in ^{13}C abundance between diets is used but can be considerable for plant material isotopically enriched above natural abundance, depending on the desired enrichment level and pulse dose size. Growing plants can be labelled *in vivo* in flexible isotope assimilation chambers on field but typically does not allow frequent labelling. Continuous *in vivo* labelling in a greenhouse is possible and particularly interesting for producing highly and uniformly enriched plant material (Gorissen et al., 1996; Ippel et al., 2004). The nutrient composition of an isotopic labelled diet might differ to some extent from the bulk material although modern climate-control isotope assimilation chambers allow real-time simulation of the climatic conditions on field.

Isotope analysis by mass spectrometry is sensitive and compound specific, allowing for quantitative isotope determination in minor dietary components such as fatty acids and alkanes (Bezabih et al., 2011a,b) by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). Alternatives to mass spectrometry are available and often less expensive, although sometimes less accurate for low concentrations and specific for single compounds, such as emission spectrometry for $\delta^{15}N$ analyses and infrared absorption spectroscopy for $\delta^{13}C$ analyses in gases.

Conclusions

Passage kinetics can be estimated by the use of several digesta markers. In contrast to conventionally used marker techniques, intrinsic stable isotope labelling offers the opportunity to quantify nutrient-specific passage kinetics. Such information accommodates a more mechanistic approach to quantify nutrient supply and model animal response in relation to optimal animal performance, environmental pollution and animal health. Stable isotope labelled nutrients are subjected to the same digestive mechanisms as the nutrients from the ingested bulk feed, and can be regarded as promising biological markers to investigate nutrient passage in ruminants.

Chapter 3

Passage kinetics of concentrates in dairy cows measured with carbon stable isotopes

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Abstract

Fractional passage rates form a fundamental element within modern feed evaluation systems for ruminants, but knowledge on feed-specific fractional passage is largely lacking. Commonly applied marker techniques based on externally applied markers, such as chromium-mordanted fibre (Cr-NDF), have been criticised for behaving differently to feed particles. This study describes the use of the carbon stable isotope ratio (^{13}C:^{12}C) as an internal digesta marker to quantify the fractional passage rate of concentrates through the digestive tract of dairy cows. In a crossover study, five dairy cows were fed low (24.6%) and high (52.6%) levels of concentrates (DM basis) and received a pulse dose Cr-NDF and ^{13}C isotopes. The latter was administered orally by exchanging part of the dietary concentrates of low ^{13}C natural abundance with a pulse dose of maize bran based concentrates of high ^{13}C natural abundance. Fractional passage rates from the rumen (K_1) and from the large intestine (K_2) were determined from faecal marker concentrations of Cr-NDF and of ^{13}C in the dry matter (^{13}C-DM), NDF (^{13}C-NDF) and neutral detergent soluble (^{13}C-NDS). No differences in K_1 estimates were found for the two concentrate levels fed but significant differences between markers ($P < 0.001$) were observed. Faecal Cr-NDF excretions gave lower K_1 estimates (0.037–0.039/h) than ^{13}C-DM (0.054–0.056/h) and ^{13}C-NDF (0.061–0.063/h). The ^{13}C-NDS was calculated by the difference of ^{13}C in the DM and NDF, and K_1 values (0.039–0.043/h) were comparable to Cr-NDF. Total mean retention time (TMRT) was considerably higher for Cr-NDF (40.9–42.0 h) as compared to ^{13}C-DM and ^{13}C-NDF (32.0–33.5 h; $P < 0.001$). The accuracy of the curve fits for Cr-NDF and ^{13}C-DM and ^{13}C-NDF was overall good (mean prediction error of 9.9–13.9%). Fractional passage rate of Cr-NDF was comparable to studies where this marker was assumed to represent the fractional passage of roughages. However, K_1 estimates based on the ^{13}C:^{12}C ratio varied considerably from studies based on external markers. Our results suggest that the use of ^{13}C isotopes as digesta passage markers can provide feed component specific K_1 estimates for concentrates and provides new insight into passage kinetics of NDF from technologically treated compound feed.

Introduction

Fractional passage of feed determines the time feed is retained in the digestive tract. More importantly, fractional passage specifies the site and extent of degradation of feed particles and the efficiency of microbial protein synthesis (Dijkstra et al., 2007). Knowledge on fractional passage rates is therefore essential to predict the amount of volatile fatty acids (VFA) and microbial protein synthesised in the rumen, which are the major sources of absorbed energy and protein, respectively. Protein supply and VFA production predictions were found to be very sensitive to changes in fractional passage rates (Bannink et al., 1997; Seo et al., 2006b), emphasising the need of an accurate estimation of fractional passage of concentrates.

Accuracy of passage rate estimation can vary considerably with marker choice. Predominantly, studies on passage dynamics have employed external digesta markers although their use has been criticised for their representativeness (Tamminga et al., 1989b; Owens and Hanson, 1992; Pellikaan et al., 2013). In contrast, internal markers would be preferred as they undergo the same biological processes to which the feed fractions under investigation are exposed to. Stable isotopes are present in low amounts in feedstuffs and can be used as an internal marker. Differences in natural abundance of the stable isotope ^{13}C relative to the major carbon isotope ^{12}C, as occurs between carbon-fixating C_3 and C_4 plant species (Boutton et al., 1988), can be used to determine fractional passage rates (Südekum et al., 1995; Sponheimer et al., 2003). Recent studies further emphasised the advantages of stable isotopes in estimating feed-specific fractional passage rates (Huhtanen and Hristov, 2001; Pellikaan et al., 2013). In the latter study, ^{13}C labelled grass silage yielded lower fractional rumen passage rates compared to the external marker chromium-mordanted fibre (Cr-NDF), likely because the latter marker typically associates to the small indigestible rumen particle pool (Bosch and Bruining, 1995). Such marker discrepancies were also reported between Cr-NDF and the internal marker NDF following rumen evacuations (Robinson et al., 1987). However, it is not known whether differences among small-particle external markers and internal markers persist for concentrates, which are typically provided as relatively fine material.

Passage of feed particles has been found to be feed and animal specific (e.g. Colucci et al., 1990; Lund et al., 2006, Ahvenjärvi et al., 2010) but effects of concentrate level in the diet on passage of feed are less clear. For instance, fractional passage of protein and starch in roughage and concentrates are fixed in the Dutch protein evaluation system for ruminants,

whereas that for NDF is estimated from the fractional NDF degradation rate (van Duinkerken et al., 2011). However, fractional passage rate of forages was found to increase with an increasing concentrate proportion in the diet (review of Offer and Dixon, 2000). Similarly, fractional passage of concentrates was reported to increase from 0.050/h to 0.069/h when concentrate levels increased from less than 20% up to 50% in the diet, whilst a further increase in concentrate level up to 80% gave a decrease in the fractional rate of passage to 0.034/h (Owens and Goetsch, 1986). Colucci et al. (1990) observed decreased fractional rumen passage rates of a Cr-mordanted concentrate meal with increasing levels of concentrates in the diet with this relationship depending strongly on feed intake. Ahvenjärvi et al. (2010) further reported a significant effect of the type of diet on fractional rumen passage of forage but not on that of a concentrate meal based on the external marker lanthanum.

The aim of this study was to determine feed component specific fractional passage rates for concentrates provided to dairy cows at low and high levels. In this study, the principle of higher ^{13}C abundance in C_4 plants as compared to C_3 plants was used. A shift in ^{13}C enrichment was created by exchanging part of the C_3 basal concentrates by a pulse dose of C_4 maize bran in the diet. Passage kinetics of concentrates were then quantified from changes in the ^{13}C:^{12}C-ratio in different feed components in faeces.

Material and Methods

Animals, Diet and Experimental Design

The experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University and executed in accordance with the Dutch legislation on the use of experimental animals. Six Holstein-Friesian dairy cows in their second to third lactation, fitted with a permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond, Parma, ID), were individually housed in tie stalls. After an initial adaptation period to the experimental diet, one animal was excluded from the experiment due to general health issues. At the onset of the experiment, cows were 267 ± 8 days in milk (mean ± SEM), averaged 574 ± 9 kg in body weight and produced 14.6 ± 0.96 kg milk/d. Animals received a basal diet (Table 1) containing whole-plant silage and concentrates. Whole-plant silage was prepared from ensiled barley and grass silage as a 1:1 (w/w) mixture on product basis. Concentrates were prepared from C_3 and C_4 plant ingredients (60:40; w/w), selected to achieve low natural level of ^{13}C abundance (Table 1), and fed at two levels to obtain concentrate levels of 230 g/kg DM (low

concentrate level; LC) and 520 g/kg DM (high concentrate level; HC) in the diet. Concentrates were provided on top of the roughage to ensure that all concentrates were eaten. Animals were paired based on parity and milk production and among pairs animals were randomly assigned to receive either a LC or a HC diet over two experimental periods in a crossover design. Both experimental periods consisted of an adaptation period of 15 days and a measuring period of 5 days. Animals were fed three times per day at 0615, 1415 and 2115 h. Feed intake for all animals was restricted based on the animal with the lowest individual feed intake, thereby minimising feed residues and ensuring equal concentrate and nutrient uptake. Animals had constant access to water and minerals (KNZ, AkzoNobel). Animals were milked twice daily at 0615 and 1645 h.

Markers

Fractional passage rates of carbon stable isotopes (^{13}C) and chromium-mordanted fibre (Cr-NDF) were determined from their faecal excretion patterns following a pulse dose on day 16. The ^{13}C pulse dose consisted of 9.0 kg pelleted maize bran based concentrates (ground to 2.75 mm; Table 1) fed to animals in two equal portions. The first ^{13}C portion (4.5 kg maize bran) was administered orally at 0600 h and after 30 min non-ingested pelleted maize bran was quantitatively administered intra-ruminally via the cannula. At the same moment, 90 g Cr-NDF (45.9 g Cr/kg Cr-NDF; <0.5 mm) was pulse-dosed intra-ruminally. Thereafter, the second ^{13}C portion (4.5 kg maize bran) was administered the same way as described above. Feeding time was postponed to 0700 h on the day of pulse dose administration. To ensure equal concentrate uptake after adding additional DM from the maize bran bulk to the rumen DM pool through the maize bran pulse dose, the LC group did not receive concentrates during the morning and afternoon feeding, and the HC group did not receive concentrates during the morning feeding. Rumen pH measurements were regularly taken over a 72-h cycle after pulse dosing at faecal sampling times (18 measurements per animal) to have some indication of rumen fermentation during passage kinetics measurements.

Feed digestibility was determined by applying the continuous infusion method (Faichney, 1975), employing a continuous infusion of cobalt ethylenediamine tetraacetic acid (Co-EDTA) in the rumen. Starting at day 7, Co-EDTA (1.14 g Co/d) was dissolved in 12 L water and infused into the rumen at a constant rate (0.5 L/h). The Cr-NDF and Co-EDTA were prepared as described by Udén et al. (1980); Cr-NDF was prepared from wheat straw, thoroughly washed, dried and ground to pass a 0.5-mm screen.

Table 1. Ingredients and chemical composition of the experimental diet fed to dairy cows

Item[1]	WPS[2]	Concentrates, pelleted[3]	Maize bran, pelleted[3]
Ingredients			
Wheat	—	54.1	—
Palm kernel expeller	—	150.0	—
Sunflower seed	—	126.2	—
Soybean hulls	—	26.5	—
Coconut expeller	—	175.0	—
Sugarbeet pulp	—	200.0	—
Potato starch	—	150.0	—
Phosphoric acid limestone	—	7.6	—
Salt	—	3.0	—
Minerals[4]	—	7.5	—
Molasses	—	100.0	100.0
Maize bran	—	—	900.0
Chemical composition			
Dry matter (g/kg)	468	900	873
Organic matter	912	909	950
Crude protein	122	167	141
DVE[5]	50	100	78
OEB[5]	20	9	−29
Neutral detergent fibre	463	287	373
Starch	124	172	255
Sugar	54	74	19
OMd (g/kg)[5]	752	805	780
NE_L (MJ/kg DM)[6]	6.0	7.0	7.3

[1]g/kg dry matter, unless mentioned otherwise.
[2]WPS = whole plant silage (1:1 mixture, w/w, of barley and grass silage); determined by near-infrared spectroscopy (Blgg AgroXpertus, Wageningen, The Netherlands).
[3]Ingredients and chemical composition determined through linear programming based on table values from CVB (2000).
[4]Mineral premix on basis of limestone, Mervit Rundvee 31, Pre-Mervo, Utrecht, The Netherlands.
[5]DVE = intestinal digestible protein; OEB = rumen degraded protein balance; OMd = organic matter digestibility; all units in the Dutch protein evaluation system (van Duinkerken et al., 2011).
[6]NE_L = net energy lactation (calculated as described by van Es, 1975).

Measurements and Chemical Analyses

Milk samples were taken twice daily during the measurement period and pooled per day. Milk composition was analysed by mid-infrared spectroscopy and pooled per animal over each sampling period (Milk Control Station, Zutphen, The Netherlands).

Feed residues were collected daily before the morning feeding, oven-dried at 70°C and ground over a hammer mill to pass a 1-mm screen (Peppink 100 AN, Olst, The Netherlands). Feed samples and residues were pooled per animal over each sampling period. Contents of

DM and ash were determined gravimetrically after 4 h drying in a forced air stove at 103°C (ISO 6496) and after incineration for 3 h in an oven at 550°C (ISO 5984), respectively. Nitrogen was determined using the Kjeldahl method with copper(II) sulfate as catalyst (ISO 5983) and CP was calculated as 6.25 × N. Starch content was determined enzymatically according to the method of Brunt et al. (1998). Ash free neutral detergent fibre (NDF) was determined by a modified method of Van Soest et al. (1991) with the use of heat stable amylase but without sodium sulphite (ISO 16472).

Directly after administration of markers into the rumen, 20 spot samples of faeces were collected after defecation in sampling blocks of three hours each to determine fractional passage rates. Faecal samples were collected at average times t = 0, 4, 5, 7, 9, 11, 13, 16, 17, 20, 23, 31, 35, 41, 48, 53, 60, 72, 83, 99 h after pulse dose administration. For an optimal curve fit, the interval between collection times during the first 24 h after pulse dose administration were shortened such to ensure that sufficient data points were obtained before the anticipated marker peak concentrations time. Faeces were weighed, homogenised and a representative sample (300 g) taken. Samples were stored at −20°C, freeze-dried, ground (<1 mm; Peppink 100 AN, Olst, The Netherlands) and stored at 4°C pending analyses. Feed digestibility was determined by collecting faeces in sampling blocks of three hours each over three sampling days, thereby covering a 24-h period, which was representative for an entire feeding cycle. Samples were processed as described above.

Faecal marker excretion patterns were established for Cr-NDF and for ^{13}C in the DM fraction (^{13}C-DM), NDF fraction (^{13}C-NDF) and neutral detergent soluble fraction (^{13}C-NDS). All fractions, including Cr and Co concentrations, were analysed as described in detail by Pellikaan et al. (2013). The NDS fraction was obtained by calculating the difference between the DM and NDF. The relative ^{13}C-enrichment is expressed as the ^{13}C:^{12}C ratio in the samples relative to the ^{13}C:^{12}C ratio of the international Vienna Pee Dee Belemnite standard. After correction for natural ^{13}C abundance, faecal excretion patterns of excess ^{13}C were established.

Curve Fitting and Statistical Analyses

Fractional passage rates were derived from faecal excretion patterns of ^{13}C-DM, ^{13}C-NDF, ^{13}C-NDS and Cr-NDF, fitted iteratively with non-linear compartmental models. These models were two-compartmental models with progressively higher orders of age dependency (GnG1; Matis, 1972) and a multicompartmental age-independent model (Dhanoa et al., 1985). Model accuracy was assessed by comparing observed to predicted marker concentrations using the root mean square prediction error, which was decomposed into errors due to overall bias of

prediction, errors due to deviation of the regression slope from unity, and errors due to random variation (Bibby and Toutenberg, 1977), and scaled to the observed mean (mean prediction error, MPE).

The multicompartmental model gave overall the best fit between observed and predicted faecal marker concentrations as shown by its low MPE (13.3 ± 0.85% across markers) and high coefficient of determination (R^2 = 0.977 across markers), and all 40 curves converged. The higher-order GnG1 (n = 3–5) models had overall higher MPE values (ranging from 26.7 ± 1.50% for the G3G1 model to 19.9 ± 1.09% for the G5G1 model), a lower R^2 (ranging from 0.915 for the G3G1 model to 0.953 for the G5G1 model) and curves had a poorer convergence ranging from a total of eight non-converged curves (G3G1) to one non-converged curve (G5G1). The multicompartmental model was therefore selected as the most suitable model to derive passage kinetics in this study and was defined as (Eq. 1):

$$C_t = A \times e^{-K_1 \times t} \times \exp\left[-(N-2) \times e^{-(K_2 - K_1) \times t}\right] \quad [1]$$

where C_t denotes the faecal marker concentration at time = t (h); t is the average time span of collection after marker administration; K_1 and K_2 (/h) refer to the fractional rate constants for the compartment with the longest (reticulorumen) and the second longest retention time (large intestine) in the digestive tract, respectively; N refers to the model-derived number of mixing compartments; and A forms a scalable parameter dependent on K_1, K_2 and N. Before curve fitting, faecal marker concentrations were scaled to the marker peak concentration, following the procedure of Sponheimer et al. (2003). Curve fitting was performed using non-linear least squares regression procedures of SAS (version 9.2, Cary, NC) based on the least square Levenberg-Marquardt algorithm. Initial values for the iterative procedure were obtained through a grid search and curve fits were normally solved after 6 to 10 iterations.

Transit time (TT) and moment of peak concentration (PCT) were derived from the estimated parameters based on Eq. 1 as described by Dhanoa et al. (1985). Total mean retention time (TMRT) in the gastro-intestinal tract was calculated as the sum of the reciprocals of K_1 and K_2 including retention times associated to the remaining compartments (assumed to be TT).

All model parameters were log transformed by taking the natural logarithm due to asymmetrical distribution patterns of residuals. Log-transformed values were tested by analyses of variance as a split plot with a crossover arrangement of treatments for main plots with mixed model procedures of SAS (version 9.2, Cary, NC), according to the model (Eq. 2):

$$Y_{ijkm} = \mu + A_i + P_j + D_k + (A \times P \times D)_{ijk} + M_m + (D \times M)_{km} + \varepsilon_{ijkm} \quad [2]$$

where Y_{ijkm} is the dependent variable; μ is the overall mean; A_i (animal effect; i = 5), P_j (period effect; j = 2), and D_k (effect for concentrate level in the diet; k = 2) represent effects related to the main plots; M_m (marker effect; m = 4), and the interaction term $(D \times M)_{km}$ represent effects related to the subplots. Main plot variables were tested against the interaction term $(A \times P \times D)_{ijk}$ and subplot variables were tested against the pooled residual error (ε_{ijkm}). Covariance parameters were estimated using the residual maximum likelihood (REML) method and denominator degrees of freedom (DDFM) were estimated using the Satterthwaite approximation. Differences between marker types were assessed using orthogonal contrasts. Tables show back-transformed values (geometric means). The standard error of the mean (SEM) was calculated by multiplying the log-transformed SEM by its geometric mean. Animal performance data were statistically analysed as crossover arrangement of treatments as outlined in Eq. 2 but omitting subplots for marker effects.

Results

Feed Intake and Animal Performance

Feed intake, digestibility and milk production data are given in Table 2. Passage kinetics were studied for diets fed to dairy cows at low levels (LC) and high levels (HC) of concentrates. The final concentrate intake levels on DM basis were 4.0 kg/d (24.6% of total DM intake) for LC diets and 9.2 kg/d (52.6%) for HC diets. Animals receiving an HC diet realised a slightly higher daily DM intake (17.5 kg DM/d) compared to the LC fed group (16.4 kg DM/d; P = 0.057). The difference in DM intake was mainly due to two animals in the LC treatment group that had somewhat higher feed residues (2.3 ± 0.23 kg product per day) after pulse dosing maize bran than during other days. However, feed residues did not include concentrates as these were provided on top of the roughage. Intake of crude protein and starch were higher with HC diets (P = 0.003), and intake of NDF tended to differ (P = 0.089) between diets. Dry matter digestibility was 3%-units higher (P = 0.021) for HC than LC diets whereas NDF digestibility remained similar across treatments.

Fat and protein corrected milk (FPCM) production tended to be higher (2.0 kg/d; P = 0.068) for animals fed a HC diet. Milk fat and protein concentrations were higher (P = 0.011

and P = 0.033, respectively) in the HC group, resulting in somewhat higher absolute fat and protein yields for the HC group (P = 0.043 and P = 0.057, respectively).

Table 2. Animal performance of dairy cows fed a mixture of whole plant barley and grass silage at low levels (LC) and high levels of concentrates (HC) in the diet

Item	LC	HC	SEM	*P*-value		
				Cow	Period	Diet
Intake						
Concentrates (kg/d)	4.0	9.2	0.03	0.395	0.634	<0.001
Concentrate allowance (%)[1]	24.6	52.6	0.49	0.839	0.724	<0.001
Dry matter (kg/d)	16.4	17.5	0.25	0.360	0.557	0.057
Organic matter (kg/d)	15.0	15.9	0.23	0.370	0.498	0.061
Crude protein (g/d)	2180	2537	30	0.362	0.504	0.003
Neutral detergent fibre (g/d)	6901	6472	121	0.401	0.531	0.089
Starch (g/d)	2226	2602	30	0.363	0.504	0.003
Digestibility						
Dry matter (g/kg)	626	657	4.7	0.395	0.037	0.021
Neutral detergent fibre (g/kg)	572	585	7.4	0.149	0.103	0.304
Milk yield and composition						
Milk (kg/d)	13.1	14.3	0.41	0.044	0.282	0.142
FPCM (kg/d)[2]	14.7	16.7	0.50	0.037	0.524	0.068
Fat (g/kg)	48.3	52.4	0.50	0.003	0.035	0.011
Protein (g/kg)	37.4	38.5	0.21	0.003	0.016	0.033
Lactose (g/kg)	41.7	42.8	0.32	0.004	0.038	0.087
Fat (g/d)	637	745	22.3	0.022	0.732	0.043
Protein (g/d)	489	546	13.1	0.029	0.529	0.057

[1]% of total dry matter intake.
[2]FPCM: fat and protein corrected milk.

Passage Kinetics

Fractional passage rates were estimated for ^{13}C-DM, ^{13}C-NDF, ^{13}C-NDS and Cr-NDF based on faecal marker excretion curves following a pulse dose of maize bran and Cr-NDF into the rumen. Among markers, the mean prediction error (MPE) was highest for ^{13}C-NDS (17.8–21.3%; P < 0.001) but comparable between Cr-NDF and ^{13}C-DM and ^{13}C-NDF (9.9–13.9%; results not shown). The largest proportion of the MPE was due to random variation (94.6%)

and only a minor part was explained by an error in the overall mean (2.7%) or regression bias (2.8%) (results not shown).

Concentrate level in the diet did not affect any model fit parameter. On the other hand, marker choice affected fractional passage rates from the reticulorumen, (K_1; $P < 0.001$) and from the large intestine (K_2; $P = 0.003$) (Table 3). The ^{13}C-DM and ^{13}C-NDF gave considerably higher K_1 estimates than ^{13}C-NDS and Cr-NDF ($P < 0.001$). In contrast, K_2 estimates were lower when measured with ^{13}C-DM and ^{13}C-NDF than ^{13}C-NDS and Cr-NDF ($P = 0.001$). Model parameters A and N as estimated by the multicompartmental model were on average 3.3 (SEM 0.54; $P < 0.001$) and 70 (SEM 16.0; $P < 0.001$) across markers, respectively (results not shown).

Marker peak concentration time (PCT) and marker transit time (TT) were not significantly different among markers and among diets (Table 3). The total mean retention time in the gastro-intestinal tract (TMRT) was therefore highly variable among markers ($P < 0.001$) and was on average 8.2 h longer for Cr-NDF than for ^{13}C-DM and ^{13}C-NDF. Among ^{13}C marked feed components the ^{13}C-NDS had the highest TMRT (on average, 3.5 h longer).

Mean rumen pH values of 180 measurements taken over 72 hours after pulse dosing maize bran were 6.66 (SD 0.37) for the LC diet and 6.61 (SD 0.38) for the HC diet. No effect of concentrate level in the diet on pH was observed. Hours below pH 6.2, the critical level for depression of fibrolytic bacteria activity (Mould et al., 1983), were on average 2.6 (SD 1.61) for the LC diet and 3.3 (SD 1.84) for the HC diet per 72 h. It can be therefore assumed that the maize bran pulse dose did not adversely affect rumen fermentation kinetics.

Discussion

Accuracy of Curve Fits

The present study quantifies fractional passage rates based on the faecal excretion patterns of Cr-NDF and ^{13}C isotopes in the DM, NDF and NDS fractions. The approach used in this study is a relatively inexpensive marker technique to determine digesta passage kinetics in dairy cows. By switching from C_3 concentrates to C_4 concentrates with a different natural ^{13}C enrichment level, the often laborious and expensive preparation of external markers or isotopic labelled plant material above natural enrichment can be evaded.

Table 3. Passage kinetics of different markers in dairy cows fed low levels (LC) and high levels of concentrates (HC) in the diet[1]

Marker[2]	K_1		K_2		PCT		TT		TMRT	
	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC
^{13}C-DM	0.056	0.054	0.320	0.358	19.3	19.4	11.3	12.1	32.8	33.5
^{13}C-NDF	0.063	0.061	0.288	0.317	20.3	19.2	12.0	11.5	32.0	32.8
^{13}C-NDS	0.039	0.043	0.386	0.444	18.7	17.8	11.0	11.0	37.3	36.8
Cr-NDF	0.039	0.037	0.366	0.408	20.3	19.8	12.1	12.3	40.9	42.0
SEM[3]	0.0050		0.0305		0.80		0.78		1.67	
P-values[4]										
Cow	0.325		0.612		0.016		0.191		0.137	
Period	0.406		0.849		0.985		0.778		0.342	
Diet (D)	0.993		0.184		0.297		0.890		0.783	
Marker (M)	<0.001		0.003		0.144		0.417		<0.001	
D × M	0.696		0.994		0.846		0.849		0.931	
(^{13}C-DM, ^{13}C-NDF) *vs.* (^{13}C-NDS, Cr-NDF)	<0.001		0.001		0.478		0.781		<0.001	
^{13}C-DM *vs.* ^{13}C-NDF	0.136		0.177		0.643		0.914		0.534	
^{13}C-NDS *vs*. Cr-NDF	0.301		0.406		0.032		0.104		0.003	

[1]Passage kinetics: K_1 = fractional passage rate constant (/h) for the reticulorumen; K_2 = fractional passage rate constant (/h) for the large intestine; PCT = peak concentration time (h); TT = transit time (h); TMRT = total mean retention time (h).

[2]Markers: ^{13}C-DM = ^{13}C labelled dry matter; ^{13}C-NDF = ^{13}C labelled NDF; ^{13}C-NDS = ^{13}C-labelled neutral detergent soluble; Cr-NDF = chromium-mordanted NDF.

[3]Standard error of the mean.

[4]Analysis of variance based on log-transformed means.

Costs of the latter techniques can be high, particularly for large ruminants requiring an accordingly large marker dosage. However, the approach used in this study is only suitable for testing comparable diets with a sufficiently large difference in their natural carbon isotope enrichment.

Passage kinetics of Cr-NDF from wheat straw was chosen as a point of reference as it has been widely used in passage studies. Differences in marker type material and preparation, especially with regard to particle size and density and possible migration of particulate marker to particles not originally labelled, may influence rate constants (Owens and Hanson, 1992). These limitations need to be considered in passage studies using different types of markers as well as when rate constants among studies are compared. Passage kinetics of ^{13}C isotopes were determined from the specific carbon isotope ratio (^{13}C:^{12}C ratio) at several time intervals after marker dosage. Pellikaan et al. (2013) showed that the ^{13}C:^{12}C ratio did not change with fermentative degradation in the rumen and, therefore, can be used to assess feed specific and feed component specific passage kinetics.

A pulse dose of naturally enriched ^{13}C feed source maize bran was offered orally and non-ingested portions were injected into the rumen. The intra-ruminally administered dose was on average 1.98 kg product (SEM 0.57) or 22% of the total pulse-dosed maize bran. Although this relatively minor part of the maize bran was not subject to the normal chewing during eating, this is not expected to significantly affect passage behaviour of maize bran since the bran was pelleted. The total pulse dose time was on average 94 min (SEM 9). Marker excretion curves for individual animals and markers (Figure 1) were characterised by a sharply ascending curve phase, a distinct peak at 20 h after marker dosage and a slowly descending curve phase. Marker excretion patterns were comparable to studies in dairy cattle in which a ^{13}C pulse dose was mixed with previously fully evacuated rumen content (Pellikaan et al., 2013). The different routes of marker administration need to be considered when comparing markers. Compared to intra-ruminal marker administration, oral administration might slow down the marker accumulation in the rumen and markers might require more mixing time before reaching the respective peak concentration, thereby affecting the shape of the curve peak. Figure 1 suggests that this was not the case in the present study as pelleted maize bran of fine particle size was used.

The mean prediction error (MPE) provides the goodness of fit of the model describing the observed faecal marker excretion patterns. Accuracy of curve fits was similar among the markers Cr-NDF, ^{13}C-DM and ^{13}C-NDF with rather low MPE values of 8.9–13.9%. Somewhat higher MPE values were obtained for ^{13}C-NDS (17.8–21.3%). The latter was

calculated as the difference between ^{13}C concentration in the DM and NDF, which may have contributed to the larger MPE values of ^{13}C-NDS since any measurement errors may accumulate in the ^{13}C-NDS fraction.

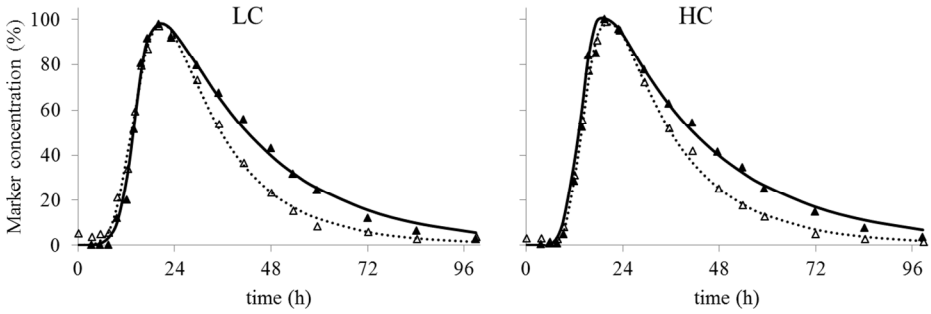

Figure 1. Faecal mean marker concentrations relative to the marker peak concentration with model fits in dairy cows fed a diet with low (LC) and high levels (HC) of concentrates for chromium-mordanted NDF (Cr-NDF; ▲) and^{13}C labelled NDF (^{13}C-NDF; △).

Passage Kinetics of Concentrates

Fractional passage rates were derived from faecal marker excretion patterns for the compartment with the longest (K_1; reticulorumen) and second longest retention time (K_2; large intestine). Estimates for K_1 based on Cr-NDF are in the range of rate constants reported by Colucci et al. (1990) for dairy cows fed a lucerne-based diet at maintenance level and receiving comparable levels of concentrates. They reported fractional passage rates of 0.046/h and 0.031/h for inclusion levels of 20% and 45% concentrates in the diet, respectively. For animals fed ad libitum at a comparable feed intake as in our study, rumen fractional passage was more than doubled (on average, 0.083/h) and considerably higher than our observed K_1 estimates. Colucci et al. (1990) used Cr-mordanted soybean meal as compared to Cr-mordanted wheat straw in our study. Possible difference in particle size, shape and density, especially when compared to the ingested ^{13}C maize bran, might explain some of the observed discrepancies in fractional passage rates.

In the present study, the ^{13}C markers were introduced by replacing part of the concentrates with maize bran originating from C_4 carbon fixating plants, which have a higher natural ^{13}C enrichment than the concentrates originating from C_3 plants. The cell wall fractions (^{13}C-

NDF) of the maize bran passed out quicker from the reticulo-rumen than Cr-NDF and non-cell wall fractions (^{13}C-NDS) and slightly quicker than ^{13}C-DM, which is in contrast to passage studies on grass silages (Pellikaan et al., 2013). In contrast to K_1, estimates for K_2 were higher for Cr-NDF than ^{13}C-DM and ^{13}C-NDF. This is in disagreement to findings on roughages using similar markers (Pellikaan et al., 2013) as well as to findings on concentrates using external markers (Collucci et al., 1990). These differences may partly result from a differential passage behaviour of fibres originating from different feed source, although possible differences in particle size and density between Cr-NDF and ^{13}C-NDF originating from ingested maize bran pellets might be confounding factors in the present study.

As to the lower K_1 estimates for ^{13}C-DM as compared to ^{13}C-NDF in the present concentrate diets, a contribution of ^{13}C loaded microbial biomass in the rumen to faecal DM cannot be excluded. Rumen bacteria might incorporate some of the ^{13}C added to the rumen DM and NDS pool and eventually exit the reticulo-rumen at different flow rates depending on the digesta phase they are associated with. Furthermore, Brito et al. (2006) observed different isotopic enrichment levels for liquid- and solid-associated bacteria, with the latter being lower enriched in ^{15}N isotopes. If the same can be assumed for ^{13}C isotopes, this ^{13}C recycling by rumen bacteria in combination with a differential passage of the rumen bacteria and a potentially substantial contribution of microbial biomass in faeces (van Vliet et al., 2007), might influence the ^{13}C:^{12}C ratio in the faecal DM to some degree. In contrast, passage of ^{13}C-NDF will not be affected by microbial ^{13}C recycling as shown by Pellikaan et al. (2013) as NDF is not part of microbial matter. Future research should address the potential effect of bacterial incorporation of isotopes on their passage kinetics; for instance, by quantifying the faecal bacterial biomass in dairy cows fed similar concentrate levels, or by assessing the passage behaviour of labelled plant components, such as cuticular alkanes which are components typically not degraded in the rumen and known to associate well to the rumen particulate DM pool (Mayes et al., 1997).

Estimates of TMRT of Cr-NDF for our concentrate mixture (40.9 and 42.0 h for LC and HC, respectively) were comparable to Cr-mordanted soybean meal for dairy cows of a similar DM intake and fed concentrate levels similar to our LC and HC treatment (Colucci et al., 1990; 35.0–35.9 h). Ahvenjärvi et al. (2010) though reported a considerably lower TMRT of 24.7 h for lanthanum labelled rapeseed meal for dairy cows of a similar DM intake fed 40% concentrates in silage-based diets. The internal markers ^{13}C-DM and ^{13}C-NDF had a lower TMRT than the external marker Cr-NDF. In general, the TMRT of the ^{13}C markers were considerably lower for maize bran in the present study compared to roughage from previous

^{13}C passage studies: on average, TMRT for maize bran was 31 h faster than grass silage and 40 h faster than fresh grass (Pellikaan, 2004). However, TMRT of Cr-NDF was comparable throughout all passage studies cited despite qualitative contrasts of the diets used. These results suggest that Cr-NDF is less sensitive to dietary changes than ^{13}C This is in line with Bosch and Bruining (1995) who concluded that Cr-NDF ground to 0.2–1.0 mm associates to the small indigestible rumen particle pool and can be considered to be only representative of that specific pool. In contrast, ^{13}C isotopes, incorporated as natural components in all plant components, represent the entire fraction in which they are analytically recovered and better suitable to measure differences between diets.

Levels of concentrates in the diet (24.6% *vs*. 52.5% concentrate proportion) did not influence fractional passage rates and kinetics of concentrates. Our findings agree with studies on ruminants based on external markers. Colucci et al. (1990) found no differences of fractional passage rates and total mean retention time of Cr-mordanted soybean meal particles between 20 and 45% concentrates in the diet, comparable to our LC and HC treatments, for animals fed comparable amounts of DM. Poore et al. (1990) confirmed these findings with rare earth element labelled sorghum grain in steers receiving concentrate levels comparable to our LC and HC treatments. They reported fractional rumen passage rates of 0.053 and 0.051/h for 30 and 60% concentrate diets, respectively. In contrast to the absence of effect of concentrate level on fractional passage rate, roughage characteristics may well affect fractional passage rate. Ahvenjärvi et al. (2010) observed no differences in fractional passage rates of lanthanum labelled rapeseed meal for dairy cows receiving a 40% concentrate allowance. They reported rumen fractional passage rates for rapeseed meal ranging from 0.057 to 0.061/h for different silages that were prepared either from early or late cut grass, or red clover, or whole barley. These results suggest that passage of concentrates is considerably less sensitive to changes in diet than forage for dairy cows with high feed intake. Furthermore, passage kinetics based on ^{13}C-NDF across studies suggest that fibres show a different passage behaviour depending on the feed source. Hence, feed source and feed component specific fractional passage rate constants determined with stable isotopes might considerably enhance the accuracy of effective fibre degradability.

Isotope determined passage of fibres across studies suggest that is possible to discriminate between fibres from roughage and fibres from technologically treated compound feed. Further research based on isotope labelled cell walls, either determined as fibre fractions of common gravimetric fibre analysis methods or preferably as isolated individual structural polysaccharides will provide a better understanding of the differential passage behaviour of

fibres from different feed sources. The use of stable isotopes as a digesta passage marker should be further developed to analytically determine the fractional passage of the non-cell wall components, in particular starch and protein fractions.

Conclusions

Fractional rumen passage rates of concentrates determined with the external marker Cr-NDF were lower as compared with the internal marker ^{13}C measured both in the DM or in the NDF. An increase in the concentrate proportion from 24.6 to 52.6% did not influence fractional passage rates in the digestive tract of dairy cows. Our results suggest that stable isotopes can estimate feed component specific fractional passage rates of concentrates providing new insight into fractional passage of concentrates as compared to when external markers are used.

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Chapter 4

Passage kinetics of ^{13}C labelled maize silage components through the gastrointestinal tract of dairy cows

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Abstract

Fractional passage rates form a fundamental element within modern feed evaluation systems for ruminants but knowledge on feed type and feed component specific passage rates are largely lacking. This study describes the use of carbon stable isotopes (^{13}C) to assess component specific passage kinetics of six intrinsically ^{13}C labelled maize silages varying in quality (two cultivars × three maturity stages) in a 6 × 6 Latin square design using six rumen-fistulated lactating dairy cows. An increase in maturity increased starch and decreased neutral detergent fibre and acid detergent fibre content of maize silages. Passage kinetics were assessed for an external (chromium mordanted fibre; Cr-NDF) and an internal marker (^{13}C isotopes) collected in faeces and omasal digesta. The best fit was obtained with a deterministic multicompartmental model compared to stochastic G*n* and G*n*G1 models with increasing order of age dependency (n = 1–5) for both sampling sites. The Cr-NDF marker yielded higher rumen fractional passage rates (K_1) than ^{13}C in the dry matter (^{13}C-DM) in faeces (0.042/h *vs*. 0.023/h). Omasal marker excretion patterns support the conclusions based on conventional faecal marker excretions. Component specific passage was assessed for the acid detergent fibre (^{13}C-ADF) in faeces and for starch (^{13}C-ST) in omasal digesta. The fractional passage rate based on faecal ^{13}C-DM and ^{13}C-ADF did not differ. Omasal ^{13}C-ST provided higher K_1 values (0.042/h) than omasal ^{13}C-DM (0.034/h) but lower values than omasal Cr-NDF (0.051/h). Fractional passage rates from the proximal colon-caecum (K_2) based on faecal marker concentrations showed trends similar to K_1, with Cr-NDF providing a value (0.425/h) more than twice as high than that of ^{13}C-DM (0.179/h) and ^{13}C-ADF (0.128/h). Total mean retention time in the gastrointestinal tract was approximately double for ^{13}C-DM (64.1 h) and ^{13}C-ADF (77.6 h) than for Cr-NDF (36.4 h). Maize silage quality did not affect any of the estimated passage kinetic parameters. Fractional *in situ* degradation rates did not differ among maize silages, except for a decreased fractional degradation rate of starch with advancing maturity. Results suggest that isotope labelling allows to assess component specific passage kinetics of carbohydrate fractions in maize silage.

Introduction

The competition between the processes of degradation and passage in the rumen has a major impact on digestibility of nutrients (Faichney, 1980; Allen and Mertens, 1988) and, therefore, on the formation of volatile fatty acids (VFA) and microbial protein, the cow's major sources of absorbed energy and protein, respectively. Hence, the protein supply to the animal largely depends on the balance between fractional degradation and fractional passage rates and is feed specific (Robinson et al., 1986). The fractional passage rate determines the time feed is retained in the gastrointestinal tract and is a major determining factor of the site and extent of degradation as well as efficiency of microbial protein synthesis (Dijkstra et al., 2007).

Rumen degradation has been assessed for a wide range of maize silage qualities (De Boever et al., 2002) and rumen escape starch was not related to chemical composition or maturity. In contrast, Philippeau and Michalet-Doreau (1997) reported starch degradation rates to decrease with increasing maize maturity due to a concomitant increase in grain vitreousness, although this decrease with increased vitreousness may depend on particle size of maize kernels (van Zwieten et al., 2008). Maize silage is commonly fed to dairy cows in intensive dairy systems, but quantitative information on fractional passage rate of maize silage is limited. In the Dutch protein evaluation system for ruminants a fixed fractional passage rate of protein and starch for forage is assumed independent of type of forage and forage quality, whereas the fractional passage rate constant for neutral detergent fibre is estimated from its fractional degradation rate (van Duinkerken et al., 2011). Rumen passage of forages has been reported to depend on forage type (Lund et al., 2006) and to increase with increasing maturity of the grass silage (Rinne et al., 1997a).

Fractional passage rates have been traditionally estimated using external markers collected in faeces or in digesta from intestinally cannulated animals. External markers can describe passage kinetics of a specific rumen particle pool, as shown by Bosch and Bruining (1995) for chromium mordanted fibre (Cr-NDF), but do not provide component specific fractional passage rates. Stable isotopes have been proposed as internal markers to study rumen passage rates in ruminants (Südekum et al. 1995; Huhtanen and Hristov, 2001; Sponheimer et al., 2003), and recently carbon isotopes (^{13}C:^{12}C ratio) were shown to be suitable to determine component specific fractional passage rates for grass silages (Pellikaan et al., 2013).

The aim of this study was to estimate feed component specific fractional passage kinetics of two cultivars of maize silage, harvested at three maturity stages, through the

gastrointestinal tract of dairy cows. Maize silages were intrinsically labelled with ^{13}C by growing the maize plants under continuous elevated $^{13}CO_2$ conditions. In addition to the external marker Cr-NDF, ^{13}C isotopes were used as internal markers to specifically assess component specific passage kinetics for starch and acid detergent fibre based on the ^{13}C:^{12}C ratio in glucose in omasal digesta and acid detergent fibre in faeces, respectively.

Materials and Methods

Animals and Diet

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands) and carried out under the Dutch Law on Animal Experimentation. Six multiparous Holstein-Friesian dairy cows in their second to fourth lactation, fitted with a permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond Inc., Parma, ID), were individually housed in tie stalls. At the start of the experiment, cows were 53 ± 11 DIM (mean ± SD), averaged 571 ± 66 kg in bodyweight and produced 39.1 ± 4.9 kg milk/d. Animals were fed a total mixed ration consisting of 625 g/kg DM roughage (a 1:1 mixture of maize silage and grass silage on DM basis) and 375 g/kg DM compound feed (Table 1). The compound feed ingredients originated from cool season C_3 plants to keep background level of ^{13}C enrichment low and similar to that of the natural enrichment level of the grass silage mixed in the experimental diet. Maize silages were prepared from two maize cultivars, each harvested at three maturity stages (Table 1). Maize cultivars were Aastar (Limagrain Advanta BV, Rilland, The Netherlands) and Baleric (Syngenta Seed, Merelbeke, Belgium). Maturity stages were set to obtain a target DM content between 260–280 g/kg (early), 320–340 g/kg (mid) and 380–400 g/kg (late). Maize plants were sown on the 1st of May 2009 on sandy soil at Unifarm, Wageningen, The Netherlands, and were harvested with a precision chop harvester during early September to early October 2009, and ensiled in sealed 30 kg bales.

Maize silage treatments were randomly distributed over six animals and six experimental periods according to a Latin square design with 2 × 3 factorial arrangement of treatments. Each experimental period lasted 21 d starting with a 12 d adaptation period to the diet. From day 9 onwards, animals were fed 95% of the individual DM intake measured during the preceding adaptation period to minimize feed refusals during the measuring days. Animals received their daily rations in two equal meals at 0600 and 1700 h. The diet was prepared

twice weekly and stored in a cooling unit at 8°C from April onwards. Feed samples were collected each time the diet was prepared. Feed residues were collected daily before the morning feeding. Feed samples and residues were pooled per animal over each experimental period. Animals were milked twice daily during feeding times and milk samples were collected from day 13 through 21. Feed intake and milk yield determined from day 13 through 21 of each experimental period were averaged per period for statistical analysis.

Table 1. Chemical composition of two maize silage cultivars harvested at three maturity stages, grass silage and compound feed

Item	Aastar			Baleric			Grass silage[3]	Compound feed[4]
	early	mid	late	early	mid	late		
Growing days[1]	124	139	152	125	140	154	—	—
DM content, g/kg	285	330	375	270	345	402	617	873
Chemical composition[2]								
Organic matter	956	963	964	950	954	960	907	952
Crude protein	87	81	78	83	78	76	207	253
Starch	286	375	426	295	368	400	—	216
NDF	410	357	341	407	376	361	485	248
ADF	229	197	188	234	208	198	254	158
DVE[3]	59	62	65	57	63	64	86	163
OEB[3]	−34	−45	−49	−36	−47	−50	56	8
OMD[3]	0.77	0.79	0.79	0.75	0.77	0.78	0.80	0.71
NE_L, MJ/kg DM[4]	6.8	7.1	7.1	6.6	6.8	6.9	6.7	7.0

[1]Interval in days from sowing to harvest date of maize silage.

[2]Chemical composition: NDF = ash free amylase-treated neutral detergent fibre; ADF = ash free acid detergent fibre; DVE = intestinal digestible protein; OEB = rumen degraded protein balance; OMD = organic matter digestibility coefficient; NE_L = net energy lactation. Values expressed as means in g/kg DM, unless specified otherwise, of six experimental periods (starch not determined for grass silage).

[3]Values determined by near-infrared spectroscopy (Blgg AgroXpertus, Wageningen, The Netherlands).

[4]Based on table values from CVB (2007); ingredients (g/kg DM): wheat (54.1), sunflower seeds (126.2), soybean hulls (26.5), palm kernel expeller (150.0), soybeans (100.0), sugar beet pulp (100.0), potato starch (200.0), MervoBest rumen-protected soybean meal (225.0), phosphoric acid limestone (7.6), salt (3.0), mineral premix (7.5).

Marker Preparation

Chromium mordanted fibre (Cr-NDF) and the stable isotope of carbon (^{13}C) were used as external and internal passage markers, respectively. The Cr-NDF was prepared as described by Udén et al. (1980) from wheat straw, dried and ground to pass a 0.5-mm screen. The ^{13}C markers were prepared as intrinsically labelled maize silage as described above for the field maize silage (two maize cultivars × three maturity stages) under greenhouse conditions as

applied by Huhtanen and Hristov (2001). Maize seeds were sown in hermetically sealed and climate-controlled assimilation chambers, specifically designed to enable homogeneous atmospheric isotope labelling of herbage resulting in uniformly labelled plants (Gorissen et al., 1996). Maize plants were continuously enriched under high levels of $^{13}CO_2$ (released from ^{13}C-bicarbonate) from plant emergence onwards by IsoLife BV, Wageningen, The Netherlands. Growing maize plants were exposed to continuous moderate ventilation by a rotating fan to resemble wind-induced mechanical stress of field plants (Biddington, 1986). At harvest, the DM contents of the labelled maize plants were within the targeted range as described above for the field maize plants. Kernels were gently broken using a mortar pestle to simulate kernel bruising of harvested field maize plants, and stems and leaves cut with a paper cutter into pieces of 1 cm. The collected maize plant portions were placed inside several bags of larger mashed grit gauze (pore size 212 µm; PA-74, Sefar Nytal, Heiden, Switzerland) and distributed over silage bales to be ensiled together with field maize plants. After an 8-week ensiling period, the ^{13}C labelled marker material was removed and stored at −20°C in sealed plastic bags.

Marker Administration, Sampling and Measurements

Marker Administration. On day 13, animals received a ruminal pulse dose of corresponding ^{13}C labelled maize silage (30 g DM) and Cr-NDF (100 g; 45.9 g Cr/kg Cr-NDF). Pulse dosing started at 0600 h with cow 1 and continued after an interval of 30 min for each consecutive animal. The mean degree of atom percentage ^{13}C (At%^{13}C) as a proportion of total carbon in the DM of the labelled maize silage ranged between 8.68 and 12.36 At%^{13}C for the different treatments (n = 6) whereas the natural level of enrichment of the unlabelled maize silage was 1.10 At%^{13}C (SD = 0.002). Prior to pulse dosing, the frozen and cut ^{13}C labelled marker material was further cut to pieces of approximately 0.5 cm to resemble ingested bulk maize silage particles. From day 9 through 17, cobalt ethylenediamine tetraacetic acid (Co-EDTA; Udén et al., 1980) was continuously infused (0.5 L/h; 1.14 g Co/d dissolved in 12 L water) in the rumen after Faichney (1975) to measure feed digestibility (from day 13 through 17), preceded by a primer dose of 1.5 times the daily dose.

Faecal Sampling. From day 13 onwards, directly after administration of markers into the rumen, 22 spot samples of faeces were collected after defecation. Faecal samples were collected in sampling blocks of three hours each at average times t = 0, 3, 7, 11, 15, 19, 23, 29, 35, 41, 48, 54, 60, 66, 73, 79, 85, 91, 98, 104, 110, 116 h after pulse dose administration. Faeces were weighed, thoroughly homogenised by hand and a representative sample of 300–

400 g fresh matter was stored at −20°C. An aliquot of 1% fresh faeces was taken during each collection time and pooled over one experimental period to determine total tract feed digestibility.

Omasal Digesta Sampling. At the same collection time as indicated above for faeces, omasal digesta leaving the reticulorumen was collected by means of the omasal sampling technique as described by Huhtanen et al. (1997). The sampling device, introduced 24 h prior to pulse dosing, was designed based on modifications of Ahvenjärvi et al. (2000) and Sterk et al. (2012) to prevent blockage of the sampling device by coarse digesta. Blocking of the holes was prevented by injecting small volumes of pressurised carbon dioxide prior to sampling. The location of the sampling device in the omasal canal was confirmed each day before the morning feeding as described by Brito et al. (2007). Omasal digesta samples (~750 mL) were collected and pH was measured. Collected omasal digesta was weighed, quantitatively poured into containers and stored at −20°C immediately after collection.

Rumen Liquid Sampling. During the first 24 h after administration of markers into the rumen, approximately 350 mL rumen liquid was collected proportionally from a cranial, middle and caudal direction after omasal sampling. Per treatment and cow, a total of six rumen fluid samples were collected after average times t = 0, 3, 7, 11, 15, 19, 23 h relative to moment of marker introduction. Subsamples of 0.75 mL each were taken for VFA, ammonia (NH_3) analyses, and rumen pH was measured immediately using an electronic pH meter (pH electrode HI1230, Hanna Instruments BV, IJsselstein, The Netherlands). The VFA and NH_3 samples were stored at −20°C in 85% phosphoric acid (1:1, v/v) and 10% trichloroacetic acid (1:1, v/v), respectively. Excess fluid was immediately returned to the rumen.

Rumen Degradability of Maize Silage

Rumen degradation of fresh maize silage was assessed per individual animal and per silage type treatment *in situ* as described by Tas et al. (2006) over two consecutive series. The short-term incubations (0, 2, 4, 8, 12, 24, 48, 72 h; all-out procedure) of the first incubation series started at 60 hours after administration of the passage rate markers to ensure similar conditions of degradation and passage rates. A previous isotope marker study (D. Warner et al., unpublished data)[1] showed that faecal baseline concentration of ^{13}C after a pulse dose was reached at approximately four days after pulse dosing. Hence, the *in situ* incubations did not interfere with fractional passage rates. The prolonged incubation of 336 h was conducted on

[1] Chapter 3 in this thesis

different rumen-cannulated animals fed a similar diet to the experimental diet after Tas et al. (2006). Effective rumen degradability (ED) was calculated as described by van Duinkerken et al. (2011).

Chemical Analyses

Samples were freeze-dried and ground over a hammer mill to pass a 1-mm screen (Peppink 100 AN, Olst, The Netherlands). Dry matter, ash, crude protein, starch, NDF and ADF were analysed as described by Abrahamse et al. (2008a,b). Concentrations of VFA were determined by gas chromatography with isocaproic acid as an internal standard using an EM-1000 column (30 m × 0.53 mm; Alltech, Deerfield, IL) and hydrogen as mobile phase. Concentrations of NH_3 were determined calorimetrically using a spectrophotometer (Cary 50, Varian, Palo Alto, CA) based on the Berthelot reaction as described by Searle (1984) after deproteinising the supernatant by addition of 10% trichloroacetic acid. Milk composition was analysed by mid-infrared reflection spectroscopy (Milk Control Station VVB, Nunspeet, The Netherlands).

Faecal and omasal Co and Cr concentrations were determined using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) after oxidation with wet-destruction. Faecal and omasal excretion patterns of ^{13}C were determined in the DM fraction (^{13}C-DM), ADF fraction (^{13}C-ADF; faeces only) and starch fraction (^{13}C-ST; omasal digesta only). The ADF fraction was obtained by washing the dried and ground material with acid detergent in filter bags (Type F57, porosity 25 µm, Ankom Technology, Macedon, NY) but omitting the final combustion. The DM and ADF fractions were pulverised in a bullet mill (MM2000, Retsch, Haan, Germany) for 3 min at 85 Hz. The starch fraction was chemically isolated and ^{13}C enrichment determined in the purified fraction by elemental analyses as suggested by Wanek et al. (2001). Briefly, after extraction of soluble sugars with ethanol (40%, v/v), solubilisation by autoclaving and enzymatic hydrolyses with amyloglucosidase, the glucose extract was acidified by adding 3 N HCl to pH 2 and deionised by ion exchange (Amberlite MB-6113, Merck, Darmstadt, Germany) to separate amino acids, minerals and acetic acid from glucose. Glucose was collected, washed three times with Millipore water to dispose of remaining acids, brought into tin cups and evaporated. Amino acid analyses on random samples confirmed that collected residues did not contain amino acids. All fractions were analysed for ^{13}C enrichment by elemental analyses using an isotope ratio mass spectrometer (Finnigan MAT CN, Fisons Instruments, Milan, Italy). The relative ^{13}C enrichment is expressed as the ^{13}C:^{12}C ratio in the samples relative to the ^{13}C:^{12}C ratio of the international

Vienna Pee Dee Belemnite standard. After correction for natural ^{13}C abundance, faecal and omasal excretion patterns of At%^{13}C excess were established.

Curve Fitting and Statistical Analyses

Fractional passage rates for the compartment with the longest retention time (K_1; assumed to be the reticulorumen) and for the compartment with the second longest retention time (K_2; assumed to be the proximal colon-caecum for faecal samples) were derived from excretion patterns of At%^{13}C and Cr, fitted iteratively with nonlinear compartmental models (Table 2). Because of the inherently different profiles of faecal and omasal excretion curves, the following models were selected: one-compartmental models without (G1 model) and with age dependency (G2 model); two-compartmental models without (G1G1 model) and with age dependency with increasing order of gamma distribution (G*n*G1 models; n = 2 to 5; Matis, 1972; Pond et al., 1988); and a multicompartmental model (MC model; Dhanoa et al., 1985). Predicted marker concentrations were compared to the observed values using the root mean square prediction error, which was decomposed into errors due to overall bias of prediction, errors due to deviation of the regression slope from unity, and errors due to random variation (Bibby and Toutenberg, 1977), and scaled to the observed mean (mean prediction error, MPE).

Table 2. Nonlinear compartmental models for fractional passage rate estimation

Model type[1]	Equation[2]
G1	$C_t = A \times e^{-K_1 \times T}$
G2	$C_t = A \times K_1 \times T \times e^{-K_1 \times T}$
G1G1	$C_t = A \times K_1 \times [e^{-K_1 \times T} - e^{-K_2 \times T}]/(K_2 - K_1)$
G*n*G1	$C_t = A \times \{\delta^n \times e^{-K_1 \times T} - [e^{-\lambda_2 \times T} \times \{\sum_{i=1}^{n} \delta^i \times (\lambda_2 \times T)^{n-1}/(n-i)!\}]\}$
MC	$C_t = A \times e^{-K_1 \times t} \times \exp[-(N-2) \times e^{-(K_2 - K_1) \times t}]$

[1]Model type: G1 = one-compartmental age-independent model; G2 = one-compartmental age-dependent model; G1G1 = two-compartmental age-independent model; G*n*G1 = two-compartmental models with increasing order of age dependency (n = 2 to 5); MC = multicompartmental age-independent model.

[2]Model parameters: C_t = marker concentration at time = t (h); A = scalable parameter; K_1 = fractional rate constant for the compartment with the longest retention time (/h); K_2, λ_2 = fractional rate constant for the compartment with the second longest retention time (/h); N = model-derived number of mixing compartments; T = (t – *TD*), where *TD* = time delay (h); $\delta = \lambda_2/(\lambda_2 - K_1)$.

Before curve fitting, marker concentrations were scaled to the marker peak concentration following the procedure of Sponheimer et al. (2003). Excretion curves were superimposed

and values were considered outliers if they deviate by more than twice the SD from the mean per sampling time point (n = 36). Curve fitting was performed using nonlinear least squares regression procedures of SAS (version 9.2, Cary, NC) based on the least square Levenberg-Marquardt algorithm. Initial values for the iterative procedure were obtained through a grid search and curve fits were solved after, on average, 18 to 24 iterations for faecal marker concentrations and 13 to 30 iterations for omasal marker concentrations. Based on model fit parameters estimated with the MC model, transit time (TT; i.e. moment of first appearance of the marker in the faeces) and moment of peak concentration (PCT) were calculated for faecal marker excretion patterns as described by Dhanoa et al. (1985). Total mean retention time (TMRT) in the reticulorumen (i.e. for omasal digesta) was calculated as the sum of the estimated retention times; TMRT in the entire gastrointestinal tract (i.e. for faeces) was calculated as above including retention times associated to the remaining compartments (assumed to be TT). Total marker clearance time was calculated as three times the TMRT as suggested by France et al. (1993). *In situ* degradation residues were fitted as described above using a first order model with lag time as described by Robinson et al. (1986).

Passage kinetic parameters were log transformed due to asymmetrical distribution patterns of residuals and tested by analyses of variance in a Latin square split-plot design, with a 2 × 3 factorial arrangement of treatments within main plots and type of marker assigned to subplots, with mixed model procedures of SAS (version 9.2, Cary, NC) according to the model:

$$Y_{ijklm} = \mu + A_i + P_j + C_k + M_l + (C \times M)_{kl} + (A \times P \times C \times M)_{ijkl} + T_m + (C \times M \times T)_{klm} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent variable; μ is the overall mean; A_i (animal; i = 6), P_j (period; j = 6), C_k (cultivar; k = 2), M_l (maturity; l = 3) and its interaction term $(C \times M)_{kl}$ represent effects assigned to the main plots in a Latin square; T_m (type of marker; m = 3 per sampling site) and $(C \times M \times T)_{klm}$ represent effects related to the subplots. Main plot variables were tested against the interaction term $(A \times P \times C \times M)_{ijkl}$ and subplot variables were tested against the pooled residual error (ε_{ijklm}). Covariance parameters were estimated using the residual maximum likelihood (REML) method and denominator degrees of freedom were estimated using the Satterthwaite approximation. Differences between marker types were assessed using orthogonal contrasts. Tables report back-transformed values (geometric means). Standard errors of the mean (SEM) were calculated by multiplying the log-transformed SEM by its geometric mean. Rumen pH, VFA and NH_3 concentrations were considered repeated measurements and were analysed after Yandell (1997) as a Latin square split-plot design as

described above, with T_m (time after marker administration; $m = 7$) as the subplots effects. All other data were tested by analysis of variance as a Latin square with a 2 × 3 factorial arrangement of treatments ($Y_{ijkl} = \mu + A_i + P_j + C_k + M_l + (C \times M)_{kl} + \varepsilon_{ijkl}$).

Results

Diet and Animal Characteristics

Maize Silage Quality and Rumen Fermentation End-Products. The chemical composition of the six maize silages tested is presented as means over six experimental periods in Table 1. Within treatments, the starch and fibre content differed on average by 11 g/kg DM (SD) throughout the six experimental periods. On average, the Aastar cultivar had a higher crude protein and starch content but a lower NDF and ADF content than Baleric. This pattern is reflected as well in the fermentation end-products present in the rumen liquid with a significantly lower rumen pH ($P = 0.023$) and tendency for lower acetic acid ($P = 0.062$) and higher propionic acid molar proportions ($P = 0.079$) for cultivar Aastar compared with Baleric (Table 3). Starch content increased markedly with prolonged maturation, which was more pronounced for Aastar. The contents of crude protein, NDF and ADF in the maize silage decreased with maturity; the decrease in fibres being more pronounced for Aastar. Despite these changes in the nutrient composition towards increased starch with maturing maize cultivars, no effects on rumen fermentation end-products were observed, except for a decrease in butyric acid with increasing maturity ($P = 0.036$).

Animals. Animal performance was not different between maize cultivars. However, DMI and milk fat proportion were affected by the maturity of the maize plants (Table 4). The DMI increased by 0.6 and 0.8 kg DM/d from early to late harvest for Aastar and Baleric, respectively ($P = 0.006$). Although not significant, the highest DMI intake was observed for mid maturity maize. Both DMI and milk yield decreased from the third experimental period onwards with advancing lactation stage ($P \leq 0.034$ for factor period). Milk fat proportion decreased with advancing maturity of the maize silage (−1.7 g/kg milk from early to late maturity; $P = 0.007$), whereas milk protein was not affected. Milk yield tended to be higher for dairy cows fed the most mature maize silage by 1.3 kg milk/d ($P = 0.087$). As a result, milk fat and protein yields did not change with maize silage maturity. Total tract feed digestibility after a continuous Co-EDTA infusion was higher highest for cultivar Baleric for

all nutrients ($P < 0.001$) except starch (Table 4). Digestibility coefficients were not affected by maturity stage.

Table 3. Rumen liquid characteristics of dairy cows fed different maize silage qualities[1]

Cultivar	Maturity	pH	HAc	HPr	HBu	HVa	HBc	tVFA	NGR	NH_3
Aastar	early	6.13	65.4	18.7	11.8	1.48	2.54	124.0	4.29	3.87
	mid	6.10	64.2	20.7	11.1	1.46	2.57	122.4	3.88	3.51
	late	6.12	64.9	20.1	11.0	1.50	2.53	123.6	3.96	3.76
Baleric	early	6.17	65.0	19.1	12.0	1.50	2.47	120.1	4.24	3.98
	mid	6.19	66.0	18.9	11.0	1.42	2.62	116.7	4.28	4.02
	late	6.23	66.2	18.7	11.1	1.44	2.62	116.9	4.31	4.23
SEM[2]		0.040	0.56	0.64	0.36	0.037	0.109	0.15	0.147	0.292
P-values[3]										
Animal		<0.001	0.030	0.003	0.047	0.027	0.014	0.004	0.004	0.000
Period		0.002	0.333	0.540	0.646	0.903	0.308	0.012	0.417	<0.001
Cultivar (C)		0.023	0.062	0.079	0.833	0.398	0.761	0.033	0.068	0.123
Maturity (M)		0.760	0.713	0.374	0.036	0.383	0.693	0.684	0.437	0.707
C × M		0.719	0.124	0.201	0.981	0.551	0.751	0.896	0.275	0.753
Time (T)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
C × M × T		0.981	0.599	0.523	0.981	0.967	0.643	0.927	0.671	0.990

[1]Rumen liquid parameters: HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HVa = valeric acid; HBc = branched-chain volatile fatty acids (*iso*-butyric + *iso*-valeric acid); tVFA = total volatile fatty acids (in mmol/L; HAc + HPr + HBu +HVa + HBc); NGR = non-glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric) / (HPr + HVa + *iso*-valeric)]; NH_3 = ammonia (mmol/L). All values expressed in mol/100 mol unless specified otherwise.

[2]SEM = standard error of the mean.

[3]Analyses of variance based on log-transformed means with subplot T (= time; 0, 3, 7, 11, 15, 19, 23 h after marker administration) within factorial main plots in a Latin square.

Passage Kinetics

Model Assessment and Accuracy of Curve Fits. Faecal marker peak concentration (PCT) occurred on average at 17.1 h, 29.5 h and 36.3 h after marker dosage for Cr-NDF, ^{13}C-DM and ^{13}C-ADF, respectively (Table 5). In general, faecal samples were almost fully depleted of Cr-NDF by the time sampling was terminated 116 h after pulse dose administration as shown by the estimated total marker clearance time (109 h) from the gastrointestinal tract. Concentration of ^{13}C at 116 h was still above natural abundance level, as also indicated by the total marker clearance time of 192 h for ^{13}C-DM and 233 h for ^{13}C-ADF. Omasal marker excretion patterns differed from faecal excretion curves by a markedly shorter PCT occurring at 4.4 h, 10.0 h and 9.5 h after marker dosage for Cr-NDF, ^{13}C-DM and ^{13}C-ST, respectively (data not shown).

Table 4. Animal performance data and total tract feed digestibility coefficients for six dairy cows fed rations containing two maize silage cultivars harvested at three maturity stages[1]

Cultivar	Maturity	DMI (kg/d)	Total tract feed digestibility coefficients					Milk						
			OM	CP	Starch	NDF	ADF	Yield (kg/d)	Fat (g/kg)	Protein (g/kg)	Lactose (g/kg)	Urea (mg/dL)	Fat (g/d)	Protein (g/d)
Aastar	early	21.4	0.666	0.554	0.949	0.568	0.463	34.4	43.5	34.3	45.6	25.4	1472	1171
	mid	22.1	0.667	0.550	0.961	0.558	0.460	35.6	42.7	34.9	45.7	25.6	1499	1228
	late	22.0	0.675	0.568	0.931	0.574	0.467	36.1	42.0	34.0	46.1	25.8	1522	1229
Baleric	early	20.9	0.729	0.640	0.955	0.664	0.573	34.5	43.8	34.5	45.7	26.7	1506	1187
	mid	22.0	0.747	0.658	0.959	0.673	0.593	35.3	42.1	33.8	45.8	26.0	1474	1180
	late	21.8	0.769	0.696	0.950	0.707	0.622	35.4	41.9	34.0	46.4	25.2	1470	1194
SEM[2]		0.26	0.0107	0.0152	0.0103	0.0150	0.0155	0.56	0.46	0.28	0.23	0.75	28	19
P-values														
Animal		<0.001	0.199	0.022	0.675	0.022	0.025	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Period		0.006	0.413	0.251	0.246	0.251	0.290	0.034	0.009	<0.001	0.333	<0.001	0.005	0.185
Cultivar (C)		0.193	<0.001	<0.001	0.364	<0.001	<0.001	0.482	0.724	0.243	0.446	0.534	0.532	0.163
Maturity (M)		0.006	0.088	0.081	0.189	0.081	0.232	0.087	0.007	0.347	0.057	0.773	0.943	0.235
C × M		0.865	0.371	0.179	0.602	0.179	0.374	0.817	0.591	0.083	0.906	0.489	0.312	0.242

[1]DMI = dry matter intake; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre.
[2]SEM = standard error of the mean.

Due to the different marker excretion patterns between faeces and omasal digesta, several compartmental models were evaluated and their accuracy estimated by calculating the mean prediction error (MPE) and the coefficient of determination (R^2) (Figure 1). Accuracy of fit was highest when the observed marker concentrations were fitted with a multicompartmental model (MC). One-compartmental models without (G1; MPE of 61.7–89.5%) and with age dependency (G2; MPE of 38.3–54.0%) did not adequately fit the observed omasal marker concentrations and did not converge when faecal marker concentrations were used. Two-compartmental models with increasing order of age dependency (G*n*G1 models) performed somewhat better for omasal marker concentrations. The G2G1 model appeared to fit best among G*n*G1 models. A further increase in age dependency to $n = 5$ generally reduced model accuracy. The G1G1 model frequently did not converge when faecal marker concentrations were used. The MC model had generally the lowest MPE values for omasal and faecal marker concentrations. Furthermore, the MC model converged for all 36 curves for Cr-NDF and ^{13}C-DM, respectively, showing a high model robustness to faecal marker excretion patterns. Therefore, passage kinetics will be further discussed for fractional passage rates estimated by the MC model.

Among markers, MPE for faecal marker excretion profiles was lowest for Cr-NDF (7.1%; $P < 0.001$) compared to the ^{13}C-DM (32.5%) and ^{13}C-ADF (29.4%). For omasal marker excretion profiles, MPE was similar among markers with values ranging from 24.9% for Cr-NDF, 26.0% for ^{13}C-DM and 34.6% for ^{13}C-ST ($P = 0.266$). The largest proportion of the MPE for faecal and omasal marker concentrations was due to random variation (96.9–97.2%) and only a minor part was explained by an error in the overall mean (1.2–1.3%) or regression bias (1.6–1.8%) (data not shown).

Fractional Passage Rates through the Gastrointestinal Tract. Marker choice affected faecally determined fractional passage kinetics ($P < 0.001$; Table 5). Fractional passage rates from the reticulorumen (K_1) were on average 1.9 times higher for Cr-NDF than for the ^{13}C markers ($P < 0.001$) but were not different among the ^{13}C markers. Fractional passage rates from the proximal colon-caecum (K_2) were highest for Cr-NDF ($P < 0.001$) and, among ^{13}C markers, lowest for ^{13}C-ADF ($P = 0.008$). Model parameters A and N, as estimated by the MC model, averaged 2.5 (SEM 0.70; $P = 0.042$) and 40 (SEM 36.5; $P < 0.001$) across markers, respectively (data not shown). Marker PCT and transit time (TT) were lowest for Cr-NDF ($P < 0.001$). Total mean retention time (TMRT) in the gastrointestinal tract was higher for the ^{13}C markers compared to Cr-NDF ($P < 0.001$), and, among individual markers, TMRT was

highest for ^{13}C-ADF being 41.2 h longer than Cr-NDF and 13.5 h longer than ^{13}C-DM (P = 0.001). Maize silage quality did not affect passage kinetic parameters.

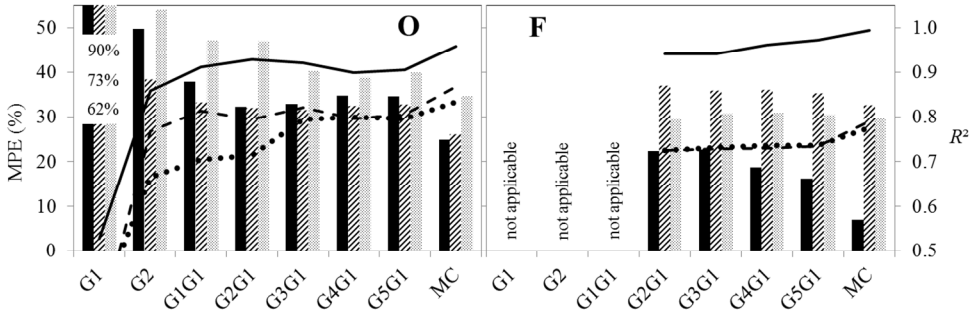

Figure 1. Model accuracy for different markers in omasal digesta (O) and faeces (F) based on one-pool age-independent (G1) and age-dependent models (G2), two-pool age-independent (G1G1) and age-dependent models with increasing order of gamma distribution (G*n*G1, n = 1–5) and an age-independent multicompartmental model (MC). Bars: mean prediction error (MPE); lines: coefficient of determination (R^2). Markers: chromium mordanted fibre (Cr-NDF; solid bars and lines), ^{13}C in dry matter (^{13}C-DM; dashed bars and lines), ^{13}C in omasal starch (^{13}C-ST) or faecal acid detergent fibre (^{13}C-ADF) (dotted bars and lines). G1, G2 and G1G1 models not shown for faeces due to poor model convergence.

Rumen Fractional Passage Rates. Rumen fractional passage rates for starch from maize silages were determined as ^{13}C-ST from marker excretion in the omasal digesta (Table 6). No effects of maize silage cultivar or maturity were found. However, K_1 estimates were affected by choice of marker (P < 0.001) with omasal Cr-NDF having the highest rates. Omasal ^{13}C-ST had higher K_1 estimates than ^{13}C-DM (P = 0.017) but lower rates than omasal Cr-NDF (P = 0.001). Estimates of K_2 (data not shown) were 2.91/h, 1.07/h and 1.33/h for Cr-NDF, ^{13}C-DM and ^{13}C-ST, respectively (SEM 1.480/h; P = 0.006). TMRT was highly affected by marker choice (P < 0.001) and TMRT of ^{13}C-ST in the reticulorumen was on average 7.9 h shorter than ^{13}C-DM and on average 6.6 h longer than Cr-NDF. Model parameters A and N were 1.5 (SEM 0.29; P = 0.031) and 6 (SEM 3.2; P = 0.283) across markers, respectively (data not shown). Mean pH of omasal digesta was 6.52 (SEM 0.035).

Table 5. Passage kinetics and residence times of different markers in the gastrointestinal tract of dairy cows fed rations containing two maize silage cultivars harvested at three maturity stages determined by faecal sampling[1]

Cultivar	Maturity	K_1	K_2	PCT	TT	TMRT
		Chromium mordanted fibre (Cr-NDF)				
Aastar	Early	0.042	0.383	16.8	9.1	35.8
	Mid	0.039	0.507	15.8	9.4	37.5
	Late	0.037	0.465	16.2	9.4	39.0
Baleric	Early	0.047	0.391	17.8	10.5	34.7
	Mid	0.043	0.422	17.1	10.1	35.8
	Late	0.044	0.382	17.5	9.9	35.5
		^{13}C isotopes in dry matter (^{13}C-DM)				
Aastar	Early	0.022	0.196	29.1	14.6	66.2
	Mid	0.022	0.153	29.3	12.1	66.6
	Late	0.025	0.168	28.5	13.3	60.8
Baleric	Early	0.023	0.175	31.4	16.0	67.2
	Mid	0.025	0.162	30.4	14.6	61.6
	Late	0.022	0.219	24.8	11.1	62.0
		^{13}C isotopes in acid detergent fibre (^{13}C-ADF)				
Aastar	Early	0.020	0.140	29.1	11.5	70.8
	Mid	0.016	0.202	26.0	10.6	79.5
	Late	0.022	0.088	41.9	17.1	75.8
Baleric	Early	0.024	0.143	37.1	19.8	68.9
	Mid	0.023	0.122	39.0	18.9	77.4
	Late	0.020	0.069	52.5	25.0	93.3
SEM[2]		0.0061	0.0831	4.26	2.45	8.36
P-values[3]						
Animal		0.112	0.941	0.435	0.276	0.060
Period		0.757	0.911	0.983	0.727	0.941
Cultivar (C)		0.162	0.383	0.029	0.003	0.904
Maturity (M)		0.730	0.325	0.280	0.396	0.620
C × M		0.433	0.744	0.669	0.434	0.636
Marker type (T)		<0.001	<0.001	<0.001	<0.001	<0.001
C × M × T		0.887	0.397	0.058	0.025	0.673
Cr-NDF *vs.* (^{13}C-DM, ^{13}C-ADF)		<0.001	<0.001	<0.001	<0.001	<0.001
^{13}C-DM *vs.* ^{13}C-ADF		0.180	0.008	<0.001	0.010	0.001

[1]Fractional passage kinetic parameters: K_1 = fractional passage rate constant (/h) for the reticulorumen; K_2 = fractional passage rate constant (/h) for the proximal colon-caecum; PCT = marker peak concentration time (h); TT = transit time (h); TMRT = total mean retention time ($1/K_1 + 1/K_2$ + TT; h).

[2]SEM = standard error of the mean.

[3]Analyses of variance with orthogonal contrasts based on log-transformed means in a split-plot arrangement with factorial main plots in a Latin square.

Table 6. Rumen passage of chromium mordanted fibre (Cr-NDF) and ^{13}C isotopes in the dry matter (^{13}C-DM) and starch (^{13}C-ST) for dairy cows fed rations containing two maize silage cultivars harvested at three maturity stages determined by omasal sampling[1]

Cultivar	Maturity	K_1			TMRT		
		Cr-NDF	^{13}C-DM	^{13}C-ST	Cr-NDF	^{13}C-DM	^{13}C-ST
Aastar	Early	0.055	0.028	0.039	18.6	37.0	27.2
	Mid	0.057	0.053	0.044	18.3	24.4	26.1
	Late	0.044	0.027	0.042	22.8	39.0	24.4
Baleric	Early	0.053	0.031	0.042	19.3	35.6	26.6
	Mid	0.045	0.024	0.048	22.9	43.5	21.7
	Late	0.053	0.039	0.039	20.0	28.7	30.0
SEM[2]			0.0096			7.11	
P-values[3]							
Cow			0.509			0.819	
Period			0.842			0.523	
Cultivar (C)			0.709			0.506	
Maturity (M)			0.626			0.990	
C × M			0.069			0.283	
C × M × T			0.569			0.770	
Marker type (T)			<0.001			<0.001	
Cr-NDF *vs.* (^{13}C-DM, ^{13}C-ST)			0.001			<0.001	
^{13}C-DM *vs.* ^{13}C-ST			0.017			0.021	

[1]Fractional passage kinetic parameters: K_1 =: fractional passage rate constant (/h) for the reticulorumen; TMRT = total mean retention time ($1/K_1 + 1/K_2$ + transit time; h), where transit time = 0.
[2]SEM = standard error of the mean.
[3]Analyses of variance with orthogonal contrasts based on log-transformed means in a split-plot arrangement with factorial main plots in a Latin square.

Rumen Degradability

In situ rumen degradation characteristics of maize silages are presented in Table 7. The potentially degradable fraction (*D*) increased, whereas the undegradable (*U*) and washable (*W*) fractions decreased with advancing maturity for all nutrients ($P < 0.001$) except for *U* of NDF, which showed the opposite effect ($P = 0.007$). Fractional degradation rate of the *D* fraction (K_D) decreased with maturity for the starch fraction ($P < 0.001$) but did not change for all other nutrients. Maize silage cultivar did not affect K_D. No direct relationship was observed between fractional degradation and marker passage rates (data not shown). The highest observed R^2 values were 0.14 for K_D of starch and NDF with K_1 of faecal Cr-NDF.

Based on the experimentally determined K_D values, effective rumen degradability (Figure 2) was calculated either from *in vivo* measurements (i.e. experimentally determined K_1

estimates based on Cr-NDF and ^{13}C isotopes) or from K_1 estimates as assumed by the Dutch protein evaluation system for ruminants (DVE/OEB system; van Duinkerken et al., 2011). The DVE/OEB K_1 values are fixed to 0.045/h for crude protein and starch, whereas K_1 for NDF is estimated from its K_D. Effective nutrient degradability calculated from DVE/OEB K_1 values was similar to the effective degradability measured *in vivo* with faecal Cr-NDF. Effective rumen crude protein degradability was lower with the DVE/OEB K_1 values (49.4%) than with faecal ^{13}C-DM (55.8%). Similarly, effective rumen NDF degradability was lower using the DVE/OEB equation (20.6%) than when measured *in vivo* with faecal ^{13}C-ADF (28.4%). In contrast, effective starch degradability calculated *in vivo* with omasal ^{13}C-ST (58.9%) was in close agreement with effective degradability based on the DVE/OEB K_1 values (57.7%).

Table 7. Rumen degradation kinetics of different maize silage qualities

Degradation parameter[1]	Aastar			Baleric			SEM[2]
	early	mid	late	early	mid	late	
			Organic matter				
K_D	0.024	0.023	0.021	0.022	0.026	0.025	0.0015
D	62.0	66.1	66.8	56.1	58.9	66.7	0.61
W	17.1	17.7	15.4	19.0	19.0	12.9	0.57
U	21.0	16.2	17.8	24.9	22.1	20.4	0.26
lag	1.8	0.7	1.0	0.9	4.3	5.3	1.03
			Crude protein				
K_D	0.028	0.021	0.022	0.025	0.024	0.022	0.0025
D	41.3	42.9	46.1	31.7	37.9	42.2	0.79
W	36.2	40.6	33.4	42.2	38.5	36.4	0.72
U	22.6	16.5	20.5	26.1	23.6	21.5	0.19
lag	3.0	1.0	6.8	5.3	10.8	11.2	2.79
			Starch				
K_D	0.042	0.045	0.033	0.052	0.042	0.034	0.0019
D	68.5	71.1	75.4	51.8	70.3	85.7	1.87
W	31.6	29.0	24.6	48.2	29.7	14.4	1.87
lag	0.3	0.6	0.8	1.1	3.2	3.1	0.82
			Neutral detergent fibre (NDF)				
K_D	0.018	0.016	0.017	0.021	0.020	0.020	0.0035
D	66.6	68.7	60.9	59.5	58.0	55.5	1.69
U	33.4	31.3	39.1	40.5	42.0	44.5	1.69
lag	6.3	11.6	20.6	12.9	10.1	15.7	7.24

[1]Fractional degradation kinetic parameters: K_D = fractional degradation rate constant (/h) of *D* fraction; *D* = potentially degradable and insoluble fraction (%; $D = 100 - W - U$); *U* = undegradable fraction at $t = \infty$ (%; assumed zero for starch); *W* = washable fraction (%; assumed zero for NDF); *lag* = lag time (h).
[2]SEM = standard error of the mean.

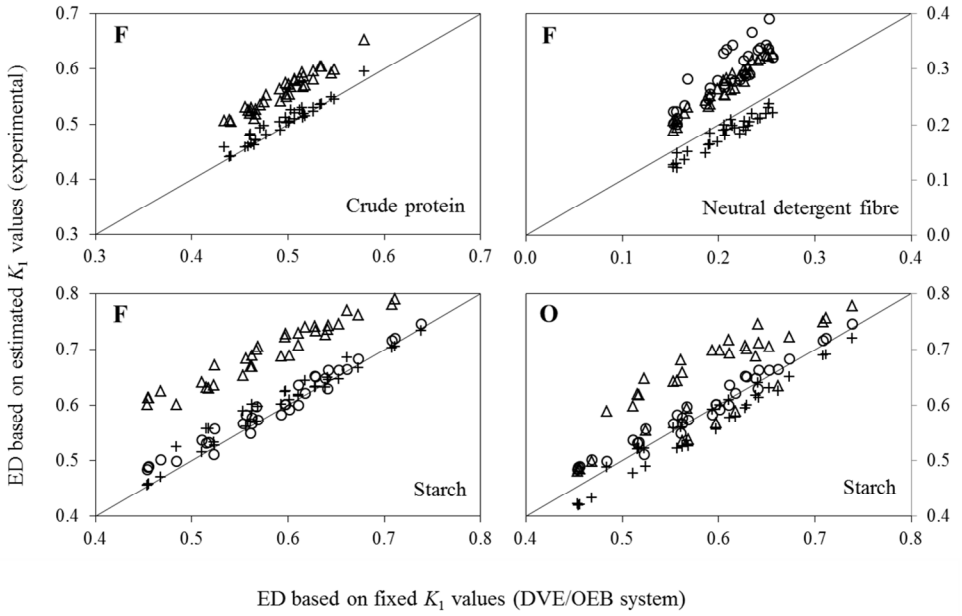

Figure 2. Relations between different calculations of effective rumen degradability (ED) of different maize silage qualities. The X-axis shows a reference ED based on fixed fractional rumen passage rates (K_1) as assumed by the Dutch protein evaluation system (DVE/OEB system; van Duinkerken et al., 2011); the Y-axis shows ED based on K_1 estimates experimentally determined from faecal (F) or omasal (O) excretion of chromium mordanted fibre (Cr-NDF; +),^{13}C in the dry matter (^{13}C-DM; △), and ^{13}C in the respective feed component (○) either as omasal starch (^{13}C-ST) or faecal acid detergent fibre (^{13}C-ADF).

Discussion

Animal Performance

Fractional passage rates were reported to depend on the level of feed intake (Huhtanen and Kukkonen, 1995). In this study, daily mean DMI intake (21.7 ± 0.26 kg DM) increased, and milk fat (42.7 ± 0.46 g/kg milk) decreased, with advancing maturity (Table 4). The observed deviations in DMI and milk fat content can be explained by the higher inclusion of starch in the diet due to the maturing maize plants. Our findings are in line with observations of Phipps et al. (2000) and Khan et al. (2012). The increase in milk yield with advancing maturity agrees with observations reported by Sutton et al. (2000). An inconsistent response of molar proportions of VFA and ratio of non-glucogenic to glucogenic VFA was observed, despite the

marked increase in starch with maturing maize silage, which is, however, coherent to findings of Sutton et al. (2000). Maize maturity had no clear effect on feed digestibility, which is however in line with data on maize silage at comparable maturity stages (Sutton et al., 2000). Total tract apparent digestibility was slightly higher for starch and crude protein in their study compared to our observations, but no maturity effect was observed, except for a significant although small decrease for starch.

Passage Dynamics of Maize Silages

Excretion patterns of an external (Cr-NDF) and an internal marker (^{13}C isotopes) were established from faecal and omasal digesta grab samples upon a pulse dose into the rumen. A previous marker study showed the suitability of ^{13}C as an internal marker to assess component specific passage kinetics of grass silage (Pellikaan et al., 2013). In our study, externally applied Cr-NDF from wheat straw (<0.5 mm) was used as a reference passage rate marker for maize silage as it has been widely used in passage studies. The particle size of Cr-NDF was chosen to be similar to the mean particle size in the rumen observed for maize silages of different maturity (approximately 0.5 mm; Fernandez and Michalet-Doreau, 2002). However, differences in marker type preparation and material, especially with regard to particle size, particle density, particle entrapment in the rumen and possible migration of particulate marker to particles not originally labelled (Owens and Hanson, 1992), need to be considered when comparing markers within the present study and between studies.

Rumen Passage Kinetics of Maize Silage. Passage kinetics were assessed from marker concentrations in faeces and omasal digesta based on the multicompartmental model. The model assigns a slow fractional passage rate to the compartment with the highest retention time (K_1) and a fast fractional passage rate to the compartment with the lowest retention time (K_2). With regard to faecal marker excretion, K_1 and K_2 are generally associated to the reticulorumen and proximal colon-caecum, respectively (Dhanoa et al., 1985). With regard to omasal marker excretion, K_2 most probably represents rumen-related processes, such as mixing time and gas entrapment of marker particles, rather than a major second particle-retaining compartment per se.

Passage kinetics did not change with maize silage maturity. However, marker type had a large effect on faecal and omasal K_1 estimates, with the highest mean K_1 estimates observed for the external marker Cr-NDF for both sampling sites. Similar observations were reported for grass silages using Cr-NDF, ^{13}C-DM and ^{13}C labelled NDF in faeces (Pellikaan et al., 2013). Our observed mean K_1 values based on faecal Cr-NDF (0.042/h) were slightly lower

than for grass silage (0.048–0.050/h; Pellikaan et al., 2013). With regard to faecal ^{13}C-DM, mean K_1 values for maize silage (0.023/h) reported here were distinctly lower than for grass silage (0.035–0.039/h). This in agreement with Mambrini and Peyraud (1994) who reported lower K_1 values for maize silage (0.041/h) than for fresh grass (0.053/h) using rare earth elements as passage rate markers. However, their reported rate constants should be treated with caution as stage of lactation and concentrate levels differed among their dietary treatments. Mulligan et al. (2002) reported K_1 values of 0.020/h for maize silage based on faecal Cr-NDF excretion but DMI was considerably lower (13.5 kg/d).

Interestingly, the mean K_1 of fibres based on faecal ^{13}C-ADF (0.021/h) was similar to that of ^{13}C-DM (0.023/h; P = 0.180), and K_1 of starch based on omasal ^{13}C-ST was comparable (0.042/h) to that of faecal Cr-NDF although lower than K_1 of omasal Cr-NDF (0.051/h). Pellikaan et al. (2013) observed higher differences between the ^{13}C markers for grass silages with K_1 values for fibres (0.015–0.017/h as ^{13}C labelled NDF) being half the value of ^{13}C-DM. The different amplitude in variation between markers in the two studies might be due to the specific physical structure of the two forages. Maize silage fibre was reported to cause a higher rumen fill than grass silage fibre (Mulligan et al., 2002). Furthermore, physical structure, derived by the ruminating index, was reported to be lower for maize silage than for grass silage (De Boever et al., 1993a). This might induce a shorter retention time of maize silage fibres in the reticulorumen as compared to grass silage fibres, which is shown by the higher K_1 value of maize silage fibre (this study) compared to grass silage fibre (Pellikaan et al., 2013). Data on individual structural polysaccharides intrinsically labelled with ^{13}C might provide a better understanding of the differential passage of fibres between those two forages.

Total Tract Passage Kinetics. Passage behaviour of markers in the two main mixing compartments was comparable, with K_2 showing a similar trend to K_1, which was reflected in their TMRT through the gastrointestinal tract. Based on faecal Cr-NDF, no differences between TMRT of our maize silage (36.4 h) and that of grass silage (35.8–37.4 h; Pellikaan et al., 2013) could be detected, whereas TMRT based on faecal ^{13}C was on average 26 h longer (^{13}C-DM) and 29 h shorter (^{13}C-ADF) than that of grass silage. These results suggest that the external Cr-NDF marker is less sensitive to the nutritional quality of the forages and confirms early reservations on the use of Cr-NDF as not fully representing the rumen particle pool (Bosch and Bruining, 1995).

Evaluation of Sampling Sites. Sampling site had a considerable effect on fractional passage rates of maize silages. Estimates of K_1 were 15% (Cr-NDF) and 21% (^{13}C-DM) higher in omasal digesta than in faeces. The higher amplitude in variation for ^{13}C-DM between

sampling sites is in line with the higher retention time of ^{13}C-DM in the proximal colon-caecum (equivalent to a lower K_2) as compared to Cr-NDF. Therefore, differences between sampling sites might be due to particle retention in the proximal colon-caecum. Similar discrepancies among sampling sites were observed between faecal and duodenal sampling (Wylie et al., 2000; Huhtanen and Hristov, 2001), and between faecal and ruminal sampling (Beauchemin and Buchanan-Smith, 1989). Estimated K_1 values of Cr-NDF were moderately related between faeces and omasal digesta (R^2 = 0.51; P < 0.001) but not the K_1 values of ^{13}C-DM (R^2 = 0.10; P = 0.07). Possible factors that contributed to the low R^2 value between faecal and omasal ^{13}C-DM might be the small pulse dose size of ^{13}C labelled maize silage as compared to Cr-NDF resulting in a differential mixing behaviour of the two markers in the rumen.

Model Comparison. Marker concentrations in faeces and omasal digesta upon a ruminal pulse dose were fitted using several nonlinear compartmental models. As profiles of excretion curves differed between sampling sites, several stochastic and one deterministic compartmental model were chosen to best describe fractional passage rates. The first type of models assumes a time (or age) dependency of ingested particles required for their escape from the reticulorumen (Matis, 1972) in line with observations made on required fermentative particle size reduction (Poppi et al., 1980) and buoyancy characteristics of particles (Sutherland, 1988). Age-dependent turnover of particles was described by an increasing order of discrete gamma distribution lifetimes (Gn; $n \geq 1$) for one-compartmental (Gn) and two-compartmental systems (GnG1). Because of the quickly occurring peak time and fast initial increase in marker concentrations in the omasum upon ruminal pulse dosage, one-compartmental models without (G1) or with age dependency (G2) would suggest an adequate curve fit allowing an estimation of the rumen fractional passage rates from the descending curve phase. However, the mean prediction error (MPE) as an indicator of the goodness of fit of the model suggests a poor accuracy for both the G1 (74.7% across markers) and the G2 model (47.3%) with omasal marker excretion. Similar observations were reported for faecal excretion of Cr mordanted hay particles (Moore et al., 1992), although G1 and G2 models did not converge based on our faecal marker concentrations. Increasing the age dependency generally improved the model accuracy for faecal marker to a certain extent. Similar observations have been reported for ruminally and abomasally dosed rare earth elements in duodenal chyme and faeces (Wylie et al., 2000). On the other hand, the multicompartmental (MC) model is based on a deterministic model approach and fitted the marker concentrations best among chosen models. The overall MPE for faecal and omasal marker excretion with the

MC model was 23.0 and 28.5%, respectively. Similarly, Bernard et al. (1998) concluded that the K_1 estimates of external markers in the duodenum and faeces of sheep were, in general, more accurately determined with the MC model than the G2G1 model when compared to the algebraic sum method which served as their reference method.

Model Accuracy. Model accuracy for parameter estimates from the MC model was in general better for faecal than omasal marker excretion due to the larger variability of marker excretion profiles in the omasal digesta, a circumstance also described for duodenal chyme by Wylie et al. (2000). Model accuracy seemed to be affected by marker choice and was generally better for the external than internal markers. Model accuracy was somewhat less accurate for faecal ^{13}C isotopes (29.4–32.5%) than for ^{13}C labelled grass silage (11.8–17.0%; Pellikaan et al., 2013) and concentrates (9.9–13.9%; D. Warner et al., unpublished data)[2]. The conditions for the different studies varied, particularly with regard to the pulse dose size, the ^{13}C enrichment, and the route of marker administration. The present study used the smallest (30 g DM) but a continuously and high enriched pulse dose (8.68–12.36 At%^{13}C). In contrast, Pellikaan et al. (2013) used a larger (170–252 g DM grass silage) but lower enriched pulse dose (1.65–1.97 At%^{13}C), and D. Warner et al. (unpublished data)[2] used the largest (9 kg of DM maize bran) but low enriched pulse dose (i.e. the difference of natural ^{13}C abundance of C_4 maize bran relative to that of a C_3 concentrate mixture) which was administered orally. Model accuracy for faecal Cr-NDF was comparable among the three studies, which all applied a similar large ruminal dose (90–100 g) of 0.5-mm-ground Cr-NDF. Based on these results it is suggested to increase the pulse dose size of ^{13}C labelled material to obtain a better mixing of the marker with the rumen digesta.

An uneven distribution of isotopes in growing plants not labelled continuously might restrict the use of isotopes as passage rate markers (Owens and Hanson, 1992). Previous studies applied ^{13}C labelled forage enriched under field conditions, either as alfalfa labelled on one single occasion (Svejcar et al., 1993), or as grass silage labelled on six to eight occasions (Pellikaan et al., 2013). Although the latter study did not find any effects of their labelling procedure on rumen passage rates based on faecal grab samples, early time points before PCT in their faecal excretion curves were affected. This might restrict the use of isotopes if they were to be collected more proximate to the reticulorumen (e.g. in the omasal digesta) as omasal marker excretion curves were shown to rather follow an exponential decay curve or having a quickly ascending phase in our study. The procedure used in our study

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based on continuous isotope dosing of growing plants resulting in a homogeneous distribution of isotopes (Gorissen et al., 1996) will, therefore, circumvent any potential effects of differential ^{13}C enrichment in plant tissue on the passage kinetics (Owens and Hanson, 1992) from omasal digesta. Conversely, a closer resemblance of labelled plant material to unlabelled field plants might be more easily achieved under field conditions as the growing and climate conditions are more alike. Nonetheless, Huhtanen and Hristov (2001) proved the suitability of intrinsically labelled alfalfa under greenhouse conditions to assess passage kinetics of alfalfa. In our study, conditions between labelled and unlabelled maize plants were kept similar by inducing mechanical stress on plant cell walls to resemble wind stress of field plants (Biddington, 1986), and by ensiling labelled plants with the unlabelled field plants.

Effective Rumen Degradability

Rumen degradability of the maize silages by *in situ* nylon bag incubation revealed a considerable effect ($P \leq 0.007$) of maize silage maturity on *D*, *U* and *W* fractions but less on K_D. Estimates for K_D decreased for the starch fraction with advancing maturity ($P < 0.001$) but did not change for all other nutrients. An *in vitro* incubation experiment, in which the same maize silages were used (J. Oerlemans, D. Warner, W.F. Pellikaan, unpublished data), confirmed our findings from the *in situ* experiment and revealed no effect of maize silage maturity on *in vitro* OM disappearance after 12 h (0.68–0.71) or 72 h (0.82–0.88) of incubation in rumen fluid, or on gas production as an indicator of *in vitro* fermentation measured at 12 h (207–230 ml/g OM) and 72 h (291–328 ml/g OM). It can be assumed that degradation is linked to passage kinetics as a faster rumen degradation was associated with a higher rumen escape of feed particles (Welch, 1986; Hristov et al., 2003) by altering the buoyancy properties of feed particles (Sutherland, 1988). However, no direct link could be established for *in vivo* maize silage degradation and passage in our study, probably due to the low number of experimental units required for the comparison.

Effective rumen degradability measured *in vivo* with Cr-NDF was comparable to that calculated from the DVE/OEB equations. This is in line with our expectations as feed evaluation systems typically rely on passage studies using external markers. The ^{13}C isotopes gave higher effective rumen degradability values for crude protein and NDF although comparable values for starch relative to the DVE/OEB equations. Results on effective crude protein degradability should be considered with caution as ^{13}C isotopes might not best describe component specific fractional passage of feed proteins and the use of ^{15}N isotopes should be rather considered. Nonetheless, these results suggest that although the use of stable

isotopes should be considered to estimate component specific fractional passage rates, the rumen escape of nutrients might not be affected, depending on the component of interest and type of feed.

Conclusions

In conclusion, intrinsic isotope labelling of maize silages allowed to assess component specific fractional passage rates. Maize silage cultivar and stage of maturity did not affect passage dynamics although the chemical composition and *in situ* rumen degradability were affected to a large extent. Fractional passage rate from the rumen estimated with an external marker (Cr-NDF) was higher than that estimated with ^{13}C feed components used as internal passage rate markers. Among ^{13}C markers, fractional passage rate from the rumen estimated with labelled maize silage DM (^{13}C-DM) did not differ from that with labelled fibres (^{13}C-ADF) but was lower than that of labelled starch (^{13}C-ST).

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Chapter 5

Passage kinetics of stable isotope labelled grass silage fibre and fibre-bound protein through the gastrointestinal tract of dairy cows

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Abstract

Fractional passage rates are required to predict nutrient absorption in ruminants but data on nutrient-specific passage kinetics are largely lacking. With the use of stable isotopes we assessed passage kinetics of fibres and fibre-bound nitrogen (N) of intrinsically labelled grass silage from faecal and omasal excretion patterns of $\delta^{13}C$ and $\delta^{15}N$. In a 6 × 6 Latin square, lactating dairy cows received grass silages (455 g/kg total diet DM) in a 2 × 3 factorial arrangement from low (45 kg N/ha) or high (90 kg N/ha) fertilised ryegrass swards harvested at three maturity stages. Feed intake and milk yield were increased at high N fertilisation and at decreasing maturity. Nutrient digestibility decreased with increasing plant maturity, in particular at the high N fertilisation level, essentially reflecting dietary treatment effects on the nutritional composition of the grass silage. Fractional rumen passage rates (K_1) were highest and total mean retention time in the gastrointestinal tract (TMRT) was lowest when based on the external marker Cr-NDF (0.047/h and 38.0 h, respectively). Faecal ^{13}C-ADF provided the lowest K_1 (0.023/h) and the highest TMRT (61.1 h) and highest peak concentration time (PCT; 24.3 h) among markers. In comparison, faecal ^{15}N-ADF had a considerably higher K_1 (0.034/h) and lower TMRT (46.4 h) than ^{13}C-ADF. Fractional passage rates from the proximal colon-caecum (K_2; 0.37/ h) were highest and PCT (17.4 h) was lowest for the total N, measured with ^{15}N-DM, among markers. Fractional passage rates tended to increase with the high N fertilisation level and generally decreased with advancing plant maturity at low N fertilisation. In contrast, passage kinetics generally increased with advancing plant maturity at high N fertilisation. Omasal digesta sampling largely confirmed results based on faecal sampling. Results suggest that the use of ^{13}C and ^{15}N stable isotopes can describe fibre-specific passage kinetics of forage.

Introduction

Quantitative knowledge on fractional rumen passage rates (K_1) is required to determine ruminal volatile fatty acid and microbial protein yields, and to determine site and extent of degradation (Dijkstra et al., 2007). Therefore, K_1 is an essential parameter in several feed evaluation systems and mechanistic rumen models that predict absorption of nutrients in the small intestine as well as excretion of nutrients with respect to environmental and metabolic load (Kebreab et al., 2009). Fractional rumen passage rates are conventionally determined by marker techniques, involving external or inert internal markers. External markers are not inherent to the feed and have therefore been criticized for not fully representing the passage behaviour of the diet (Smith, 1989; Tamminga et al., 1989b). In particular, external markers do not provide feed chemical component specific passage rates. In principle, internal markers are preferred as they are inherent to the feed ingested by the animal; however, inert markers require time- and labour-intensive rumen evacuations to determine the rumen pool size. By labelling specific feed fractions with stable isotopes, fractional passage rates can be determined from stable isotopes as internal markers determined in faeces (Südekum et al., 1995). In particular, fractional passage rates of fibre-bound nitrogen (N) were determined from ^{15}N labelled lucerne (Huhtanen and Hristov, 2001). A subsequent study has shown that the carbon isotope ratio (^{13}C:^{12}C), i.e. $\delta^{13}C$, can be used to assess fibre passage kinetics of a ^{13}C labelled grass silage and that the $\delta^{13}C$ did not change with microbial rumen fermentation under *in vitro* conditions (Pellikaan et al., 2013). Disappearance of the labelled fibre fractions in the gastrointestinal tract did therefore not affect respective passage kinetics estimated from the $\delta^{13}C$ in the apparent undigested faecal fraction. This has been validated *in vivo* by quantifying the carbon isotopes from feed and faecal output (L.M.M. Ferreira et al., unpublished data) resulting in an equal $\delta^{13}C$ between the isotope pool flowing into the rumen and that in faeces.

In the study of Pellikaan et al. (2013), changing the diet from a low to high digestible grass silage had no effect on the passage kinetics based on two cows. Previous studies based on external or inert markers suggest that grass silage quality affects passage kinetics, although effects were not clear and results from literature range from slightly negative or no effects (Mambrini and Peyraud, 1994; Lamb et al., 2002; Lund et al., 2006; Kuoppala et al., 2009; Bayat et al., 2010; Bayat et al., 2011) to clearly positive effects (Gasa et al., 1991; Bosch et al., 1992a; Rinne et al., 1997a; Rinne et al., 2002) of advancing plant maturity on K_1. Effects of N fertilisation were not specifically investigated with regard to passage kinetics but results from *in*

situ degradation studies indicate clear effects of N fertilisation level on fractional degradation rates and the potentially rumen digestible fraction (van Vuuren et al., 1991; Valk et al., 1996; Peyraud et al., 1997).

Previously, we used intrinsic isotope labelling of maize silages varying in nutritional quality to assess component specific fractional passage rates (Warner et al., in press[3]). The aim of the present study was to assess feed component specific passage kinetics of grass silage from early through late maturity from ryegrass swards fertilised at two different N levels. Ryegrass plants were uniformly labelled with ^{13}C and ^{15}N under greenhouse conditions, and passage kinetics of fibre and fibre-bound N were determined based on the respective δ value, determined in faecal and omasal digesta samples, and compared with the external marker Cr-NDF.

Materials and Methods

Animals and Diet

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands) and carried out under the Dutch Law on Animal Experimentation. Six multiparous Holstein-Friesian dairy cows in their second to fourth lactation, fitted with a permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond Inc., Parma, ID), were individually housed in tie stalls. At the start of the experiment, cows were 63 ± 13 DIM (mean ± SD), averaged 549 ± 64 kg in bodyweight, had an average daily feed intake of 17.2 ± 2.60 kg dry matter (DM), and produced 33.1 ± 5.76 kg milk/d. Animals were fed a total mixed ration consisting of 455 g/kg DM grass silage, 195 g/kg DM maize silage and 350 g/kg DM compound feed (Table 1). The compound feed ingredients mainly originated from cool season C_3 plants to keep the background level of ^{13}C enrichment low and similar to that of the natural enrichment level of the grass silage mixed in the experimental diet. Grass silage was prepared from ryegrass (a 70:30 mixture of late-heading tretraploid and diploid ryegrass, *Lolium perenne*) fertilised at two N fertilisation levels and harvested at three maturity stages (Table 1). Levels of N fertilisation were either 45 kg N/ha (N45), or 90 kg N/ha (N90) per cut, and applied as a NPK complex. Maturity stages were set to obtain a target DM yield in the range of 1800–2000 kg/ha (early), 3200–3400 kg/ha (mid) and 4600–4800 kg/ha (late). The field received 50 kg N/ha prior to the first

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regrowth. Fresh grass from the second (mid to late maturity) and third regrowth (early maturity) was harvested from August to September 2010, wilted and ensiled.

Table 2. Chemical composition of the diet consisting of grass silage at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity, maize silage and compound feed

Item[1]	N45			N90			Maize silage	Compound feed[4]
	early	mid	late	early	mid	late		
Growing days	35	54	70	25	47	68	—	—
DM yield (kg/ha)	1840	3107	4734	2020	3366	4860	—	—
DM content	366	670	723	567	354	520	390	889
Chemical composition[2]								
OM	879	906	914	907	904	903	961	933
CP	197	175	137	249	206	168	77	262
Starch	—	—	—	—	—	—	403	218
NDF	432	543	545	429	438	556	356	293
ADF	294	317	296	257	266	326	201	151
DVE[3]	63	75	72	92	64	60	61	176
OEB[3]	77	29	−3	86	76	36	−45	33
OMD (g/kg)[3]	686	642	651	733	714	645	788	768
NE_L (MJ/kg DM)[3]	6.3	5.7	5.7	6.7	6.6	5.7	7.0	7.9

[1]DM = dry matter; OM = organic matter; CP = crude protein; NDF = ash free amylase treated neutral detergent fibre; ADF = ash free acid detergent fibre; DVE = intestinal digestible protein; OEB = rumen degraded protein balance; OMD = organic matter digestibility; NE_L = net energy lactation.

[2]g/kg DM, unless specified otherwise, of six experimental periods (starch not determined for grass silage).

[3]Units determined by near-infrared spectroscopy (Blgg AgroXpertus, Wageningen, The Netherlands).

[4]Ingredients (g/kg DM): wheat (80.0), sunflower seeds (140.0), soybean hulls (26.5), palm kernel expeller (90.0), soybeans (185.0), sugar beet pulp (75.0), potato starch (200.0), MervoBest rumen-protected soybean meal (185.0), phosphoric acid limestone (7.5), salt (3.0), mineral premix (8.0).

Grass silage treatments were randomly distributed over six animals and six experimental periods according to a Latin square design with 2 × 3 factorial arrangement of treatments. Each experimental period lasted 21 d starting with a 14-d adaptation period to the diet. From day 12 onwards, animals were fed 95% of the individual DM intake measured during the adaptation period to minimize feed refusals during the measuring days. Animals received their daily rations in two equal meals at 0600 and 1700 h. The diet was prepared twice weekly and stored in a cooling unit at 8°C from April onwards. Feed samples were collected each time the diet was prepared. Feed residues were collected daily before the afternoon feeding. Feed samples and residues were pooled per animal over each experimental period. Animals were milked twice daily during feeding times and milk samples were collected from day 15 through

21. Feed intake and milk yield determined from day 15 through 21 of each experimental period were averaged per period for statistical analysis. One animal was removed from the experiment in Period 5 and 6 due to general health issues not related to the dietary treatments nor experimental conditions, reducing the treatment combinations to $n = 34$.

Markers

Chromium-mordanted fibre (Cr-NDF) and stable isotope labelled grass silage components were used as external and internal passage markers, respectively. The Cr-NDF (45.9 g Cr/kg DM) was prepared as described by Udén et al. (1980) from wheat straw, dried and ground to pass a 0.5-mm screen. The isotope enriched internal markers were prepared as ^{13}C and ^{15}N labelled grass silage originating from the field that also provided the bulk grass silage. In brief, representative ryegrass shoots from the second regrowth were randomly collected from the field and grown on hydroponics (8.6 shoots per m²) under climate-controlled greenhouse conditions in hermetically sealed isotope assimilation chambers (IsoLife BV, Wageningen, The Netherlands), specifically designed for homogeneous atmospheric isotope-labelling (Gorissen et al., 1996). From plant emergence onwards, grass was continuously enriched under high levels of $^{13}CO_2$ and by injecting plant pots a $K^{15}NO_3$ solution. The labelled grass plants were exposed to similar conditions to the field plants; e.g., by adjusting the light schedule in the greenhouse to the field conditions, and inducing wind stress to the labelled plants. Grass plants received the identical fertilisation regimen and were harvested at a similar physiological stage (172 g, 322 g and 498 g DM/m² from early through late maturity) as the field plants. Plants were subsequently wilted, cut to size (2 cm), placed into several bags of larger mashed grit gauze (pore size 212 µm; PA-74, Sefar Nytal, Heiden, Switzerland) and distributed over silage bales to be ensiled together with the field plants over an 8-week period. The mean degree of atom% as a proportion of total C or N in the DM of the labelled grass silage ranged between 6.74 and 8.20 atom%^{13}C excess and between 16.93 and 18.11 atom%^{15}N excess for the different treatments ($n = 6$).

On day 15, animals received a ruminal pulse dose of corresponding isotopic labelled grass silage (15 g DM) and Cr-NDF (100 g DM). Pulse dosing started at 0900 h with cow 1 and continued after an interval of 30 min for each consecutive animal. Prior to pulse dosing, the frozen and cut labelled marker material was further cut to pieces of approximately 0.5 cm to resemble ingested bulk grass silage particles. From day 11 through 19, cobalt ethylenediamine tetraacetic acid (Co-EDTA; Udén et al., 1980) was continuously infused (0.5 L/h; 1.14 g Co/d

dissolved in 12 L water) in the rumen after Faichney (1975) to measure feed digestibility (from day 15 through 19), preceded by a primer dose of 1.5 times the daily dose.

Sampling and Measurements

Faeces. Directly after administration of markers into the rumen, 20 spot samples of faeces were collected after defecation. Faecal samples were collected in sampling blocks of three hours each at times t = 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, 54, 60, 72, 78, 84, 96, 102, 108, 120 h upon pulse dose administration. Faeces were weighed, thoroughly homogenized by hand and a representative sample of approximately 400 g fresh matter was stored at −20°C. An aliquot of 1% fresh faeces was taken from each collection time and pooled over one experimental period to determine total tract feed digestibility.

Omasal Digesta. Fifteen spot samples (~750 mL) of omasal digesta were collected as described in Warner et al. (in press)[4] in sampling blocks of three hours each at average times t = 0, 3, 5, 9, 12, 14, 17, 24, 30, 36, 47, 59, 71, 96, 120 h upon pulse dose administration and pH was measured immediately using an electronic pH meter (pH electrode HI1230, Hanna Instruments BV, IJsselstein, The Netherlands).

Rumen Liquid. On day 21, approximately 350 mL rumen liquid was collected proportionally from a cranial, middle and caudal direction. Per treatment and animal, a total of four rumen fluid samples were collected at times t = 2, 6, 8, 10 h after morning feeding. Subsamples of 0.75 mL each were taken for volatile fatty acid (VFA) and ammonia (NH_3) analyses, and rumen pH was measured immediately. The VFA and NH_3 samples were stored at −20°C in 85% phosphoric acid (1:1, v/v) and 10% trichloroacetic acid (1:1, v/v), respectively.

Rumen Degradability. On day 18 through 21, rumen degradation of fresh grass silage was determined *in situ* per individual animal and per silage type treatment *in situ* as described by Warner et al. (in press)[4]. Incubations (0, 2, 4, 8, 24, 48, 72 h) started with the 72-h incubation bags according to the all-out procedure at 80 h upon marker administration to ensure similar conditions of degradation and passage rates. Previous studies showed that faecal marker concentrations were close to baseline concentrations after 4 d (D. Warner et al., unpublished data).[5]

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[5] Chapter 3 in this thesis

Chemical Analyses

Samples were freeze-dried and ground over a hammer mill to pass a 1-mm screen (Peppink 100 AN, Olst, The Netherlands). Dry matter, ash, crude protein (CP), starch, NDF and ADF were analysed as described by Abrahamse et al. (2008a,b). Concentrations of VFA and NH_3 were determined as described by Warner et al. (in press)[6]. Milk composition was analysed by mid-infrared reflection spectroscopy (Milk Control Station VVB, Nunspeet, The Netherlands).

Faecal and omasal Co and Cr concentrations were determined using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) after oxidation with wet-destruction as described by Pellikaan et al. (2013). Isotopic excretion patterns in faeces and omasal digesta were determined for fibre-bound ^{13}C and ^{15}N in the ADF fraction, and for ^{13}C and ^{15}N in the total DM. The ADF fraction was obtained by washing dried and ground test samples with acid detergent in filter bags (Type F57, porosity 25 µm, Ankom Technology, Macedon, NY) but omitting the final combustion. The DM and ADF fractions were pulverized in a bullet mill (MM2000, Retsch, Haan, Germany) for 3 min at 85 Hz and analysed for $\delta^{13}C$ and $\delta^{15}N$ by elemental analyses using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Bremen Germany). The relative isotopic enrichment is expressed as $\delta^{13}C$ and $\delta^{15}N$ (the respective ^{13}C:^{12}C and ^{15}N:^{14}N ratio) in the samples relative to the isotopic ratio of the respective international standards Vienna Pee Dee Belemnite and air. After correction for natural isotopic abundance, faecal and omasal excretion patterns of atom%^{13}C and ^{15}N excess were established, and scaled to marker peak concentration to improve model fit.

Curve Fitting and Statistical Analyses

Curve fitting procedures and statistical analyses are outlined in Warner et al. (in press)[6]. In brief, fractional passage rates were derived from faecal and omasal excretion patterns of ^{13}C, ^{15}N and Cr, fitted iteratively with a nonlinear multicompartmental model (Dhanoa et al., 1985). The multicompartmental model provided the most satisfactory curve fits for maize silage in faeces and omasal digesta (Warner et al., in press),[6] compared to two compartmental models with age dependency with increasing order of gamma distribution (G*n*G1 models; n = 2 to 5). The multicompartmental model assigns a slow fractional passage rate to the compartment with the highest retention time (K_1; *viz*. reticulorumen) and a fast fractional passage rate to the compartment with the lowest retention time (K_2; *viz*. proximal colon-

[6] Chapter 4 in this thesis

caecum). With regard to omasal marker excretion, K_2 most probably represents rumen-related processes, such as mixing time and gas entrapment of marker particles, rather than a particle-retaining compartment per se (Warner et al., in press).[7] Predicted marker concentrations were compared to the observed values using the root mean square prediction error, which was decomposed into errors due to overall bias of prediction, errors due to deviation of the regression slope from unity, and errors due to random variation (Bibby and Toutenberg, 1977), and scaled to the observed mean (mean prediction error, MPE). Log-transformed model parameters were tested by analyses of variance in a Latin square split-plot design, with a 2 × 3 factorial arrangement of treatments (two N fertilisation levels × three maturity stages) within main plots and type of marker assigned to subplots by mixed model procedures of SAS (version 9.2, SAS Institute, Cary, NC), as described in detail by Warner et al. (in press).[7] Differences between marker types were assessed using orthogonal contrasts. Rumen pH, VFA and NH_3 concentrations were considered repeated measurements and were analyzed as a Latin square split-plot design with repeated measurements as the subplots effects. *In situ* degradation residues were fitted as described by Tas et al. (2006) using a first order model with lag time (Robinson et al., 1986). *In situ* degradation parameters and animal performance data were tested by analysis of variance as a Latin square with a 2 × 3 factorial arrangement of treatments.

Results

Characteristics of the Grass Silage

Diet. Six nutritionally contrasting grass silages differing particularly in their CP and NDF content (Table 1), were fed to dairy cows. The CP content was on average 38 g/kg DM units higher for N90 and decreased considerably with increasing maturity stage. The lowest absolute value was observed for N45 of late maturity (137 g/kg DM). The NDF content increased with increasing maturity stage although differences were less clear than for CP. The NDF content of N45 was comparable between the last two maturity stages (mid to late), whereas that of N90 was comparable between the first two maturity stages (early to mid). From early to mid maturity, N90 showed a particular quick regrowth performance producing a respective average daily amount of 28 kg and 14 kg DM/ha more than N45, whereas at late maturity the regrowth performance was comparable between N fertilisation levels.

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Animals. Animals had daily average feed intake of 16.7 ± 0.48 kg DM (mean ± SEM) and produced 26.7 ± 0.92 kg milk/d (Table 2). Advancing maturity stage generally decreased feed intake and milk yield with the absolute lowest values observed for N90 of late maturity. Nitrogen fertilisation increased feed intake and milk yield, except for the late maturity grass silage, and a significant interaction between fertilisation level and maturity occurred. A similar trend was observed for total tract CP digestibility (on average, 0.69 ± 0.013). Total tract digestibility of OM (0.75 ± 0.010), NDF (0.70 ± 0.014) and ADF (0.62 ± 0.016) decreased with advancing maturity stage but a significant interaction between maturity and fertilisation level indicated that this decline was more pronounced at N90 than N45. Milk protein content (30.5 ± 0.26 g/kg) decreased for N90 but not with N45 with advancing maturity stage, whereas effects were not significant for milk fat content (42.5 ± 1.21 g/kg). The urea content (37.6 ± 1.39 mg/dL milk) was higher for N90.

Rumen Fermentation End-Products. The acetic acid content in rumen liquid generally increased with advancing maturity of the grass silage but effects of N fertilisation levels on acetic acid were less clear. A similar but opposite trend was observed for valeric acid and the branched-chain VFA, whereas all other VFA, rumen pH and ammonia content were not affected by basal diet treatments, although significant interaction between fertilisation and maturity was present for butyric acid molar proportion (Table 3).

Rumen Degradation. Rumen *in situ* degradability of the six grass silage treatments (Table 4) showed generally higher fractional degradation rates (K_D) for N90 for the different feed fractions, whereas grass silage maturity had less clear effects on K_D. The potentially degradable fractions (*D*) and washable fractions (*W*) generally decreased, whereas the undegradable fractions (*U*) and the lag time generally increased with advancing maturity for all nutrients.

Passage Kinetics

Faeces. Passage kinetics estimated by marker techniques from faecal samples were affected by the type of marker. Fractional passage rates for the compartment with the highest retention time (K_1; *viz*. reticulorumen) were highest for Cr-NDF (0.047 ± 0.0030/h; Table 5). Among stable isotope markers, K_1 differed with lower values for ^{13}C-ADF (0.023 ± 0.0017/h) compared to ^{13}C-DM (0.034 ± 0.0023/h). No difference between fractions were observed for ^{15}N. Similarly, fractional passage rates for the compartment with the second highest retention time (K_2; *viz*. proximal colon-caecum) differed among markers with higher K_2 values for ^{15}N than ^{13}C (Table 5).

Table 2. Animal performance data for six dairy cows fed rations containing grass silages at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity[1]

Fertilisation	Maturity	BW (kg)	DMI (kg/d)	Total tract digestibility coefficients					Milk						
				DM	OM	CP	NDF	ADF	Yield (kg/d)	Fat (g/kg)	Protein (g/ kg)	Lactose (g/ kg)	Urea (mg/dL)	Fat (g/d)	Protein (g/d)
N45	Early	590	16.7	0.72	0.75	0.68	0.71	0.62	28.1	40.9	30.4	45.6	35.2	1156	852
	Mid	571	16.0	0.72	0.74	0.68	0.71	0.63	24.9	43.2	30.8	45.7	35.9	1075	764
	Late	597	15.6	0.71	0.73	0.65	0.68	0.60	24.5	43.0	30.4	45.3	37.0	1062	741
N90	Early	578	18.6	0.77	0.78	0.73	0.77	0.69	29.8	44.0	32.0	45.8	38.1	1320	955
	Mid	567	18.6	0.74	0.76	0.72	0.71	0.63	29.9	43.5	30.5	45.3	37.7	1323	911
	Late	571	14.5	0.68	0.70	0.65	0.65	0.56	23.3	40.3	28.8	44.7	41.7	946	678
SEM[2]		12.8	0.48	0.011	0.010	0.013	0.014	0.016	0.92	0.12	0.03	0.03	1.39	57	26
P-values															
Animal		<0.001	<0.001	0.548	0.412	0.493	0.263	0.531	<0.001	<0.001	<0.001	0.017	0.004	<0.001	<0.001
Period		0.230	0.075	0.303	0.288	0.172	0.431	0.515	0.001	<0.001	<0.001	0.005	<0.001	<0.001	0.012
Fertilisation (F)		0.159	0.006	0.238	0.407	0.007	0.510	0.345	0.015	0.776	0.648	0.418	0.008	0.035	0.006
Maturity (M)		0.329	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.339	<0.001	0.085	0.080	0.001	<0.001
F × M		0.671	0.001	0.004	0.007	0.112	0.013	0.005	0.005	0.056	<0.001	0.387	0.524	0.007	0.001

[1]BW = body weight; DMI = dry matter intake; DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre.

[2]SEM = standard error of the mean.

Table 3. Rumen liquid characteristics of dairy cows fed two grass silage types (N45: 45 kg N/ha; N90: 90 kg N/ha) harvested at early through late maturity[1]

Fertilisation	Maturity	pH	HAc	HPr	HBu	HVa	HBc	tVFA	NGR	NH_3
N45	Early	6.30	65.0	19.0	11.8	1.49	2.68	106.0	4.29	3.4
	Mid	6.08	67.5	18.2	11.0	1.19	2.17	108.4	4.58	3.9
	Late	6.08	67.3	17.9	11.6	1.14	1.97	106.9	4.65	4.2
N90	Early	6.14	65.4	18.7	11.8	1.36	2.73	115.4	4.38	5.3
	Mid	6.23	64.2	18.5	12.9	1.53	2.92	108.8	4.42	3.6
	Late	6.18	67.3	17.7	11.1	1.30	2.51	103.8	4.60	4.3
SEM[2]		0.044	0.58	0.55	0.358	0.064	0.127	3.07	0.154	0.53
P-values[3]										
Animal		0.010	0.066	0.026	0.566	0.107	0.018	<0.001	0.030	0.591
Period		<0.001	0.016	0.233	0.061	0.233	0.037	0.008	0.224	0.530
Fertilisation (F)		0.350	0.037	0.949	0.117	0.019	<0.001	0.339	0.714	0.191
Maturity (M)		0.119	0.002	0.156	0.203	0.008	0.003	0.184	0.151	0.417
F × M		0.003	0.004	0.792	0.004	0.002	0.020	0.097	0.692	0.070
Time (T)		<0.001	<0.001	<0.001	0.972	<0.001	<0.001	<0.001	<0.001	<0.001
F × M × T		0.793	0.524	0.382	0.966	0.529	0.546	0.536	0.354	0.286

[1]Rumen liquid parameters: HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HVa = valeric acid; HBc = branched-chain volatile fatty acids (*iso*-butyric + *iso*-valeric acid); tVFA = total volatile fatty acids (in mmol/L; HAc + HPr + HBu +HVa + HBc); NGR = non-glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric) / (HPr + HVa + *iso*-valeric)]; NH_3 = ammonia (mmol/L). All values expressed in mol/100 mol unless specified otherwise.
[2]SEM = standard error of the mean.
[3]Analyses of variance based on log-transformed means with subplot T (= time; 2, 6, 8, 10 h after morning feeding) within factorial main plots in a Latin square.

Marker peak concentration time (PCT) was higher for the ADF (21.2 and 24.3 h for ^{15}N and ^{13}C, respectively) than the DM fractions (17.4 and 21.1 h for ^{15}N and ^{13}C, respectively; Table 6). PCT was lowest for ^{15}N-DM among markers (17.4 ± 0.38 h). Marker transit time (TT) was similarly affected by marker choice ($P < 0.001$; data not shown). Total mean retention time (TMRT) was lowest for Cr-NDF (38.0 ± 1.53 h) and considerably longer for ^{13}C-ADF (61.1 ± 2.80 h) compared to ^{13}C-DM (45.6 ± 1.91 h; Table 6). No difference was observed between the DM and ADF fractions for ^{15}N (42.0 and 46.4 h, respectively). Faecal samples were almost fully depleted of Cr-NDF by the time sampling was terminated 120 h after pulse dose administration, as shown by the estimated mean marker clearance time (114 ± 4.6 h) from the gastrointestinal tract. Concentration of ^{15}N and ^{13}C at 120 h was still above natural abundance level, as indicated by the total marker clearance time of 126–137 h for the DM fraction and 139–183 h for the ADF fraction.

Table 4. Rumen degradation kinetics of dairy cows fed rations containing grass silages at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity

Degradation parameter[1]	N45			N90			SEM[2]
	early	mid	late	early	mid	late	
			Organic matter				
K_D	0.035	0.032	0.032	0.048	0.049	0.052	0.0055
D	59.0	69.9	63.1	57.9	56.6	51.3	3.64
W	15.3	19.1	24.5	14.8	17.7	34.3	3.28
U	25.8	11.0	12.4	27.3	25.7	14.4	1.47
lag	0.4	0.0	2.1	2.4	5.4	6.6	1.94
			Crude protein				
K_D	0.060	0.042	0.052	0.072	0.059	0.063	0.0069
D	34.5	54.0	50.6	45.4	33.3	32.4	1.88
W	17.0	19.3	30.4	12.7	14.6	30.5	0.95
U	48.5	26.7	19.0	41.9	52.2	37.0	1.53
lag	0.0	0.0	0.0	2.7	0.0	2.5	1.36
			Neutral detergent fibre (NDF)				
K_D	0.028	0.034	0.024	0.040	0.048	0.050	0.0070
D	87.5	77.1	79.7	81.9	75.5	59.5	5.24
U	12.5	22.9	20.3	18.2	24.5	40.5	5.24
lag	2.6	3.2	4.4	6.1	9.2	9.4	1.97
			Acid detergent fibre (ADF)				
K_D	0.030	0.036	0.022	0.030	0.042	0.041	0.0050
D	80.0	75.5	79.4	85.2	77.8	60.3	5.19
U	20.0	24.5	20.6	14.8	22.2	39.7	5.19
lag	1.6	5.7	4.7	5.3	8.2	7.6	2.26

[1]K_D = fractional degradation rate constant (/h) of *D* fraction; *D* = potentially degradable and insoluble fraction (%; D = 100 – *W* – *U*); *U* = undegradable fraction at t = ∞ (%); *W* = washable fraction (%; assumed zero for the NDF and ADF fraction); *lag* = lag time.
[2]SEM = standard error of the mean.

Medians for model parameters *A* and *N*, as estimated by the multicompartmental model, were 2.3 ($P < 0.001$) and 24 ($P > 0.10$) across markers, respectively (data not shown). The mean prediction error (MPE) as an indicator of curve fit accuracy was lowest for Cr-NDF (9.7 ± 0.72%; $P < 0.001$) among markers. Interestingly, MPE was lower with ^{15}N (11.0–13.7%) than with ^{13}C (22.1–24.0%; $P < 0.001$). The largest proportion of the MPE across markers was due to random variation (96.5%) and only a minor part was explained by mean bias (1.5%) or regression bias (1.9%) (data not shown). Basal diet treatment generally affected passage kinetics except for K_1 which tended to be higher for N90 (0.035 ± 0.0019/h) compared to N45 (0.031 ± 0.0020/h). A significant fertilisation × maturity interaction for K_1 was observed, and generally K_1 decreased with maturity with N45 but increased with maturity with N45. A similar interaction effect was observed for K_2, PCT and TMRT.

Table 5. Fractional passage rates of different markers as determined by faecal sampling from dairy cows fed rations containing grass silages at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity[1]

Fertilisation	Maturity	K_1					K_2				
		Cr-NDF	^{13}C-DM	^{13}C-ADF	^{15}N-DM	^{15}N-ADF	Cr-NDF	^{13}C-DM	^{13}C-ADF	^{15}N-DM	^{15}N-ADF
N45	Early	0.045	0.044	0.020	0.035	0.033	0.29	0.18	0.17	0.25	0.23
	Mid	0.043	0.034	0.025	0.034	0.030	0.34	0.22	0.21	0.67	0.16
	Late	0.046	0.025	0.018	0.024	0.029	0.28	0.18	0.23	0.24	0.17
N90	Early	0.043	0.035	0.029	0.025	0.023	0.34	0.22	0.25	0.30	0.27
	Mid	0.056	0.033	0.020	0.046	0.048	0.30	0.31	0.28	0.48	0.44
	Late	0.050	0.034	0.030	0.043	0.035	0.27	0.47	0.19	0.46	0.31
SEM[2]				0.0075					0.062		
P-values[3]											
Animal				0.069					0.570		
Period				0.205					0.773		
Fertilisation (F)				0.079					<0.001		
Maturity (M)				0.394					0.011		
F × M				0.034					0.452		
Marker type (T)				<0.001					<0.001		
F × M × T				0.298					<0.001		
Cr *vs.* (^{13}C, ^{15}N)				<0.001					0.084		
^{13}C *vs.* ^{15}N				0.024					<0.001		
^{13}C: DM *vs.* ADF				<0.001					0.195		
^{15}N: DM *vs.* ADF				0.615					<0.001		

[1]Fractional passage parameters: K_1 = fractional passage rate constant (/h) for the compartment with the highest retention time in the gastrointestinal tract (*viz.* reticulorumen); K_2 = fractional passage rate constant (/h) for the compartment with the second highest retention time in the gastrointestinal tract (*viz.* proximal colon-caecum). Markers: chromium mordanted fibre (Cr-NDF); ^{13}C and ^{15}N isotopes in the dry matter (^{13}C-DM; ^{15}N-DM) and acid detergent fibre fraction (^{13}C-ADF; ^{15}N-ADF).

[2]SEM = standard error of the mean.

[3]Analyses of variance with orthogonal contrasts between markers based on log-transformed means in a split-plot arrangement with factorial main plots in a Latin square.

Table 6. Marker residence time in the gastrointestinal tract as determined by faecal sampling of dairy cows fed rations containing grass silages at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity[1]

Fertilization	Maturity	PCT					TMRT				
		Cr-NDF	^{13}C-DM	^{13}C-ADF	^{15}N-DM	^{15}N-ADF	Cr-NDF	^{13}C-DM	^{13}C-ADF	^{15}N-DM	^{15}N-ADF
N45	Early	21.8	23.2	26.1	19.9	24.1	39.4	40.5	64.9	41.9	48.1
	Mid	21.0	22.0	23.9	14.7	22.2	39.4	46.4	56.1	39.4	47.4
	Late	21.6	26.6	31.8	21.0	28.8	38.4	59.3	82.7	55.8	54.8
N90	Early	19.8	22.8	22.6	20.4	22.7	38.5	45.9	52.8	53.5	59.8
	Mid	20.6	17.6	20.6	14.5	14.6	34.7	43.0	66.7	32.4	32.2
	Late	22.7	16.1	22.5	15.0	17.8	37.5	40.9	49.2	34.4	41.3
SEM[2]				1.48					6.46		
P-values[3]											
Animal				0.303					0.174		
Period				0.966					0.217		
Fertilisation (F)				<0.001					0.006		
Maturity (M)				<0.001					0.055		
F × M				0.006					0.003		
Marker type (T)				<0.001					<0.001		
F × M × T				<0.001					0.061		
Cr *vs.* (^{13}C, ^{15}N)				0.396					<0.001		
^{13}C *vs.* ^{15}N				<0.001					<0.001		
^{13}C: DM *vs.* ADF				<0.001					<0.001		
^{15}N: DM *vs.* ADF				<0.001					0.100		

[1]Residence time parameters: PCT = marker peak concentration time in faeces (h); TMRT = total mean retention time in the gastrointestinal tract (h). Markers: chromium mordanted fibre (Cr-NDF); ^{13}C and ^{15}N isotopes in the dry matter (^{13}C-DM; ^{15}N-DM) and acid detergent fibre fraction (^{13}C-ADF; ^{15}N-ADF).

[2]SEM = standard error of the mean.

[3]Analyses of variance with orthogonal contrasts between markers based on log-transformed means in a split-plot arrangement with factorial main plots in a Latin square.

Omasum. In general, passage kinetics (K_1, TMRT) based on omasal grab sampling showed a similar trend to that based on faecal grab sampling (Table 7). Passage kinetics were not affected by maturity stage but K_1 was higher for N90 (0.037 ± 0.0017/h) than for N45 (0.030 ± 0.0016/h). Nitrogen fertilisation decreased TMRT from 34.7 h (N45) to 28.3 h (N90). Marker effect was significant for K_1 and TRMT. Estimates of K_2 averaged 1.96 ± 2.441/h (P > 0.10 for marker effect). Medians for model parameters A and N were 1.1 (P < 0.001) and 4 (P > 0.10) across markers, respectively (data not shown). The MPE for Cr-NDF curve fits was 24.7 ± 1.56% and in range with that for ^{13}C and ^{15}N curve fits (16.6–27.8%; data not shown). Mean pH of omasal digesta was 6.51 ± 0.012.

Table 7. Rumen passage kinetics of different markers as determined by omasal sampling of dairy cows fed rations containing grass silages at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha) and harvested at early through late maturity[1]

Marker[2]	K_1	K_2	TMRT
Cr-NDF	0.042	1.35	25.7
^{13}C-DM	0.034	2.18	29.7
^{13}C-ADF	0.024	1.38	42.8
^{15}N-DM	0.036	2.01	29.3
^{15}N-ADF	0.034	1.12	31.6
SEM[3]	0.0059	2.441	9.84
P-values[4]			
Animal	0.034	0.746	0.007
Period	0.239	0.437	0.335
Fertilisation (F)	0.012	0.162	0.009
Maturity (M)	0.257	0.506	0.272
F × M	0.057	0.739	0.060
Marker type (T)	<0.001	0.386	<0.001
F × M × T	0.028	0.153	<0.001
Cr *vs*. (^{13}C, ^{15}N)	<0.001	0.466	<0.001
^{13}C *vs*. ^{15}N	<0.001	0.593	0.016
^{13}C: DM *vs*. ADF	<0.001	0.222	<0.001
^{15}N: DM *vs*. ADF	0.534	0.133	0.419

[1]Passage kinetic parameters: K_1 = fractional passage rate constant (/h) for the compartment with the highest retention time in the reticulorumen; K_2 = fractional passage rate constant (/h) for the compartment with the second highest retention time in the reticulorumen; TMRT = total mean retention time in the reticulorumen (h).
[2]Cr-NDF = chromium mordanted fibre; ^{13}C-DM = ^{13}C in the dry matter; ^{13}C-ADF = ^{13}C in the acid detergent fraction; ^{15}N-DM = ^{15}N in the dry matter; ^{15}N-ADF = ^{15}N in the acid detergent fibre fraction.
[3]SEM = standard error of the mean.
[4]Analyses of variance with orthogonal contrasts between markers based on log-transformed means in a split-plot arrangement with factorial main plots in a Latin square.

Discussion

Effect of Grass Silages with Increasing Plant Age and Nitrogen Fertilisation Levels on Feed and Animal Characteristics

As expected, the grass silage treatments imposed had a clear effect on CP content. Grass silage maturity decreased CP content at an average rate of 1.8 g/kg DM per d from early through late maturity. Nitrogen fertilisation increased CP content by, on average, 38 g/kg DM with N90 relative to N45. The NDF content was little affected by N fertilisation but more by maturity stage, and is essentially in agreement with previous findings (Valk et al., 1996; Peyraud and Astigarraga, 1998). Effects on the fibre content were less clear. With N45, the NDF content increased from early to mid but did not increase from mid to late maturity; for N90, the NDF content did not increase from early to mid but increased from mid to late maturity.

The nutritional composition of the grass silage fed had a substantial effect on feed intake. Nitrogen fertilisation increased feed intake by 2.2 kg DM/d for early and mid maturity grass silage, which is in line with observations on fresh grass (Mackle et al., 1996). Advancing maturity decreased feed intake and was lowest for N90 of late maturity (reduced by 4.1 kg DM/d relative to preceding maturity stages). A similar effect on feed intake was observed by Bosch et al. (1992b) and Tahir et al. (2013) for grass silages with similar nutritional contrasts. Based on the chemical composition of the grass silage, it is not clear what induced this particular drop in feed intake for N90. No deficiency in ruminal degradable protein (as indicated by the positive OEB value) for optimal microbial fermentation was observed, which could impair ruminal digestion and consequently feed intake (Romney and Gill, 2000). The drop in feed intake for N90 matches with a particularly low total tract CP and OM digestibility observed for that particular treatment combination. A similar trend was observed for milk, milk fat and milk protein yields. The decline of milk and milk protein yield with advancing plant maturity might be caused by the reduced CP digestibility and supply as reviewed by Huhtanen and Hristov (2009). Grass silage maturity increased acetic acid molar proportion in the rumen but did not change propionic acid molar proportion and the non-glucogenic to glucogenic VFA ratio, which agrees with literature findings as reviewed by Rinne et al. (1997b).

Passage and Degradation Kinetics of Grass Silage

Passage through the gastrointestinal tract was generally faster for the high fertilised N90 treatment. In contrast, grass silage maturity had not a clear effect on passage kinetics and there was a strong interaction between plant maturity and N fertilisation on K_1. For the low fertilised N45, passage generally slowed down with advancing maturity, in particular when measured with ^{15}N markers. For the high fertilised N90, the opposite effect was observed and passage kinetics appeared to be faster for grass silage of late maturity than grass silage of early maturity. Results from literature are ambiguous and seem to depend on the plant material and resulting specific particle size. While some studies reported reduced or unchanged K_1 values for late *vs*. early maturing grass (Mambrini and Peyraud, 1994; Lamb et al., 2002; Lund et al., 2006; Kuoppala et al., 2009; Bayat et al., 2010; Bayat et al., 2011), other studies showed increased K_1 values with advancing maturity of grass silage (Gasa et al., 1991; Bosch et al., 1992a; Rinne et al., 1997a; Rinne et al., 2002).

The interaction between plant maturity and N fertilisation observed in our study could explain the inconclusive findings observed in an earlier study (Pellikaan et al., 2013). In that study, high digestible grass silage (100 kg N/ha; 39 d regrowth) did not result in a measurable change in passage kinetics compared to a low digestible grass silage (50 kg N/ha; 81 d regrowth), as could have been expected based on the large nutritional contrasts of the grass silages. Furthermore, Owens et al. (2008a,b) reported increased K_1 values of fresh ryegrass harvested at a later stage for the high CP (165–192 g/kg DM) autumn regrowth but not so for the low CP (99–116 g/kg DM) spring regrowth. Rumen conditions could explain some of the variation in passage kinetics. Tafaj et al. (2005) observed reduced K_1 values along with a reduced rumination activity and fibre digestibility for low-fibre ytterbium labelled hay when concentrate levels were increased. In our study, rumen conditions did not appear to deteriorate by increasing N fertilisation but fibre digestibility was clearly impaired, which might explain the increase in K_1 (Owens and Goetsch, 1986) observed with ^{15}N for N90 of late *vs*. early maturity. Furthermore, K_1 is not expected to be associated to a potential change in the cell wall structure as the composition of the cell wall fraction, in particular fibre-bound N, seem to be unaffected by the level of N fertilisation (Wilman et al., 1977).

Digesta particle size was not measured in this study but a smaller digesta particle size with grass silage of advancing plant maturity was associated with an increased K_1 (Rinne et al., 2002; Krämer et al., 2013a). These studies reported a faster particle breakdown rate and an increased K_1 for the small particle pool in the rumen for a late-cut compared with an early-cut

grass silage. In particular, Rinne et al. (2002) observed higher fragility or brittleness of the more lignified particles from late-cut grass silage. Furthermore, maturity stage of grass silage considerably decreased the proportion of large particles (>1 mm) but increased that of small particles (0.2–0.5 mm) in faeces (Jalali et al., 2012). It can be hypothesized that the larger leaf blade size upon increased N fertilisation (Wilman and Pearse, 1984) in combination with a higher brittleness of leaves often observed with the more mature plant material (Ulyatt, 1983), resulted in a considerable particle size reduction of the ^{15}N-DM and ^{15}N-ADF from leaves. This might have resulted in increased passage kinetics of the ^{15}N fractions with advancing plant maturity. In addition, stem morphology was not altered upon increased N fertilisation (Harris et al., 1996), and earlier studies suggested that leaves are more prone to particle size reduction than stems of grass (Poppi et al., 1981; Cherney et al., 1991). This might explain why passage kinetics of the ^{13}C fractions did not change with advancing plant maturity like the ^{15}N fractions.

Degradation kinetics of the six grass silages revealed similar trends to passage kinetics. The *U* fraction generally increased and the *W* fraction decreased with advancing maturity, essentially in line with degradation characteristics of grass silage reported by Tamminga et al. (1991) and Bosch et al. (1992b); and the *W* fraction was higher with elevated N fertilisation as observed by Peyraud et al. (1997). In contrast, the *D* fraction of OM and CP increased with maturity for N45 but decreased for N90. The fractional degradation rate (K_D) of the *D* fraction of CP decreased with maturity at both N fertilisation levels, essentially in line with van Vuuren et al. (1991), but for other nutrients K_D increased with advancing maturity for the high fertilised N90 only. The *D* fraction of the ryegrass CP fraction decreased with maturity of N90, which is in line with observations on fresh ryegrass (Peyraud et al., 1997). The K_D of the ryegrass CP fraction increased with the high fertilised N90 treatment, which was also observed by van Vuuren et al. (1991) and Valk et al. (1996) who reported somewhat higher values (0.078–0.084/h and 0.061–0.133/h, respectively) for fresh ryegrass fertilised at comparable N levels relative to our ryegrass silage. In general, K_D was not well correlated to K_1 based on our dataset ($r \leq 0.40$; $n = 34$). The highest correlation coefficients were obtained among fibre K_D and K_1 based on ^{13}C-ADF and ^{15}N-ADF ($r = 0.25$ to 0.40) but with regard to other nutrients, stable isotopes did not particularly result in higher r-values than Cr-NDF. In a previous experiment with maize silage and using ^{13}C-DM and ^{13}C-ADF as internal markers,

no direct link could be established between silage degradation and passage rates (Warner et al., in press).[8]

Passage Kinetics of Stable Isotopes in Faeces and Omasal Digesta

Passage kinetics from faecal and omasal digesta samples were estimated from a deterministic multicompartmental model (Dhanoa et al., 1985). Passage kinetics of the grass silage was highly dependent on the type of marker used from both faecal and omasal digesta samples ($P < 0.001$). In particular, the external marker Cr-NDF provided approximately 0.016/h units higher K_1 values and, in turn, a 10.8-h lower TMRT than the internal ^{13}C and ^{15}N markers. Comparable findings were reported for Cr-NDF and ^{13}C from labelled grass silage (Pellikaan et al., 2013) and maize silage (Warner et al., in press).[8] The ADF fraction had considerable slower passage kinetics than the DM fraction when ^{13}C was used (K_1 reduced by 0.011/h; TMRT increased by 15.5 h; PCT increased by 3.3 h). In contrast, differences in K_1 between the two fractions were almost absent with maize silage (K_1 of 0.023/h and 0.021/h for ^{13}C-DM and ^{13}C-ADF respectively; Warner et al., in press).[8] With grass silage, differences were higher in the study of Pellikaan et al. (2013) to some extent, although dependent on the silage quality (difference of −0.019 and −0.023/h for high and low digestible grass silage, respectively). We found a similar silage quality effect on the relation between ^{13}C-DM and ^{13}C-ADF with differences in K_1 being amplified for the low fertilised grass (difference of −0.008 and −0.014/h for N90 and N45, respectively). These results may reflect the different physical structure of fibres between the two forage types (De Boever et al., 1993a,b) and between forage quality.

When ^{15}N was used, the ADF fraction had similar passage kinetics to the DM fraction, except for a somewhat reduced K_2 (by 0.125/h) and a considerably increased PCT (3.9 h). The use of ^{15}N provided a more detailed insight into the passage kinetics of the cell walls. Cell wall bound ^{15}N appear to pass out at a somewhat higher rate from the reticulorumen (K_1 increased by 0.009/h) and from the gastrointestinal tract (TMRT reduced by 14.1 h) than the cell walls. Similarly, total proteins based on ^{15}N-DM appeared to have increased passage kinetics to some extent relative to the non-protein fraction based on ^{13}C-DM. Overall, the experimentally determined K_1 of ^{15}N-DM (0.034 ± 0.0021/h) is lower than what is assumed by the Dutch feed evaluation system (0.045/h; van Duinkerken et al., 2011), which is based on passage studies using external markers. Similarly, K_1 of ^{15}N-DM is lower than when

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calculated based on the equations used in the UK Feed-into-Milk system (0.043/h; Thomas, 2004) but in line with results based on the equations used in the NRC feed evaluation system (0.031/h; NRC, 2001) and the Nordic feed evaluation system (0.035/h; Volden, 2011). The K_1 of Cr-NDF in the present study (0.047 ± 0.0030/h) was well in line with the assumption in the Dutch and UK system (0.045 and 0.043, respectively).

A bias in K_1 due to marker migration often observed with rare earth elements (Owens and Hanson, 1992) might also occur with stable isotopes if they are incorporated into microbial biomass (Firkins et al., 1998; Pellikaan et al., 2013). The acid detergent treatment circumvents this problem as none or only negligible amounts of digestible fractions are analytically recovered in this fraction. Digestible fractions are more prone to bias, and it has been shown that up to 8 g C/kg of faecal OM were of microbial origin (van Vliet et al., 2007) and that the faecal microbial biomass increased with decreasing grass silage maturity. Hence, marker migration via microbial biomass might in theory introduce some bias in DM or intracellular matter but overall effects on the K_1 of digestible fractions will be probably minor.

Sampling site had little effect on passage kinetics suggesting that faecal sampling provide reliable ruminal passage kinetics. In addition, the same marker contrasts as well as plant maturity × N fertilisation level interaction (data not shown) observed in faecal samples were detected in omasal digesta. Faecal marker K_1 and TMRT were moderately correlated with those based on omasal digesta sampling (on average, r = 0.47 and r = 0.56, respectively) with little difference between Cr-NDF and stable isotope markers, except for ^{13}C-ADF for which no correlation was observed.

Curve fit accuracy based on the MPE was in the range of previous studies using ^{13}C labelled forage (Pellikaan et al., 2013; D. Warner et al., unpublished data)[9] but MPE values were almost double compared to those reported for ^{13}C labelled concentrates pulse dosed at high amounts (9 kg; D. Warner et al., unpublished data).[9] Interestingly, curve fit accuracy for Cr-NDF based on faecal sampling was considerably better than that for stable isotopes, but accuracy considerably declined for Cr-NDF with omasal digesta sampling and was even slightly worse than that of ^{15}N isotopes. These findings suggest that the small ruminal pulse dose size of stable isotopes probably affected its curve fit accuracy, and that omasal sampling provided somewhat less reliable results than faecal sampling. Interestingly, curve fit accuracy of stable isotopes was not particularly worse based on omasal digesta compared to faecal sampling as it could have been expected based on the results for Cr-NDF. This suggests that a

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satisfactory marker distribution in the rumen was very probably attained well before the first omasal samples were taken. It should be furthermore noted that a lower number of omasal than faecal samples were collected and submitted to analyses. Visual inspection of curve fits confirmed that the presence of a few possible outlying values weakened the fit of omasal excretion curves to a higher extent than that of faecal excretion curves, where a higher number of available data points per curve improved its fit. The increased costs associated with collecting and submitting a higher number of samples to isotope analyses might be avoided by using a larger but lower enriched pulse dose size. Most likely, the marker distribution within the rumen content will be accelerated, as it is probably the case for the finely ground Cr-NDF particles, and lower the effect of possible outlying values on curve fit accuracy and consequently passage kinetics.

Conclusions

The use of ^{13}C and ^{15}N stable isotopes from intrinsically labelled ryegrass silage allowed the assessment of passage kinetics of fibres and fibre-bound N. Fractional passage rates decreased with advancing plant maturity for the low N fertilised grass silage but increased for the high N fertilised grass silage. Fractional rumen passage rates were higher for the high compared with the low N fertilisation level. Passage kinetics of ^{13}C fibres differed from those of the external marker Cr-NDF and fibre-bound ^{15}N. Sampling site, *viz*. faeces *vs*. omasum, did hardly affect rumen fractional passage rate estimates.

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Chapter 6

Stable isotope labelled plant wax *n*-alkanes to determine digesta passage through the gastrointestinal tract of ruminants

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Abstract

We describe the use of carbon stable isotope (^{13}C) labelled *n*-alkanes as a potential internal marker to assess digesta passage kinetics in ruminants. Plant cuticular *n*-alkanes originating from intrinsically ^{13}C labelled ryegrass plants were pulse dosed intraruminally in four rumen-cannulated lactating dairy cows receiving four contrasting ryegrass silage treatments that differed in nitrogen (N) fertilisation level (45 or 90 kg N ha^{-1}) and maturity (early or late). Passage kinetics through the gastrointestinal tract were derived from the $\delta^{13}C$ (ratio ^{13}C:^{12}C) in apparently undigested faecal material. Isotopic enrichment was observed in a wide range of long-chain *n*-alkanes (C_{27}–C_{36}) and passage kinetics were determined for the most abundant C_{29}, C_{31} and C_{33} *n*-alkanes, for which a sufficiently high response signal was detected by combustion isotope ratio mass spectrometry. Basal diet treatment and carbon chain length of *n*-alkanes did not affect fractional passage rates from the rumen (K_1) among individual *n*-alkanes (3.71–3.95 %/h). Peak concentration time and transit time showed a quantitatively small, significant ($P \leq 0.002$) increase with carbon chain length. K_1 estimates were comparable to those of the ^{13}C labelled ryegrass dry matter fraction (3.38 %/h; $r = 0.61$ to 0.71; $P \leq 0.012$). Literature has shown that *n*-alkanes are not fermented by microorganisms in the rumen and affirms no preferential depletion of ^{13}C versus ^{12}C. Our results suggest that ^{13}C labelled *n*-alkanes can be used as digesta passage markers and support the reliability of the $\delta^{13}C$ signature of digestible feed nutrients as a tool to measure nutrient-specific passage kinetics.

Introduction

Production animals need to be fed according to their nutritional requirements in order to reach their maximum performance, reduce the loss of waste products into the environment from undigested feed nutrients, and prevent nutrient-related disorders due to an unbalanced or insufficient supply of nutrients. Knowledge on the behaviour of ingested feed nutrients in the different compartments of the gastrointestinal tract is essential to understand the fate of nutrients and related digestive mechanisms (Dijkstra et al., 2007). In ruminants, which are particularly adapted to yield energy from poor-quality forages, the reticulorumen is the main site of fermentative degradation of nutrients through the action of microorganisms, mixing of the ingesta and particle size reduction. Once ingested feed particles reach a specific particle size (Poppi et al., 1980) and specific density (Siciliano-Jones and Murphy, 1986), they pass in aboral direction into the following digestive compartment at specific fractional rates (i.e., as a fraction per hour; %/h) depending on a number of feed and animal characteristics (Owens and Goetsch, 1986; Pellikaan et al., 2013). If quantified accurately, knowledge on the fractional passage rate from the reticulorumen may be used to predict the extent of degradation and excretion of nutrients (Dijkstra et al., 2007). Fractional passage rates are, therefore, an essential part of modern feed evaluation systems as well as of mechanistic models describing the dynamics of microbial population in the rumen and their contribution to methane emissions from ruminants (Kebreab et al., 2004). Yet, quantitative knowledge on feed- and nutrient-specific passage kinetics are limited.

Passage kinetics are commonly estimated by marker techniques and, recently, the carbon isotope signature of feed nutrients ($\delta^{13}C$; i.e. the ratio of the stable isotopes ^{13}C to ^{12}C) has been proposed as an internal passage marker (Pellikaan et al., 2013). In particular, $\delta^{13}C$ in faecally excreted undigested fibres allowed researchers to quantify nutrient-specific passage in dairy cows. It was further proposed to use stable isotopes in combination with *n*-alkanes as an internal passage marker (Dove and Mayes, 2006). *n*-Alkanes are saturated aliphatic hydrocarbon chains naturally present in plant cuticular wax. They have a high faecal recovery in ruminants depending on the carbon chain length (Doves and Mayes, 1991; Ferreira et al., 2009), are neither degraded nor synthesised in the rumen (Bartley et al., 1971; Keli et al., 2008), and their analytical determination is specific and well described (Dove and Mayes, 2006); hence, they possess close-to-ideal marker characteristics.

The objective of this study was to evaluate whether the $\delta^{13}C$ signature of *n*-alkanes can be used to estimate passage kinetics in ruminants. To our knowledge no literature is available on the application of stable isotope labelled *n*-alkanes as fractional passage rate markers, although $\delta^{13}C$ of individual *n*-alkanes were successfully employed to assess feed digestibility of ruminants (Bezabih et al., 2011b). The present study describes for the first time *in vivo* ^{13}C labelled *n*-alkanes and their relevance to passage kinetics studies in dairy cattle.

Material and Methods

Animals and Housing

The present study was part of a larger experiment on passage kinetics of nutrients through the gastrointestinal tract of dairy cows (D. Warner et al., unpublished data),[10] approved by the Institutional Animal Care and Use Committee of Wageningen University. Four multiparous Holstein-Friesian dairy cows in their second to fourth lactation, fitted with a permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond, Parma, ID), were individually housed in tie stalls. Animals averaged (mean ± SEM) 561 ± 13 kg in bodyweight, and, during measurement weeks (n = 4), had a dry matter (DM) intake of 17.0 ± 0.35 kg/d and produced 26.2 ± 0.87 kg milk/d. Animals were milked twice daily during feeding times.

Diet and Treatments

Animals were fed a total mixed ration consisting of 455 g/kg DM ryegrass silage, 195 g/kg DM maize silage and 350 g/kg DM of a specifically designed compound feed (Table 1). The compound feed ingredients originated from cool-season C_3 plants to keep the background level of ^{13}C low and similar to that of the natural levels of the grass silage mixed in the experimental diet. Grass silage was prepared from perennial ryegrass (*Lolium perenne*) from the second regrowth, fertilised at two nitrogen (N; as potassium phosphorus nitrate) levels, and harvested at two maturity stages. Levels of fertilisation were either 45 kg N/ha (N45), or 90 kg N/ha (N90). Maturity stages were set to obtain a target DM yield in the range of 1800–2000 kg/ha (early) and 4600–4800 kg/ha (late). The grass plants were harvested in September 2010, wilted and ensiled. Animals were offered daily rations of the diet as two equal meals at 0600 and 1700 h, and had free access to water. The diet was prepared twice weekly; feed

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ingredient samples were collected each time the diet was prepared and pooled per animal over each experimental period.

Table 1. Chemical composition of the diet consisting of grass silage of early or late maturity at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha), maize silage and compound feed

Chemical composition (g/kg dry matter)	N45		N90		Maize silage	Compound feed[1]
	early	late	early	late		
Dry matter (g/kg fresh)	366	723	567	520	—	—
Dry matter (kg/ha)	1840	4734	2020	4860	—	−—
Organic matter	879	912	907	903	961	933
Crude protein	197	137	249	168	77	262
Starch	—	—	—	—	403	218
Neutral detergent fibre	432	545	429	556	356	293
Acid detergent fibre	294	296	257	326	201	151

[1]Ingredients (g/kg DM): wheat (80.0), sunflower seeds (140.0), soybean hulls (26.5), palm kernel expeller (90.0), soybeans (185.0), sugar beet pulp (75.0), potato starch (200.0), MervoBest rumen-protected soybean meal (185.0), phosphoric acid limestone (7.5), salt (3.0), mineral premix (8.0).

Marker Preparation

The external marker Cr-NDF was prepared as described by Udén et al. (1980) from wheat straw, dried and ground to pass a 0.5-mm screen. The ^{13}C marker was prepared from ^{13}C labelled and ensiled ryegrass originating from the field that also provided the basal experimental diet. In brief, representative ryegrass shoots from the second regrowth were randomly collected from the field and grown on hydroponics (8.6 shoots per m²) under climate-controlled greenhouse conditions in hermetically sealed isotope assimilation chambers, specifically designed for homogeneous atmospheric isotope-labelling (Gorissen et al., 1996). Grass was continuously enriched under high levels of ^{13}carbon dioxide (released from 99.98 atom% ^{13}C bicarbonate) from plant emergence onwards in a commercial facility (IsoLife BV, Wageningen, The Netherlands). The labelled grass plants were exposed to similar conditions to the field plants to account for potential known sources of variation for an altered cell wall structure and *n*-alkanes content in temperate forages (Dove et al., 1996); e.g., by adjusting the light schedule in the greenhouse to the field conditions, and inducing wind stress to the labelled plants. Grass plants received the identical fertilisation regime and harvested at a similar physiological stage (172 g DM/m² and 498 g DM/m² for early and late maturity stage, respectively) as the field plants. Plants were subsequently wilted, cut to size (2 cm), placed into several bags of larger mashed grit gauze (pore size 212 µm; PA-74, Sefar

Nytal, Heiden, Switzerland) and distributed over silage bales to be ensiled together with the field plants over an 8-week period. The stable isotope labelling was successfully terminated by collecting ensiled ryegrass plants enriched up to 7.75–9.21 atom% ^{13}C for the different treatments relative to a background level of 1.01 atom% ^{13}C (SD 0.001) for the unlabelled field plants.

Sampling and Measurements

Four experimental periods of three weeks each were used, which included an adaptation after diet changeover from day 1 through 14, followed by faecal sampling from day 15 through 19. From day 12 onwards, animals were fed 95% of the individual DM intake measured during the preceding adaptation days to minimise feed residuals. Feed and water uptake were monitored daily and animals were milked twice daily.

Animals received a ruminal pulse dose of 100 g DM Cr-NDF (45.9 g Cr/kg DM Cr mordant) and 15 g DM ^{13}C labelled grass silage. Prior to pulse-dosing, the labelled marker material was cut to pieces of about 0.5 cm to resemble ingested bulk grass silage particles.

From day 15 onwards, directly after administration of markers into the rumen (0900 h), 20 spot samples of faeces were collected after defecation in sampling blocks of three hours each. Ten faecal samples collected at times t = 0, 12, 18, 24, 30, 36, 48, 72, 96 and 120 h after pulse dose administration were analysed for their marker concentrations. Faeces were weighed, thoroughly homogenised by hand and a representative sample of about 400 g fresh matter was stored at −20°C pending analyses.

Chemical Analyses

All samples were freeze-dried and ground over a hammer mill to pass a 1-mm screen (Peppink 100 AN, Olst, The Netherlands). Dry matter, ash, crude protein, starch, NDF and ADF were analysed as described by Abrahamse et al. (2008a,b). Faecal Cr concentrations were determined using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) after oxidation by wet-destruction as described by Pellikaan et al. (2013). *n*-Alkane extraction was carried out after Mayes et al. (1986), following modifications after Oliván and Osoro (1999) and Bezabih et al. (2011a) and using tetratriacontane (C_{34}) as an internal standard. In brief, test samples were pulverised (MM2000, Retsch, Haan, Germany; 3 min at 85 Hz) prior to *n*-alkane extraction. Full base line separation of *n*-alkanes (C_{27} to C_{36}) was achieved using a gas chromatograph (GC; Finnigan Trace GC Ultra, Milan, Italy) fitted with a

capillary column (40 m x 0.32 mm i.d. fused silica capillary SPB-1 and 0.10 µm film thickness) and using helium as a carrier gas at a constant flow of 2.5 ml/min. *n*-Alkane extracts, previously diluted with 125 µL of heptane, were injected using a split/splitless-type injector operating on split mode (split ratio of 1:5). The temperature for the injector was 270°C. The oven temperature program started at 210°C (maintained for 1 min), increased at a rate of 7.2°C/min to a temperature of 300°C (maintained for 6 min). To determine the $\delta^{13}C$ of individual *n*-alkanes, the column outlet was fitted to a combustion interface (Thermo Finnigan GC Combustion III, Bremen, Germany) that was connected to an isotope ratio mass spectrometer (IRMS; Delta V Advantage, Thermo Scientific, Bremen Germany). For $\delta^{13}C$ analyses of the DM fraction (^{13}C-DM), a test sample was pulverised, and $\delta^{13}C$ was determined by elemental analysis using an IRMS as describe above. The relative atom% ^{13}C in the substrate is expressed as the ^{13}C:^{12}C ratio in the samples relative to the ^{13}C:^{12}C ratio of the international Vienna Pee Dee Belemnite standard, and presented as $\delta^{13}C$. After correction for natural ^{13}C abundance, faecal excretion patterns of atom% ^{13}C excess were established.

Curve Fitting and Statistical Analyses

Fractional passage rates were derived from marker excretion patterns, fitted iteratively with a nonlinear multicompartmental model (Dhanoa et al., 1985):

$$C_t = A \times e^{-K_1 \times t} \times \exp\left[-(N-2) \times e^{-(K_2 - K_1) \times t}\right]$$

where C_t denotes the faecal marker concentration at time = t; t is the average time span of collection after marker administration; K_1 and K_2 refer to the fractional rate constants for the compartment with the longest (reticulorumen) and the second longest retention time (proximal colon-caecum) in the gastrointestinal tract, respectively; N refers to the model-derived number of mixing compartments; and A forms a scalable parameter dependent on K_1, K_2 and N.

Before curve fitting, faecal marker concentrations were scaled to the marker peak concentration as described by Sponheimer et al. (2003). Curve fitting was performed using nonlinear least squares regression procedures of SAS (version 9.2, Cary, NC) based on the least square Levenberg-Marquardt algorithm. Initial values for the iterative procedure were obtained through a grid search and curve fits were solved after, on average, 6 to 9 iterations. Transit time (TT; i.e. moment of first appearance of the marker in the faeces) and moment of peak concentration (PCT) were derived for faecal marker excretion patterns from the

estimated parameters as described by Dhanoa et al. (1985). Total mean retention time (TMRT) in the reticulorumen was calculated as the sum of the reciprocals of K_1 and K_2 plus TT; total marker clearance time was calculated as described by France et al. (1993). Accuracy of curve fits were evaluated by comparing predicted marker concentrations with observed values using the root mean squared prediction error relative to the observed mean, thus obtaining the mean prediction error (MPE). The MPE was decomposed into errors due to random variation, errors of central tendency and errors due to regression (Bibby and Toutenber, 1977).

Model parameters were log transformed due to asymmetrical distribution patterns of residuals and tested by analysis of variance in a split plot, with factorial main plots in a Latin square and subplots representing the type of *n*-alkane, using the mixed model procedure of SAS (version 9.2, Cary, NC), according to the model:

$$Y_{ijklm} = \mu + A_i + P_j + S_k + M_l + (S \times M)_{kl} + (A \times P \times S \times M)_{ijkl} + Alk_m + (S \times M \times A)_{klm} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent variable; μ is the overall mean; A_i (animal; $i = 4$), P_j (period; $j = 4$), S_k (silage; $k = 2$), M_l (maturity; $l = 2$) and its interaction term $(S \times M)_{kl}$ represent effects assigned to the main plots in a Latin square; Alk_m (type of *n*-alkane; $m = 3$) and $(S \times M \times Alk)_{klm}$ represent effects related to the subplots. Main plot variables were tested against the interaction term $(A \times P \times S \times M)_{ijkl}$ and subplot variables were tested against the pooled residual error (ε_{ijklm}). Covariance parameters were estimated using the residual maximum likelihood (REML) method and denominator degrees of freedom were estimated using the Satterthwaite approximation. Tables report back-transformed values. Passage kinetics of ^{13}C-*n*-alkanes were compared to the commonly used external tracer Cr-NDF and the internal tracer ^{13}C-DM originating from the same substrate by means of the Pearson correlation coefficient *r*.

Results

Concentrations of ^{13}C isotopes were detected in a wide range of *n*-alkanes (Table 2). A high signal amplitude voltage by combustion isotopic ratio mass spectrometry was obtained for the odd-chain *n*-alkanes C_{29}, C_{31} and C_{33}, which were also used to assess fractional passage (in total, 48 curve fits). The signal amplitude voltage obtained for the even-chain *n*-alkanes (C_{28}–C_{36}; amplitude always below 0.3 V) and for the odd-chain *n*-alkanes C_{27} and C_{35} (amplitude

always below 0.5 V, with up to 72% of values below 0.3 V) were below or close to the limit of quantitation (defined here as 0.3 V) and considered too low to obtain a reliable $\delta^{13}C$ based on the analytical procedure and equipment used (see details in Figure 1). Background levels of $\delta^{13}C$ (i.e. natural enrichment) decreased with carbon chain length of odd-chain *n*-alkanes (Table 2). Differences between $\delta^{13}C$ background and peak levels were generally higher for the odd-chain *n*-alkanes and highest for C_{29}, C_{31}, C_{33} and C_{35}.

Table 2. Mean background and peak concentrations of delta (δ) ^{13}C for individual *n*-alkanes in faeces as a mean of four grass silage treatments[1]

n-Alkane	Background ($\delta^{13}C$)	Peak ($\delta^{13}C$)
Even-chain		
C_{28}	−33.07 (0.592)	−29.84 (0.279)
C_{30}	−35.10 (0.526)	−30.29 (0.751)
C_{32}	−33.81 (0.753)	−30.03 (0.579)
C_{36}	−29.51 (0.302)	−27.86 (0.271)
Odd-chain		
C_{27}	−32.90 (0.577)	−29.23 (0.611)
C_{29}	−35.58 (0.241)	−29.25 (0.714)
C_{31}	−36.08 (0.195)	−27.57 (0.888)
C_{33}	−36.69 (0.223)	−29.95 (0.800)
C_{35}	−37.27 (0.518)	−30.37 (0.840)

[1]Standard error of the mean given in parenthesis; C_{34}: internal standard. $\delta^{13}C$ refers to the relative atom% ^{13}C in the sample relative to the atom% ^{13}C of the international Vienna Pee Dee Belemnite standard.

Faecal excretion patterns of ^{13}C-*n*-alkanes were characterised by an initial quickly ascending phase until moment of marker peak concentration (PCT) followed by a slowly descending phase (Figure 2). The ^{13}C concentrations in *n*-alkanes were close to their natural abundance when faecal sampling was terminated as shown by the mean total marker clearance time of 134 ± 4.9 h (mean ± SEM). The 48 curve fits established for ^{13}C-*n*-alkanes showed a mean prediction error (MPE) of 9.9 ± 4.68% (Table 3), of which 92.7 ± 1.14% were related to errors due to random variation, 2.7 ± 0.45% to errors of central tendency and 4.6 ± 0.70% to errors due to regression. *n*-Alkane carbon chain length did not affect MPE of curve fits.

Fractional passage rates representing the outflow of marker from the reticulorumen (K_1) and from the proximal colon-caecum (K_2), marker PCT and transit time (TT) were not affected by the basal diet treatment (Table 3). *n*-Alkane carbon chain length did not affect the respective K_1 estimates (3.71–3.95 %/h). Quantitatively small, significant changes in some

passage kinetic parameters occurred with increasing carbon chain length, such as a decrease of K_2 (P = 0.002) and an increase in PCT (P < 0.001) and TT (P = 0.047). Total mean retention time (TMRT) was not different among *n*-alkanes (43.9–45.3 h; P = 0.088). The model parameters N (model-derived number of mixing compartments) and A (scalable model parameter) as obtained after fitting excretion data with a multicompartmental model were 13 ± 2.5 and 3.8 ± 0.42, respectively (data not shown). Determination of ^{13}C in the faecal DM fraction revealed a mean K_1 value of 3.38 ± 0.315 %/h, a K_2 value of 24.1 ± 1.84 %/h, and a TMRT of 47.1 ± 2.52 h. Determination of faecal Cr-NDF revealed a mean K_1 value of 5.25 ± 0.490 %/h, a K_2 value of 31.2 ± 2.39 %/h, and a TMRT of 35.7 ± 1.91 h (data not shown).

Table 3. Passage kinetics of ^{13}C labelled odd-chain *n*-alkanes (C_{29}, C_{31}, C_{33}) in dairy cows fed grass silages of early and late maturity at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha)[1]

Marker	K_1	K_2	PCT	TT	TMRT	MPE
n-Alkanes						
C_{29}	3.95 (0.158)	21.3 (2.81)	22.6 (2.93)	13.4 (0.37)	43.9 (0.74)	9.7 (2.08)
C_{31}	3.71 (0.148)	20.8 (2.74)	22.7 (2.94)	13.0 (0.36)	44.9 (0.75)	7.8 (1.66)
C_{33}	3.93 (0.157)	18.8 (2.47)	23.9 (3.10)	14.0 (0.39)	45.3 (0.76)	9.8 (2.10)
Nitrogen fertilisation						
N45	4.05 (0.204)	17.0 (3.13)	26.7 (4.89)	13.8 (0.40)	45.0 (1.24)	8.4 (2.40)
N90	3.67 (1.849)	24.2 (4.46)	19.9 (3.64)	13.1 (0.44)	44.3 (1.22)	9.8 (2.81)
Maturity stage						
Early	3.76 (0.232)	20.6 (4.65)	22.6 (5.07)	13.1 (0.48)	45.1 (1.15)	12.9 (4.53)
Late	3.96 (0.244)	20.0 (4.51)	23.5 (5.27)	13.7 (0.50)	44.3 (1.13)	6.4 (2.23)
	Model evaluation for *n*-alkanes (P-values)[2]					
Main plots						
Animal	0.370	0.673	0.614	0.265	0.472	0.746
Period	0.010	0.908	0.771	0.147	0.010	0.424
Fertilisation (F)	0.216	0.225	0.299	0.332	0.612	0.709
Maturity (M)	0.618	0.936	0.920	0.535	0.680	0.264
F × M	0.940	0.523	0.379	0.651	0.178	0.667
Subplots						
n-Alkane (Alk)	0.120	0.002	<0.001	0.047	0.088	0.100
Alk × F × M	0.050	0.173	0.022	0.167	0.018	0.167

[1]Passage kinetics: K_1: fractional passage rate constant (%/h) from the reticulorumen; K_2: fractional passage rate constant (%/h) from the proximal colon-caecum; PCT: marker peak concentration time (h); TT: marker transit time (h); TMRT: total mean retention time (h); MPE: mean prediction error (% of observed mean); values represent means (n = 16 per *n*-alkane type) and respective standard error of the mean in parenthesis.
[2]Analyses of variance on log-transformed means.

Discussion

The Labelling and Analytical Determination of Carbon Isotope *n*-Alkanes

The present study is the first describing stable isotope (^{13}C) labelled *n*-alkanes from *in vivo* isotopic labelled plant material and its application in digesta passage studies in ruminants. A first attempt to estimate fractional rumen passage rates in small ruminants from radioactive isotope ($^{14}C/^{3}H$) labelled *n*-alkanes (originating from fresh perennial ryegrass) has been made by Mayes et al. (1997) and was published as a conference proceedings abstract. Details on the labelling procedure were not provided but appear to involve immersion of fresh grass in a solution of ^{14}C labelled acetate followed by a short exposure to a high-intensity light source (Dove and Mayes, 1991) for temporary ^{14}C assimilation.

We quantified passage kinetics for the most abundant ^{13}C-*n*-alkanes C_{29}, C_{31} and C_{33} in apparent undigested faeces. The use of a combustion isotope ratio mass spectrometer allowed detection of $\delta^{13}C$ for the long-chain $C_{27\text{-}36}$ *n*-alkanes. The even-chain and the odd-chain C_{27} and C_{35} *n*-alkanes offered particular weak $\delta^{13}C$ signals, most probably because of their low natural concentrations generally observed in plant biomass and faeces (Mayes et al., 1986). In ryegrass species, concentrations of the most abundant *n*-alkanes C_{29}, C_{31} and C_{33} typically ranged from 77 to 338 mg/kg DM (Ali et al., 2005; Ferreira et al., 2012). The GC-IRMS was set up to present a high amount of sample for analyses (see details in Figure 1) and thus allow for high analytical sensitivity. A higher sensitivity of the low enriched *n*-alkanes was not feasible due to the relatively high enrichment levels and corresponding strong signal response of some of the adjacent *n*-alkanes approaching the upper analytical detection limit for $\delta^{13}C$.

Continuous intrinsic isotope labelling applied in our study was shown to provide uniformly labelled plant material (Gorissen et al., 1996; Ippel et al., 2004). Differences in $\delta^{13}C$ background levels between *n*-alkanes (Table 2) appeared to depend on the respective chain length and suggest that carbon isotope discrimination occurs during the enzymatic *n*-alkane biosynthesis by decarbonylase in the plant cuticula. Similar observations were reported for the biosynthesis of lignin (Benner et al., 1987) and starch (Scott et al., 1999) in plant tissue. Differential carbon allocation as observed in the plant tissue does not occur in the animal organism due to the absence of ruminal synthesis and degradation of *n*-alkanes (Bartley et al., 1971; Keli et al., 2008).

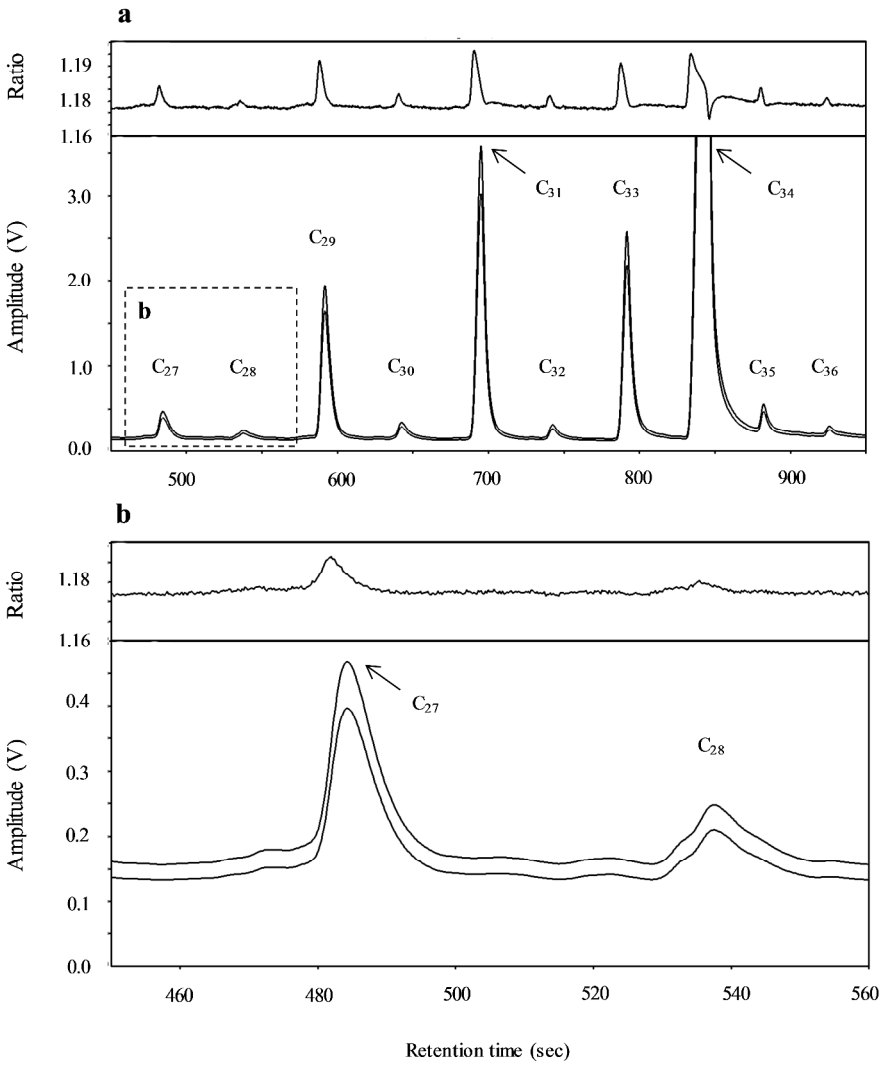

Figure 1. Mass spectra for ^{13}C labelled *n*-alkanes (C_{27} to C_{36}) collected in faeces upon an intraruminal pulse dose. Plots a–b show mass chromatograms with signal amplitude voltage (V; lower plot segments) of individual *n*-alkanes for the carbon isotopes ^{12}C and ^{13}C (from lowest to highest concentration, respectively), and their respective ^{13}C:^{12}C ratio (upper plot segments). C_{34} is the internal standard. Mass spectra illustrate the distinctive peaks of the most abundant *n*-alkanes C_{29}, C_{31} and C_{33} originating from a highly enriched faecal sample collected at peak concentration time (mean $\delta^{13}C$ −28.84; SEM 1.736). Although signals were detected and distinctive peaks were identified for lesser abundant *n*-alkanes (plot 1a), the ^{13}C:^{12}C ratio indicates that the respective $\delta^{13}C$ levels were below or close to quantitation limit (0.3 V; plot 1b). Lower enriched faecal samples often provided no response signal for lesser abundant *n*-alkanes. Test samples were pre-concentrated by reducing the amount of solvent to 125 µL, and were injected at a split ratio 1:5 using a split/splitless-type injector operating on split mode to obtain a high peak resolution.

Microbial fermentation in the hindgut of ruminants is minor (Illius and Gordon, 1992) and a hypothetical *n*-alkane degradation and preferential carbon isotope disappearance from the hindgut is therefore unlikely to affect passage kinetics estimations of *n*-alkanes from faecal samples. Disappearance of some ingested *n*-alkanes in the gastrointestinal tract has been reported and generally decreased with carbon chain length (Doves and Mayes, 1991; Ferreira et al., 2009). However, literature suggests that absorption from the small intestine rather than ruminal degradation accounts for the main loss of *n*-alkanes (Dove and Mayes, 1991).

Passage Kinetics Assessment of Isotope Labelled n-Alkanes

Basal diet treatments had no effect on passage kinetics in our study despite a considerable change in the dietary nutritional composition. The carbon chain length of *n*-alkanes affected various passage kinetic parameters but did not affect K_1 estimates. Overall, TMRT and total marker clearance time from the gastrointestinal tract were similar among *n*-alkanes. When compared to a commonly used external digesta passage marker, passage kinetics of ^{13}C-*n*-alkanes differed considerably from those of Cr-NDF (on average 3.86 and 5.25 %/h, respectively). No significant correlations between the two markers were observed for K_1 and TMRT ($P \geq 0.10$; Figure 3).

The discrepancy in TMRT between the two markers is in line with observations on $^{14}C/^{3}H$ labelled *n*-alkanes and Cr-NDF in small ruminants (Mayes et al., 1997). The discrepancy in K_1 between the two markers might be explained by potential differences in particle size (Bruining and Bosch, 1992) and the increased feed particle density (Ehle et al., 1984) with a resulting reduced buoyancy (Sutherland, 1988) commonly observed for Cr-NDF particles. The degree of marker association with the particulate matter might be a confounding factor throughout passage studies (Owens and Hanson, 1992), although Mayes et al. (1997) found a nearly full affinity (0.98) of those markers.

When compared to an alternative internal digesta passage marker, passage kinetics of ^{13}C-*n*-alkanes was comparable to that of ^{13}C-DM originating from the same labelled plant material (Figure 3). *n*-Alkanes were shown to associate well to the particulate DM pool (Mayes et al., 1997), which sustains the overall satisfactory resemblance in passage kinetics observed for ^{13}C-*n*-alkanes and ^{13}C-DM. Pearson correlation coefficient *r* ranged from 0.61 to 0.71 ($P \leq 0.012$; $n = 16$) for K_1 (on average 3.38 %/h), and from 0.75 to 0.80 ($P \leq 0.001$; $n = 16$) for TMRT (47.1 h). Our K_1 estimates are in line with recent data on ^{13}C labelled ryegrass silage of high and low digestibility (Pellikaan et al., 2013). They reported mean K_1 estimates of 3.52–3.85 %/h and 4.76–5.03 %/h for ^{13}C-DM and Cr-NDF, respectively. For even-chain *n*-

alkanes sprayed onto ryegrass leaves or stems, K_1 values were considerably higher (7.5–9.5 %/h; Giráldez et al., 2006), whereas K_1 values for Cr-NDF (4.0 %/h) were in line with studies using Cr-NDF in dairy cows (Bosch and Bruining, 1995; Pellikaan et al., 2013). These exceptionally high rates for forages were partly explained by the binding association of the sprayed *n*-alkanes onto the plant cell wall matrix, which can be low for a synthetic matrix (Dove and Mayes, 1991), resulting in migration of sprayed *n*-alkanes to the liquid phase (Bulang et al., 2008).

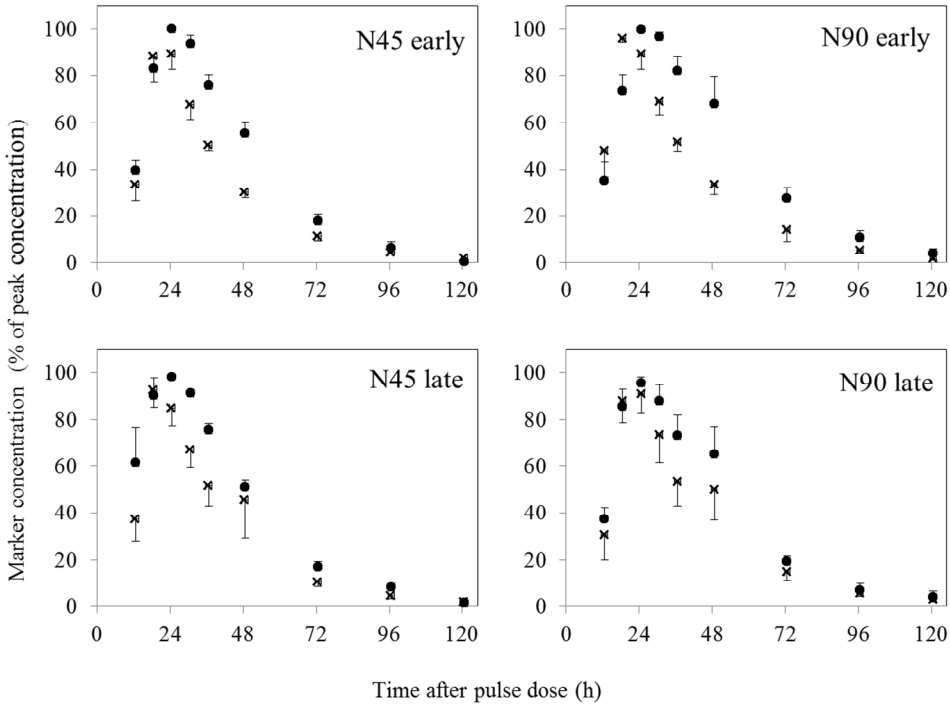

Figure 2. Mean faecal marker concentrations with standard error bars of the $\delta^{13}C$ labelled C_{31} *n*-alkane (●) and chromium mordanted fibre (×) upon an intraruminal pulse dose in dairy cows fed grass silages of early and late maturity at two nitrogen (N) fertilisation levels (N45: 45 kg N/ha; N90: 90 kg N/ha).

Observations by Mayes et al. (1997) further suggest a considerably lower binding association of dosed even-chain *n*-alkanes (0.92) compared to natural or $^{14}C/^{3}H$ labelled *n*-alkanes from ryegrass plants (0.98). In contrast to the findings of Giráldez et al. (2006), Mayes et al. (1997) reported considerably lower K_1 values of 2.85–2.98 %/h (based on even-chain *n*-alkanes sprayed onto ryegrass leaves and stems), and 2.54–2.56 %/h (based on

$^{14}C/^{3}H$-*n*-alkanes from ryegrass) for small ruminants. Assuming that animal species have a minor effect on K_1 (Colucci et al., 1990; Cannas and Van Soest, 2000), differences in feed intake level between the various studies might explain the somewhat higher K_1 values for Cr-NDF in our study. Reservations on the spraying technique, which implies an external application of the even-chain *n*-alkanes, have been expressed with regard to rare earth elements as it was observed that plant tissue did not uniformly absorb the sprayed-on marker (Ellis et al., 1982). Passage kinetics are therefore highly dependent on the absorption capacity of the plant cell wall matrix. In contrast, intrinsically isotope labelled plants will circumvent this problem, as the ^{13}C isotopes are homogeneously distributed in the plant tissue when continuously labelled in a greenhouse (Gorissen et al., 1996; Ippel et al., 2004).

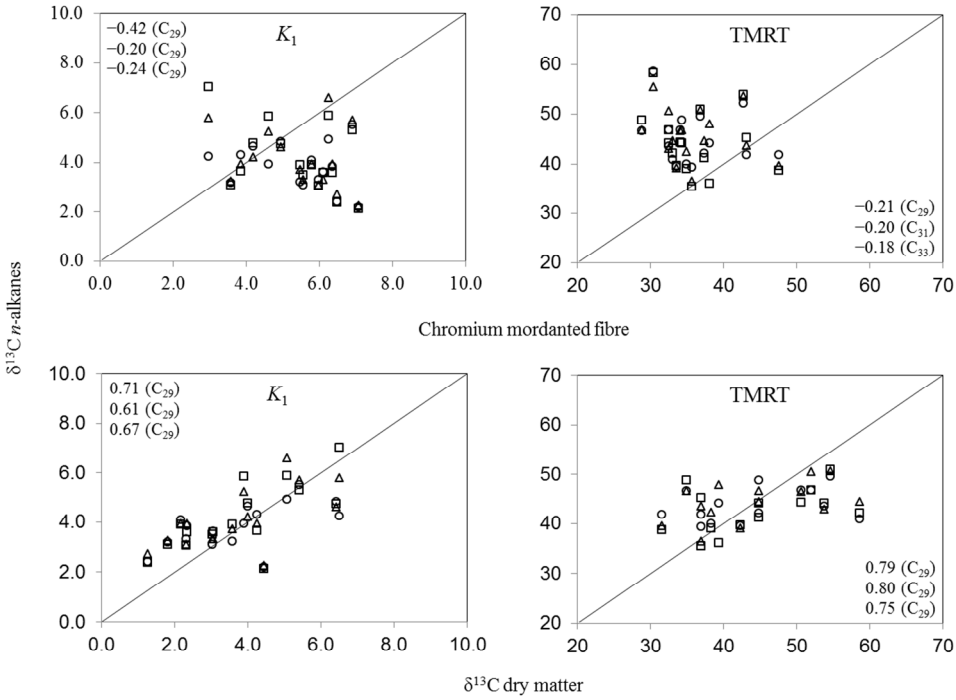

Figure 3. Relation of fractional rumen passage rate (K_1, %/h) and total mean retention time (TMRT, h) between chromium mordanted fibre or $\delta^{13}C$ labelled dry matter and $\delta^{13}C$ labelled *n*-alkanes (□ C_{29}; ○ C_{31}; Δ C_{33}). Pearson correlation coefficients shown in plots. Mean K_1 is (mean ± SEM) 3.38 ± 0.315 %/h for $\delta^{13}C$ labeled dry matter and 5.25 ± 0.490 %/h for chromium mordanted fiber; mean TMRT is 47.1 ± 2.52 h for $\delta^{13}C$ labeled dry matter and 35.7 ± 1.91 h for chromium mordanted fiber.

The Use of Carbon Isotope Labelled *n*-Alkanes for Digesta Passage Kinetic Studies

Various studies have shown that isotopes from intrinsically labelled plants can be detected in various undigested fractions of faeces and digesta of ruminants, such as in various fibre fractions (Huhtanen and Hristov, 2001; Pellikaan et al., 2013) and starch (D. Warner et al., unpublished data).[11] In addition to the plant cuticular *n*-alkanes, isotopes can be potentially detected in further plant wax components, such as in long-chain alcohols and very long-chain fatty acids, which are typically present in considerably higher concentrations than *n*-alkanes in perennial ryegrass (Ali et al., 2005; Ferreira et al., 2012). This could be of particular interest for quantifying passage kinetics of a diet composed of plant species containing low concentrations of *n*-alkanes such as some of the common temperate grass species (e.g. *Phleum pratense* and *Dactylis glomerata*; Dove and Mayes, 1991) and some tropical forages (Bezabih et al., 2011a,b).

The use of isotopic *n*-alkanes could be of particular interest for comparative passage rate studies to study the rumen physiology and its evolutionary mechanisms (Clauss and Lechner-Doll, 2001; Clauss et al., 2003) of domestic and wild ruminants (Hatt et al., 1989; Lechner-Doll and Engelhardt, 1989). The ^{13}C-*n*-alkane dose can be adjusted to the original type of diet preferably consumed on pasture by wild herbivores (Bezabih et al., 2011a,b) or by using confined herbivores or captive zoo animals under controlled housing and dietary conditions.

n-Alkanes share the linear aliphatic hydrocarbon chain with fatty acids. As the latter pass through the reticulorumen with the particle phase (Dijkstra et al., 2000), it has been suggested that also the flow characteristics of *n*-alkanes through the gastrointestinal tract might be alike (Ohajuruka and Palmquist, 1991). Passage kinetics of *n*-alkanes through the reticulorumen might be therefore similar to that of the common long-chain C_{10}-C_{18} fatty acids present in dietary lipids. These specific dietary fatty acids can be taken up by the rumen microorganisms for their own growth and the unsaturated long-chain fatty acids can undergo substantial transformation through hydrogenation by microbial enzymes (Dijkstra et al., 2000). A direct measurement of their fractional rumen passage rate is difficult because of the substantial transfer of carbon isotopes from the labelled dietary fatty acid into newly formed fatty acid compounds or microbial lipids. Knowledge on passage kinetics of *n*-alkanes might be therefore a useful indicator of rumen passage kinetics of dietary long-chain fatty acids bypassing the reticulorumen.

[11] Chapter 4 in this thesis

Incorporation of carbon isotopes into *n*-alkanes in the rumen was considered negligible (Mayes et al., 1988), providing further evidence as to the potential use of isotope labelled *n*-alkanes to measure rumen digesta passage. An early *in vivo* study with dairy cows (Bartley et al., 1971) suggested that rumen bacteria may incorporate some of the intra-ruminally dosed ^{14}C-*n*-alkane C_{18}, although no further metabolic process was observed for that specific labelled *n*-alkane by McCarthy (1964) and Bartley et al. (1971). Yet, no evidence for microbial incorporation is available with regard to the more common natural long-chain forage *n*-alkanes C_{27} to C_{35} used in our study. A recent *in vitro* study, in which ^{14}C labelled perennial ryegrass was incubated in buffered rumen fluid (Keli et al., 2008), suggested the complete absence of ruminal degradation and synthesis of long-chain *n*-alkanes by ruminal bacteria. *n*-Alkanes feature hence ideal attributes of a digesta passage marker. Furthermore, an analytical procedure has been described (Dove and Mayes, 2006) based on gas chromatography allowing for a sensitive and specific determination of *n*-alkanes.

In contrast, dietary nutrients are often subjected to extensive fermentative degradation in the rumen. Isotopes originating from an isotopic labelled diet may be incorporated into rumen microbial protein and volatile fatty acids (Alexander et al., 1969b). A bias in the prediction of K_1 can occur if marker migration occurs; for instance, if stable isotopes from dietary nutrients are incorporated into microbial biomass (Firkins et al., 1998; Pellikaan et al., 2013). Furthermore, imperfect experimental *in vivo* conditions, such as an inhomogeneous isotope distribution in the diet, non-steady state conditions in the rumen or irregular nutrient uptake, might result in a different $\delta^{13}C$ of the indigested nutrients relative to that of the original nutrients ingested by the animal, thereby affecting respective fractional passage rates. The overall satisfactory resemblance in passage kinetics of ^{13}C-*n*-alkanes and dietary ^{13}C-DM observed in this study supports earlier studies on the use of stable isotopes to measure rumen passage of dietary feed nutrients (Pellikaan et al., 2013; D. Warner et al., unpublished data).[12]

Conclusions

Passage kinetics of ^{13}C labelled *n*-alkanes are rather comparable to that of the dietary dry matter originating from stable isotope labelled ryegrass plants but differs considerably from that of the external marker Cr-NDF. In combination with evidence from literature as to the absence of microbial involvement in the passage of *n*-alkanes, our results suggest that stable

[12] Chapter 3–5 in this thesis

isotopes are an appropriate tool to assess passage kinetics of *n*-alkanes and dietary nutrients through the gastrointestinal tract of ruminants.

Acknowledgments

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Chapter 7

General discussion

Introduction

Rumen fractional passage rates (K_1) are key parameters in the empirical equations used in current feed evaluation systems for ruminants, and also are key parameters in mechanistic models of rumen fermentation processes. While various feed evaluation systems seek a more dynamic mechanistic approach to model animal response (Dumas et al., 2008), data on nutrient-specific K_1 are scarce and are often hampered by the marker techniques currently in use.

Investigations on suitable markers to estimate retention times in ruminants started as early as in the 1920s with simple marker techniques, such as dyes to stain feed particles (Balch, 1950). Thereafter, investigations focused primarily on external markers as they are not inherent to the ingested feed and can be easily distinguished from the bulk material. However, this may also imply that external marker do not always reflect the passage behaviour of the feed particles they are associated with. In particular, external markers are likely to affect feed particle degradability and they may dissociate from feed particles (Smith, 1989; Owens and Hanson, 1992). The use of internal markers is thus preferred but requires time- and labour-intensive rumen evacuations or slaughter trials, which do not provide information on the kinetics of passage. Both techniques described above may be strictly relevant for the particle pool they are associated with and are not suitable to describe nutrient-specific passage. Nonetheless, external markers are commonly used to estimate K_1 in some common feed evaluation systems, such as in the NRC system (NRC, 2001), the CNCPS system (Fox et al., 2004), the FiM system (Thomas, 2004) and the DVE system (van Duinkerken et al., 2011). The rumen evacuation technique is the reference method for estimating K_1 of the NDF fraction in the NorFor system (Volden, 2011).

In contrast to external markers, stable isotopes are present in the feed in small amounts and it was suggested to use faecal ^{13}C concentrations in faeces to determine digesta passage (Svejcar et al., 1993; Südekum et al., 1995). This technique was further applied to ^{15}N isotopes (Huhtanen and Hristov, 2001). These studies limited the use of stable isotopes to strictly indigestible material and were not based on the relative abundance of the respective stable isotope in relation to the main isotope (i.e. the ratio ^{13}C:^{12}C or ^{15}N:^{14}N, commonly denoted as δ). It has been shown that no preferential disappearance of ^{13}C in the gastrointestinal tract (GIT) occurred upon microbial degradation *in vitro* (Pellikaan et al., 2013) and *in vivo* (L.M.M. Ferreira et al., unpublished). The $\delta^{13}C$ was hence recommended as a tool to determine digesta passage kinetics.

In this general discussion, major research findings on the use of stable isotope labelled feed components in relation to conventional marker techniques (Chapter 2) and as a tool to assess feed component specific passage kinetics of concentrates (Chapter 3), maize silage (Chapter 4), grass silage (Chapter 5) and compound specific passage of plant *n*-alkanes (Chapter 6) will be discussed. Implications of stable isotopes for feed evaluation and compartmentalisation of the digestive tract will be discussed and recommendations for future research will be provided.

Use of Stable Isotopes as Passage Markers

Stable Isotopes in Relation to Conventional Marker Techniques

In the preceding chapters, $\delta^{13}C$ and $\delta^{15}N$ were used to assess passage kinetics of different feed types and feed components. In Figure 1, passage kinetic parameters are summarised for fibre and total DM originating from grass silage, maize silage and concentrates based on $\delta^{13}C$, and compared with a conventionally used external marker (chromium mordanted fibre; Cr-NDF). Results across experiments suggest that Cr-NDF gave higher K_1 values than $\delta^{13}C$ for forages (Figure 1). The same trend was observed when Cr-NDF was compared with an indigestible internal marker following rumen evacuations in small and large ruminants as reviewed by Aitchison et al. (1986) and Krizsan et al. (2010a), respectively.

Overall, Cr-NDF appears to be less sensitive to changes in feed type than $\delta^{13}C$ and rather reflects the passage behaviour of the indigestible small-particle rumen pool as suggested by Bosch and Bruining (1995). Passage kinetics for $\delta^{13}C$ further provided useful information specific to the individual marker behaviour in the GIT. Although differences in K_1 between forage types were small when measured with Cr-NDF and ^{13}C labelled fibre fractions (Figure 1) and in line with previous studies (Mulligan et al., 2002; Krämer et al., 2013b), differences in passage kinetics were more clear than K_1: fractional passage from the proximal colon-caecum (K_2) were higher with grass silage marker compared with maize silage, peak concentration time (PCT) total mean retention time (TMRT) were higher with maize silage; the latter was in line findings of Krämer et al. (2013b). These results suggest that the fibre structure considerably differs among the two forages. This is in line with observations by De Boever et al. (1993a,b) and Mulligan et al. (2002), who observed a higher rumination index and rumen fill for grass silage than for maize silage, and with observations by Bruining et al. (1998), who observed a lower particle size reduction rate for grass silage compared to maize silage. It should be noted

that feed intake levels between the two experiments differed (16.7 *vs*. 21.7 kg DM/d for grass silage and maize silage, respectively), and previously a higher feed intake level was reported to increase K_1 (Robinson et al., 1987; Tamminga et al., 1989a; Colucci et al., 1990). However, in a completely randomised block design, Krämer et al. (2013b) did not observe significant effects of forage type on K_1 at equal feed intake levels. Potential differences in forage particle length might explain some of the variation in K_1 between forage types, although literature suggest that maize particle size has only marginal effects on K_1 (Turnbull and Thomas, 1987).

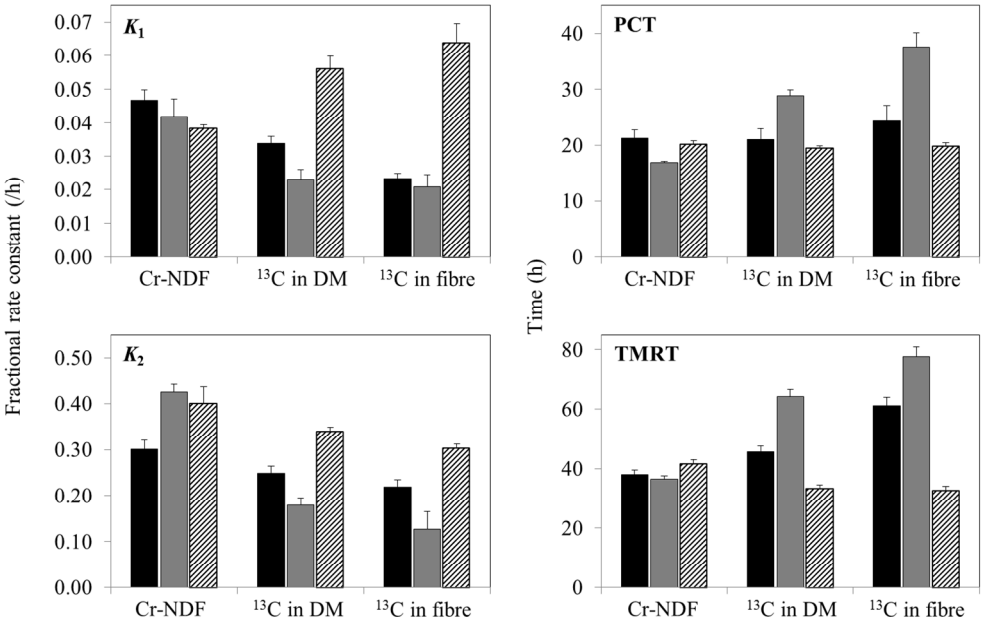

Figure 1. Fractional passage rates from the reticulorumen (K_1) and from the proximal colon-caecum (K_2), marker peak concentration time (PCT) and total mean retention times (TMRT) with standard error bars for an external marker (chromium mordanted fibre, Cr-NDF) and for the internal marker ^{13}C in the dry matter (DM) and fibre fractions from faecal marker excretions. Solid black bars, grass silage (Chapter 5); solid grey bars, maize silage (Chapter 4); striped bars, concentrates (Chapter 3).

Interestingly, in comparing passage behaviour of grass silage versus maize silage, the opposite effect was observed with Cr-NDF compared to stable isotopes (Figure 1). Using Cr-NDF as a marker, TMRT appeared to be slightly higher for maize silage than for grass silage, which is in line with results (numerical only) using rare earth elements (Krämer et al., 2013b).

Furthermore, Cr-NDF provided lower K_1 values than $\delta^{13}C$ for concentrates. Separation into the total DM and fibre fraction based on $\delta^{13}C$ revealed that fibre passage of concentrates was high and similar to that of the more digestible DM fraction.

The particle size differed among markers, which might explain part of the observed differences between markers. Bruining and Bosch (1992) reported large differences in ruminal passage rates being faster with decreasing Cr-NDF particle size. They further recommended a marker particle size distribution comparable to that of the rumen particle pool associated with the marker. The internal markers $\delta^{13}C$ and $\delta^{15}N$ were thus introduced into the rumen as enriched forage plants chopped to pieces of approximately 0.5 cm to resemble ingested feed particles in the rumen, whereas the small-particle marker Cr-NDF was ground to 0.5 mm to resemble the particle size distribution of the small-particle pool leaving the reticulorumen. The smaller particle size of Cr-NDF compared to the ^{13}C marker might therefore explain to a big part the higher K_1 values observed for Cr-NDF with forages. However, the opposite was observed with concentrates, which were administered orally in pelleted form (ground to 2.75 mm). As the difference between Cr-NDF particle size and the (hypothetical) ^{13}C particle size was even lower for concentrates than forages, it can be assumed that other factors were more important. For instance, particle density was observed to be high for concentrates and to depend on feedstuff (Ramanzin et al., 1994), which might explain the high K_1 values observed for concentrates. Potential differences in particle density between concentrate ^{13}C and Cr-NDF might explain part of the observed variation in K_1 among markers.

The external marker Cr-NDF was considered a reference passage marker for particulate matter, as some common feed evaluation systems (e.g. Thomas, 2004; van Duinkerken et al., 2011; Volden, 2011) entirely or partly predict K_1 based on studies using Cr-NDF. Other particle or solid phase associated external markers in addition to Cr-NDF were not evaluated in this thesis. Liquid phase associated external markers were not evaluated either but might be of interest to assess the passage of soluble nutrients, peptides or rumen fermentation end-products (reviewed by Seo et al., 2006a). Chromium mordanted NDF (ground to 0.5 mm in this thesis) most probably reflects passage of small particles of that defined pool size (Bosch and Bruining, 1995). In contrast, Cannas and Van Soest (2000) postulated that Cr-NDF should be considered as a passage marker of the NDF fraction as chromium is typically mordanted to the fibre fraction (Udén et al., 1980). However, data on stable isotope labelled forage fibre rather support the theory based on particle size proposed by Bosch and Bruining (1995).

Difference in particle density may additionally explain some of the observed differences between markers. The use of markers may affect density of particles that the marker is

associated with. For instance, the chromium complex may increase the density of the mordanted feed particle (Udén et al., 1980; Bruining and Bosch, 1992; Owens and Hanson, 1992), resulting in a higher probability of escape (Sutherland, 1988).

Opportunities and Limitations of Using Stable Isotopes as Passage Markers

Dietary nutrients can be quite extensively fermented in the rumen by microorganisms, and isotopes originating from an isotopic labelled diet may be transferred to rumen microbial protein and volatile fatty acids (Alexander et al., 1969b). If this extensive microbial activity causes a shift in the isotopic ratio (e.g. ^{13}C to ^{12}C), fractional passage rates of $\delta^{13}C$ cannot be accurately quantified. Residues upon *in vitro* fermentation of a ^{13}C labelled grass silage were shown not to be depleted in ^{13}C relative to ^{12}C (Pellikaan et al., 2013). However, conclusions based on *in vitro* data could be questioned in an *in vivo* situation with more extensive dynamics in the microbial population or for a less controlled environment (such as for non-steady state conditions in the rumen or in case of irregular nutrient uptake due to diet selection or a low feeding frequency). For instance, ruminal microorganisms might incorporate ^{13}C loaded nutrients for their own maintenance and growth, and pass from the reticulorumen at a different fractional passage rate than the undigested nutrients, resulting in marker migration from cellular to microbial protein. It was suggested that with high-energy diets less than 2% of the total carbon excreted in faeces was of microbial origin but may increase to 12–24% when damaged microbial material (mainly originating from the rumen) is included (van Vliet et al., 2007). However, passage data of stable isotope labelled *n*-alkanes from grass silage (Chapter 5) suggest that marker migration may have a rather small confounding effect with forages. The latter components are not degraded in the rumen in contrast to the ^{13}C labelled grass silage DM fraction, although disappearance from the small intestine was reported for *n*-alkanes (Dove and Mayes, 1991), but both components had comparable passage kinetics. Furthermore, L.M.M. Ferreira et al. (unpublished) quantified carbon isotopes in *n*-alkanes by total faecal collection in goats receiving forages with ^{13}C labelled *n*-alkanes and results indicate that the $\delta^{13}C$ did not change in the GIT. However, these results are not validated for the specific diets used in this thesis and microbial activity has been found to depend on diet quality (van Vliet et al., 2007). The latter might explain why fractional passage rates of concentrates were not lower for the slower degradable ^{13}C-NDF compared with the quicker degradable ^{13}C-DM as was observed for the forages.

Two different marker application procedures were described in this thesis. With regard to concentrates (Chapter 3), ^{13}C was introduced by exchanging a concentrate diet originating

from cool-season C_3 plant ingredients (low ^{13}C abundance) with a concentrate diet originating from warm-season C_4 plant ingredients (high ^{13}C abundance). This procedure allowed the administration of a large pulse dose (9 kg DM administered orally) with low enrichment. With regard to forages, stable isotopes originated from either maize silage (Chapter 4) or grass silage (Chapter 5) enriched above natural abundance under greenhouse conditions. This procedure allowed the administration of a higher enriched but small pulse dose (≤ 30 g DM administered intraruminally). Figure 2 summarises the mean prediction error (MPE; Bibby and Toutenburg, 1977) as a measure of curve fit accuracy for various markers used in the experiments outlined in this thesis. While Cr-NDF showed the highest curve fit accuracy, its MPE was comparable across studies indicating no effect of how markers were administered (oral *vs*. intraruminal). In contrast, ^{13}C had considerably higher MPE values when forages were fed, indicating a poorer curve fit. This was not the case for concentrates, however (Figure 2). These results suggest that curve fit accuracy is related to the pulse dose size, probably due to a differential mixing behaviour in the rumen.

If a larger pulse dose is not available, an alternative could be to manually mix the marker with (partially) emptied rumen content. However, this could affect the rumen content stratification during the first few hours of passage estimation. If an insufficient marker distribution is expected (e.g. with inhomogeneous material such as maize silage; De Boever et al., 1993a), manual mixing in pre-evacuated rumen content may be considered nonetheless. Figure 2 indicates a higher MPE for faecal ^{13}C from maize silage compared with that from grass silage, which might be due to a more difficult rumen mixing behaviour of the maize silage dosed intraruminally in low amounts. The use of a highly enriched small pulse dose may be however of advantage if pulse dosing a large amount of labelled feed is expected to affect the microbial degradation in the rumen. Furthermore, it adds some flexibility to the labelling procedure under greenhouse conditions. In principle, the feed marker should be of similar quality of the basal diet. Feeding low amounts of a highly enriched marker will less likely affect the rumen environment and potential differences between the labelled and unlabelled diet may become less important.

The isotope signature of labelled feed is currently the sole available procedure to label and measure passage of digestible feed components. The intrinsic stable isotope labelling procedure described in this thesis is particularly adapted to produce uniformly enriched plant material (Gorissen et al., 1996; Ippel et al., 2004). However, the labour input and costs of a continuous labelling procedure can be considerable, in particular with respect to photosynthetic ^{13}C assimilation procedures, and prohibit its application in large-scale animal

experiments. Labelling costs can be reduced by pulse labelling plants on several occasions during the growing period. Pellikaan et al. (2013) and Smith and Erdman (1986) reported uniform isotopic distribution with a labelling regimen of six to eight (^{13}C labelled grass silage) and four times (^{14}C labelled lucerne), respectively.

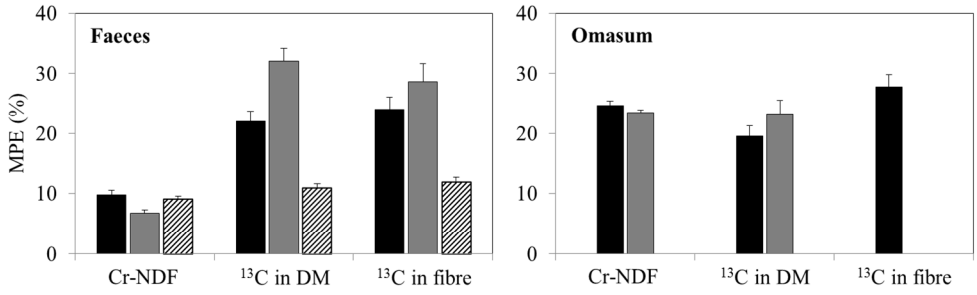

Figure 2. Curve fit accuracy as measured by the mean prediction error (MPE) with standard error bars for an external marker (chromium mordanted fibre, Cr-NDF) and for the internal marker ^{13}C in the dry matter (DM) and fibre from faecal and omasal marker excretions. Bars show ^{13}C labelled forages and concentrates pertaining to various experiments: grass silage (solid black bars; Chapter 5), maize silage (solid grey bars; Chapter 4), and concentrates fed to dairy cows (striped bars; Chapter 3). Forages were labelled above natural enrichment and administered intraruminally at small doses (≤ 30 g DM); concentrates were at natural enrichment and administered orally at high doses (9 kg DM).

Compartmentalising the Digestive Tract

The GIT of a ruminant can be mechanistically described as a series of mixing compartments, each with distinctive mixing and passage processes, and flow segments. The reticulorumen plays a central role within the GIT and essentially acts as a continuous-flow mixing compartment (Penry and Jumars, 1987) controlling the passage of ingested feed by selective retention (Allen and Mertens, 1988). Ruminal passage of feed particles is counterbalanced by the microbial ruminal degradation competing for the same substrate. This competitive passage is less an issue in post-ruminal compartments essentially being pure flow segments, except for the proximal colon-caecum in which digesta is mixed and some microbial fermentation occurs (Dixon et al., 1982). Conventionally, K_1 is estimated from the decay in faecal concentrations of a digesta marker following an initial pulse dose. Disappearance of the marker upon oral or intraruminal administration typically follows a nonlinear dilution curve. K_1 in the different GIT segments can be either estimated by linear regression following linearization through

logarithmic transformation, or, more accurately, by nonlinear regression using mathematical compartmental models.

Choice of Compartmental Model

Various models were developed to represent the mixing compartments and flow segments within the GIT and estimate passage kinetics through the main mixing compartments. The first model following a sequential approach was proposed by Blaxter et al. (1956) describing digesta mixing in two compartments (representing a slow and fast pool) and digesta flow between the fast pool and faeces. The two compartments were later identified as the reticulorumen and the proximal colon-caecum, respectively (Grovum and Williams, 1973). Based on this concept, several deterministic models were proposed and modified such to accommodate 3 or 4 mixing compartments (France et al., 1985) or *n* compartments with a multicompartmental model (MC model; Dhanoa et al., 1985), and some specific aspects such as viscosity and diffusion (France et al. 1993; Thornley et al. 1995).

A derivative of the Blaxter model was proposed (G*n* model; Ellis et al., 1979) based on the notion of increasing gamma time dependency in the first compartment to describe age-discriminating processes involved in particle outflow from the reticulorumen (Matis, 1972). Later the G*n* model was modified to accommodate a two-pool system (G*n*G1 models; Pond et al., 1988) as well as more specific aspects such as the probability of backflow of small particles into the rumen raft pool (Poppi et al., 2001). In contrast to the original Blaxter model, the G*n* type models have a stochastic approach based on increasing gamma age dependency ($n \geq 1$). The most suitable G*n* type model is typically chosen based on the best fitting accuracy and on the purpose and conditions of the experiment, regardless if the biological system reflects a one-pool, two-pool or higher *n* pool system. Model choice can then become challenging and less of a straightforward procedure because increasing the order of gamma age dependency increases retention time in the age-dependent pool.

In Figure 3, gradually increasing age dependency (gamma *n*) in G*n* type models improved the curve fit accuracy in faecal marker dilution curves (decreased MPE), eventually approaching a MC model in curve shape and fitting accuracy, but the MC model remained superior. However, the opposite was observed for omasal marker dilution curves, although they were again best fitted with the MC model. Sampling site affected thus the fitting accuracy of G*n* type models and higher order G*n* type models did not by default improve the curve fit when sampling more proximate to the reticulorumen, which is line with observations

of Wylie et al. (2000). Furthermore, fitting accuracy of G*n* type models depended on the marker used (Figure 3).

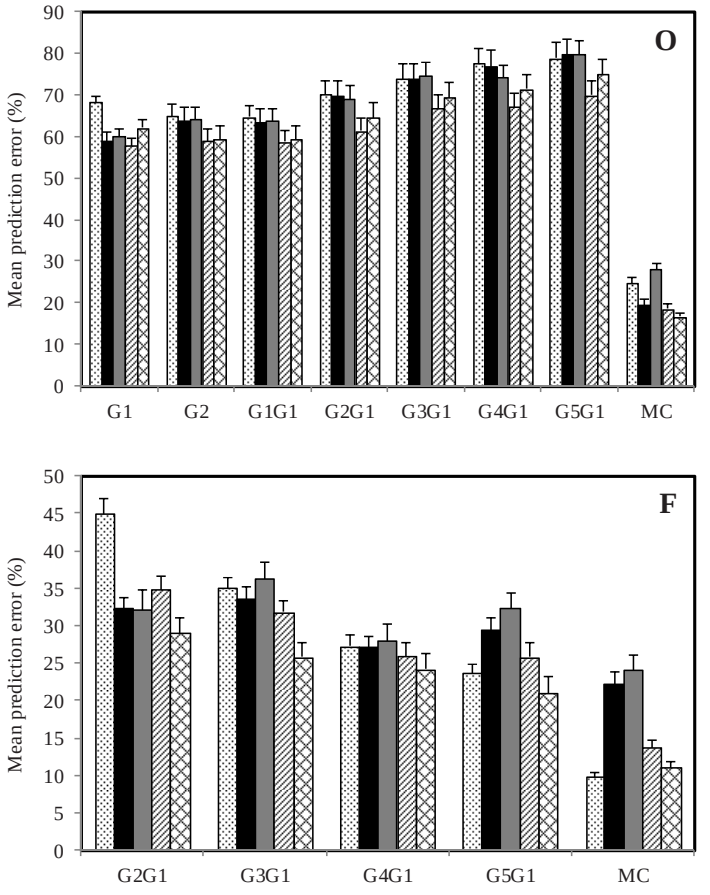

Figure 3. Model accuracy (mean prediction error with standard error bars based on 34 curve fits per marker) for different markers in omasal digesta (O) and faeces (F) from dairy cows fed grass silage (data from Chapter 5). Models tested were one-pool age-independent (G1) and age-dependent models (G2), two-pool age-independent (G1G1) and age-dependent models with increasing order of gamma distribution (G*n*G1, *n* = 1–5; Pond et al., 1988), and an age-independent multicompartmental model (MC; Dhanoa et al., 1988). Markers: chromium mordanted fibre (Cr-NDF; dotted bars), ^{13}C in dry matter (^{13}C-DM; black bars), ^{13}C in acid detergent fibre (^{13}C-ADF; grey bars), ^{15}N in dry matter (^{15}N-DM; striped bars), ^{15}N in acid detergent fibre (^{15}N-ADF; diamonds). G1, G2 and G1G1 models not shown for faeces due to poor model convergence.

Hence, increasing age dependency is not based on biological mechanisms but simply on each particular model's fitting accuracy. This implies that marker dilution curves established under different experimental conditions could be in principle each fitted with a different G*n* type model, depending on the marker, sampling site and animal. Furthermore, these observations indicate a certain bias in estimating the retention time of the age-dependent pool with G*n* type models (essentially K_1 with a MC model), as higher order G*n*G1 models by default improve the fit of faecal dilution curves. The underlying concept of such models differs thus from that of a deterministic compartmental model such as the MC model, which gives a point estimate for fractional passage in the succeeding compartments.

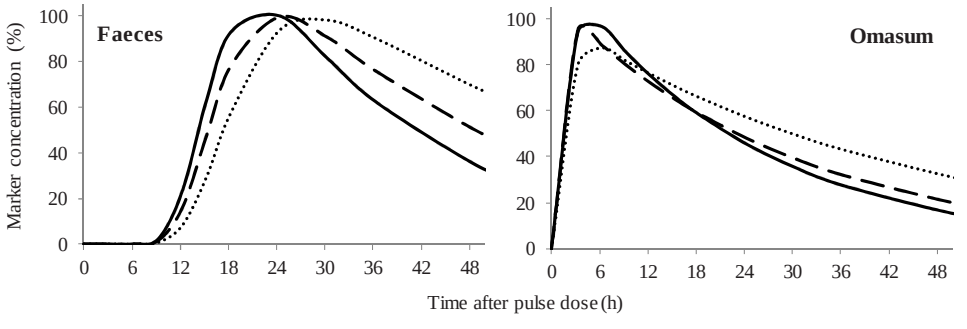

Figure 4. Marker excretion curves based on mean faecal and omasal digesta marker concentrations (n = 34 per marker) in dairy cows fed diets containing various grass silage treatments (data from Chapter 5). Markers: chromium mordanted fibre (Cr-NDF; solid line), ^{13}C in dry matter (^{13}C-DM; dashed lined), ^{13}C in acid detergent fibre (^{13}C-ADF; dotted line). Marker concentrations were scaled to the peak concentration and fitted with a multicompartmental model (Dhanoa et al., 1985).

Effects of model type on K_1 are not shown but can be considerable (Pond et al., 1988; Moore et al., 1992). If such values are reported, they must be treated with caution as biological models should be chosen based on their applicability and underlying biological meaning rather than on the their estimated output parameters. Results across experiments suggest that the deterministic MC model was the most universally applicable model describing K_1 of different feed types both from faecal and omasal digesta samples (see Chapter 3, Chapter 4, and Figure 3). The MPE was always lowest with the MC model compared with the G*n* type models. These results were confirmed for omasal sampling (Chapter 4; Figure 3). Despite the unique shape of omasal dilution curves with a particularly

short ascending curve phase (0.66–1.34 h across markers) (Figure 4), they could be best fitted with the MC model. Similar observations were made for faecal and ileal excretion patterns by Pellikaan (2004), who further reported a better model robustness for the MC model. Overall, from a purely model performance point of view, our findings indicate a better fitting accuracy and applicability of the MC model.

Effect of Sampling Site on Fractional Passage Rates and Implications for Digestive Tract Compartmentalisation

Rumen passage kinetics are conventionally estimated from marker excretions patterns in faecal grab samples in intact animals. It is generally assumed that the compartment with the highest retention time is associated to the reticulorumen, but effects related to particle retention in succeeding segments might interfere when rumen K_1 is estimated from faecal excretion patterns. This can be circumvented by sampling more proximate to the reticulorumen; e.g. by sampling intestinal chyme or by sampling omasal or reticular digesta. Intestinal sampling requires surgical intestinal cannulation and is therefore an invasive technique that may further lead to post-surgical complications. Advantages and limitations of different intestinal cannulation techniques have been reviewed elsewhere (Harmon and Richards, 1997; Titgemeyer, 1997). In contrast to intestinal sampling, omasal and reticular sampling can be performed through the rumen fistula from intact animals with intestines not translocated from their normal position and are more desirable with respect to animal welfare issues. Reticular sampling has been shown to provide comparable flow estimates to omasal sampling (Krizsan et al., 2010b) but frequent sampling required for passage rate estimations is hampered by the difficulty of fixing a sampling device permanently into the reticulum. In contrast, omasal sampling is done via a sampling device fixated into the omasal canal and, hence, allows frequent sampling without the necessity to open the rumen fistula (Huhtanen et al., 1997). A limitation of this technique is a sometimes frequently blocked sampling device, particularly with coarse diets as confirmed by Brito et al. (2007). This could explain the somewhat lower curve fit accuracy obtained with omasal compared with faecal sampling (overall, MPE increased by 4.4% for omasal marker excretion curves). Interestingly, while curve fit accuracy of faecal markers excretion patterns was lowest for Cr-NDF, curve fit accuracy of omasal excretion patterns did not seem to depend on marker type (Figure 2). These results indicate that K_1 estimations derived from omasal sampling were to some extent more variable than those derived from faecal sampling.

As discussed above, the MC model ascribes fractional passage rates to two distinct pools, most likely representing the reticulorumen (K_1; "slow" compartment) and the proximal colon-caecum (K_2; "fast" compartment) with faecal sampling (Dhanoa et al., 1985). In contrast, stochastic two-compartmental G*n*G1 models ascribes a first *K* value to a "slow" turnover pool (essentially K_1 in the MC model) representing particle escape from the reticulorumen, and a second *K* value (conventionally denoted λ_2 but essentially K_2 in the MC model) to a "fast" rumination pool representing age-dependent lag fermentation processes in the reticulorumen (Wylie et al., 2000). Hence, particle retention in the large intestine is disregarded. Post-ruminal mixing is defined as transit time and accounts for approximately 10 h (Wylie et al., 2000). If faecally derived K_2 were to reflect age-dependent rumination processes, sampling more proximal to the reticulorumen should in principle yield comparable K_2 values. However, the numerical similarities observed between faecally and omasally derived K_1 in this thesis (although relationships were moderate for different grass silage qualities: on average, r = 0.47; Chapter 5), rather support the deterministic concept of the MC model assigning K_2 to a postruminal mixing compartment and assigning transit time to tubular digesta flow (i.e. flow between the reticulorumen and the proximal colon-caecum).

These findings are supported by data of Pellikaan (2004), who compartmentalised the digestive tract based on various sampling (ileum, faeces) and dosing sites (rumen, abomasum, ileum). With faecal sampling, TT increased from ileal to abomasal and to ruminal dosing, hence likely reflecting tubular digesta flow. Retention time in the "slow" compartment upon ileal and abomasal dosing was similar and matched well with the retention time in the "fast" compartment upon ruminal dosing. Similar findings were reported based on faecal excretion of external markers (Wylie et al., 2000), which provided a comparable retention time in the "slow" compartment upon abomasal dosing and in the "fast" compartment upon ruminal dosing. In the present thesis, K_2 upon omasal digesta sampling was associated to rumen-related processes comparable to λ_2 (i.e. the "fast" compartment) in G*n*G1 models upon faecal sampling. This assumption is in part supported by Figure 4 showing that omasal marker PCT is in line with the starting point of the ascending faecal marker excretion phase. Extrapolation of these results should be avoided however, as omasal K_2 estimates were based on an average of only 2 to 4 data points depending on marker type, with obvious implications for omasal PCT estimation. Overall, these results suggest that retention time in the "fast" compartment reflects post-ruminal mixing and varies with marker type as confirmed in this thesis.

Implications for Feed Evaluation

The Relationship between Ruminal Degradation and Passage Kinetics

Ruminal degradation of feed particles occurs within the timeframe particles are retained in the rumen. Passage of feed particles depends on their effective particle density or buoyancy characteristics (Sutherland, 1988). The probability of escape is considerably lower for buoyant particles and is determined by the amount of fermentation gases entrapped within particles. This implies that the release of gases, including carbon dioxide and methane, upon microbial fermentation can decrease the specific gravity of feed particles and delay passage. A higher fermentative degradation rate of feed particles may thus reduce their probability of escape from the reticulorumen. Table 1 summarises significant ($P \leq 0.10$) linear relationships between K_1 of various tested passage markers and rumen fractional degradation rates (K_D) of feed fractions from maize silages (n = 6) and grass silages (n = 6), estimated from *in situ* nylon bag incubations. Generally, coefficients of determination (R^2) for linear regressions were low. No relationship between the starch K_D and K_1 were observed. Stable isotope based K_1 values appeared to be somewhat better related to K_D than Cr-NDF based K_1, particularly with respect to the cell wall fractions. $\delta^{15}N$ was the major determinant of ADF in particular. K_1 estimated from omasal digesta samples was not particularly better related to K_D than faecally determined K_1. The weak linear relationships observed here might be due to the low number of treatments tested. It has been further suggested that particle density alone might not fully describe rumen escape and that particle density is correlated to particle size (Sutherland, 1988). This might explain the week relationship observed between K_D and K_1 of Cr-NDF for which the particle size is defined (<0.5 mm).

Prediction of Effective Rumen Degradability and Protein Supply

Common feed evaluation systems integrate knowledge on fractional degradation and passage to evaluate the protein status and to predict protein supply to the small intestine. Prediction of the amount of protein available for digestion depends on the effective rumen degradability (ED) and may hence vary considerably with K_1. The Dutch feed evaluation system (DVE system; van Duinkerken et al., 2011) assumes a fixed K_1 for the protein and starch fraction (0.045/h for forages), whereas K_1 of the NDF fraction is empirically determined from the respective K_D. Other feed evaluation systems are more dynamic to some extent as reviewed in Chapter 2. In Figure 5, ED coefficients for CP and NDF are illustrated based on variable K_1

values. Coefficients were calculated based on the experimentally derived K_1 of maize silage and grass silage, and regressed with ED coefficients based on K_1 values as assumed by the DVE system. Overall, ED of our test forages was lowest when based on fixed passage rates (DVE). Based on the latter, ED coefficients (g/g DM) for CP and NDF were 0.49 and 0.21 for maize silage, respectively, and 0.58 and 0.35 for grass silage, respectively. Coefficients based on the external marker Cr-NDF were always lower, and closer to those based on fixed K_1 values (DVE), compared with stable isotopes. Small-particle markers like Cr-NDF were typically used as reference markers in feed evaluation systems, which explains the closer association observed for Cr-NDF in Figure 5. It should be noted that for grass silage NDF, low correlations among ED coefficients were observed.

Table 1. Linear relationship between the fractional degradation rate (K_D) of various feed fractions and fractional passage rate (K_1) of various markers

Linear regression model	Marker	Site	R^2
Dry matter			
0.016 (0.0081) + 0.366 (0.1675) × K_1	Cr-NDF	Faeces	0.11*
0.020 (0.0057) + 0.444 (0.1717) × K_1	^{13}C-DM	Faeces	0.15*
0.020 (0.0052) + 0.369 (0.1345) × K_1	^{13}C-DM	Omasum	0.16**
Organic matter			
0.023 (0.0071) + 0.371 (0.2157) × K_1	^{13}C-DM	Faeces	0.07†
0.020 (0.0064) + 0.411 (0.1642) × K_1	^{13}C-DM	Omasum	0.14*
Crude protein			
0.016 (0.0110) + 0.592 (0.2275) × K_1	Cr-NDF	Faeces	0.15*
0.024 (0.0079) + 0.628 (0.2376) × K_1	^{13}C-DM	Faeces	0.15*
0.026 (0.0073) + 0.504 (0.1874) × K_1	^{13}C-DM	Omasum	0.16*
Neutral detergent fibre (NDF)			
0.018 (0.0073) + 0.386 (0.1874) × K_1	^{13}C-DM	Omasum	0.10*
Acid detergent fibre (ADF)			
0.015 (0.0097) + 0.584 (0.2952) × K_1	^{15}N-ADF	Faeces	0.14†
0.018 (0.0080) + 0.431 (0.2087) × K_1	^{15}N-DM	Omasum	0.15*
0.023 (0.0061) + 0.296 (0.1610) × K_1	^{15}N-ADF	Omasum	0.12†

Model: K_D = intercept + slope × K_1. Standard errors of the mean given in parenthesis.
Markers: Cr-NDF = chromium mordanted fibre; ^{13}C-DM = ^{13}C in the dry matter; ^{15}N-DM = ^{15}N in the dry matter; ^{15}N-ADF = ^{15}N in the acid detergent fibre.
† $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; only relationships with $P \leq 0.10$ are shown.

Overall, the calculated ED values are low when compared to the observed total tract apparent digestibility of starch and fibre. In theory, hindgut fermentation should be particularly high for total digestive tract degradation (ED + contribution from hindgut fermentation) to approximate total tract digestibility of starch and NDF. However, contributions from hindgut fermentation (estimated from digestibility studies) were observed

to account up to 13% of total tract fermentation for starch from maize silage (Sutton et al., 2000) and to be minor for NDF from fresh perennial ryegrass (Owens et al., 2008a; Peyraud et al., 1997).

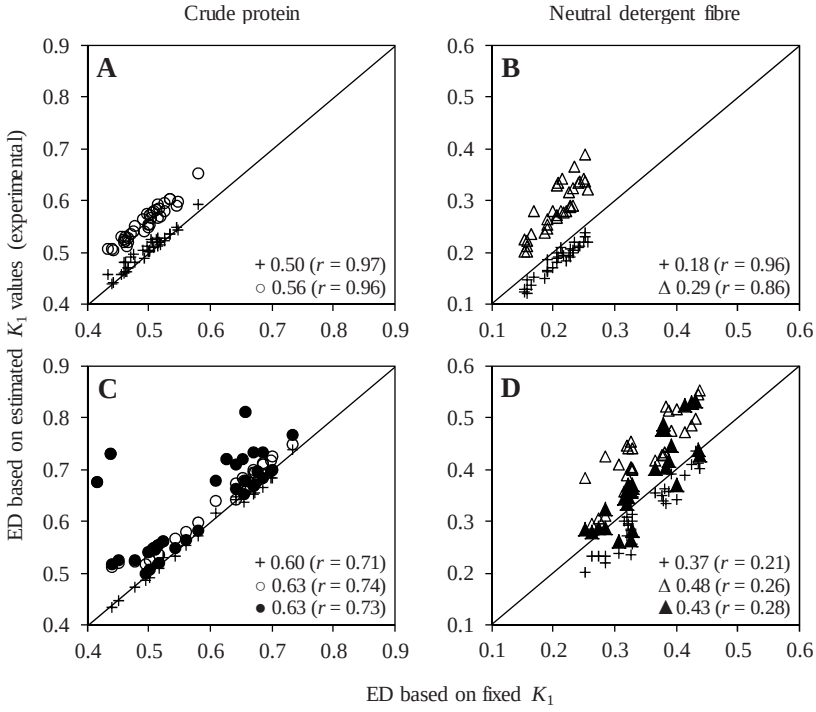

Figure 5. Relationship between different calculations of effective rumen degradability (ED; g/g DM) of maize silage (A-B) and grass silage (C-D) of varying quality. The X-axis shows a reference ED coefficient based on fractional rumen passage rates (K_1) as assumed by the Dutch protein evaluation system (DVE; 0.045/h for forage CP; established from the respective fractional degradation rate for forage NDF); A = 0.49, B = 0.21; C = 0.58, D = 0.35. The Y-axis shows estimated ED coefficients based on experimentally determined K_1 from faecal excretion of chromium mordanted fibre (Cr-NDF; +), ^{13}C in the dry matter (^{13}C-DM; ○), ^{13}C in the acid detergent fibre (^{13}C-ADF; Δ), ^{15}N in the dry matter (^{15}N-DM; ●), or ^{15}N in the acid detergent fibre (^{15}N-ADF; ▲). ED coefficients were calculated according to and DVE K_1 values were derived from van Duinkerken et al. (2011). Data from Chapter 4 (maize silage) and Chapter 5 (grass silage).

Based on the varying ED coefficients observed above, it can be assumed that K_1 has a large impact on the actual protein supply. Amounts of undegraded feed protein escaping degradation in the rumen (RUP) and intestinal digestible RUP were calculated after van

Duinkerken et al. (2011) for low digestible (a combination of late maturity and low N fertilisation) and high digestible grass silage (a combination of early maturity and high N fertilisation) (Figure 6). Both RUP and intestinal digestible RUP are highly sensitive to K_1. Both parameters were generally highest with the fixed DVE derived K_1 (0.045/h) and lowest with the variable stable isotope derived K_1 values. Overall, these results imply that the amount of protein available for digestion might be higher when K_1 is estimated with a small-particle marker. Stable isotopes appear to be a promising tool to improve the predictions of protein supply in the equations used in current feed evaluation systems. These findings should be validated over a wider range of dietary and experimental conditions.

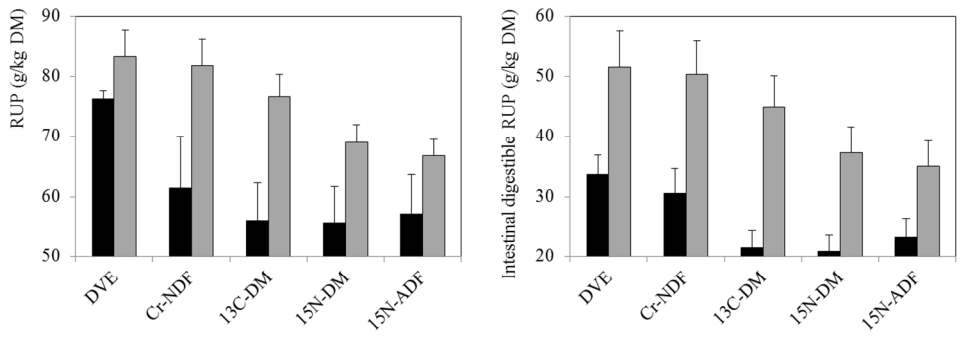

Figure 6. Amounts of rumen undegraded protein (RUP) and intestinal digestible RUP with respective standard error bars estimated with variable fractional rumen passage rates (K_1). Effects of low digestible (black bars) *vs.* high digestible grass silage (grey bars) are shown (data from Chapter 5). K_1 values were derived from the Dutch feed evaluation system (DVE; 0.045/h) or experimentally derived from various markers: chromium mordanted fibre (Cr-NDF); ^{13}C and ^{15}N isotopes in the dry matter (DM) and acid detergent fibre fraction (ADF). RUP values were calculated according to and DVE K_1 values were derived from van Duinkerken et al. (2011).

General Conclusions

Assessing fractional digesta passage has received much attention due to its importance for nutrient supply prediction. Modern feed evaluation systems seek for a more dynamic mechanistic approach to model animal response to dietary nutrient changes. However, quantitative information on nutrient-specific fractional passage is lacking. Conventional passage marker techniques do not provide information on nutrient-specific passage kinetics.

An alternative technique is hence required and involves the use of intrinsic stable isotopes labelled feed components as internal passage markers. Stable isotopes are inherent to the feed

and can be essentially determined in all dietary compounds. Furthermore, stable isotopes do not affect degradation and passage behaviour of labelled feed components. They qualify thus as a tool to specifically measure passage kinetics of digestible feed components. In contrast to externally applied markers, stable isotopes undergo the same digestive mechanism as the feed particles they are associated with and offer a comprehensive representation of feed passage.

Based on this concept, passage kinetics of various cell wall fractions (fibres, fibre-bound protein) and non-cell wall fractions (starch, total protein) were assessed for a common dairy ration consisting of grass silage, maize silage and concentrates. Stable isotopes were able to quantify feed and nutrient specific fractional passage rates. In contrast, a simultaneously used external marker (chromium mordanted fibre) was not able to discriminate among the various feed types. By intrinsically labelling plants above natural enrichment, a homogeneous isotope distribution and high enrichment levels can be achieved. This allows reducing the marker dosage which can be of advantage if a particular feed marker fed in large amounts is expected to affect the rumen environment.

Stable isotopes can be determined in dietary compounds present in particular small concentrations, such as plant wax *n*-alkanes, due to their highly sensitive and specific analytical determination. This gives the opportunity to assess passage kinetics of a wide range of dietary compounds of interest as long as measurable concentrations are recovered in faecal or digesta samples. The isotopic signature of purified fibre and protein fractions such as cellulose, hemicellulose, and soluble proteins, offers scope for the future for a more detailed insight into the passage kinetics of feed nutrients.

Detailed quantitative information on nutrient specific passage rates accommodates a more mechanistic approach to quantify nutrient supply and model animal response in relation to optimal animal performance, environmental and animal-health issues. The use of variable nutrient specific fractional rumen passage rates will ultimately improve the predictions of protein supply to ruminants in response to changes in diet composition and quality.

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Summary – Samenvatting – Zusammensetzung

Summary

Ruminants possess a unique digestive system to digest fibre-rich diets. The time feed is retained in the rumen determines the time feed is exposed to fermentative degradation by the microorganisms in the rumen. Rumen fractional passage rate (the reciprocal of retention time) determines thus the volatile fatty acid and microbial protein yield, which are the main energy and protein source to the ruminant, respectively. Quantitative knowledge on rumen fractional passage rates is required to predict nutrient supply in the small intestine. Fractional passage is therefore a key parameter in feed evaluation systems and in mechanistic rumen models describing the fate of nutrients with respect to animal performance, nutrient waste into the environment and animal-health issues. Such models require information on feed type and feed component specific fractional passage kinetics. Quantitative data on feed type and feed component specific passage kinetics are, however, scarce. Measurements of passage kinetics are conventionally conducted by marker techniques involving mainly externally applied markers. Such markers are not inherent to the feed and may therefore not best reflect passage of feed and nutrients. Inert internal markers are preferred but require rumen evacuations and do not provide feed type specific passage kinetics. The main aim of this thesis was to acquire quantitative data on passage kinetics of common feed types used in an intensive dairy system (grass silage, maize silage, concentrates), with emphasis on their nutrient-specific passage, using stable isotopes as an internal passage marker.

In Chapter 2, conventional passage marker techniques were compared with a promising novel technique involving stable isotope labelled feed as an internal passage marker. Some major limitations on the use of stable isotopes were discussed, including a potentially differential disappearance of isotopes upon microbial degradation in the rumen, a potential marker migration, and inhomogeneous isotope distribution. The first limitation may not apply to steady-state conditions as literature indicates that the isotopic ratio (δ) is not affected by microbial degradation under stable *in vitro* and *in vivo* conditions. The latter limitation can be circumvented by intrinsically labelling feed with stable isotopes. Marker migration from cellular to microbial matter may overestimate to some extent the fractional passage of digestible feed fractions based on current knowledge but effects appear to be minor. Because stable isotopes are inherent to the feed and present in digestible as well as indigestible feed fractions, they offer scope for the future to assess passage kinetics of digestible feed fractions.

In Chapter 3, passage kinetics of concentrates were assessed based on faecal $\delta^{13}C$ of maize bran. In a crossover study, five dairy cows received low (24.6%) and high (52.6%) levels of concentrates (DM basis). The natural difference in $\delta^{13}C$ between C_3 concentrate ingredients and C_4 maize bran was used to administer orally ^{13}C isotopes. Fractional passage kinetics were determined from faecal marker concentrations of an internal marker (chromium mordanted fibre; Cr-NDF) and of the internal marker ^{13}C in the dry matter (^{13}C-DM), neutral detergent fibre (^{13}C-NDF) and neutral detergent soluble (^{13}C-NDS). Passage kinetics were not affected by concentrate proportion in the diet but differences between markers were considerable. Fractional passage rates from the reticulorumen (K_1) of ^{13}C-NDF (0.061–0.063/h) and ^{13}C-DM (0.054–0.056/h) was high and considerably higher than that of Cr-NDF (0.037–0.039/h) and ^{13}C-NDS (0.039–0.043/h). In contrast, fractional passage rates from the proximal colon-caecum (K_2) were lowest with ^{13}C-NDF and ^{13}C-DM. Total mean time (TMRT) was considerably higher for Cr-NDF (40.9–42.0 h) as compared to ^{13}C-DM and ^{13}C-NDF (32.0–33.5 h). The results suggest that ^{13}C isotopes can provide feed component specific passage kinetics for concentrates and provide new insights into passage kinetics of fibre from technologically treated compound feed.

A different procedure was used to measure passage kinetics of forages. Maize and grass plants were intrinsically labelled with, respectively, ^{13}C and $^{13}C/^{15}N$ above natural enrichment under greenhouse conditions, ensiled and administered intraruminally. In Chapter 4, intrinsically labelled maize silage was used to assess passage kinetics of the acid detergent fibre fraction (^{13}C-ADF) in faeces and starch (^{13}C-ST) in omasal digesta. In a 6 × 6 Latin square design, six rumen-fistulated dairy cows received maize silages in a 2 × 3 factorial arrangement of treatments (two cultivars × three maturity stages). An increase in maturity increased starch content and decreased NDF and ADF content but did not affect fractional passage and fractional degradation rates, except for a decreased fractional degradation rate of starch with advancing maturity. The Cr-NDF marker provided higher K_1 estimates than ^{13}C-DM in faeces (0.042/h *vs*. 0.023/h). Omasal marker excretion patterns confirm these observed differences. Faecal K_1 estimates did not differ between ^{13}C-DM and ^{13}C-ADF. Omasal ^{13}C-ST provided higher K_1 values (0.042/h) than omasal ^{13}C-DM (0.034/h) but lower values than omasal Cr-NDF (0.051/h). Faecal K_2 estimates showed trends similar to K_1, with Cr-NDF providing a value (0.425/h) more than twice as high than that of ^{13}C-DM (0.179/h) and ^{13}C-ADF (0.128/h). The TMRT in the gastrointestinal tract was approximately double for ^{13}C-DM (64.1 h) and ^{13}C-ADF (77.6 h) than for Cr-NDF (36.4 h). The results indicate that passage of starch from maize silage is similar to that assumed by the Dutch feed evaluation system (0.045/h; DVE/OEB

system), but passage of fibre from maize silage is smaller, giving rise to higher effective degradability values. Overall, it was shown that intrinsic ^{13}C labelling of maize plants allows to measure passage kinetics of the maize fibre and starch fraction.

In Chapter 5, intrinsically labelled grass silage was used to assess passage kinetics of fibre (^{13}C-ADF) and fibre-bound nitrogen (^{15}N-ADF) in faeces and omasal digesta. In a 6 × 6 Latin square design, six rumen-fistulated dairy cows received grass silages in a 2 × 3 factorial arrangement of treatments (two N fertilisation levels × three maturity stages). Faecal K_1 estimates were considerably higher for Cr-NDF (0.047/h) compared to ^{13}C isotopes. Faecal ^{13}C-ADF provided the lowest K_1 (0.023/h) and the highest TMRT (61.1 h) among markers. In comparison, faecal ^{15}N-ADF appeared to have a higher K_1 (0.034/h) and a lower TMRT (46.4 h) than ^{13}C-ADF. Fractional passage rates tended to increase when N fertilisation level was increased from 45 to 90 kg N/ha, and generally decreased with advancing plant maturity at low N fertilisation. In contrast, passage kinetics generally increased with advancing plant maturity at high N fertilisation. Omasal digesta sampling confirmed results based on faecal sampling. The results suggest that passage kinetics fibre and fibre-bound N can be assessed with ^{13}C and ^{15}N stable isotopes.

In Chapter 6, the use of stable isotopes was extended to minor dietary components, such as cuticular plant *n*-alkanes. In contrast to the dietary components tested in Chapter 3 through 5, *n*-alkanes are not degraded by microorganisms in the rumen. The carbon isotopic signature of C_{29}, C_{31} and C_{33} *n*-alkanes from grass silage were used to estimate respective K_1 values from faecal samples. Other *n*-alkanes were not tested due to the low ^{13}C content in feed and faeces. Carbon chain length of *n*-alkanes did not affect K_1 among individual *n*-alkanes (0.037–0.040/h). This experiment confirmed that stable isotopes can be determined in a wide range of minor dietary compounds quantifiable in residues. Passage kinetics of $\delta^{13}C$ *n*-alkanes were comparable to $\delta^{13}C$ of the grass silage DM, which in contrast to *n*-alkanes is degraded in the rumen. These findings suggest that stable isotope migration from cellular to microbial matter is minor.

The isotope signature of labelled feed allows to assess feed type and feed component specific fractional passage kinetics, in particular those of digestible feed components. The use of stable isotopes as a digesta passage marker is currently the sole available method to label and measure passage of digestible feed components and should be further developed to analytically determine the fractional passage of purified fibre and protein fractions such as cellulose, hemicellulose, and soluble proteins. The use of variable nutrient specific fractional rumen passage rates will ultimately provide a more comprehensive prediction of energy and protein

supply and microbial protein efficiency to ruminants in response to changes in diet composition and quality.

Samenvatting

Herkauwers bezitten een uniek verteringsapparaat wat hen in staat stelt om vezelrijke voeders te verteren. De tijdsduur dat het voer in de pens verblijft is bepalend voor de tijd dat het voer blootgesteld staat aan fermentatieve afbraak onder invloed van de aanwezige micro-organismen. De fractionele passagesnelheid (de inverse van de verblijftijd) is daarmee bepalend voor de vluchtige vetzuur productie en microbiële eiwitsynthese, die respectievelijk de belangrijkste energie- en eiwitbronnen voor de herkauwer zijn. Om de aanvoer van nutriënten in de dunne darm te kunnen voorspellen is het van belang kwantitatieve kennis te hebben van de fractionele passagesnelheden. De fractionele passagesnelheid is dan ook een van de essentiële parameters binnen voederwaarderingssystemen en zo ook in mechanistische modellen waarmee de metabolische omzettingen van nutriënten wordt beschreven in relatie tot de gerealiseerde productie, de uitscheiding van nutriënten naar het milieu en diergezondheid gerelateerde aspecten. Zulke modellen vereisen informatie over het type voer en de daaraan verbonden voercomponent-specifieke passagekinetiek, maar kwantitatieve informatie hierover is echter in beperkte mate beschikbaar.

Het belangrijkste doel van dit promotieonderzoek was om kwantitatieve informatie te verkrijgen van de passagekinetiek van een aantal voedermiddelen (gras- en maissilage, en krachtvoer) welke algemeen aangewend worden in intensieve melkveehouderijsystemen. Hierbij lag de nadruk op het verkrijgen van informatie over de nutriënt-specifieke passagesnelheid van deze voedermiddelen. Voor het schatten van de passagekinetiek wordt de markeerstofmethode gebruikt waarbij traditioneel externe markeerstoffen worden ingezet. Dit type markeerstof vormt echter geen inherent bestanddeel van het voedermiddel en geven daardoor niet de beste afspiegeling van de werkelijke nutriëntenpassage. Daarnaast zijn er ook inerte interne markeerstoffen die bovengenoemd nadeel niet hebben. Interne markeerstoffen hebben dan ook de voorkeur, maar vereisen dat de pensinhoud volledig geëvacueerd wordt; ze geven geen inzicht in de voersoort-specifieke passagekinetiek.

Hoofdstuk 2 beschrijft een literatuurstudie waarbij de traditioneel gebruikte markeerstoftechniek vergeleken wordt met een veelbelovende nieuwe techniek waar gebruik gemaakt wordt van stabiele isotopen als een interne markeerstof om passage te schatten. Hierbij worden een aantal beperkingen van de nieuwe techniek bediscussieerd, waaronder de mogelijke verdwijning van stabiele isotopen onder invloed van microbiële afbraak, de mogelijke migratie van de stabiele isotopen en de mogelijke effecten van een niet-uniforme verdeling van stabiele

isotopen over de voercomponenten. Het eerste punt lijkt niet van invloed te zijn onder 'steady-state' condities op basis van *in vitro* en *in vivo* onderzoek waarin beschreven wordt dat de microbiële afbraak de isotoop ratio (δ) niet beïnvloedt. Het laatstgenoemde punt, de niet-uniforme verdeling van isotopen, kan worden ondervangen door het voer te verrijken onder condities waarbij de isotoopconcentratie continue verhoogd is. De migratie van stabiele isotopen van de celwandfracties naar de microbiële fracties zou kunnen leiden tot een overschatting van de fractionele passage van de verteerbare fractie, maar deze effecten lijken verwaarloosbaar. Omdat stabiele isotopen een inherent bestanddeel vormen van het voer en zowel aanwezig zijn in de verteerbare als onverteerbare fracties, bieden ze perspectief voor het schatten van de passagekinetiek van de verteerbare voercomponenten.

In hoofdstuk 3 is de passagekinetiek van krachtvoer in kaart gebracht op basis van fecale excretie $\delta^{13}C$ in maïszemelgrint. In een kruisproef met twee voerbehandelingen werden vijf melkkoeien aan een rantsoen toegekend met een laag (24,6 %) of hoog (52,6 %) niveau van krachtvoergift. Door gebruik te maken van het natuurlijke verschil in $\delta^{13}C$ tussen C_3 plantcomponenten (basis krachtvoer) en C_4 plantcomponenten (maiszemelgrint krachtvoer) werd een contrast in $\delta^{13}C$ gecreëerd door het C_3-krachtvoer eenmalig uit te wisselen met het C_4-krachtvoer. De fractionele passagesnelheden werden bepaald op basis van de fecale markeerstofconcentraties van de externe markeerstof (chroom-NDF) en van de interne markeerstof ^{13}C in de droge stof fractie (^{13}C-DS), de celwandfractie verkregen na hydrolyse in een neutraal detergens (^{13}C-NDF) en de niet-celwandfractie (^{13}C-NDS). Schattingen van fractionele passagesnelheden vanuit de pens (K_1) werden niet beïnvloed door het niveau van krachtvoergift, maar de markeerstoffen verschilden onderling aanzienlijk. Fractionele passagesnelheden vanuit de pens van ^{13}C-NDF (0,061–0,063/uur) en ^{13}C-DS (0,054–0,056/uur) lagen aanzienlijk hoger dan die van Cr-NDF (0,037–0,039/uur) en ^{13}C-NDS (0,039–0,043/uur). In tegenstelling hiermee waren de fractionele passagesnelheden vanuit het proximale colon-caecum (K_2) juist het laagst voor ^{13}C-NDF en ^{13}C-DS. De totale gemiddelde verblijftijd van markeerstoffen in het maagdarmkanaal (TMRT) was voor Cr-NDF (40,9–42,0 uur) aanzienlijk hoger in vergelijk met die van ^{13}C-DS en ^{13}C-NDF (32,0–33,5 uur). Deze resultaten suggereren dat ^{13}C isotopen de mogelijkheid geven om specifieke informatie te verkrijgen over de passagekinetiek van de celwandfractie afkomstig van de krachtvoercomponent in het rantsoen.

Voor het meten van de passagesnelheden van componenten uit ruwvoeders werd een andere procedure dan in hoofdstuk 3 gebruikt. Als ruwvoer zijn maïs- en grasplanten intrinsiek verrijkt met ^{13}C en $^{13}C/^{15}N$ respectievelijk in een klimaat-gecontroleerde kas. De verrijkte planten

werden na oogsten geënsileerd om vervolgens te kunnen worden toegediend in de pens van de koe.

In hoofdstuk 4 zijn ^{13}C-verrijkte maïssilages gebruikt voor het bepalen van de passagekinetiek van de celwandfractie verkregen na hydrolyse in een zuur detergens (^{13}C-ADF) en van de zetmeelfractie (^{13}C-ST). De passagekinetiek van de ^{13}C-ADF werd geschat op basis van fecale excretiepatronen, die van ^{13}C-ST op basis van markeerstof excretiepatronen in de chymus van de boekmaag. In een 6 × 6 Latijns vierkant ontvingen zes pens-gefistuleerde melkkoeien rantsoenbehandelingen met verschillende maïssilages in een 2 × 3 factoriële proefopzet van behandelingen (twee cultivars × drie afrijpingsstadia). Maissilages lieten een verhoging in zetmeelgehalte en een afname in NDF en ADF zien bij een toenemend afrijpingsstadium. Oogststadium liet geen effecten zien op de fractionele passage- en afbraakconstanten, met uitzondering van een afname in de fractionele afbraaksnelheid van zetmeel bij een oplopend afrijpingsstadium. De externe markeerstof Cr-NDF gaf een hogere schatting voor K_1 dan ^{13}C-DS (0,042/uur versus 0,023/uur) op basis van fecale excretiepatronen. Excretie patronen in chymus van de boekmaag bevestigden deze waargenomen verschillen. Schattingen van K_1 bepaald vanuit de fecale excreties lieten geen verschillen zien tussen de ^{13}C-DS en ^{13}C-ADF. In boekmaagchymus liet ^{13}C-ST (0,042/uur) een hogere waarde voor K_1 zien dan ^{13}C-DM (0,034/uur), maar beide fracties gaven lagere K_1-waarden dan Cr-NDF (0,051/uur). Schattingen voor K_2 op basis van fecale uitscheidingspatronen gaven een vergelijkbare tendens, waarbij de K_2 van Cr-NDF (0,425/uur) meer dan tweemaal zo hoog was dan die van ^{13}C-DS (0,179/uur) en ^{13}C-ADF (0,128/uur). De TMRT in het maagdarmkanaal was voor ^{13}C-DS (64,1 uur) en ^{13}C-ADF (77,6 uur) ongeveer tweemaal zo lang dan voor Cr-NDF (36,4 uur). De resultaten geven aan dat de fractionele passagesnelheden van zetmeel uit maïssilage vergelijkbaar is met de waarden die in het Nederlandse voederwaarderingssysteem worden aangenomen (0,045/uur; DVE/OEB systeem). De resultaten lieten zien dat het gebruik van met isotoop verrijkte maïssilage het mogelijk maakt om de passagekinetiek van verschillende koolhydraatfracties (celwandfractie versus zetmeelfractie) vast te stellen.

In hoofdstuk 5 zijn ^{13}C-verrijkte grassilages gebruikt om de passage kinetiek van celwandfracties (^{13}C-ADF) en celwand-gebonden stikstof (^{15}N-ADF) vast te stellen vanuit excretiepatronen in de feces en boekmaagchymus. In een 6 × 6 Latijns vierkant ontvingen zes pens-gefistuleerde melkkoeien verschillende grassilages in een 2 × 3 factoriële proefopzet met twee N-bemestingsniveaus en drie oogststadia. Schattingen voor de K_1 op basis van fecale excreties waren aanzienlijk hoger voor Cr-NDF (0,047/uur) dan de met ^{13}C verrijkte fracties. Van de markeerstoffen gaf ^{13}C-ADF de laagste K_1 (0,023/uur) en de hoogste TMRT (61,1 uur).

Een vergelijking tussen fecale excretiepatronen van ^{13}C en ^{15}N liet zien dat ^{15}N-ADF een aanzienlijk hogere K_1 (0.034/uur) had en een lagere TMRT (46,4 uur) dan ^{13}C-ADF. Fractionele passagesnelheden neigden naar hogere waarden bij een oplopend N bemestingsniveau (45 versus 90 kg N/ha), en lieten een dalende tendens zien bij een toenemende ouderdom (oogststadium) bij gras met een laag N bemestingsniveau. Gras met een hoog bemestingsniveau liet een tegenovergesteld beeld zien waarbij oogststadium en passage positief gecorreleerd bleken. Excretiepatronen in boekmaagchymus bevestigden de bevindingen gedaan op basis van fecale excreties. De resultaten lieten zien dat de passagekinetiek van de celwandfractie en celwand-gebonden N kan worden vastgesteld met behulp van zowel ^{13}C als ^{15}N.

In hoofdstuk 6 is het gebruik van stabiele isotopen verder uitgewerkt voor *n*-alkanen, een natuurlijk voorkomende (secundair) plantcomponent dat de waslaag (cuticula) op de plant vormt en geen nutritionele waarde heeft. In tegenstelling tot de voedercomponenten en fracties zoals beschreven in de voorgaande hoofdstukken, worden de *n*-alkanen niet afgebroken door de micro-organismen in de pens. De ^{13}C isotoop-verrijking van C_{29}, C_{31} en C_{33} *n*-alkanen van verschillende grassilages is gebruikt om hun respectievelijk K_1-waarden te schatten. De overige *n*-alkanen zijn niet in de analyse meegenomen vanwege hun te lage concentratie in de voeders en feces. Dit experiment bevestigde dat stabiele isotopen kan worden bepaald in een brede range van (secundaire) plantcomponenten mits deze kwantitatief te isoleren zijn. De passagekinetiek van $\delta^{13}C$ in *n*-alkanen waren vergelijkbaar met de $\delta^{13}C$ in de droge stof van grassilage, die in tegenstelling tot de *n*-alkanen wordt afgebroken in de pens. Onze bevindingen bevestigden dat de migratie van stabiele isotopen van de celwandfractie naar de microbiële fractie gering is.

De isotoop samenstelling van verrijkte voederbestanddelen maakt het mogelijk om onderscheid te maken tussen het type voer en om voercomponent-specifieke fractionele passagesnelheden vast te leggen, en met name die van de verteerbare voercomponenten. Het gebruik van stabiele isotopen als markeerstof voor het meten van digesta passage is momenteel de enige beschikbare methode om verteerbare voercomponenten te verrijken en de passage van te schatten. Deze methode kan in de toekomst verder ontwikkeld worden om de fractionele passage van geïsoleerde celwandcomponenten (bijv. cellulose en hemicellulose) en eiwitfracties (bijv. oplosbare eiwitten) te onderzoeken. Het gebruik van variabele voercomponent-specifieke fractionele passagesnelheden zal uiteindelijk zorgdragen voor een gedetailleerdere voorspelling van de eiwitvoorziening van herkauwers bij veranderingen in rantsoensamenstelling en -kwaliteit.

Zusammenfassung

Wiederkäuer sind in der Lage, faserreiches Grobfutter mit Hilfe von mikrobiellen Enzymen im Pansen abzubauen. Dabei spielt die Verweilzeit von Futterpartikeln im Verdauungstrakt eine zentrale Rolle, da diese die Dauer, der Futterpartikel der mikrobiellen Fermentation im Pansen ausgesetzt sind, bestimmt. Die fraktionelle ruminale Passagerate (Kehrwert der Verweilzeit) ist somit für die Bildung von flüchtigen Fettsäuren und für die Bildung von Eiweiß mikrobieller Herkunft, der jeweiligen Hauptbezugsquelle von Energie und Eiweiß für den Wiederkäuer, ausschlaggebend. Die quantitative Erfassung der fraktionellen ruminalen Passagerate bestimmt des Weiteren den Nährstoffffluss zum Dünndarm. Fraktionelle Passageraten sind die Grundlage für die Bestimmung von Nährstoffzufuhr und -ausscheidung in Futtermittelbewertungssystemen und mechanistischen Pansenmodellen für Wiederkäuer besonders mit Hinblick auf eine optimale Nährstoffverwertung für Tier und Umwelt. Um den Futterwert der Rationen und deren Nährstoffzufuhr genauer zu erfassen, sind Informationen zum Passageverhalten einzelner Futtermittel und -nährstoffen nötig. Entsprechende Daten sind jedoch mit Hilfe der vorhandenen Prozeduren schwer zu erfassen. Herkömmliche Prozeduren umfassen meist externe Marker, die nicht natürlicher in Futterrationen vorkommen und dementsprechend deren Passageverhalten nicht optimal wiedergeben. Interne Marker sind zwar natürlicher Bestandteil von Futterrationen, bedürfen jedoch aufwendiger Pansenentleerungen und geben keinen Aufschluss über einzelne Futtermittel und -nährstoffen, sondern nur über die aufgenomme Gesamtration. Daher war es Ziel meiner Studie, stabile Isotopen als alternative Passagemarker anzuwenden, um das Passageverhalten gängiger Grobfuttermittel (Grass- und Maissilage) und Kraftfutter mit besonderem Hinblick auf einzelne enthaltene Nährstoffe für Wiederkäuer zu erfassen.

In der ersten Studie (Kapitel 2) wurden konventionelle externe Passagemarker mit stabilen Isotopen als alternative Passagemarker verglichen. Letztere umfassen isotopisch markierte Futternährstoffe und haben den Vorteil, dass der Marker denselben Fermentationsbedingungen wie die der natürlich aufgenommen Nährstoffen obliegt. Stabile Isotopen sind nicht-radioaktive und in nur sehr geringen Mengen natürlich vorkommende Elemente und bereits kleine Veränderungen im natürlichen Isotopenverhältnis lassen sich analytisch genau erfassen. Zu den Nachteilen stabiler Isotopen gehören eine mögliche Veränderung im natürlichen Isotopenverhältnis (δ) aufgrund mikrobieller Pansenfermentation, eine potenzielle Markermigration und eine inhomogene Isotopenverteilung im

Ausgangssubstrat. Ersterer spielt eine geringe Bedeutung solange sich das Verdauungssystem im Fließgleichgewicht befindet, da die mikrobielle Fermentation das Isotopenverhältnis nicht beeinflusst. Eine potenzielle Isotopenmigration von zellulären zu mikrobiellen Bestandteilen kann sich negativ auf eine korrekte Erfassung der Passageraten auswirken, scheint jedoch von geringem Einfluss zu sein. Letzterer kann durch die Anwendung von kontinuierlich isotopenmarkierten Futtermittelkomponenten umgangen werden. Da stabile Isotopen in allen Futtermitteln und in sowohl pansenbeständingen als auch pansenunbeständigen Futterfraktionen vorkommen, können diese erstmals Aufschluss über beide Futterfraktionen geben.

In der zweiten Studie (Kapitel 3) wurde das Passageverhalten von Kraftfutter berechnet. In einem Crossover-Versuch erhielten fünf pansenfistulierte Milchkühe eine Futterration mit niedrigem (24,6 %) bzw. hohem Kraftfutteranteil (52,6 % auf TS-Basis). Dabei wurde Kraftfutter mit natürlichem niedrigen ^{13}C-Anteil (C_3 Pflanzen) durch Kraftfutter mit natürlichem hohen Anteil an ^{13}C (Maiskleie; C_4 Ursprung) getauscht, um eine orale ^{13}C-Dosis zu verabreichen. Fraktionelle Passageraten wurden anhand von Markerausscheidungsprofilen eines externen Markers (chromgebeizte Faser bzw. Cr-NDF) und ^{13}C-Isotopen als interne Marker in der Trockensubstanz (^{13}C-DM), in Neutral-Detergenz-Faser (^{13}C-NDF) und in der neutralen Detergenzien-löslichen Fraktion (^{13}C-NDS) berechnet. Der Kraftfutteranteil in der Futterration hatte keinen Einfluss auf die Passagekinetik. Letztere war jedoch stark von der Markerwahl geprägt. Die fraktionelle Passageraten vom Reticulorumen (K_1) von ^{13}C-NDF (0,061–0,063/h) und ^{13}C-DM (0,054–0,056/h) waren beträchtlich höher als jene von Cr-NDF (0,061–0,063/h) und ^{13}C-NDS (0,039–0,043/h). Im Gegensatz dazu waren die fraktionellen Passageraten vom Dickdarm (eigentlich, proximaler Colon-Caecum; K_2) von Cr-NDF und ^{13}C-NDS am niedrigsten. Die Gesamtverweilzeit (TMRT) von Cr-NDF war beträchtlich höher (40,9–42,0 h) im Vergleich zu jener von ^{13}C-DM and ^{13}C-NDF (32,0–33,5 h). Den Ergebnissen zufolge lassen sich mit Hilfe von ^{13}C-Isotopen nährstoffspezifische Passageraten für Kraftfutter berechnen.

Die Passageraten für Grobfutter wurden mit Hilfe von isotopisch angereicherter Maissilage (^{13}C) und Grassilage (^{13}C/^{15}N) berechnet. Das in einem Gewächshaus angereicherte Pflanzenmaterial wurde als Silage intraruminal verabreicht und die fraktionellen Passageraten anhand von Markerausscheidungsprofilen in Kuhkotproben und ruminal verdauter Nahrung im Omasum berechnet. In der dritten Studie (Kapitel 4) lieferte isotopisch angereicherte Maissilage Passageraten zur Säure-Detergenz-Faser in Kotproben (^{13}C-ADF) und Stärke im Omasum (^{13}C-ST). Sechs pansenfistulierte Milchkühe erhielten jeweils eine von sechs

verschiedenen Maissilagen (zwei verschiedene Maisvarietäten mit jeweils drei verschiedenen Reifegraden) in einem Lateinischen Quadrat. Mit steigendem Reifegrad erhöhte sich der relative Stärkeanteil und verminderte sich der relative NDF- und ADF-Anteil. Die fraktionelle Passage- und Abbaubarkeitsrate waren nicht betroffen, außer einer verminderten Abbaubarkeitsrate von Stärke bei steigendem Reifegrad. Die höchsten K_1-Werte ergaben sich mit dem Marker Cr-NDF (0,042/h), gefolgt von ^{13}C-DM (0,023/h). Dieser Markerkontrast wurde mit den omasalen Proben bestätigt. Es konnte kein signifikanter Unterschied zwischen ^{13}C-DM und ^{13}C-ADF festgestellt werden. Der K_1-Wert von ^{13}C-ST im Omasum war mit 0,042/h höher als jener von ^{13}C-DM (0,034/h), jedoch niedriger als jener von Cr-NDF (0,051/h) im Omasum. Gleichermaßen waren die K_2-Werte höher für Cr-NDF (0,425 /h) als für ^{13}C-DM (0,179/h) und ^{13}C-ADF (0,128/h) in den Kotproben. TMRT von ^{13}C-DM (64,1 h) und ^{13}C-ADF (77,6 h) waren wesentlich höher als jene von Cr-NDF (36,4 h). Den Ergebnissen zufolge war die Passagerate von Maisstärke jenem Wert ähnlich, der gegenwärtig im niederländischen Futtermittelbewertungssystem angenommen wird (0,045/h; DVE/OEB System). Die Passagerate von Maisfaser (^{13}C-ADF) war jedoch geringer, und dies hatte zur Folge, dass deren effektive Abbaubarkeit wesentlich höher war als jene die vom DVE/OEB-System abgeleitet wurde. Die Ergebnisse lassen darauf schließen, dass isotopenmarkierte Maispflanzen Rückschluss auf die nährstoffspezifische Passagerate von Maisstärke und –faser geben können.

In der vierten Studie (Kapitel 5) wurden Passageraten von Fasern (^{13}C-ADF) und fasergebundenem Stickstoff (^{15}N-ADF) von isotopenmarkierter Grassilage berechnet. Sechs pansenfistulierte Milchkühe erhielten jeweils eine von sechs verschiedenen Grassilagen (zwei verschiedene Stickstoffdüngungsraten mit jeweils drei verschiedenen Reifegraden) in einem Lateinischen Quadrat. Die ^{13}C-Isotopen hatten wesentlich geringere K_1-Werte als Cr-NDF (0,047/h). Der absolut niedrigste K_1-Wert mit 0,023/h und gleichzeitig höchste TMRT von 61,1 h ergab sich mit ^{13}C-ADF. Im Vergleich dazu ergaben sich für ^{15}N-ADF ein etwas höherer K_1-Wert von 0,034/h und ein niedrigerer TMRT von 46,4 h. Bei Erhöhung der Düngungsrate von 45 zu 90 kg N/ha tendierten die fraktionelle Passageraten zuzunehmen. Bei einer Düngungsrate von 45 kg N/ha nahmen die fraktionellen Passageraten bei steigendem Reifegrad im Allgemeinen zu. Bei einer Düngungsrate von 90 kg N/ha traf jedoch das Gegenteil zu. Die omasalen Proben bestätigten diesen Verlauf. Die Ergebnisse lassen darauf schließen, dass die stabilen Isotopen ^{13}C und ^{15}N Rückschluss auf die Passagekinetik von Fasern und faserngebundenem N geben können.

In der fünften und letzten Studie (Kapitel 6) wurden Passageraten von isotopenmarkierten Pflanzen-*n*-Alkanen berechnet. *n*-Alkane sind sekundäre Pflanzeninhaltsstoffe und fester Bestandteil der Pflanzencuticula, jedoch nur in geringen Mengen vorhanden. Im Gegensatz zu denen in Kapitel 3 bis 5 untersuchten Futterkomponenten sind *n*-Alkane nicht mikrobiell im Pansen abbaubar. Stabile Isotopen konnten in einer Reihe von *n*-Alkanen nachgewiesen werden, jedoch war die fäkale ^{13}C-Konzentration lediglich für die *n*-Alkane C_{29}, C_{31} und C_{33} ausreichend, um fraktionelle Passageraten abzuleiten. Die Anzahl der Kohlenstoffatome hatte keinen Einfluss auf den K_1-Wert (0,037–0,040/h). Mit diesem Versuch konnte bestätigt werden, dass stabile Isotopen in einer Reihe von Komponenten, die in geringer Konzentration im Futter enthalten sind und sich in Kuhkotproben quantifizieren lassen, nachweisbar sind. Die Passagekinetik von *n*-Alkanen war vergleichbar mit jener von ^{13}C-DM, die, im Gegensatz zu *n*-Alkanen, im Pansen von Mikroben abgebaut werden kann. Dies lässt darauf schließen, dass eine potenzielle Markermigration von zellulärer zu mikrobieller Substanz in pansenverdauten Nährstoffen von geringem Ausmaß ist.

Den Ergebnissen dieser Dissertationsschrift zufolge eignen sich isotopenmarkierte Futterkomponenten für die Erfassung von nährstoff- und futtermittelspezifischen Passageraten. Die Anwendung von stabilen Isotopen als Passagemarker ist zur Zeit die einzige Möglichkeit, die Passageraten für potenziell verdauliche Nährstoffe, die im Organismus den üblichen Verdauungsmechanismen ausgesetzt sind, zu berechnen. Die Prozedur zur analytischen Erfassung von stabilen Isotopen in Hauptfutterkomponten soll demnach auf jene Inhaltsstoffe, die in geringerer Konzentration im Futter enthalten sind, ausgeweitet werden; so zum Beispiel für chemisch isolierte Kohlenhydrate und Eiweißstoffen, wie Zellulose, Hemizellulose und lösliche Proteine. Die Anwendung von flexiblen nährstoffspezifischen Passageraten in Futtermittelbewertungsssystemen ermöglicht eine umfassende Bewertung von Energie- und Proteinzufuhr sowie der mikrobiellen Eiweißsynthese für unterschiedliche Futterrationen in Wiederkäuern.

Acknowledgements

"As we acquire more knowledge,
things do not become more comprehensible,
but more mysterious." (*Albert Schweitzer)*

This classical quote did, regrettably, not make it to one of my thesis propositions as I believe it has often enough been recycled – yet, I think this quote defines very well how I have personally developed during my stay in Wageningen as a PhD researcher. When I had been first asked if I were interested in conducting the very PhD research outlined in this book… I had my doubts. Not about the fact that research would be involved, nor about the overall theme (ruminant nutrition and forage evaluation have always been and will, in one way or the other, keep being my research interest) but about the very precise research topic being somewhat 'abstract'. When I had been first told that the project is going to focus on isotopes, my first thought was (shame on me), "Oh my god, I've never been good in physics, I just wanna work with animals and do some fancy research". True enough, this work does not cover one of those current impressive earth-saving research topics of vast societal impact, and from the very start it was clear to me that this is going to be difficult but exciting research to explain to friends and foes. Yet, it was quite reassuring to know that publishing quickly would not be a major issue due to a somewhat less fierce competition in this field. Either way, the more I dived into this topic and the more knowledge I acquired, things have indeed become more and more 'mysterious' – probably not the way A. Schweitzer had meant it, but I have discovered for myself how satisfying it can be to be driven by curiosity in science and in life, and how encouraging it can be to be able to explore in more detail these two fields and to know I actually do not yet comprehend enough.

Having said this, it has become time to thank all those who in one way or the other contributed to this piece of work and gave me the opportunity to develop myself personally and professionally during my stay at Wageningen. First of all, I would like to express my gratitude to my promoter and my supervisors for offering me the opportunity to work within the Animal Nutrition Group and for giving me support in challenging times, each of them in his own way. It has been a pleasure discussing and publishing with you and learning from your experience. **Wouter**, I would like to thank you in particular for considering the logistic problems we have experienced, for supporting this project during dire straits and for getting me in touch with the Quarisma project, which helped me finalising my thesis within a short time after the four-year PhD period.

Jan, it has been a real pleasure working with you although 'sharing' you with so many other PhD students made it often challenging to discuss things with you in limited time. The meetings with you have always been extremely fruitful but also pleasant as it is always remarkable to see how you manage to connect things apparently that easily. You made me start thinking in 'flow charts', a very effective way of structuring thoughts and ideas. Your comments on manuscript drafts were impressive, both in quality and in quantity!

Wilbert, as my daily supervisor – sometimes I had the feeling you were rather my hourly supervisor – you were for sure the person I have been working the closest with. I could always count on you when I encountered (un)expected problems during my experiments, even if this meant waking you up in the middle of the night – this was very reassuring indeed, although, I fear, it was less reassuring for your own family… I hope that Joanna and your wonderful daughters do not think bad of me when I deliberately took you away from them for entire evenings and weekends. The frequent gatherings at your homeplace have always been extremely enjoyable albeit somewhat challenging the following day… Nonetheless, your positive attitude and sincere smile both at and outside work always gave me a boost in motivation when I needed it. The (usually non-scheduled) meetings with you have always been motivating. I appreciated very much how you managed to steer the discussion from a heavy professional conversation into a more pleasant and sometimes private chat when it was necessary. Often enough I returned to my desk with more questions than answers. Yet, this gave me enough freedom to work out things all by myself and to dive deeply into the mistery of isotopes.

A particular satisfying task during my PhD work at Wageningen University was the opportunity to co-supervise **thesis students**. Linda, Peter, Dirk, Ruud, Judith, Nelleke, Tichaona, Laurent, Jan and Jean-Baptiste, although each of you was naturally different and it took me quite some efforts to adapt my working style to your needs, it was a pleasure to work with you! You have all done your share of work, including heavy night shifts while your mates were enjoying a night out in the pubs.

To all my colleagues at the **Animal Nutrition Group** and particularly to those that helped sampling and taking measurements, a big thank you! Sadly, I coud not acknowledge all my little helpers in the peer-reviewed papers. A special thank-you goes therefore to Francine, Bayissa and Aníbal, and to the technical support staff from both the animal research facilities (formerly, de Ossekampen) and the Animal Nutrition Group. In particular, Tamme, Sven and Joswita: thank you, not only for helping taking measurements but also for your priceless advice on some fancy technical equipment. I further wish to express my graditude to the

laboratory staff of the Animal Nutrition Group. Aadrian, Xuan, Erika, Saskia and Leon, thank you for assisting with and advising on laboratory analyses, and for carrying out some of the analyses. I would like to thank in particular Michel and Jane-Martine for your input on stable isotope analysis. Needless to say, without your help, this work would have been impossible!

To all my **PhD colleagues**. Mubarak, Wouter, we started and finished our PhD work around the same time. It was a nice feeling to know that one is not alone and can share moments of high stress and happiness! To my office mates: Sonja and Harma, it has always been extremely enternaining with you in the office but also quite useful statistic-wise (Sonja!) and publishing-wise (Harma!). All the best with finalising your thesis! To all of you out there in the common PhD room: the ruminant connection (Sanne, Sabrina, Bayissa, Kasper, Nguyen, Genet, Felicidade and Jacqueline), the piggy connection (Yvonne, Tetske, Hsuan, Sergio and Esther), the chicken connection (Shafqat, Pierre), the somewhere-in-between-ruminant-monogastric connection (Myrthe), the pet connection (Lotte), the magic mushroom connection (Sandra), the Matrix connection (Henk), and… the horsey connection (Geronda), thanks for sharing cakes, coffee, barbecue evenings and jokes! Although the PhD group has considerably grown in number since I first joined, it is nice to see that a few things have not changed and I am confident that the group will maintain the willingness to help each other. I have realised how important it is to push each other or to pull one back when necessary.

I would like to thank my **paranymphs** Leon and Mascha. You have been both working on your own PhD and particularly fun to work with. Mascha, you have been a dear friend to me and always been ready when I needed to talk to somebody, I hope we will manage to keep in touch!

My research interests on ruminant nutrition and forages and my overal interest in reseach have developed during my first research thesis as an undergraduate, for which I am grateful to Prof Karl Buchgraber and Dr Andreas Steinwidder of AREC Raumberg-Gumpenstein, Austria, and to the Mountain Agriculture team of the Research Centre for Agriculture and Forestry Laimburg, Italy. I would further like to thank Prof Michael Kreutzer and Dr Florian Leiber from the ETH Zurich, Switzerland, who gave me the opportunity to join an experiment for two months during my PhD study and to co-author a publication on beef meat quality in high altitudes.

To my **family**: Es isch net leicht in kurzen Worten zu fossen, wie sehr i enk ollen donkbor bin. Vor ollem, weil es mir die Gelegenheit geben hobts, meinen eigenen Willen zu folgen und eps nuies kennen zu lernen, um mi weiter zu entwickeln. Es wor definitiv net olm leicht, vor ollem zu wissen, dass viele Holländer pa ins in den Südtiroler Bergen Urlaub mochen,

während i oft orbeitsbedingt in Holland bleiben hon miassen... Als kloaner Trost, hon i als Titelfoto koane klassische holländische Schwarzbunte auf einer flochen saftigen Weide ausgsuacht, ober stottdessen oan Bild, dass mir dahoam af dor Villanderer Alm aufgnummen hobn. Ma, leider hosch du net dabei sein kennen, obr i bin mir sicher, du hosch die gonze Zeit glücklich und vergnügt af mi obigschaugt!

Enfin, **Isa**, ça c'est fait :) Tu as été vraiment formidable, tu as été toujours là pour moi ! Je me souviens encore comme tu étais ravie de m'aider à remuer les excréments de vaches en pleine nuit avec un estomac vide ! Ou quand je suis revenu avec une mauvaise odeur de fluide ruminal après une nuit avec mes animaux. Je te suis naturellement très reconnaissant pour tous les week-end auxquels tu as renoncé pour m'aider préparer mes expérimentations ou prendre des échantillons. Tu as souffert avec moi et tu m'as toujours donné ton magnifique sourire même quand je t'ai embêté avec mes problèmes de luxe de PhD ! Now, it is your turn to finalise your PhD research at the ETH Zurich. It was wonderful to see you working with your beef cattle high up on the Swiss mountains, always on full throttle but always with a smile on your face! This book is definitely yours as well !!

Wageningen, September 2013

'de Ossekampen' research farm, Wageningen

Curriculum vitae

About the Author

Daniel Warner was born on March 26, 1982 in Bozen/Bolzano, Italy. He attended high school education at his birthplace. In 2004, he obtained a BSc degree in Agricultural Techniques and Economics with a specialisation in Mountain Farming at the Free University of Bozen/Bolzano, Italy. Here, he developed his research interests in ruminant nutrition, forages and alpine farming. After a brief intermezzo with professional internships at the Agricultural Research Centre Raumberg-Gumpenstein, Austria, and on a cattle station in Australia he pursued a graduate study on Animal Sciences at Wageningen University. He worked on two MSc theses on the forage quality and nutritional aspects of various alternative and conventional forage species. In 2008, he obtained a MSc degree in Animal Sciences with a specialisation in Animal Nutrition. In the same year, he joined the Animal Nutrition Group of Wageningen University to conduct a PhD study as a member of the Wageningen Institute of Animal Sciences (WIAS). The results are described in this thesis. From January to August 2013 he was appointed a postdoctoral researcher at VION Fresh Meat North GmbH in Düsseldorf, Germany, within the Marie Curie fellowship project on Quality and Risk Management in Meat Chains (QUARISMA). During the PhD study he investigated the potential of stable isotopes as passage markers to estimate fractional passage rates of various feed components in dairy cows.

Publications

List of Scientific Publications Included in this Thesis

Warner, D., J. Dijkstra, W.H. Hendriks, and W.F. Pellikaan. 2013. Passage kinetics of ^{13}C-labeled corn silage components through the gastrointestinal tract of dairy cows. J. Dairy Sci. 96: 5844–5858.

Warner, D., L.M.M. Ferreira, M.J.H. Breuer, J. Dijkstra, and W.F. Pellikaan. 2013. Stable isotope labeled *n*-alkanes to assess digesta passage kinetics through the digestive tract of ruminants. PLOS ONE, in press (doi: 10.1371/journal.pone.0075496).

Warner, D., J. Dijkstra, W.H. Hendriks, and W.F. Pellikaan. 2013. Passage kinetics of stable isotope labelled grass silage fibre and fibre-bound protein through the gastrointestinal tract of dairy cows. J. Dairy Sci., accepted for publication.

Warner, D., J. Dijkstra, S. Tamminga, and W.F. Pellikaan. 2013. Passage kinetics of concentrates in dairy cows measured with carbon stable isotopes. Animal, accepted for publication.

Warner, D., J. Dijkstra, W.H. Hendriks, and W.F. Pellikaan. Stable isotope labelled feed nutrients to assess nutrient-specific feed passage kinetics in ruminants. Submitted.

List of Other Published Work During the PhD Period not Included in this Thesis

Warner, D., A. Elgersma, and R.J. Dewhurst. 2009. Effect of freeze-thaw treatment of herbage on the biohydrogenation of α-linolenic acid. In: Chilliard, Y., F. Glasser, Y. Faulconnier, F. Bocquier, I. Veissier, and M. Doreau (eds.), *Ruminan Physiology. Digestion, Metabolism, and Effects of Nutrition on Reproduction and Welfare*, Wageningen Academic Publishers, Wageningen, The Netherlands, p. 396.

Warner, D., S.K. Jensen, J.W. Cone, and A. Elgersma. 2010. Fatty acid composition of forage herb species. In: H. Schnyder, J. Isselstein, F. Taube, K. Auerswald, J. Schellberg, M. Wachendorf, A. Herrmann, M. Gierus, N. Wrage, and A. Hopkins (eds.), *Grassland in a Changing World*, Mecke Druck und Verlag, Duderstadt, Germany, pp. 491–493.

Warner, D., W.F. Pellikaan, H. Boer, S. Tamminga, P. Gregorini, B. Tas, and J. Dijkstra. 2011. Passage characteristics of ^{13}C-labelled carbohydrate fractions in dairy cows fed high

and low level of concentrates. In: *Advances in Animal Biosciences, Vol. 2, Part 2.* Cambridge University Press, Aberystwyth, UK, p. 387.

Warner, D., J. Dijkstra, and W.F. Pellikaan. 2012. Passage kinetics of maize silages determined with ^{13}C isotopes and Cr-mordanted fibre. In: J.W. Cone (ed.), *Proceedings of the 37th Animal Nutrition Research Forum*, Wageningen, The Netherlands, pp. 61–62.

Warner, D., J. Dijkstra, and W.F. Pellikaan. 2013. Passage of feed through the gastrointestinal tract of dairy cows. In: *Proceedings of the WIAS Science Day*, Wageningen, The Netherlands, p. 8.

Gangnat, I.D.M., J.O. Zeitz, D. Warner, M. Kreuzer, and F. Leiber. 2013. Influence of different grassland vegetation types on ruminal protozoa and ammonia in beef cattle. *Fourth EAAP International Symposium on Energy and Protein Metabolism and Nutrition*, Sacramento, CA.

Completed Training and Supervision Plan

Courses and scientific exposure (58 ECTS*)

The basic package (3.0 ECTS)
- Course on Ethics and Philosophy of Animal Science, Wageningen, 2009
- WIAS Introduction Course, Wageningen, 2009

International conferences (3.9 ECTS)
- ISRP, 11th Int. Symposium on Ruminant Physiology, Clermont-Ferrand, France, 2009
- ISNH, 8th Int. Symposium on the Nutrition of Herbivores, Aberystwyth, Wales, UK, 2011
- EGF, 16th European Grassland Federation Symposium, Irdning, Austria, 2011
- BASIS meeting, Benelux Association for Stable Isotope Scientists, Gent, Belgium, 2013

Seminars and workshops (3.9 ECTS)
- 7th Int. Workshop on Modelling Nutrient Digestion and Utilization in Farm Animals, Paris, France, 2009
- 5th Annual Dairy Solutions Symposium, Utrecht, 2010
- Int. Symposium on Dairy Cattle Nutrition, Wageningen, 2011
- Animal Nutrition Research Forum, Belgium & The Netherlands, 2009–12
- WIAS Science Day, Wageningen, 2009–13

Presentations (6.0 ECTS)
- 11th ISRP, 2009 (poster,)
- 23rd EGF Meeting, Kiel, Germany, 2010 (poster)
- 8th ISNH, 2011 (poster)
- 37th Animal Nutrition Research Forum, Wageningen, 2012 (oral)
- WIAS Science Day, 2013 (oral)
- BASIS meeting, 2013 (oral)

In-depth studies (6.8 ECTS)
- Design of Animal Experiments, Wageningen, 2009
- Analytical Work and Possibilities within Animal Nutrition Sciences, Wageningen, 2009
- Statistics of the Life Sciences, Wageningen, 2009
- Fatty Aicds in Dairy Cattle in Relation to Product Quality and Health, Gent, Belgium, 2012
- Reaction Kinetics in Food Sciences, Wageningen, 2012

Statuary courses (3.0 ECTS)
- Use of Laboratory Animals, Utrecht, 2009

Professional skills training (5.9 ECTS)
- PhD Competence Assessement, 2009
- Project and Time Management, 2010
- Information Literacy, 2011
- Communicating Science with Press, Policy and Public, 2011
- Techniques for Writing and Presenting a Scientific Paper, 2011
- High-Impact Writing in Science, 2012

Research skills training (5.6 ECTS)
- Introduction to R for Statistical Analysis, 2009
- Preparing PhD research proposal, 2009
- External training period, ETH Zurich, Switzerland, 2012

Didactic skills training (19.5 ECTS)
- Lecturing: Animal Nutrition and Physiology, Wageningen (ANU-30806), 2010/13
- Supervising practicals: Principles of Animal Nutrition, Wageningen (ANU-20306), 2013
- Tutorship: Research Master Cluster, Wageningen (YAS-60312), 2009–10
- Supervising thesis students (9 MSc, 1 BSc), 2009–13

**one ECTS credit equals a study load of approximately 28 hours*

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