Methodic investigations on the suitability of plant and synthetic *n*-alkanes as markers to predict feed intake and digestibility in horses

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List of abbreviations

Apart from abbreviations of the International System of Units (8th ed., 2006, updated 2014; available at http://www.bipm.org/en/publications/si-brochure/) and abbreviations for chemical elements and compounds, the following abbreviations were used. Abbreviations that were used in equations or statistical models are specified at the respective passages.

1-C24-OH	1-tetracosanol
1-C26-OH	1-hexacosanol
1-C28-OH	1-octacosanol
1-C30-OH	1-triacontanol
ACP	acyl carrier protein
AFR	alkane faecal recovery
AIA	hydrochloric acid-insoluble ash
AS	acceptance score
AT	acceptance test
Avg.	average
B	bolus
BCS	body condition score
BK	Biegekraft
BT	bending test
BW	bodyweight
C18	*n*-octadecane
C19	*n*-nonadecane
C21	*n*-heneicosane
C24	*n*-tetracosane
C26	*n*-hexacosane
C27	*n*-heptacosane
C28	*n*-octacosane
C29	*n*-nonacosane
C30	*n*-triacontane
C31	*n*-hentriacontane
C32	*n*-dotriacontane
C33	*n*-tritriacontane
C35	*n*-pentatriacontane
C36	*n*-hexatriacontane
C38	*n*-octatriacontane
C40	*n*-tetracontane
C22-acid	docosanoic acid
C24-acid	tetracosanoic acid
C26-acid	hexacosanoic acid
C28-acid	octacosanoic acid
C30-acid	triacontanoic acid

C32-acid	dotriacontanoic acid
CF	crude fibre
CL	chain length
CP	crude protein
CRD	controlled-release device
$\delta^{13}C$	ratio of stable isotopes ^{13}C:^{12}C
d	day
DDF	daily defecation frequency
DE	digestible energy
DFQ	daily faeces quantity
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
DMO	dry matter output
DPA	digesta phase association
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
EM	energy metabolizability
ER	endoplasmatic reticulum
FAE	fatty acid elongase
FAS	fatty acid synthase
FID	flame ionization detector
FL	flexural load
GC	gas chromatography
GCA	gas chromatographic analysis
gft.	gefriergetrocknet
HPMC	hydroxypropyl methylcellulose (hypromellose)
K	fractional passage/residence rate
LCFA	long-chain fatty acid
LCOH	long-chain fatty alcohol
LF	labelled feed
LFP	laboratory filter paper
LSM	least squares means
ME	metabolizable energy
MFS	mealtime frequency and sequence
n.d.	not detectable
n.g.	not given
OM	organic matter
OMD	organic matter digestibility
P	placebo
pcdAA	pre-caecal digestible amino acids
pcdCP	pre-caecal digestible crude protein
pcdLYS	pre-caecal digestible lysine
pcdMET + CYS	pre-caecal digestible methionine + cysteine
pcdTHR	pre-caecal digestible threonine
RD	relative difference

RFG	Restfeuchtegehalt
RM	residual moisture
S	suspension
SFQ	single faeces quantity
SI	soil intake
ST	storage test
TD	Trocknungsdauer
TGA	thermogravimetric analysis
TMRT	total mean retention time
TT	transit time
V	verum
VK	Verdaulichkeitskoeffizient
W	wax

1. Introduction

In horses and other farm animals, feed intake, feed, energy and nutrient digestibility, the selection of feeds, the composition of the ingested diet and digesta passage kinetics in the digestive tract are key indicators for the assessment of the nutritive value of feedstuffs and their utilization. The thorough determination of feed intake and digestibility provides information of energy and nutrient supply and utilization by the animal. Such information is necessary to assess the fulfilment of maintenance and performance requirements and to improve management decisions particularly in pasture-based husbandry systems. It furthermore enables the monitoring and prevention of supply gaps, resulting performance depression and resulting health risks, which is particularly important in growing animals. The determination of feed selection and composition of the ingested diet refines the assessment of supply, particularly in free-ranging animals, and allows for investigation of animals' intrinsic ambition to a continuous supply balance (Thomas *et al.*, 2007; Edouard *et al.*, 2010). The determination of digesta passage kinetics adds information of the quantity of feed components in the gut or segments of the gut and the efficiency of their utilization (Marais, 2000), which supports the assessment of digestibility, water balance, or digestive disorders in the animals under test (Rosenfeld *et al.*, 2006).

The direct determination of such indicators is laborious or impractical in specific situations like the investigation of free-ranging farm animals. Among a wide range of estimation methods, it is a logical result to apply dietary markers, which preferentially are immanent to the feed plants. Long-chain alkanes are abundant in the waxes of most of the common feed plants (Dove *et al.*, 1996), also in other natural waxes (Tulloch, 1970), and are available as synthetics. They have the potential to be applied as markers to estimate feed or nutrient intake, feed selection, feed or nutrient digestibility and passage kinetics simultaneously using a set of calculation methods with the same steps of preparation and analysis in the laboratory. However, the successful application of alkanes as dietary markers is mainly limited through their inconsistent recovery in faeces, which is equal in all other feed-internal and external markers that are known so far, although it has been shown that horses have the highest alkane faecal recovery (AFR) rates among the livestock species (Wilson *et al.*, 1999; Ferreira *et al.*, 2009b; Jurjanz *et al.*, 2014). Incomplete AFR probably arise from endogenous alkane absorption and metabo-

lism (McCarthy, 1964); an overestimation is, however, probably the result of cyclic diurnal fluctuations of faecal alkane concentrations (Giráldez *et al.*, 2004) and a deficient representativeness of faeces, feeds or boluses samples depending on the sample type (*i.e.* bulk or spot samples).

2. Background

The current work mainly deals with the use of plant and synthetic alkanes as markers to estimate feed intake and apparent digestibility in horses. However, this method enables the simultaneous estimation of feed selection and digesta passage kinetics, which is why these issues were briefly reviewed as well.

2.1. Methods for the determination of feed intake and digestibility *in vivo*

Voluntary intake of one or a few feed components can at the simplest be measured through its weighing and the weighing of orts. This is obviously very limited to a small quantity of feed components in the diet and a small quantity of animals in controlled housing conditions. It is therefore not feasible under more complex experimental conditions and in animals grazing on pasture. A variety of alternative methods of feed intake determination have been discussed in depth by Forbes (1995). A brief overview of available methods is given in the following.

The relationship between voluntary feed intake and feed intake activity traits (*e.g.* time spent eating; jaw movement oscillation) has been shown *e.g.* by Brøkner *et al.* (2008) and Bochnia *et al.* (2016) in ponies and horses. However, the estimation of feed intake on basis of the ingestive behaviour of animals may be error-prone due to a large dependency on diet properties, allometric effects and animal individuality. Moreover, it is less validated and can require special measuring equipment (Brøkner *et al.*, 2006; Bochnia *et al.*, 2016; Werner *et al.*, 2016). Related techniques of assessing feed intake behavioural traits in relation to intake estimation are summarized by Mayes and Dove (2000) and Ellis (2010).

In grazing animals, feed intake and intake rate can be derived from measuring the herbage mass reduction rate on an experimental pasture plot after a certain time of grazing (Duren *et al.*, 1989; Dowler *et al.*, 2012). Herbage mass is harvested and weighed before and after the grazing period on randomly selected subplots (Dowler *et al.*, 2012). The initial and residual herbage mass can alternatively be measured using the falling plate meter method (Rayburn and Rayburn, 1998; Glunk *et al.*, 2013). Such methods require the consideration of the re-increase in plant mass occurring over longer grazing periods, *e.g.* using pasture cages (Cayley and Bird, 1996). Moreover, short-term estimates of dry matter (DM) intake (DMI) are not suitable to extrapolate to longer periods and *vice versa*, because grazing

restriction may lead to a compensatory grazing behaviour, which has been shown by Ince *et al.* (2011) and Glunk *et al.* (2013) in ponies and horses, respectively. In grazing horses, Siciliano (2012) reported a positive linear relationship between intake rate and time of pasture access ($R^2 = 0.7$; $P < 0.001$). However, it is without consideration of herbage mass availability, plant and environmental characteristics, and the physiological status of the animals (Siciliano, 2012; Wood *et al.*, 2012).

Feed intake and intake rate can also be estimated from short-term or fairly long-term bodyweight changes (Romney *et al.*, 1996; Ince *et al.*, 2011; Longland *et al.*, 2011). However, this requires a thorough consideration of faecal and urinary excretory outputs and insensible weight losses, which continuously occur through respiratory evaporative cooling (Romney *et al.*, 1996; Ince *et al.*, 2011).

Feed intake can further be estimated from water turnover (Mayes and Dove, 2000) by sequential blood sampling following a single injection of $^{2}H_2O$ (Rogers *et al.*, 1985), $^{3}H_2O$, ^{2}H, ^{22}Na or related tracers (Silanikove *et al.*, 1987), which are diffusible into body fluids (Schoerb *et al.*, 1950). The underlying assumption is a constant ratio between free water intake and DMI (Silanikove *et al.*, 1987), which was initially found in sheep (Benjamin *et al.*, 1977). Temporal changes in blood tracer concentration can be determined by infrared spectrophotometry (Rogers *et al.*, 1985) and related methods. Total body water is then calculated from the tracer concentration time curve and the water turnover rate as the reciprocal of the slope of the time curve times total body water (Rogers *et al.*, 1985). However, since such tracers are partly radioactive, their use is restricted due to legal and safety reasons (Mayes and Dove, 2000).

Besides such methods, the estimation of total feed, diet component or nutrient intake from knowledge of the faecal output and inverse digestibility has widely been applied in livestock nutrition studies. The equation is provided in the formulary (Equation 1, Appendix). However, a thorough determination of faecal output and apparent digestibility is essentially required. Faecal output can be quantified *via* total collection by default, or estimated using external markers (Dove and Mayes, 2006). Determination of apparent digestibility is specified below.

The concomitant use of feed-internal digestibility markers and external output markers replacing total collection of faeces is in the focus of scientific interest for decades. For intake estimation purposes in horses and cattle, respectively, Fleurance *et al.* (2001) and Schmidt *et al.* (2004) used faecal nitrogen as the esti-

mator of apparent digestibility (see Schmidt *et al.*, 1999), coupled with faecal output quantification either by total collection or external alkanes. Krull (1984) estimated forage intake in grazing horses using chromium (Cr) sesquioxide (Cr_2O_3) as output marker and lignin to estimate digestibility. A significant simplification of the method was the algebraic combination of these separate estimates and the simultaneous use of synthetic and plant alkanes, which require for one and the same analytical process (Mayes *et al.*, 1986) The equation is provided in the formulary (Equation 2, Appendix).

Natural markers such as titanium (Ti) and hydrochloric acid-insoluble ash (AIA) may furthermore be helpful to estimate soil intake, which can be an issue of interest in grazing animals that ingest soil by choice as a source of trace- and macro-elements or inadvertently (Mayes and Dove, 2000; Jurjanz *et al.*, 2014). Since silica is also an immanent compound of feed plants (Mayland *et al.*, 1975), AIA is an estimator with restriction. To determine soil contamination in vegetation, Ti that is naturally absent in plants might be a useful marker, but representative soil sampling can be limited (Mayes and Dove, 2000).

Feeding mixed diets basally containing forage, supplemented with concentrate, is common practice in horse husbandry. Substitution effects alter intake and digestion rate of the basal forage (Mayes and Dove, 2000). Edouard *et al.* (2008) found a positive relationship between dietary fibre content and feed intake in individual horses. Usually, supplement intake is known. Is the supplement is considered as part of a mixed diet, its intake is estimable from diet component proportions obtained from discrimination by specific marker patterns (see Section 2.2.; Mayes and Dove, 2000; Dove *et al.*, 2002). The equation is provided in the formulary (Equation 3, Appendix). Considering that, the use of plant-internal alkane patterns is advantageous regarding analytical effort (Dove and Mayes, 2006), but in case of common supplements (*e.g.* grains) marker patterns cannot be distinguished anyway from that of basal forage. An additional labelling (*e.g.* with beeswax alkanes) then ensures a very unique marker profile of the supplement (Mayes and Dove, 2000; Dove and Charmley, 2008).

A variety of algebraic and model-based estimations of digesta marker passage kinetics (see Section 2.3.) and marker excretion curve characteristics have been developed, from which faecal output of the animal can be calculated (France *et al.*, 1988; Galyean, 1993; Giráldez *et al.*, 2004). Feed intake then can easily be estimated according to Mayes *et al.* (1986) (Duncan *et al.*, 1999; Giráldez *et al.*, 2004,

2006), which however requires almost constant faecal background concentrations of plant alkanes. An exemplary set of equations is provided in the formulary (Equations 4 – 6, Appendix). Variation in feed intake rate, feed intake behaviour, diet digestibility, and diurnal variation of marker excretion may alter faecal alkane concentrations over time, which may bias feed intake estimation (Duncan *et al.*, 1999; Giráldez *et al.*, 2004; Molina *et al.*, 2004).

Apparent feed or nutrient digestibility is defined as the rate of digestion without consideration of endogenous excretion. The standard procedure for digestibility determination based on total faeces collection as part of a balance between intake and output of the feedstuffs or the nutrients in question. The equation is provided in the formulary (Equation 7, Appendix). The methodology of *in vivo* digestion trials was explained in brief by Zeyner (2005).

Basing on a wide range of *in vivo* studies, predictive models to estimate digestible parts of nutrients can be developed using the linear relationship to their dietary contents, at least when the nutrients in question are nutritive uniform (Lucas *et al.*, 1961). This has been performed by Zeyner and Kienzle (2002) basing on a data set from a meta-analysis of digestibility trials with horses. As a result, a predictive model to estimate digestible nutrients and digestible energy (DE) has been developed which is recommended by GfE (2003) and NRC (2007) and further expanded to be used for the estimation of metabolizable energy (ME; Kienzle and Zeyner, 2010; GfE, 2014), and pre-caecal digestible crude protein and amino acids in the feed of horses (Zeyner *et al.*, 2010; GfE, 2014; Zeyner *et al.*, 2015). However, such predictions face the problem of absent nutritive uniformity of individual nutrients (ruminants: Van Soest, 1967; horses: Zeyner and Kienzle, 2002). Moreover, nutrient supply that overwhelms physiological digestion needs to be considered by nutrient-specific restrictions. In this way, horse diets with more than 35 % crude fibre (CF) or 8 % crude fat defy the estimation of DE and ME, respectively. According to Clauss *et al.* (2014), only a feed intake rate of more than 30 g/kg $BW^{0.75}$/d enables proper digestive function in horses.

Marker-based estimation of apparent digestibility uses the proportion of dietary and faecal marker concentrations under assumption of steady-state conditions (Dove and Mayes, 2006), which can be expanded by the reverse proportion of dietary and faecal nutrient concentrations estimating digestibility of nutrients as well (Maynard and Loosli, 1962). Equations are provided in the formulary

(Equations 8 and 9, Appendix). This feature is particularly important regarding specific interrelationships between nutrients in one diet affecting each other in digestibility (Zeyner and Kienzle, 2002). For example, dietary CF tends to have a negative effect on digestibility of other proximate nutrients (Maynard and Loosli, 1962; Zeyner and Kienzle, 2002).

It is assumed that in a diet consisting of the components *a, …, n* the intakes of these components together would theoretically result in 1 kg of faeces (Dove and Moore, 1995). Resulting from the estimation of diet composition (see Section 2.2.), digestibility can be calculated using Equation 10 (Formulary, Appendix).

2.2. Methods for the determination of feed selection and composition of the ingested diet

Observational studies on feed selection by animals are viable indoors (see LaCasha *et al.*, 1999; Hadjigeorgiou *et al.*, 2003), but are less feasible when animals graze on complex plant communities. Such observations have been performed in goats and cattle (see Barroso *et al.*, 1995; Berry *et al.*, 2002; Mayer *et al.*, 2003; Leiber *et al.*, 2006) and in horses (see Fleurance *et al.*, 2001; Menard *et al.*, 2002), but they are laborious and likely to be inaccurate (Mayer *et al.*, 2003), and there is often just a very rough estimation of feed selection obtainable (see Fleurance *et al.*, 2001). A range of selectivity indices has been developed in trying to predict the degree of selection of feed plants by assessing their relative availability and utilization through the animal (Norbury and Sanson, 1992). A summary of such indices was provided by Lechowicz (1982). According to Norbury and Sanson (1992), a major disadvantage of selectivity indices is the failing response to a changing availability of a feed plant while its utilization remains constant, which generally leads to an overestimation of positive selection. A general overview of such and additional plant-based estimation methods was given by Barnes (1976).

Macroscopic and microscopic examination of ingested feeds in mouth, oesophagus and stomach contents, and in faeces, was commonly applied, which may enable accurate estimates but may also require slaughtering or fistulation of test animals and the examined feeds ingested belong only to a very short period of time (Dove and Mayes, 1996). Furthermore, a rough differentiation between the proportions of grasses and legumes in herbivore diets might be possible through

the analysis of their specific calcium contents or their natural carbon isotopic ratios (^{12}C:^{13}C; see Dove and Mayes, 1996).

A more sophisticated approach for the estimation of diet composition is based on the utilization of alkanes and other markers that are abundant in the feed plants. This approach enables to apportion the voluntary intake into its component plant groups, plant species or, theoretically, even plant parts (Dove and Mayes, 2006), or the differentiation between plant and soil intake (Jurjanz *et al.*, 2014). The method uses the principle that plants or plant parts can be discerned from a unique profile of markers evident in faeces (Dove and Mayes, 2006). The more complex the diet is, the more markers are required to avoid an increasing amount of similar and overlapping marker profiles among the species in a consumed plant community. This is why, apart from the long-chain alkanes, also long-chain alcohols (LCOH) and fatty acids (LCFA), as well as their natural ^{13}C enrichments have been applied as additional markers (Ali *et al.*, 2004; Bugalho *et al.*, 2004; Ali *et al.*, 2005; Dove and Charmley, 2008; Ferreira *et al.*, 2009a; Lin *et al.*, 2009; Ferreira *et al.*, 2010; Bezabih *et al.*, 2011a, b; Lin *et al.*, 2012; Ferreira *et al.*, 2014, 2015; Cottle, 2016). Otherwise, plants with similar marker patterns have to be grouped, which hinders a fine estimation. In pigeons, Hatt *et al.* (2001) used synthetic alkanes to estimate the selection of specifically labelled feed pellets. The application and specific challenges of the method in farm animals with emphasis on horses is described in Section 2.5.6. The non-negative least-squares optimization (Dove and Moore, 1995) is the most commonly applied method to obtain diet composition estimates using marker profiles in feed and faeces. The sum of squared discrepancies between the measured faecal proportion and the estimated dietary proportion of a marker is minimized to obtain the proportion of hypothetical intakes of the dietary components assuming that those intakes contribute and would in sum lead to 1 kg of faeces (Dove and Moore, 1995; Dove and Mayes, 2006). The general equation is provided in the formulary (Equation 11, Appendix). Further mathematical approaches and developments were described by Newman *et al.* (1995, 1998), Martins *et al.* (2002) and Barcia *et al.* (2007). The validation or complementation of the method with qualitative observation techniques (macroscopic or microscopic examination of extrusa or faeces) might be helpful (Dove and Mayes, 2006). An important advancement is the specific weighting of markers emphasizing those that differ more between plant groups, species or parts than others with a higher weight (Mayes and Dove, 2001; Cottle and Romero, 2014).

2.3. Methods for the determination of the kinetics of digesta passage

Gut passage kinetics are most commonly described by (1) the transit time (TT) of an internal or as usual an external marker, which is per definition the time delay between marker administration (either oral, intraruminal, gastral or intraluminal) and the first appearance of the marker in faeces or in chymus of any desired gut segment, and (2) its total mean retention time (TMRT), which is the mean time that the marker is restrained in the digestive tract and the associated feed component is available for digestion (Holleman and White, 1989). The phases of digesta can specifically be labelled using adequate markers, which are summarized and reviewed in the following (Sections 2.4.2. and 2.4.4). A specific summary of markers used to predict TMRT in horses was provided by Van Weyenberg *et al.* (2006).

Principally, two experimental approaches are known for the estimation of digesta passage kinetics as summarized by Van Weyenberg *et al.* (2006): Firstly, according to Castle (1956) and Todd *et al.* (1995), the external marker is administered frequently over a suitable time for adaption (*e.g.* 14 days; Castle, 1956) to reach steady-state conditions for the faecal marker concentrations, and is subsequently removed abruptly followed by a faeces sampling period; or secondly, the marker is administered onetime followed by frequent sampling of faeces, where the sampling interval can increase with time, which is nowadays the standard approach (exemplary faeces sampling schedules are given by Galyean, 1993; Duncan *et al.*, 1999; Giráldez *et al.*, 2004; or Bulang *et al.*, 2008).

To overcome erratic and diverging flow estimates for liquid and particulate digesta markers being obtained from spot sampling of digesta or faeces, Faichney (1975) proposed to apply a double-marker system labelling the liquid and the particulate phase of digesta simultaneously. However, continuous marker infusions, or oral applications, and steady-state conditions are required (Faichney, 1975). Then, it is possible to reconstitute true digesta and flow estimates for true digesta by combining liquid and particulate phases mathematically by means of liquid and particulate marker concentrations (Faichney, 1975). Water, electrolyte or nutrient flow can likewise be estimated (Faichney, 1975). However, marker migration and incomplete recovery may limit this approach (see Section 2.4.4.).

Different approaches for the algebraic calculation of TT and TMRT have been developed (Blaxter *et al.*, 1956; Faichney, 1975; Thielemans *et al.*, 1978; Todd *et al.*, 1995), of which those proposed by Faichney (1975) and Thielemans *et al.* (1978)

are among the most commonly used ones, especially in horse studies (*e.g.* in Rosenfeld *et al.*, 2006; Goachet *et al.*, 2009), but their application depends on the method of faeces sampling (see Goachet et al., 2009). For a periodic spot sampling of faeces, the approach of Thielemans *et al.* (1978) to determine TMRT is provided in the formulary (Equation 12, Appendix). According to McGreevy *et al.* (2001), basing on Cummings and Wiggins (1976), mean TT can algebraically be calculated using Equation 13 (Formulary, Appendix).

Throughout the gut, the digesta passes multiple consecutive compartments where mixing, reflux and retaining may occur. Tubular segments where no reflux and mixing occur are located interjacently (Bulang, 2005). For mathematical modelling, it has been considered that digesta particles and associated markers are restrained in one, two or an uncertain quantity (multiple) of mixing compartments for a certain time, and that fractional marker outflow is exponential (Blaxter *et al.*, 1956; Grovum and Williams, 1973). Fractional residence in the mixing compartments serves for digestion and microbial fermentation and depends on time (Bulang, 2005). Theoretically, the outflow rate of digesta particles increases with an increasing time of residence in a mixing compartment (Pond *et al.*, 1988). On that basis, models that consider time-dependency for fractional passage rates have been developed (Pond *et al.*, 1988). Time-dependency of gut passage is ideally described by γ-distribution (Matis, 1972). Passage rate models can have a single-compartmental structure with γ-time-dependency, a two-compartmental structure with γ-time-dependency in the first but time-independency in the second mixing compartment (Matis, 1972; Pond *et al.*, 1988), or a time-independent multi-compartmental structure (Dhanoa *et al.*, 1985). It is assumed that fractional passage (or fractional residence) rate (K) is, in a one-compartmental model, initially zero and then increases with time approaching a constant asymptote representing the maximal time-dependent fractional passage (or residence) rate (Pond *et al.*, 1988). In two-compartmental models, K1 and K2 are the fractional passage rates in the time-dependent and the time-independent compartment, respectively (Rosenfeld *et al.*, 2006). In the time-independent multi-compartmental model of Dhanoa *et al.* (1985), K1 and K2 represent outflow from the compartments with the longest retention times. Using these fractional passage/retention times, TT and TMRT can be calculated in turn. In horses, the single-compartmental (Holland *et al.*, 1998) or two-compartmental form (Moore-Colyer *et al.*, 2003; Rosenfeld *et al.*, 2006), and in ruminants, the multi-compartmental form have most of-

ten been applied (Giráldez *et al.*, 2004, 2006; Bulang *et al.*, 2008). Time-dependent models considering a fourth-class γ-distribution of fractional passage rates (Pond *et al.*, 1988) are recommended in horses (Moore-Colyer *et al.*, 2003; Rosenfeld *et al.*, 2006). A more complex approach for mathematical modelling of passage kinetics is provided by Aharoni *et al.* (1999). The models that are presented there consider the gut flow of solutes and fine particles and the flow of larger and coarse particles on separate parallel routes, passing an individual number of passage time-delaying mixing compartments, and they consider bypass fluxes within and between these routes (Aharoni *et al.*, 1999).

It is assumed that TT represents the residence of the marker in tubular segments outside the mixing compartments (in horses: stomach, small intestine, and rectum; Hyslop, 2003; Rosenfeld *et al.*, 2006). As shown by Van Weyenberg *et al.* (2006), and based on information provided by Drogoul *et al.* (2000), main sites of digesta separation in the digestive tract of horses are: (1) the caecum and the ventral colon, where coarse particles are selectively retained, probably by gravity-assisted segregation in the former (Rosenfeld *et al.*, 2006); (2) the *Flexura pelvina*, where coarse particles are retained; and (3) the right dorsal colon, where liquids and fine particles are selectively retained. Current mathematical modelling, however, still fails to provide a clear biological interpretation and attribution (Moore-Colyer *et al.*, 2003).

2.4. Marker methods in nutrition studies, their advantages and limitations

2.4.1. Reasons for the need of marker methods

Although the quantification of faecal output by total collection is still one of the most exact methods (Takagi *et al.*, 2002a; Bergero *et al.*, 2009), it is likewise the most limiting factor for the determination of feed intake and apparent digestibility. Total faeces collection provides most stressful conditions for the animal under test, especially when metabolism stalls are used (Huhtanen *et al.*, 1994; Bergero *et al.*, 2009). It may impair animal welfare (Parkins *et al.*, 1982) and normal feed intake behaviour of the animals (Dove and Mayes, 1996; Mayes and Dove, 2000; Glindemann *et al.*, 2009; Sales, 2012). Indirect intake determination using measured faecal output and indigestibility variables (Dove and Mayes, 1991) may furthermore be biased by inaccurate faeces collection (*e.g.* by the use of harness aprons). Hence, investigations on grazing animals are of particular complicacy,

and direct measurement of herbage intake is impractical (Glindemann *et al.*, 2009). Observations or measurements of behavioural traits are possible tools for quantifying feed intake (Romney *et al.*, 1996; Mayes and Dove, 2000), but are often impractical, and likewise stressful for the animals.

In digestion trials, it is necessary to quantify faecal output by total collection of faeces, because *in vitro* trails are unable to reflect *in vivo* digestibility sufficiently (Cochran *et al.*, 1986). Determination of digestibility *in vitro* does not consider variations in intake, animal individuality or interactions between dietary components, which all may lead to a high variability of feed and nutrient digestion *in vivo* (Dove and Mayes, 1996). Besides aforementioned limitations, a total collection trial restricts the natural or accustomed exercise, which is critical particularly in horses that are fed high-energetic test diets (Frape *et al.*, 1982) and which can alter animal physiology, behaviour (Bowers *et al.*, 1993) and the estimation of digestibility coefficients (Pagan *et al.*, 1998; Takagi *et al.*, 2002a). Moreover, a total collection of faeces is very hard to implement in livestock kept in groups or on a pasture, and it is, not to be underestimated, a laborious and time-consuming procedure (Maynard and Loosli, 1962). In large animals, large quantities of faeces have to be handled during processing and storage (Takagi *et al.*, 2002b; Sales, 2012), which can be reduced through aliquot sampling, although large quantities remain.

To determine traits of feed intake behaviour, direct observations have been performed, assisted by video monitoring, acceleration technics, pressure sensing changes for counting jaw movements, and a variety of other tools (Mayes and Dove, 2000). Measured chewing times and chewing activity have been used to quantify the intake of various roughages, variously processed types of grains, or pasture in horses (Brøkner *et al.*, 2008; Bochnia *et al.*, 2016). Potentially, feed components may roughly be discerned by that way (Dove and Mayes, 1996), which is, however, less sensitive for estimation of feed selection and diet composition in complex consisting diets. As reviewed by Norbury and Sanson (1992) and Dove and Mayes (1996), the macroscopic and microscopic visual examination of ingested feeds in mouth, oesophagus or stomach contents, or in faeces, have widely been performed, but such techniques require slaughtering or fistulation, or at least very well trained observers. Apart from this, individually ingested feeds belong only to a very short period of time, and are modified through a specific rate of digestion (Dove and Mayes, 1996). Furthermore, discrimination by calcium content or natural carbon isotopic ratio in oesophageal extrusa or faeces has been

evaluated for the estimation of proportions of grasses and legumes in herbivore diets (Playne *et al.*, 1978; Jones *et al.*, 1979; Dove and Mayes, 1996). In faeces, this method tends to underestimate intake of high-digestible plants, because less of their carbon remains in faeces, whereas it underestimates the intake of lower-digestible plants in animal tissues such as wool and hair (Mayes and Dove, 2000). The analysis of specific plant marker profiles may allow for an adequate differentiation of ingested feed plants also in complex diets (see Fraser *et al.*, 2006).

For prediction of digesta passage kinetics, feed-internal or external digesta markers are essentially required, because so far an alternative method does not exist.

2.4.2. Available markers and their classification

The marker substances that can be used to estimate feed intake, digestibility and related variables in herbivore and omnivore farm animals, pets, zoo animals or wildlife animals can generally be divided in plant-internal and external markers. A selection of the most commonly used markers is provided in Table 1. Additional information can be found in the reviews of Kotb and Luckey (1972), Owens and Hanson (1992), Marais (2000), Dove and Mayes (2006) and Sales (2012).

Table 1. Selection of plant internal and other natural markers, as well as external markers, mordants and other markers artificially bound to plant materials that have been used or might potentially be useful for the estimation of feed intake, faecal output and/or apparent digestibility in farm animals, modified and extended according to Mayes and Dove (2000)

Marker description	Marker type	Digesta association	Additional or main application
Plant markers			
Lignin, acid detergent lignin and associated preparations [2, 6, 10, 11, 12, 15]	Plant fibre fraction	Particulate	Passage kinetics
Indigestible acid detergent fibre and similar preparations [6, 10, 11, 12, 15]	Plant fibre fraction	Particulate	Passage kinetics
Indigestible neutral detergent fibre and similar preparations [6]	Plant fibre fraction	Particulate	Passage kinetics
Acid detergent insoluble nitrogen [12]	Plant fibre fraction	Particulate	Passage kinetics
Hydrochloric acid insoluble ash (usually 2 N or 4 N) [2, 7, 10, 12, 15, 16, 25]	Siliceous	Particulate	EM, SI
Silicon dioxide [1]	Siliceous	Particulate	Not known
Long-chain alkanes, alkenes, fatty acids (LCFA) and alcohols [11, 12, 15, 18, 22, 25, 27]	Wax compounds	Mainly particulate	Diet composition and feed selection
Stable isotope ratio ($\delta^{13}C$) or labels (^{13}C) of *e.g.* alkanes, LCFA, lignin [2, 12, 15, 23, 24]	Natural isotopes	Particulate	Diet composition, passage kinetics
External markers			
Chromium (Cr) sesquioxide and other trivalent metal oxides [2, 11, 12, 14, 15, 16, 18, 19, 28]	Insoluble oxide	None	Passage kinetics, CRD, EM
Titanium dioxide [2, 15, 16, 19, 21]	Insoluble oxide	None	Passage kinetics, EM, SI, rate of soil pollution
Barium sulphate [2]	Insoluble salt	None	Radiography
Plastic particles [2, 3, 9, 12]	Insoluble polymer	None	Passage kinetics
Synthetic alkanes [15, 20]	Artificial wax compounds	None	Passage kinetics, CRD
Cr- or cobalt-ethylenediaminetetraacetic acid and isotopes [3, 15, 29]	Soluble complex	Liquid	Passage kinetics
Polyethylene glycol [2, 3, 9, 12, 15]	Soluble polymer	Liquid	Passage kinetics
e.g. $^{2}H_2O$, $^{3}H_2O$, ^{2}H, ^{3}H, ^{18}O and ^{22}Na tracers or microspheres [8, 11, 15]	Tracers	Liquid, none	Water and sodium turnover
Mordants and other artificially bound markers			
Dyed feeds [2, 12]	Pigments	Mainly particulate	Passage kinetics
Cr-, cerium-, cobalt- or hafnium-mordanted fibre or concentrates [4, 5, 15, 16, 29]	Bonded insoluble oxides	Mainly particulate	Passage kinetics, EM
Lanthanides (Ce, Dy, Er, Eu, Yt, Yb) including isotopes [2, 5, 12, 15, 26]	Rare earth elements	Mainly particulate	Passage kinetics
Ruthenium-phenanthroline including isotopes [15]	Soluble complex	Mainly particulate	Passage kinetics
Beeswax, candelilla wax or synthetic alkanes [13, 17]	Bonded wax compounds	Mainly particulate	Passage kinetics

References: [1]McManus *et al.* (1967); [2]Kotb and Luckey (1972); [3]Argenzio *et al.* (1974); [4]Udén *et al.* (1980); [5]Mader *et al.* (1984); [6]Cochran *et al.* (1986); [7]Fuchs *et al.* (1987); [8]Silanikove *et al.* (1987); [9]Kaske and von Engelhardt (1990); [10]Sunvold and Cochran (1991); [11]Dove and Coombe (1992); [12]Owens and Hanson (1992); [13]Dove and Oliván (1998); [14]Austreng *et al.* (2000); [15]Marais (2000); [16]Sales and Janssens (2003); [17]Elwert and Rodehutscord (2004); [18]Dove and Oliván (2006); [19]Myers *et al.* (2006); [20]Bulang *et al.* (2008); [21]Glindemann *et al.* (2009); [22]Ferreira *et al.* (2010); [23]Warner *et al.* (2013); [24]Ferreira *et al.* (2014); [25]Jurjanz *et al.* (2014); [26]Miyaji *et al.* (2014); [27]Ferreira *et al.* (2015); [28]Kerr *et al.* (2015); [29]Hummel *et al.* (2016). CRD, controlled-release device; EM, energy metabolizability; SI, soil intake.

2.4.3. General requirements for markers in nutrition studies

Valuable reviews provided by Maynard and Loosli (1962), Faichney (1975) and Sales (2012) summarized the criteria that a marker has to fulfil is, ideally as follows: (1) The marker substance has to be non-toxic to the target animal (Sales, 2012); (2) it must not influence the physiological processes in the digestive tract (Sales, 2012), nor influence gut health through laxative, obstipant or related effects (reviewed by Glindemann *et al.*, 2009); (3) it must not alter nutrient digestibility; (4) itself has to be unaltered during the passage throughout the digestive tract (Sales, 2012), either by plant or endogenous enzymes, acid hydrolysis, or through microbial fermentation, and (5) it must not be absorbable (Faichney, 1975) by the intestinal epithelium (see McCarthy, 1964), which perhaps may be possible by phagocytosis and transcytosis through microfold cells (see Büsing *et al.*, 2011); (6) it has to be closely associated with the phase of digesta that is target to label (Faichney, 1975), or with the undigested nutrient in question, or even flow at an identical rate (Sales, 2012); (7) it has to be completely recovered in faeces, consistently over time (Glindemann *et al.*, 2009; Sales, 2012); and (8) the method for detection has to be specific, sensitive and must not interfere with other analyses (Faichney, 1975). The marker is preferably a natural constituent of the feed (Maynard and Loosli, 1962). However, there is so far no substance known, which can completely fulfil all these criteria.

2.4.4. Marker association to the liquid and particulate digesta phase, resulting and further limitations

The marker type, the way of labelling or bolus preparation, and the specific form of administration affect the association of the marker to the phases of digesta. In herbivore animals, only markers of plant origin can label the particulate phase of digesta effectively. Chemically bound mordants and other artificially bound markers are mainly particulate-associated but it is not known sufficiently whether such bonds can be undone during the passage throughout the digestive tract, perhaps due to an exposure to acidic conditions (Crooker *et al.*, 1982). There are some soluble markers and isotopic tracers known, which are useful to label the liquid phase of digesta (see Table 1). Markers, which are administered *via* boluses, are due to this form of application neither fully associated with the particulate

phase nor with the liquid phase of digesta, and can migrate between these two phases (Bulang, 2005). As reported by Mayes *et al.* (1988), 30 to 40 % of the administered quantity of the synthetic *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) alkanes were found in the supernatant fluid of digesta samples after centrifugation. A lower rate of migrated Cr (up to 6 %) and synthetic alkanes (up to 4 %) was found in the liquid phase of sheep' ruminal fluid *in vitro* (Bulang, 2005). Also Owens and Hanson (1992) described the potential risk of migrating markers, especially in rare earth elements. Evidence of an incorporation of Cr, that had been sprayed onto lucerne fibres, by intestinal microbes up to 1 % and of the synthetic alkanes C28 and C36, sprayed onto the fibre as well, up to 5 % was found by Bulang (2005) and was previously suggested by Owens and Hanson (1992) for isotopic feed labels and for chelates of the rare earth elements, too. Incorporation of feed plant and external alkanes by liquid-adherent ruminal bacteria was also reported by Keli *et al.* (2013) investigating sheep. It was further supposed that mordanted lanthanides most notably may migrate from labelled particles onto smaller particles or into solutes in the digesta, which were previously unlabelled, and can thus alter the estimation of passage kinetics (Crooker *et al.*, 1982; Ellis *et al.*, 1982; Mader *et al.*, 1984; Bulang, 2005). Thereby, the technique of forage labelling essentially affects the migrating behaviour of such markers (Mader *et al.*, 1984).

Most of the available markers show widely similar limitations of their suitability for the estimation of feed intake, feed selection, digesta passage kinetics and digestibility. One major limitation is the inconsistent and predominantly incomplete recovery in faeces, which affects the estimation of digestibility and diet composition, and less so also the estimation of feed intake when the recovery rates of two paired markers differ (Dove and Mayes, 2006). Incomplete faecal recovery has been reported, in sheep, for Cr from Cr-labelled lucerne fibre (95 % mean recovery) by Bulang *et al.* (2008), in cattle, for Ti dioxide (TiO_2; 95 % mean recovery) by Titgemeyer *et al.* (2001), in horses, for Cr mixed with concentrate prior to pelleting or top-dressed onto the concentrated feed (60 to 81 % mean recovery) by Patterson *et al.* (2002), and in pigs, for Cr_2O_3 (75 to 80 % mean recovery) and TiO_2 (97 to 98 % mean recovery), mixed into the diet, by Jagger *et al.* (1992). In contrast, faecal recovery was almost complete in the studies of Garcia *et al.* (2001) *in vitro* and Glindemann *et al.* (2009) in sheep using lanthanides and TiO_2, respectively. Incomplete faecal recovery was shown in plant and synthetic alkanes many

a time, which is explained in more detail below. Incomplete faecal recovery has especially been reported for potentially indigestible plant fibre fractions (Cochran *et al.*, 1986). Lignin, a major plant marker, can thereupon be digested to a notable extent with recovery rates from 81 to 92 % being obtained in horses (Palmgren Karlsson *et al.*, 2000; Goachet *et al.*, 2009), probably through the fermentative activity of cellulolytic microbiota. Lignin, measured as acid detergent lignin, may chemically be altered due to the formation of soluble lignin-carbohydrate complexes or otherwise (*e.g.* by acid hydrolysis) during gut passage, which is why lignin is not fully detected in the fibrous residues of faeces (reviewed by Fahey and Jung, 1983). A destruction of faecal lignin by analytical reagents has also been reported (see Fahey and Jung, 1983). However, also overestimated recovery rates were obtained (Cochran *et al.*, 1986), possibly as a result of bonded phenolic monomers that contributed to the lignin fraction isolated in animal faeces (Fahey and Jung, 1983). Dietary silicon (Si), which has been used as a marker in form of Si dioxide (SiO_2), was, in sheep, reported to be intestinally absorbed in part and re-excreted *via* the urine (Nottle and Armstrong, 1966; McManus *et al.*, 1967). Additionally, Si may be accumulated in body tissues or resides as phytolith and urolith particles in the intestine and the urinary tract, respectively (Baker *et al.*, 1961a, b; McManus *et al.*, 1967). Such a temporal intestinal accumulation of biogenic and environmental silicates may perhaps also lead to an incomplete recovery of AIA in faeces. Feed pollutions with soil, occurring particularly in roughages and silages, might have led to overestimated recovery rates in previous animal studies (see Sunvold and Cochran, 1991; Goachet *et al.*, 2009). The use of mordanted transition metals are limited in the amount that can be added to a feed because they can easily increase the density of the marked material, which then results in an altered flow (Ehle, 1984; Kaske and von Engelhardt, 1990). Moreover, mordants may alter the labelled feed chemically, which can affect its solubility and digestibility (Bulang *et al.*, 2008). Adding either Cr_2O_3, ferric oxide (Fe_2O_3) or TiO_2 to pig diets revealed little but measurable alterations of microbial ecology, *i.e.* decreasing faecal *Archaea* counts (Fe_2O_3), and digestibility of macro- and trace elements (Kerr *et al.*, 2015). During a previous study, we observed the adsorption of orally administered Cr_2O_3 at the gastric mucosa of euthanized horses, which is exemplary shown in Fig. 1. It is currently not known whether such an adsorption is temporal or permanent, but – similar to marker sedimentation – it is assumed to alter marker passage and may lead to a reduced recovery of the marker in faeces. As

indicated by Titgemeyer *et al.* (2001) and Myers *et al.* (2006), health risks owing to carcinogenic properties of Cr_2O_3 have to be kept in mind. Further detailed information about the advantages and limitations of markers are provided by Sales (2012) and other aforementioned review articles.

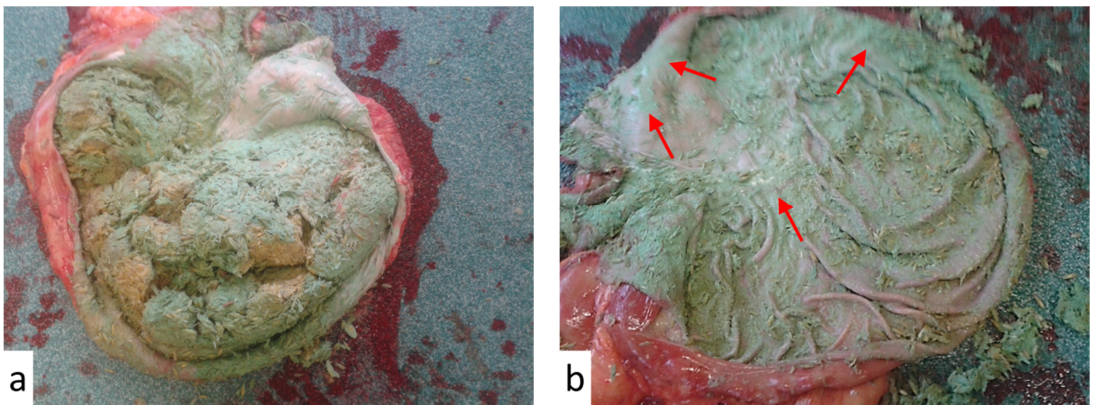

Fig. 1. (a, b) Photographs of the stomach of an euthanized horse that has previously been dosed with chromium sesquioxide *via* bolus frequently (2 times a day) over a period of 2 weeks; (a) the majority of the marker was visible in the chymus; however, (b) also considerable colouring of large areas of the mucosa were observed (as shown by arrows), which remained present even after thorough washing (source: Martin Luther University, Institute of Agricultural and Nutritional Sciences, Group Animal Nutrition).

2.5. *n*-Alkanes as dietary markers with particular emphasis on horse studies

2.5.1. Physicochemical characteristics of the *n*-alkanes

n-Alkanes represent the plainest form of saturated hydrocarbon molecules with unbranched and non-cyclic chains of single carbon-carbon and carbon-hydrogen bonds. Details regarding the molecular geometry and possible conformation of alkanes can be found in Mortimer and Müller (2007) or in similar textbooks. In alkanes, intermolecular interaction only takes place through van der Waals forces, which increase with an increasing chain length (CL) of the molecules. Alkanes are nonpolar, thus do not form hydrogen bridge bonds, are immiscible in water, and are chemically very inert (Labinger and Bercaw, 2002). However, they are *i.a.* able to react with oxygen, so can combust either completely or incompletely depending on the availability of oxygen (Mortimer and Müller, 2007), and may react also with nitrate, ferric iron and sulphate, which is used by anaerobic alkane-degrading bacterial strains or some *Archaea* (Zengler *et al.*, 1999).

2.5.2. Biosynthesis, occurrence and variability of alkanes in natural waxes

The pronounced hydrophobicity of alkanes and other aliphatic compounds is, in nature, used by all lower and higher land plants as an adaptive tool to prevent the leave surface, and secondary the surfaces of stems, fruits or petals, from becoming saturated with water (Holloway, 1969; Koch and Ensikat, 2008), which would hinder cuticular transpiration (Linskens *et al.*, 1965). However, also uncontrolled water loss through transpiration is prevented in that way (reviewed by Koch and Ensikat, 2008). Thus, epicuticular and intracuticular waxes essentially help to preserve the water balance of the plant (Eglinton and Hamilton, 1967). As reviewed by Holloway (1969), epicuticular wax compounds are responsible for water repellency, and those embedded in the cuticle are responsible for water resistance, also serving as a water transpiration barrier (Koch and Ensikat, 2008). Holloway (1969) described the wettability of different leave waxes, including *Euphorbia antisyphilitica* and *Stipa tenacissima* leave waxes, and individual wax constituents after studying the specific contact angle of distilled water on smooth model films made of each material. The contact angles ranged from 70° in α-ω-diols to a maximum of 109° in the alkane fraction and did not differ by CL within each fraction (Holloway, 1969). Despite that each of the isolated fractions were not very water repellent (Holloway, 1969), the specific composition of a plant wax and its ultrastructure define the surface's water repellent properties. Aliphatic molecules are oriented to expose the terminal methyl groups on the surface and wettability of this surface is at a minimum when the methyl groups are packed as closely as possible (Holloway, 1969), which is the case in alkanes. Thus, a high amount of alkane molecules in epicuticular waxes probably improve their water repellency and water repellency is greatest when the waxes have a crystalline or semi-crystalline ultrastructure (Holloway, 1969). Although alkanes are found to be widely distributed in plant epicuticular waxes, they occur usually in low concentrations (Koch and Ensikat, 2008). Additional functions of epicuticular and intracuticular waxes may include minimizing mechanical damage to subjacent cells, inhibiting fungal and insect attacks (*e.g.* by the self-cleaning property of superhydrophobic plant surfaces) and protecting the plant from excessive ultraviolet radiation (reviewed by Eglinton and Hamilton, 1967; Koch and Ensikat, 2008). Informative schematic representations of the cuticular layers are provided in Eglinton and Hamilton (1967) and in Kunst and Samuels (2003). Chemically, plant waxes are in the main

composed of predominantly odd-chain *n*-alkanes, mono- and di-ketones and secondary alcohols, and predominantly even-chain carboxylic acids, primary alcohols, aldehydes, α-ω-diols, hydroxy acids and dicarboxylic acids, each present in a homologous series with homologs usually ranging from 20 to 40 carbon atoms (Eglinton and Hamilton, 1967; Kunst and Samuels, 2003; Koch and Ensikat, 2008). The waxes' chemical composition and their distribution onto and within the plant surfaces is variable amongst species, plant organs and during ontogeny (Winstel and Rentschler, 1975; Jetter and Schäffer, 2001), but the adaptive response to environmental factors (*e.g.* drought or excessive light intensities) is predominantly quantitative and lesser qualitative (reviewed by Koch and Ensikat, 2008). Morphologically, the most commonly found epicuticular plant wax variants range from thin wax films to several three-dimensional structures such as crusts, granules, plates, platelets as *e.g.* in *Trifolium pratense* and *Medicago sativa* (Friedemann *et al.*, 2015), filaments, rods and tubules, usually occurring within 0.2 to 100 µm in size (reviewed by Koch and Ensikat, 2008). The *n*-alkane layers usually have a plain morphology, whilst more complex structures can be formed by molecules with terminal polar groups (*e.g.* fatty acids, aldehydes and primary alcohols), and especially by the secondary alcohols and the ketones (Koch and Ensikat, 2008). Thereby, these crystalline structures mostly appear onto an underlying wax film, which can be limited to a few molecule layers only (reviewed by Koch and Ensikat, 2008). Plant wax crystals can appear randomly distributed onto the surface or can show a specifically organized orientation (where an orthorhombic symmetry is the most common one), but ageing and erosion may lead to a wide range of intermediate forms (Koch and Ensikat, 2008). This largely affects the cuticle's permeability (Vogg *et al.*, 2004) and its transpiration barrier function. Impressive electron-microscopic images of some usual feed grasses and legumes such as *Dactylis glomerata*, *Medicago sativa*, *Vicia faba*, *Pisum sativum* and various species of clover were provided by Winstel and Rentschler (1975) and Friedemann *et al.* (2015), also showing specific alterations of the epicuticular waxes throughout the vegetation period in the former reference.

The proposed pathways of cuticular wax biosynthesis, which are localized in the epidermal cells, are schematically summarized in Fig. 2, and are reviewed and explained in much more detail by Kunst and Samuels (2003).

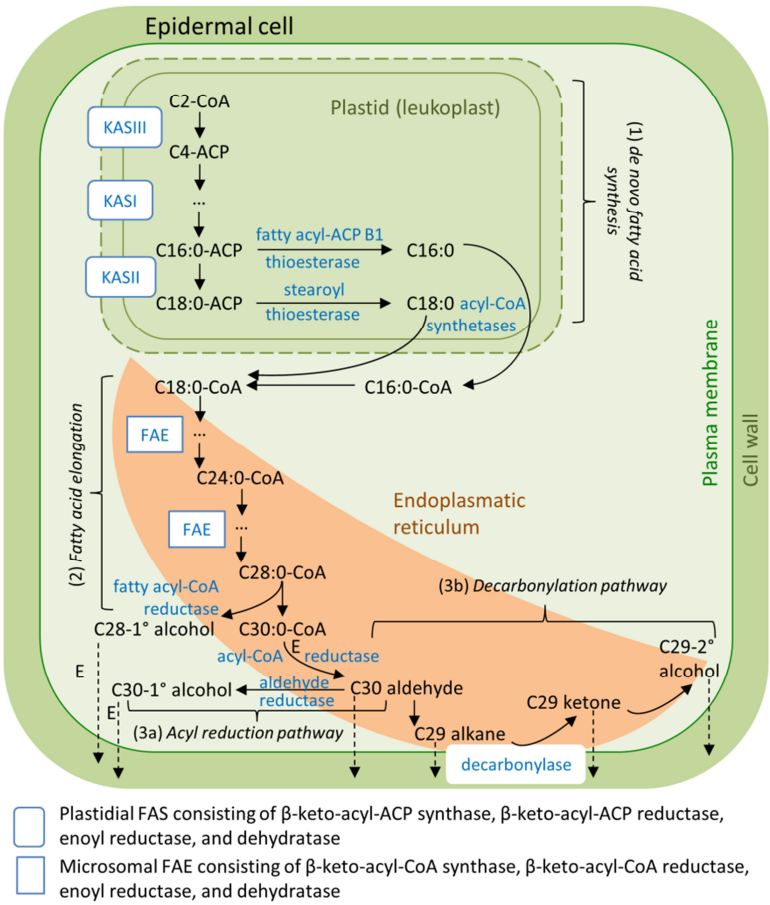

Fig. 2. Schematic and simplified description of cuticular wax biosynthetic pathways in epidermal cells as proposed and reviewed by Kunst and Samuels (2003): (1) *de novo* fatty acid synthesis in the stroma of specialized plastids by the enzymatic fatty acid synthase (FAS) complex that involves the acyl carrier proteins (ACP) as protein cofactors attaching the growing acyl chains, following the reaction series for the extension of two carbon atoms: the condensation of a C2 moiety of malonyl-ACP to acetyl-CoA, the reduction of β-ketoacyl-ACP, the dehydration of β-hydroxyacyl-ACP, and the reduction of trans-Δ2-enoyl-ACP – the types of FAS that are required for the *de novo* synthesis up to C18 fatty acids are: KASIII for C2 to C4 synthesis, KASI for C4 to C16 synthesis, and KASII for C16 to C18 synthesis – the subsequent liberation from the ACP by thioesterases, thiolesterification to CoA, and export to the endoplasmatic reticulum (ER); (2) extension of the ubiquitous C16 and C18 fatty acids to fatty acid wax precursors by extra-plastidial membrane-associated fatty acid elongases (FAE) similar to the aforementioned reaction series; (3) wax biosynthesis through (a) the enzymatic reduction of the fatty acid precursors to aldehydes followed by the reduction to primary alcohols, or a single step of the reduction to primary alcohols, and wax ester (E) synthesis by a fatty acyl CoA:fatty alcohol acyltransferase; or (b) the enzymatic decarbonylation of aldehydes to odd-chain alkanes, their hydroxylation to secondary alcohols with the further oxidation to ketones; and finally the passing of wax components throughout the plasma membrane and the hydrophilic cell wall, firstly, as surmised, *via* sites of close apposition of ER domains with the plasma membrane, or *via* vesicular traffic from the ER to the Golgi apparatus and the plasma membrane, and secondly, through the mediation of lipid transfer proteins, or through hydrophobic subdomains within the cell wall.

When using the plant wax alkanes as dietary markers in nutrition studies, it is important to know that the concentration levels and the individual patterns of alkanes vary (1) intensively among plant species, as summarized in the review articles of Dove and Mayes (1991, 1996) for a selection of the most common plant species of temperate and tropical pastures and rangelands. In spite of the large interspecies variation, it is obvious that, in general, mainly the alkanes with 25 to 35 carbon atoms can be detected emphasizing *n*-nonacosane (C29), *n*-hentriacontane (C31) and *n*-tritriacontane (C33), while alkanes with a lower number of carbon atoms are usually represented in much smaller concentrations, and the odd-numbered alkanes are, as aforementioned, usually dominant in the flowering plants (Dove and Mayes, 1991). Usually, grains do not contain considerable quantities of alkanes when prepared for feeding, and even very small quantities were found *e.g.* in the leaves of *Hordeum vulgare*, which were richer in LCOH, particularly in 1-hexacosanol (1-C26-OH; Reynhardt and Riederer, 1994). Wheat straw, by contrast, contains considerable quantities of C29 (165 mg/kg OM) and C31 (198 mg/kg OM) alkanes, and again large amounts of alcohols, particularly 1-C26-OH (236 mg/kg OM) and 1-octacosanol (1-C28-OH; 707 mg/kg OM) as reported by Dove and Charmley (2008). Additionally, the concentrations of alkanes (2) vary among different parts of the same plant, which has been shown by Dove *et al.* (1996), Smith *et al.* (2001) and Gamarra and Kahmen (2015) for some common pasture species, (3) vary during ontogeny as shown *e.g.* by Dove *et al.* (1996) for the stems of *Medicago sativa* decreasing distinctly in the concentrations of C29 and C31 alkanes as the distance from the growing point increases, and (4) vary among harvest dates, seasons (Dove *et al.*, 1996; Smith *et al.*, 2001) and vegetative cycles (Malossini *et al.*, 1990; see Fig. 3), which in part may reflect the adaptive dynamics of the plant facing continuously changing environmental influences (see above). Information about the composition of and the variations among several rangeland species and of trees and bush plants of subtropical and tropical regions are provided by Ali *et al.* (2005) and Guo *et al.* (2014), which may be of interest studying the intake, the composition of the ingested diet or the digestibility in indigenous livestock species or in exotic herbivore and omnivore animals. In addition to alkanes, considerable quantities of LCOH and LCFA can be detected and used as diet composition markers. As mentioned above, the primary alcohols and fatty acids of plant waxes are mainly the even-chain ones (the ratio of even- to odd-chain fatty acids was found to be 1:10 in *Lolium perenne* and 1:4 in *Trifolium*

repens; Ferreira *et al.*, 2009a). The variation of these wax compounds among some common pasture grass and herb species is provided in Fig. 4 as examples. Alkenes were found to be present particularly in the inflorescences of *Lolium perenne* (66 to 495 mg/kg OM on average; 25 to 29 carbon atoms) and *Phalaris aquatica* (34 to 91 mg/kg OM on average; 25 to 29 carbon atoms) grasses, or in the petioles and stolons of *Trifolium repens* (1 to 13 mg/kg OM on average; 25 to 29 carbon atoms) and *Medicago sativa* (1 to 20 mg/kg OM on average; 25 to 29 carbon atoms; Dove *et al.*, 1996). Moreover, it is possible and useful regarding the estimation of feed selection and the composition of complex consisting diets, to expand the available set of markers given by alkanes, alcohols and fatty acids by the natural carbon ($\delta^{13}C$) and hydrogen isotopic ratios of these compounds. Methods for their analysis, evaluation and data for their distribution and variability in a broad range of grassland and other plant species were provided by Meier-Augenstein (2002), Bi *et al.* (2005), Bezabih *et al.* (2011a, b) and Ferreira *et al.* (2014). To give an example: $\delta^{13}C$ was found in a range from – 36.7 ‰ for *n*-heptacosane (C27) to – 38.4 ‰ for *n*-triacontane (C30) in *Lolium perenne* (Ferreira *et al.*, 2014), or from – 18.8 ‰ on average for *n*-heneicosane (C21) to – 25.0 ‰ on average for *n*-hexacosane (C26) in *Zea mays* (Bi *et al.*, 2005).

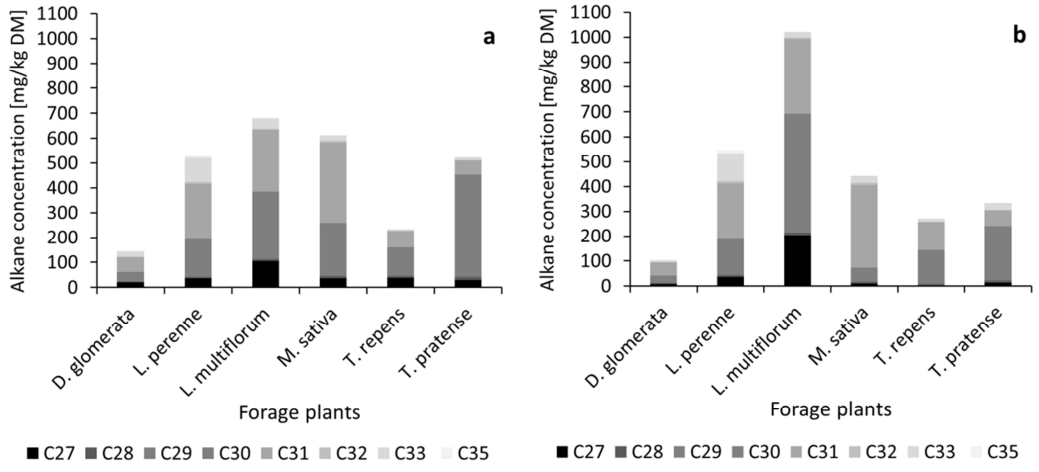

Fig. 3. Intraspecies distribution and interspecies variation of the internal alkane (C27 to C35) concentrations of some typical forage plants basing on data provided by Malossini *et al.* (1990): (a) after the first vegetative cycle; and (b) after the second vegetative cycle on the same area; abbreviations of alkanes are specified in the list of abbreviations.

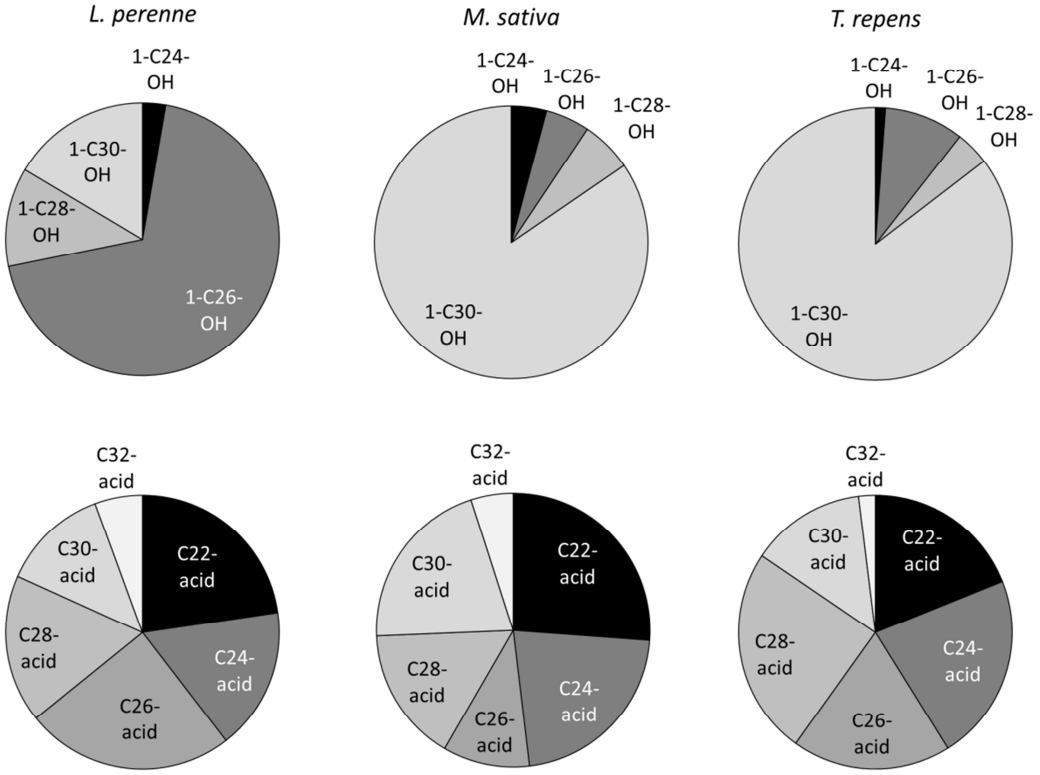

Fig. 4. Intraspecies distribution and interspecies variation of internal even-numbered long-chain fatty alcohol (1-C24-OH to 1-C30-OH) and long-chain fatty acid (C22-acid to C32-acid) concentrations [mg/kg dry matter] of some typical forage plants basing on data provided by Bugalho *et al.* (2004), Ali *et al.* (2005), Ferreira *et al.* (2009a) and Lin *et al.* (2012); abbreviations of compounds are specified in the list of abbreviations.

Furthermore, alkanes are major constituents of the waxes of honey bees, bumble bees and of various *Coccoidea*, usually occurring in a range of 20 to 35 carbon atoms while again pronouncing the odd-chain homologs (Tulloch, 1970; Brüschweiler *et al.*, 1989). In these insects, such natural waxes are used to build cells for the rearing of larvae and the storage of honey and pollen (honey bees), to coat larvae cocoons with a protective wax layer (bumble bees), to protect eggs or to protect the insect itself by a waxy body covering from predators and environmental influences (scale insects; Tulloch, 1970), or using wax coverings as part of the parasitic strategy (*e.g.* in lac insects; Mohanta *et al.*, 2014). Chikaraishi *et al.* (2013) found long-chain alkanes and alkenes, reflecting the desaturated products of analogous alkanes, in the cuticular wax of a variety of insect species, ranging from 21 to 33 carbon atoms. Particularly beeswax is predestined to be used as a cheap and easily available plant marker complex for estimation purposes in animal nutrition studies (Elwert and Rodehutscord, 2004; Elwert and Dove, 2005), and also the specific internal alkane profiles in insects may have the potential to

be used as markers in insectivorous animals (Dove and Mayes, 2006). Other known resources of wax alkanes and other wax compounds (*e.g.* lanolin, see Motiuk, 1980; and spermaceti, see Hamilton *et al.*, 1972), alkanes as compounds in insect contact pheromones (*e.g.* in *Xylotrechus colonus*; see Ginzel *et al.*, 2003; or in *Glossina morsitans*; see Carlson *et al.*, 1978) or alkanes used in chemical mimicry as a strategy of pollination (*e.g.* in *Ophrys sphegodes*; see Schiestl *et al.*, 1999) are not target for application in the current context.

2.5.3. Synthetic forms of alkanes

Besides the natural resources of long-chain alkanes, they are available as synthetics and are used in science, due to their simplicity, *e.g.* as model molecules to study much more complex organic structures. Commercial available synthetic long-chain alkanes may have their origin in petroleum or are otherwise chemically synthetized. There is, unfortunately, no information in more depth available, because it belongs to proprietary data of the manufacturers (H. Nowack, Merck KGaA, Darmstadt, Germany, Technical Service; personal communication). Further investigation has been performed for producing gaseous and liquid alkanes up to 15 carbon atoms in length from biomass in a biorefinery (Huber and Dumesic, 2006), or for synthetizing alkanes up to 17 carbon atoms by yeast (*e.g.* in *Saccharomyces cerevisiae*; Buijs *et al.*, 2015) or bacteria (*e.g. Escherichia coli* and *Bacillus subtilis*; Harger *et al.*, 2013), but whether biorefinery or biosynthetic pathways can be used to produce long-chain alkanes artificially, particularly for commercial purposes, is currently not known.

2.5.4. Labelling of feedstuffs, preparation and administration of synthetic alkanes, and major challenges in horses

Feed labelling and bolus preparation techniques that had been applied in ruminants so far are summarized by Dove and Mayes (2006). They comprise the labelling of a variety of matrices (see below) using dissolved synthetic alkanes, but also alkane suspensions and oil-in-water emulsion that need to be offered compulsorily in liquid form (Marais *et al.*, 1996; Dove and Mayes, 2006). The main objective of such preparation techniques is the complete homogenisation of the marker alkane and its carrying matrix, which then allows for a widely consistent dosing.

These approaches might enable further development of boluses, in which synthetic alkanes dissolved into a suitable oil matrix are spray-dried and encapsulated as a powder (see Turchiuli *et al.*, 2014) or directly encapsulated as an oil-in-water emulsion (see Sagalowicz and Leser, 2010). Bezabih *et al.* (2012) developed molasses-based boluses for the application in cattle that were, however, administered compulsorily as well. Charmley and Dove (2007) and other authors used dissolved beeswax, and sometimes a synthetic alkane additionally, that was sprayed onto a carrier substance (*e.g.* cottonseed meal) and then allowed to evaporate the solvent. Another invasive way of administering synthetic alkanes is to place an intra-ruminal controlled-release device that releases the marker dosage continuously at a predictable rate over up to 20 days (Dove and Mayes, 2006), but requires approximately 5 days reaching equilibrium of marker concentrations in faeces. This is so far exclusively applied in ruminants. It was described in more detail by Dove *et al.* (2002). In pigs, alkane-labelled cakes were used as boluses (Mowat *et al.*, 2001). In poultry, synthetic alkanes were mixed into a ground seed mixture that has subsequently been pelleted (Hatt *et al.*, 2001). Techniques for the labelling of feedstuffs and the preparation of boluses that have been applied to administer synthetic alkanes in horses are summarized in Article 3 – Table 1.

Those preparation techniques that use the dissolution of synthetic alkanes (according to the original method proposed by Mayes *et al.*, 1986), followed by the application onto various matrices such as feeds or feed concentrates (Dove and Oliván, 1998; Wilson *et al.*, 1999; Elwert and Dove, 2005; Chavez *et al.*, 2014), roughage fibre (Smith *et al.*, 2007), cellulose (Vulich *et al.*, 1991; Friend *et al.*, 2004), shredded paper (Mayes *et al.*, 1986; Stefanon *et al.*, 1999; Ferreira *et al.*, 2007a), filters (Giráldez *et al.*, 2004), or several kinds of biscuits (Mowat *et al.*, 2001; Kuntz *et al.*, 2006; Smith *et al.*, 2007), as well as the evaporation of the solvent and the optional packing into a further matrix (*e.g.* gelatine capsules; Vulich *et al.*, 1991), have been used most prevalently. However, the labelling of a matrix with an alkane solution increases the risk of precipitation and losses of the alkanes in part. Additionally, some of the aforementioned labelling techniques used high processing temperatures with the aim to fix the alkanes onto the bolus matrix or the substrate (Kuntz *et al.*, 2006; Smith *et al.*, 2007), but there is a considerable sensitivity of synthetic alkanes towards temperature depending on time and CL of the alkanes. This has been shown as a result of the current investigations (see Article 3). Thermolability has previously been shown in plant and other natural wax

alkanes, which led to losses during oven-drying of faeces samples (Elwert *et al.*, 2006).

It needs to be stressed that the selection of a sufficient preparation method does not depend on the animal species target to investigate but the chosen matrix does awfully well. The matrix type can have a significant effect on the site and rate of alkane release in the gut and consequently affect the diurnal variation of alkane concentration in faeces, as referred by Dove and Mayes (2006). In horses, the administration of labelled feeds or marker boluses is supposed to be much more challenging than in other livestock species, because of their high sensitivity regarding feed intake and their selection skills. Marker alkanes that are administered *via* bread pieces (Kuntz *et al.*, 2006), biscuits (Castelán-Ortega *et al.*, 2007; Smith *et al.*, 2007), pellets (Stevens *et al.*, 2002) or capsules are supposed to be regurgitated easily, separated, or ingested incompletely, which is likewise reported for markers mixed into concentrated feed (Smith *et al.*, 2007). Invasive methods, such as controlled release devices (Dove *et al.*, 1991) and compulsory marker administration (Marais *et al.*, 1996; Friend *et al.*, 2004) are not useable in horses and undesirable regarding animal welfare. Compulsory administration is particularly unfeasible with free-ranging animals.

2.5.5. Application of alkanes and previous challenges for the estimation of feed intake and apparent digestibility

Alkanes have, in the meantime, been applied in a wide range of studies estimating feed intake and digestibility in ruminants and in monogastric farm animals. Accuracy of alkane-based estimates depends on a variety of factors, of which the type of feed, the composition of the diet and the natural abundance of marker alkanes in the feed, the feeding frequency, the technique of preparing and administering external alkanes, the feed intake behaviour and animal individuality, the natural diurnal variability of faecal excretion and thus faecal alkane concentrations, the faeces sampling technique and sample preparation, and the AFR may be some examples. This results in a large variation of estimates, regardless of which animal species is focussed on. This is why the search for an optimal estimation procedure that is adopted to the animals and feeding conditions under test still goes on. Nevertheless, the general suitability of alkanes to serve as respective markers especially in pasture-based husbandry systems was confirmed, in stall-fed or graz-

ing sheep, *e.g.* by Vulich *et al.* (1991), Piasentier *et al.* (1995) and Elwert *et al.* (2004), dairy cows and beef cattle, *e.g.* by Ohajuruka and Palmquist (1991), Oliván *et al.* (2007a), Morais *et al.* (2011) and Bezabih *et al.* (2012), pigs, *e.g.* by Mowat *et al.* (2001), and poultry, *e.g.* by Hatt *et al.* (2001), mainly applying synthetic and plant wax alkanes as markers for feed intake and digestibility estimation and the double-marker method (Mayes *et al.*, 1986) for the former one. Modified methods of intake estimation basing on the labelled-supplement technique or diet composition estimates have been used by Dove and Oliván (1998), Dove *et al.* (2002) and others in sheep, Cottle and Romero (2014), Cottle (2016) and others in cattle, and Jurjanz *et al.* (2014) in poultry. A more detailed summary of the available literature is provided by the review of Dove and Mayes (2006) although not quite up to date.

Associated research performed in equids is comparatively less available. To provide some exemplary data from horse studies: Stevens *et al.* (2002) obtained estimates of DMI using either the C31:C32 or the C33:C32 alkane pair that differed between 0.0 and 1.8 kg/d from the measured reference on average, and obtained estimates of DMD using either C31, C33 or *n*-pentatriacontane (C35) plant alkanes corrected for incomplete AFR that differed between 0.3 and 9.7 % from the reference obtained through total faeces collection on average, each depending on the type of feed offered and alkanes used. Ordakowski *et al.* (2001) obtained similarly reliable DMD estimates that differed between 0.0 and maximal 1.7 % from the reference (total faeces collection) on average, again depending on what alkane has been used (recovery-corrected C25 to C33 odd-chain alkanes). By contrast, a much wider range of deviation between measured and estimated DMI was reported by Ferreira *et al.* (2007a) using various pairs of plant and synthetic alkanes (4 to maximal 46 % relative deviation) and largely dependent from the type of pairs. However, DMD estimated by C27 to C33 alkanes was not significantly different from measured DMD ($P > 0.05$) and the effect of alkane type was insignificant as well ($P > 0.05$; Ferreira *et al.*, 2007a). Interestingly, this was in huge contrast to the ruminant group studied in the same way, where DMD estimates widely differed from the measured ones ($P < 0.05$) with a large effect of the type of alkane being obtained ($P < 0.001$; Ferreira *et al.*, 2007a). The probable underlying relationship is explained below. Further data on equids can be found in Gudmundsson and Thorhallsdottir (1998), Stefanon *et al.* (1999), Friend *et al.* (2004), Peiretti *et al.* (2006), Kuntz *et al.* (2006), Castelán-Ortega *et al.* (2007), Smith *et al.* (2007) and Chavez *et al.* (2014).

The main limitation for the successful use that has been reported in agreement is the inconsistent and often incomplete AFR, which bias resulting estimates (predominantly of apparent digestibility). The experimental effort is high to compensate such bias performing total collection trials for reference and determination of AFR, respectively. An overview of mean AFR ranges in different groups of farm animals is provided in Table 2 and in Sales (2012) specific for horse studies, and the distribution of mean AFR within those ranges is shown in Fig. 5. Obviously, ruminant studies provide the major body of available data of AFR, followed by the equids group. There is a considerable lack of information in pigs and poultry. Predominantly C32 has been applied artificially, and C27, C29, C31 and C33 alkanes have been used as plant internal markers, independently from the investigated animal species. The given ranges show an intense variability of mean AFR in all livestock groups. In ruminants and poultry, it seems that the ranges of mean AFR become closer as the CL increases. Equids consistently have the lowest variation of mean AFR, independent from the CL of alkanes.

Table 2. Ranges of mean recovery (min – max) of plant, beeswax and synthetic alkanes in faeces (AFR) in different livestock groups basing on literature data

		Range		
Origin	Alkane	Ruminants	Equids	Poultry
Plant	C23	0.36 – 1.12 (5)	1.18 – 1.24 (1)	
Plant, beeswax	C25	0.26 – 1.01 (19)	0.68 – 1.15 (6)	0.23 – 0.44 (2)
Plant, beeswax	C26	0.36 – 1.08 (10)	1.06 – 1.12 (3)	
Plant, beeswax	C27	0.34 – 1.14 (22)	0.72 – 1.40 (7)	0.30 – 0.55 (2)
Plant, beeswax	C29	0.54 – 1.06 (23)	0.69 – 1.40 (7)	0.30 – 0.79 (3)
Plant, beeswax	C30	0.60 – 1.07 (17)	0.83 – 1.03 (4)	
Plant, beeswax	C31	0.60 – 1.91 (28)	0.67 – 1.41 (8)	0.37 – 0.97 (3)
Plant, beeswax	C33	0.63 – 1.16 (28)	0.72 – 1.10 (7)	0.73 – 0.88 (2)
Plant	C35	0.67 – 1.27 (12)	0.44 – 1.77 (4)	
Plant, synthetic	C28	0.34 – 1.13 (20)	0.70 – 1.03 (4)	0.17 – 0.70 (1)
Plant, synthetic	C32	0.47 – 1.09 (24)	0.81 – 1.12 (6)	0.50 – 0.81 (1)
Plant, synthetic	C36	0.70 – 1.16 (18)	0.81 – 0.99 (4)	0.79 – 0.80 (1)

References: Mayes *et al.* (1986, 1988); Ohajuruka and Palmquist (1991); Vulich *et al.* (1991); Dove and Oliván (1998); Gudmundsson and Thorhallsdottir (1998); O'Keefe and McMeniman (1998); Unal and Garnsworthy (1999); Berry *et al.* (2000); Hatt *et al.* (2001); Ordakowski *et al.* (2001); Dove *et al.* (2002); Moshtaghi Nia and Wittenberg (2002); Stevens *et al.* (2002); Valiente *et al.* (2003); Elwert *et al.* (2004); Molina *et al.* (2004); Ferreira *et al.* (2005); Elwert *et al.* (2006); Fraser *et al.* (2006); Peiretti *et al.* (2006); Ferreira *et al.* (2007a, c); Lin *et al.* (2007); Oliván *et al.* (2007a); Smith *et al.* (2007); Bulang *et al.* (2008); Elwert *et al.* (2008); Keli *et al.* (2008a); Sun *et al.* (2008); Ferreira *et al.* (2009a, b); Morais *et al.* (2011); Bezabih *et al.* (2012); Lin *et al.* (2012); Jurjanz *et al.* (2014); Kozloski *et al.* (2014); Schäfers *et al.* (2015). Pig data is exclusively provided by Wilson *et al.* (1999); ranges are therefore not available. The respective number of studies that has been considered is given in brackets. Abbreviations of alkanes are specified in the list of abbreviations.

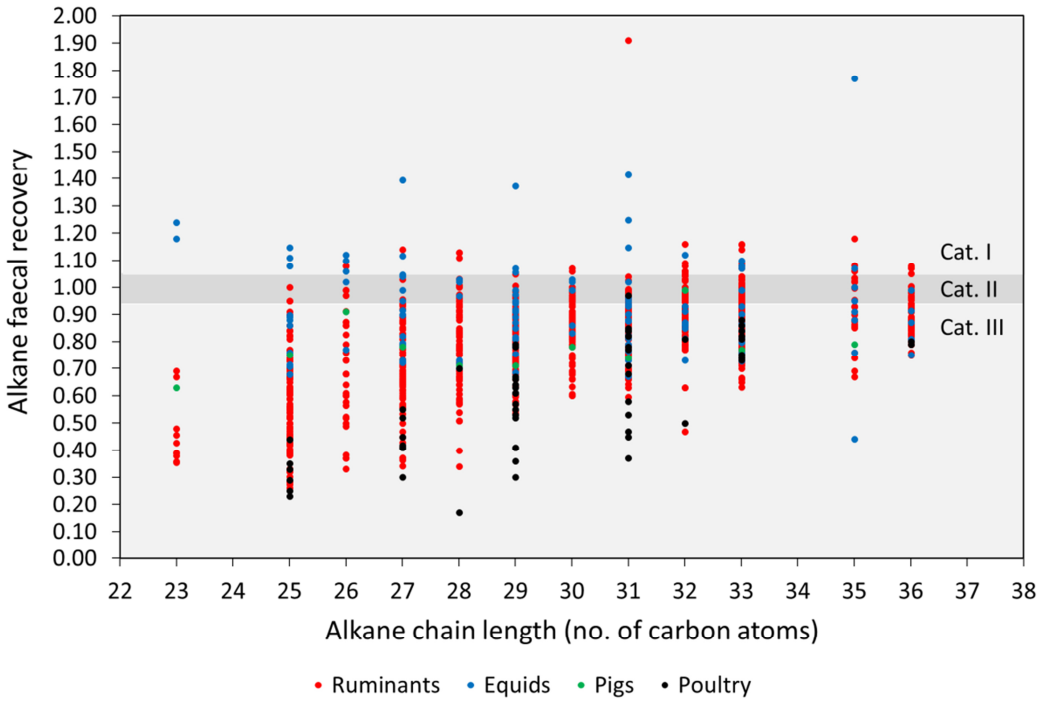

Fig. 5. Representation of the distribution of mean alkane faecal recovery (AFR) among three categories (Cat. I: AFR is unacceptably overestimated, AFR > 1.05; Cat. II: AFR is within an acceptable range, 1.05 ≥ AFR ≥ 0.95; and Cat. III: AFR is unacceptably underestimated, AFR < 0.95) in four livestock groups basing on a literature analysis (1113 means were considered in sum). References: Mayes *et al.* (1986, 1988); Ohajuruka and Palmquist (1991); Vulich *et al.* (1991); Dove and Oliván (1998); Gudmundsson and Thorhallsdottir (1998); O'Keefe and McMeniman (1998); Unal and Garnsworthy (1999); Wilson *et al.* (1999); Berry *et al.* (2000); Hatt *et al.* (2001); Ordakowski *et al.* (2001); Dove *et al.* (2002); Moshtaghi Nia and Wittenberg (2002); Stevens *et al.* (2002); Valiente *et al.* (2003); Elwert *et al.* (2004); Molina *et al.* (2004); Ferreira *et al.* (2005); Elwert *et al.* (2006); Fraser *et al.* (2006); Peiretti *et al.* (2006); Ferreira *et al.* (2007a, c); Lin *et al.* (2007); Oliván *et al.* (2007a); Smith *et al.* (2007); Bulang *et al.* (2008); Elwert *et al.* (2008); Keli *et al.* (2008a); Sun *et al.* (2008); Ferreira *et al.* (2009a, b); Morais *et al.* (2011); Bezabih *et al.* (2012); Lin *et al.* (2012); Jurjanz *et al.* (2014); Kozloski *et al.* (2014); Schäfers *et al.* (2015).

On the basis of this literature analysis, it can be inferred that: (1) in ruminants, mean AFR was in most cases (79.7 % of means) lower than a hypothetical threshold of 0.95, especially in lower molecular alkanes (C23 to C28), but did almost never exceed a hypothetical threshold of 1.05 (3.1 % of means), which both might be acceptable considering some analytical inaccuracy; (2) in equids, mean AFR were highest among all livestock species (see also Ferreira *et al.*, 2009b) with 61.3 % of means lower deviation but also 17.7 % of means overstepping the proposed hypothetical thresholds; (3) in pigs and poultry, mean AFR was consistently inacceptable low (90.9 and 98.4 % of means, respectively), however, available data are scarce; and (4) especially in the ruminants group the variation of mean AFR seem-

ingly decreased with an increasing CL of alkanes, which is in plant alkanes probably explained by an often higher abundance of the higher molecular alkanes (Dove *et al.*, 1996; Brosh *et al.*, 2003) and might in synthetic alkanes be indicative of a higher sensitivity towards preparation processes (*e.g.* the temperature treatment) or intestinal absorption in lower molecular alkanes (*e.g.* C24 and C28); in plant alkanes, there was a relatively constant variation of mean AFR not until 29 carbon atoms; mean AFR of synthetic alkanes mostly did not exceed but did frequently decline 1.05 and 0.95, respectively.

As major factors influencing AFR, the following have been identified: (1) the CL of alkanes, for which a curvilinear positive relationship has been postulated in ruminant species (Elwert *et al.*, 2006; Lin *et al.*, 2007; Oliván *et al.*, 2007a; Elwert *et al.*, 2008), *i.e.* that AFR increases as the CL increases approximating a constant at 100 % (Brosh *et al.*, 2003), but was not found in equids (Ordakowski *et al.*, 2001; Stevens *et al.*, 2002; Smith *et al.*, 2007); (2) the animal species, which might be linked to species-dependent differences in diet digestibility (Ferreira *et al.*, 2009b); (3) diet digestibility, for which a negative relationship to AFR has been identified (Ferreira *et al.*, 2005, 2009b); (4) alkane origin, where synthetic alkanes, at least in ruminants, seemed to have higher AFR than plant alkanes, which might be linked to the differences in the marker-digesta phase association and the resulting differences in marker passage rates (Oliván *et al.*, 2007a; see also Section 2.4.4. for further information); (5) the type of preparation for administration and the administration form of synthetic and other externally applied alkanes due to differences in marker bonding properties and marker exposition rates, and the preparation process of feed and faeces samples for analysis both in plant and synthetic alkanes (Molina *et al.*, 2004; Elwert and Dove, 2005; Elwert *et al.*, 2006, 2008); (6) the abundance of marker alkanes in feed plants, where low concentrations may provoke analytical errors (Brosh *et al.*, 2003; Valiente *et al.*, 2003; Elwert *et al.*, 2008); and (7) the cyclic diurnal fluctuation of feed DMI, defecation and faecal alkane concentrations, which affect the representativeness of the token faeces samples (Molina *et al.*, 2004).

Other potential effects were reported without conformity: (1) a diet composition effect (Elwert *et al.*, 2004, 2006; Lin *et al.*, 2007; Elwert *et al.*, 2008), where in the gut, the releasable and potentially absorbable amount of plant alkanes may vary between plant species, plant parts and dependent from the ontogeny (plant

age) because of a varying plant wax morphology (Lin *et al.*, 2007); (2) an effect of the feeding and the feed intake level (Molina *et al.*, 2004; Oliván *et al.*, 2007a), which might be linked to the fluctuation of DMI from day to day (Molina *et al.*, 2004) and marker passage throughout the gut (Oliván *et al.*, 2007a); a lower feed intake might cause a lower passage rate, and hence a higher rate of alkane absorption and lower AFR (Oliván *et al.*, 2007a); and (3) an effect of animal individuality (Valiente *et al.*, 2003; Oliván *et al.*, 2007a; Ferreira *et al.*, 2009b).

In dairy cows, Ohajuruka and Palmquist (1991) hypothesized that a partial loss of the ingested alkanes may occur in the rumen, because ruminally infused C32 had a notably lower AFR than C32 which was infused into the small intestine at an equal dosage (89 *vs.* 104 % AFR). Thus, a microbial fermentation of alkanes (be it through bacteria or protozoa), utilizing alkanes as sources of energy and carbon (Wentzel *et al.*, 2007), might be obvious also in the gut. This seems to be confirmed by Bulang (2005), who found small but considerable quantities of the synthetic alkanes C28 and C36 being incorporated by rumen microbes *in vitro* (up to 5 %), and by Keli *et al.* (2013), who found a considerable incorporation of alkanes by liquid-adherent ruminal bacteria in sheep. Oxidative degradation of long-chain alkanes (40 carbon atoms in length and above) to the corresponding alcohols and corresponding fatty acids, followed by chain-shortening incorporating these fatty acids into own cell lipids (see Davis, 1964) was reported in soil bacteria and other bacterial strains (Hankin and Kolattukudy, 1968; Hallas and Vestal, 1978; Wentzel *et al.*, 2007; more information on degradation pathways of alkanes in aerobes can be found in Ratledge, 1984; Berthe-Corti and Fetzner, 2002; and Wentzel *et al.*, 2007). Also bacterial degradation of alkanes under anaerobic conditions has been shown (Morikawa *et al.*, 1996), which supports the hypothesis of anaerobic alkane metabolism by the gut microbiota being conceivable. An incorporation of ^{14}C-labelled *n*-octadecane (C18) into intracellular lipids was seen in rumen bacteria, but a further metabolism has not been attested (McCarthy, 1964; Bartley *et al.*, 1971). More information on degradation pathways of alkanes in anaerobes can be found in Berthe-Corti and Fetzner (2002) and Wentzel *et al.* (2007). To be mentioned in brief, anaerobic microbes require alternative reactants (others than oxygen) serving as terminal electron acceptors and enabling the conversion of non-polar alkanes to utilizable substrates (Wentzel *et al.*, 2007). As such reactants, nitrate, sulphate and ferric iron have been identified (Zengler *et al.*, 1999; Wentzel *et*

al., 2007). However, the degradation of long-chain alkanes, the so-called microbial alkane cracking, has also been shown in methanogenic bacterial communities that are present in aquatic habitats or aged sediments where nitrate, sulphate and ferric iron (the preferred electron acceptors) are depleted (Zengler *et al.*, 1999; reviewed by Wentzel *et al.*, 2007). In addition, a link may exist to the fat content of the diet. Under laboratory conditions, Miller and Bartha (1989) showed liposome formation through encapsulation of C18 and C36 alkanes, and the rapid incorporation by pseudomonads thereafter. Consequently, encapsulation of hydrophobic compounds such as alkanes into phospholipid bilayers might facilitate their delivery to bacterial enzymes (Miller and Bartha, 1989). It may thus also be expected that fat-rich diets or supplemented fat sources in the diet additionally alter the AFR due to emulsification of alkanes in part and micelle formation in the small intestine (Ohajuruka and Palmquist, 1991). However, this has not been confirmed in sheep (Mayes *et al.*, 1986), dairy cows (Ohajuruka and Palmquist, 1991) nor in broiler chickens (Schäfers *et al.*, 2015), and has not yet been examined in horses. To my knowledge, microbial strains that are able to utilize long-chain alkanes as a source of energy or carbon have so far not been isolated from or detected in the digestive tract (neither in men nor in animals) and *in vitro* experiments failed to show any degradation of alkanes incubated with ruminal fluid (Keli *et al.*, 2008b). Although some microbes in the gut may adsorb or engulf long-chain alkanes above 18 carbon atoms (see Bartley *et al.*, 1971; Bulang, 2005; Keli *et al.*, 2013), they seem to fail in further alkane degradation and conversion (Bartley *et al.*, 1971). It is unlikely that an alkane-utilizing microbial community has established itself in evolution of the digestive tract because, compared to aerobic alkane oxidation, anaerobic alkane degradation seems to be a very slow process resulting in low growth rates (Wentzel *et al.*, 2007).

Apart from microbial metabolism of alkanes in the digestive tract, it is much more likely that alkanes can be absorbed endogenously, which is probably mediated by microfold cells (see Mabbott *et al., 2013; Büsing et al.*, 2014) in the small intestine (Mayes *et al.*, 1988), and are metabolized to fatty acids, in part, immediately during absorption (McCarthy, 1964). The absorbed alkanes are, if not metabolized immediately, taken up by the liver and metabolized mainly to phospholipids (McCarthy, 1964). However, rapid metabolism seems to be incomplete as Kolattukudy and Hankin (1966) found approximately 10 % of the administered radioactive label remained present in form of alkanes in the liver of rats. Conse-

quently, in cattle, some amount of the ^{14}C-label administered *via* C18 was found to be stored in body fat reserves (Bartley *et al.*, 1971). In rats, Kolattukudy and Hankin (1966) reported that 4 % of the ^{14}C-label administered *via n*-nonadecane (C19) was exhaled as carbon dioxide, which proves an extensive metabolic breakdown of the alkane. The remaining radioactive label was found as lipids in faeces, in chymus, in urine, in the liver tissue, in abdominal fat, in the intestinal wall, in subcutaneous fat, in the lung, in the blood, and in other excretory organs in descending order (Kolattukudy and Hankin, 1966).

In external markers, underestimation may also occur when animals do not ingest the labelled feed or bolus or at least the marker itself completely (*e.g.* after segregation of marker and matrix), or when the marker is regurgitated in part (*e.g.* in rumination, Unal and Garnsworthy, 1999, or due to a very sensible feed selection).

A considerable overestimation of AFR is a fairly uncommon but nevertheless repeatedly reported situation (see Fig. 5). It occurred more frequent in equid studies (17.7 %) than in studies with ruminants (3.1 %). It has been reported that horses have the highest AFR compared to sheep, goats and cattle (Ferreira *et al.*, 2009b). Alkane faecal recovery might physiologically be altered by microbial alkane synthesis within the digestive tract or by endogenous alkane secretion (Dove and Mayes, 1991), which both would lead to an overestimation of AFR. Biosynthesis of long-chain alkanes has been shown, besides in plants or insects (see Section 2.5.2.), also in bacteria (see Ladygina *et al.*, 2006; Lennen et al., 2010; Schirmer *et al.*, 2010), yeasts (Buijs *et al.*, 2015) and in fungi (see Ladygina *et al.*, 2006), but there is little evidence that either microbial synthesis or endogenous secretion of long-chain alkanes is acting in the gut of farm animals (Mayes *et al.*, 1988; Dove and Mayes, 1991). It may further be conceivable that alkenes or other unsaturated hydrocarbons from feed plants contribute to the detectable alkane fraction in faeces resulting from their saturation under reducing conditions in the digestive tract (Dove and Mayes, 1991; again perhaps through microbial conversion). However, there is so far no evidence that this occurs (Dove and Mayes, 1991). It might be conceivable that microbial biosynthesis of long-chain alkanes, if existing in the gut, would become visible soonest in animals with a pronounced hindgut fermentation such as equids. If this were so, effects of diet composition (Lin *et al.*, 2007) and animal individuality would be expected.

As described and discussed in Article 1, alkane concentrations in faeces follow specific dynamics resulting in a cyclic diurnal fluctuation (see also Giráldez *et al.*, 2004; Molina *et al.*, 2004; Oliván *et al.*, 2007a). Depending on the method of faeces sampling (bulk or spot samples), AFR can easily be under- or overestimated when the spot sample, and more unlikely the bulk sample, does not represent the mean faecal concentration, around which punctual concentrations fluctuate. Large non-physiological sources of bias are unrepresentative samples of the ingested feeds or boluses. Thus, the determined ingested and defecated concentrations of alkanes are incompatible. The calculation of AFR from extrapolation of the determined AFR of adjacent alkanes can lead to overestimation resulting from an incorrect assumption of linearity (Brosh *et al.*, 2003). Moreover, very low dietary and faecal concentrations may facilitate a biased determination of AFR, which affects predominantly alkanes with less than 28 carbon atoms (Brosh *et al.*, 2003).

For feed intake estimation purposes, the correction of the faecal concentrations of marker alkanes for an unbalanced AFR is not mandatory when AFR of both alkanes within the used pair is equal (Mayes *et al.*, 1986). However, then the AFR of these alkanes has to be assessed correctly, on the basis of pre-trail data (Brosh *et al.*, 2003) or literature data, which is difficult regarding the huge variation of AFR (see Table 2) and its dependency from a variety of influencing factors that have so far not been examined sufficiently. Here, the development of an adequate estimation function would probably be helpful. The correction of faecal alkane concentrations is, however, necessary for the estimation of digestibility (Dove and Mayes, 1996). Brosh *et al.* (2003) and Lin *et al.* (2007) recommended an animal and diet-specific correction to cancel out individuality and probable diet composition effects, respectively. Regarding the feasibility especially under grazing conditions, it is recommended to use at least animal-mean AFR (Brosh *et al.*, 2003) or diet-mean AFR (Oliván *et al.*, 2007b) for correction. Especially in ruminants, a correction is particularly necessary for the estimation of the selection and the composition of the ingested diet because of the relationship between AFR and CL of the alkanes (Dove and Mayes, 1996). It is, however, not necessary in equids where this relationship does not exist (López *et al.*, 2016). Another approach, which would possibly be helpful in investigations on free-ranging animals, is the specific selection of timeframes for faeces spot sampling basing on knowledge of faecal alkane concentration dynamics. This may overcome the need for an AFR correction.

2.5.6. Application of alkanes and previous challenges for the estimation of feed selection and composition of the ingested diet

The estimation of diet composition, which provides information on feed selection of the animal under test, is mainly basing on the application of plant alkanes, LCOH, LCFA and their natural ^{13}C enrichments (see Sections 2.2. and 2.5.2.). The majority of studies that were performed to develop and validate this estimation method has focussed on ruminant livestock (Duncan *et al.*, 1999; Brosh *et al.*, 2003; Valiente *et al.*, 2003; Bugalho *et al.*, 2004; Ferreira *et al.*, 2005, 2007b, c, d; Lin *et al.*, 2007; Oliván *et al.*, 2007b; Elwert *et al.*, 2008; Keli *et al.*, 2008a; Sun *et al.*, 2008). There was just very few work spent on studying feed selection and diet composition in horses on the basis of alkane markers (Ferreira *et al.*, 2007b, 2009b, 2010; Celaya *et al.*, 2011; Ferreira *et al.*, 2013; López *et al.*, 2016). Detailed data of diet composition estimates can be found in the mentioned studies.

The general results of the estimation procedures fit well with those obtained by observational studies. In consequence, it was both estimated by the use of dietary alkanes (Celaya *et al.*, 2011; Ferreira *et al.*, 2013) and observed (Fleurance *et al.*, 2001; Menard *et al.*, 2002; Edouard *et al.*, 2010) that horses preferably select grassland and herbaceous plant species, which is mainly driven by plant structure (growth stage) and less so by plant species (Fleurance *et al.*, 2001). Such a selective behaviour is likely aiming to maximize the intake of high digestible nutrients (Menard *et al.*, 2002) and to balance the protein and energy supplies in particular (Edouard *et al.*, 2010). The habitat use of horses depends on the availability of less lignified plant tissues, which changes throughout the vegetation period (Menard *et al.*, 2002; Celaya *et al.*, 2011). Approximately 5 % herbage availability was surmised to be the narrow threshold below which horses are not able to select such plant species efficiently (Celaya *et al.*, 2011). A decreasing availability thus forces the animals to switch their preference to more woody plants, in which horses respond quite flexible (Ferreira *et al.*, 2013).

Under experimental conditions where the quantity of diet components is limited and these components are well defined, the estimation of diet composition through plant wax alkanes, alcohols and fatty acids has been validated using the Kulczynski similarity index, *i.e.* the overlap of known and estimated proportions of diet components (Ferreira *et al.*, 2007b, 2009b, 2010; López *et al.*, 2016), and in

general, a high accuracy of the estimation was addressed with up to 98 % conformity (Ferreira *et al.*, 2009b; López *et al.*, 2016). Although lesser important than in ruminants, the main influence on the estimation accuracy in horses comes from the faecal recovery of markers (see Section 2.5.5.). The highest accuracy may be obtained when a diet-specific recovery correction of the faecal marker concentrations is considered (Ferreira *et al.*, 2007b, 2009b, 2010) but in horses also the renouncement of correction still lead to acceptable estimates when the relative proportions of marker concentration in faeces is not altered through different faecal recoveries (Ferreira *et al.*, 2007b). The less complex the diet is composed, the more accurate diet composition estimates can be (Ferreira *et al.*, 2010). Hence, adding additional plant markers such as fatty alcohols, fatty acids and $\delta^{13}C$ ratios might provide a more specific fingerprint to each plant species, which enhance the discriminatory power between them (Ferreira *et al.*, 2010; López *et al.*, 2016). However, this is not mandatorily the case. Rather than just to increase the number of markers, it is recommended to identify and select the most sufficient markers differing diet components through multivariate statistical analyses (*e.g.* discriminant analysis; Bugalho *et al.*, 2004).

2.5.7. Prospects and limitations of the application of alkanes to estimate digesta passage kinetics

In just a very few studies so far, long-chain synthetic alkanes were used to obtain information about passage kinetics of the particulate phase of digesta (Duncan *et al.*, 1999, and Bulang *et al.*, 2008, investigating sheep, Giráldez *et al.*, 2004, 2006, investigating goats, Warner *et al.*, 2013, investigating dairy cows, and Hatt *et al.*, 2002, investigating tortoises). Thereupon, estimations of faecal output and feed intake were deduced in turn (Duncan *et al.*, 1999; Giráldez *et al.*, 2004, 2006) as described in Section 2.2. The only study using a synthetic alkane (C32) to predict its TMRT in horses was, to my knowledge, performed by Stevens *et al.* (2002), who detected 27.9 ± 0.59 h in juvenile Thoroughbreds fed a hay-based diet with additional temporal access to pasture. This was similar to results obtained in horses by Rosenfeld and Austbø (2009) using various differently processed grains and using ytterbium as the passage rate marker (TMRT ranging from 26.4 to 27.3 h), and it was also similar to the results of Todd *et al.* (1995) using lucerne hay cubes and dysprosium as the marker (TMRT of 24.6 or 25.8 h at maintenance and 15.3 or 15.9

h at 1.4 × maintenance) indicating a significant effect of the feeding level in addition. This may be indicative for a hastened flow of synthetic alkanes, such as it is in finer feed particles or in the liquid phase of digesta (Udén *et al.*, 1982), but there are obviously many more factors influencing the passage rate of markers and the associated digesta phases. However, the results obtained by Stevens *et al.* (2002) were notably lower than TMRT estimated in adult Thoroughbreds and juvenile Shetland ponies that were fed different proportions of lucerne hay and oat straw using Cr-mordanted fibre and even using the liquid phase marker cobalt-ethylenediaminetetraacetic acid (EDTA; TMRT ranging from 38.4 to 51.9 h; Cuddeford *et al.*, 1995), and it was also lower in Thoroughbreds that were fed lucerne hay and hay cubes only or grain-supplemented diets using ytterbium as the marker, regardless of the experimental inhibition of exercise (TMRT ranging from 30.8 to 43.4 h; Pagan *et al.*, 1998). Another study investigating the effects of different hay-to-grain ratios in ponies found likewise higher TMRT both of lanthanides and of the liquid phase marker Cr-EDTA (Drogoul *et al.*, 2001).

The first main limitation of using alkanes as passage rate markers is the incomplete and impermanent binding of synthetic alkanes to the target feed component and their migration from the particulate phase to the liquid phase of digesta by tendency (Mayes *et al.*, 1997; Bulang *et al.*, 2008; see Section 2.4.4.). Therefore, estimations of passage kinetics are related to the alkanes used as markers and not necessarily related to the target feed component (Bulang *et al.*, 2008), as it is likewise in almost all other markers applied in this field. However, as surmised by Owens and Hanson (1992) and Giráldez *et al.* (2006), dissociation of synthetic alkanes may be a result of particle size reduction during feed labelling and the presence of small particles in the liquid digesta phase rather than a real migration into the liquid digesta phase. Indeed, in goats, Giráldez *et al.* (2006) found significantly higher slow (K1) and fast (K2) fractional passage rates, deriving from the multi-compartmental model that has been used (Dhanoa *et al.*, 1985), and significantly lower TMRT in synthetic C36, *n*-octatriacontane (C38) and *n*-tetracontane (C40), sprayed onto plant fibre, than in the standard marker Cr, mordanted to the plant fibre. This has also been confirmed by Bulang *et al.* (2008) in sheep for C36 sprayed onto lucerne fibre. Mordanted Cr was supposed to label the plant fibre and the particulate phase of digesta with minor dissociation (Udén *et al.*, 1980; Giráldez *et al.*, 2006). In contrast, in the tortoise study of Hatt *et al.* (2002), the TMRT of C36 differed not compared to that of mordanted Cr. The passage rate of

alkane-labelled feed particles may additionally be slowed through a reduced hydration rate due to the alkanes' hydrophobic properties (Giráldez *et al.*, 2006). It is, conclusively, impossible to predict the migrating behaviour and the specific flow rates of synthetic alkanes sufficiently, which are influenced by properties of the labelled material, the labelling technique, the feed intake rate, the animal species, conditions of animal husbandry and restriction of exercise, and animal individuality. To overcome the disadvantages of artificial labelling techniques (particle size reduction, altered flow rates, inhomogeneous distribution within the labelled material, migration), it has been tested whether plant alkanes enriched in the carbon stable isotope (^{13}C) in a greenhouse are suitable passage rate markers (Warner *et al.*, 2013), which is, however, dependent on abundance of the target alkanes in feed plants, and marker dilution due to plant biomass production during ontogeny needs to be considered (Bulang *et al.*, 2010). Unlike using the natural isotopic ratios of alkanes or other plant wax compounds (see Sections 2.5.2. and 2.5.6.), the ^{13}C enrichment provides the advantage that an abrupt change to the experimental diet can be omitted and animals can sufficiently be adapted (Svejcar *et al.*, 1993; Bulang *et al.*, 2010). Additionally, the method is independent from natural differences between alkanes in $\delta^{13}C$ background levels due to carbon isotope discrimination during alkane biosynthesis in the epidermal cell (Warner *et al.*, 2013). However, a risk of biased passage rate estimates remains if stable isotopes are incorporated by intestinal microbes as supposed by Bartley *et al.* (1971), Bulang (2005) and Warner *et al.* (2013).

The second main limitation of using alkanes as passage rate markers is the incomplete AFR. However, it is so far unknown if the difference in residence time of plant alkanes or artificially bound synthetic alkanes, which results from the usual experimental procedure providing only a single dosage of the marker and depends on the particle size distribution in the feed, affect their faecal recovery (Bulang *et al.*, 2008). Bulang *et al.* (2008) proved the impact of an assumed steadily decreasing recovery of C36 on passage kinetics in sheep and found increased K1 and K2, and rumen mean retention time was slowed.

3. Scope of the thesis

The successful application of long-chain alkanes as dietary markers can be affected by their inconsistent, unbalanced and often incomplete recovery in faeces. To obtain suitable AFR rates for the correction of faecal alkane concentrations, it is so far not recommended to use published data because of their inconsistency and the high variation of influencing factors. Hence, the determination of AFR for each trial, *i.e.* each diet type, or at least through a pre-trail, is still required and requires the total collection of faeces. If a correction for AFR is indispensable, then there is, however, currently no proper method available that helps to avoid such a laborious and by itself error-inducing procedure.

An approach that would possibly enable the researcher to quit the correction for AFR is the specific selection of timeframes for spot sampling of faeces basing on the knowledge of faecal alkane concentration dynamics. It is known that concentrations of the target marker alkanes in faeces fluctuate periodically within and across days, which mainly depends on the interval of feed intake or administration of external alkanes. It was consequently hypothesized:

(1) Periodic cycling of faecal alkane concentrations implies that during specific timeframes within the day, these concentrations would accurately reflect their average, which is the AFR that would be determined through a total faecal collection.
(2) This might enable to compensate incomplete or overestimated AFR by the selection of adjacent timeframes.
(3) This might then enable the estimation of DMI and DMD from a spot-sampling of faeces and without the correction for an unbalanced AFR.

These hypotheses have been tested indoors in horses with typical mealtime feeding of a hay-based diet. The results and discussion are provided Article 1.

This first study revealed that the administration of synthetic alkanes *via* boluses is a special challenge in horses, because horses are particularly sensitive to the presented feed and are able to select to a nicety. Because compulsory administration is undesirable and available feed-labelling techniques have some general disadvantages as well (see Section 2.5.4.), we hypothesized:

(1) For application in horses, a suitable bolus matrix would ensure a broadly consistent and high acceptance for ingestion.
(2) Such a matrix would resist microbial spoilage to be stable for storage over a sufficient period of time.

The preparation of such boluses, the results of the performed tests and their discussion are presented in Article 2.

The first study did also highlight that the accuracy and uniformity of administered alkane dosages are mandatory for a successful application and are mandatory to avoid that marker administration increase the variation among test animals as well as among and within consecutive test days. Additionally, it can easily be supposed that the easier the preparation of markers is, the easier the required accuracy and uniformity of the dosages can be achieved. Because of that we hypothesized:

(1) Melting synthetic alkanes to wax might enhance the accuracy and uniformity of subsequent bolus labelling and might further simplify it.

This study, however, showed that there is a considerable sensitivity of alkanes towards exposure to high temperatures, which has not yet been described for synthetic alkanes. The temperature sensitivity of synthetic alkanes has thus been tested in addition. The preparation of single- and multi-component synthetic alkane waxes and the results of the performed tests are presented and discussed in Article 3.

4. Original articles

The following articles build up on one another thematically and are therefore not sorted by the date of publication.

Article 1

Impact of dynamics of faecal concentrations of plant and synthetic *n*-alkanes on their suitability for the estimation of dry matter intake and apparent digestibility in horses

Published in: Journal of Agricultural Science, Cambridge (2016) 154, 1291-1305
DOI: http://dx.doi.org/10.1017/S0021859616000344
Authors: Martin Bachmann, Monika Wensch-Dorendorf, Michael Bulang, Annette Zeyner

Article 2

Bolus matrix for administration of dietary markers in horses

Published in: Livestock Science (2016) 185, 43-49
DOI: http://dx.doi.org/10.1016/j.livsci.2016.01.010
Authors: Martin Bachmann, Monika Wensch-Dorendorf, Manuela Wulf, Maren Glatter, Michèle Siebmann, Christian Bierögel, Erika Schumann, Michael Bulang, Christine Aurich, Annette Zeyner

Article 3

Preparation of synthetic alkane waxes and investigations on their suitability for application as dietary markers in farm animals

Published in: Livestock Science (2016) 185, 110-116
DOI: http://dx.doi.org/10.1016/j.livsci.2016.01.018
Authors: Martin Bachmann, Monika Wensch-Dorendorf, Karsten Mäder, Michael Bulang, Annette Zeyner

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ANIMAL RESEARCH PAPER

Impact of dynamics of faecal concentrations of plant and synthetic *n*-alkanes on their suitability for the estimation of dry matter intake and apparent digestibility in horses

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SUMMARY

In horses, the quantity of faeces and the faecal concentrations of plant and synthetic alkanes are inconsistent throughout the day. The estimation of feed intake and digestibility can additionally be limited by irregular and incomplete faecal recovery of alkanes that are used as dietary markers. The correction of alkane concentrations minimizes the bias of estimates, but requires the determination of faeces quantity by total collection. However, in consideration of the dynamics of alkane concentrations in faeces, sampling at selected timeframes throughout a day may be useful in avoiding such correction. Five adult horses were fed a hay-based diet offered three times a day in equal amounts. Horses received a bolus with similar quantities of *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) synthetic alkanes twice a day. Total faeces were quantified over 3 consecutive days. Dry matter intake (DMI), output (DMO) and digestibility (DMD) were determined from the total collection trial and additionally estimated for each of 12 equal timeframes throughout the day. The diurnal patterns of the single faeces quantity (SFQ) and faecal alkane concentrations were similar between horses and were repeated from day to day. The intra-day dynamic of SFQ was pronounced. The dynamic of the faecal concentration was much more pronounced when the alkane was administered twice instead of three times a day. The faecal recovery of alkanes that has been calculated from the total collection trial ranged from 82 ± 4·1% for C36 to 108 ± 11·1% for C28. Measured DMI was 12·0 kg/day, measured DMO was 5·9 kg/day and measured DMD was 0·51. Reliable estimates were obtained for DMI with 12·3 ± 0·79 kg/day for the combination of *n*-nonacosane (C29) and C28 and 12·1 ± 1·01 kg/day for the combination of *n*-tritriacontane (C33) and C28 at 2 h after administration, and 12·1 ± 0·96 kg/day for the combination of *n*-hentriacontane (C31) and C32 at 2 h prior to the morning meal, which included the first bolus administration. When calculated from DMO and DMD, DMI was 12·2 ± 0·89 kg/day for C29 and 12 ± 1·0 kg/day for C33 between 5 and 6 h after the morning meal. Estimates of DMD were unbiased between the 3rd and 4th hour after the morning meal with 0·52 ± 0·014 for C29 and 0·51 ± 0·021 for C33, respectively. The DMO was 5·7 ± 0·34 kg/day and 6·1 ± 0·43 kg/day when estimated 3–4 h after the 2nd meal, or prior to the 2nd bolus administration, using the product of SFQ and the daily defecation frequency or the synthetic alkanes, respectively. Knowledge of defecation dynamics might be helpful for simplifying experimental trials. They specifically followed intake dynamics, which can prospectively be used to select sampling timeframes. Based upon current results, a selection of two to three spot samples of faeces that are evenly distributed between 2 h before and 6 h after the morning meal, which was the time of bolus administration, allows for the greatest reliability. Defecation dynamics are probably less influenced by ration/bolus type, rate of exercise, or gut peristalsis, which nevertheless can result in individual shifts of optimal timeframes.

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INTRODUCTION

The cuticles of most herbage species that are used as feedstuffs for horses contain remarkable quantities of predominantly odd-chain aliphatic alkanes as compounds in integrated and superimposed waxes (Dove *et al.* 1996; Koch & Ensikat 2008). Plant and even-chain synthetic alkanes can be used to estimate feed intake and apparent digestibility (Mayes *et al.* 1986; Ordakowski *et al.* 2001; Smith *et al.* 2007), but seem to be unsuitable when faeces quantity is unknown. Particularly in digestion trials, it is necessary to correct faecal alkane concentrations because faecal recovery is irregular and often incomplete, although horses have the highest rates among livestock species (Ferreira *et al.* 2009). Faecal output quantification by total collection can impair animal welfare, restrict exercise, and can affect the estimation of digestibility (Pagan *et al.* 1998). Moreover, it is hard to implement in horses kept in groups or on a pasture.

A link may exist between the interval of mealtimes or bolus administration, gut passage dynamics, and resulting faecal concentration patterns, regardless of marker type and animal species (Haenlein *et al.* 1966; Cuddeford & Hughes 1990; Dove & Mayes 1991; Giráldez *et al.* 2004; Molina *et al.* 2004; Smith *et al.* 2007). Figure 1 provides a hypothetical explanation of the development of diurnal alkane concentration patterns in faeces after repeated administration by feed or bolus. Every single meal or bolus results in a specific excretion curve, as shown by Hyslop (2003) and Rosenfeld *et al.* (2006) in horses, that spans approximately 72 h after first appearance of the marker in faeces (Pagan *et al.* 1998). Single curves continuously follow and partly overlay one another. A specific diurnal pattern is the result of overlapped decreasing curves starting from every meal or dose of the 3 days before, and increasing curves starting from meals or doses of the actual day, considering the specific transit time of the marker. Numerous additional factors, such as diet composition (Van Weyenberg *et al.* 2006), variable profiles of alkanes in feedstuffs (Dove *et al.* 1996), exercise (Pagan *et al.* 1998), short-term stress and individual gut peristalsis may also alter marker passage and contribute to individual characteristics of alkane concentration patterns in faeces.

It was hypothesized that (i) during specific timeframes within the day, dry matter (DM) and alkane concentrations of spot samples of faeces accurately reflect their average; (ii) this context can be used to compensate incomplete alkane faecal recovery (AFR); and (iii) this context can be used to estimate DM intake (DMI) and apparent digestibility (DMD) without correction for unbalanced AFR.

The aim of the current study was to test the above-mentioned hypotheses in horses with typical mealtime feeding of a hay-based diet.

MATERIALS AND METHODS

Animals and diets

Five Saxonian Warmblood horses (three mares and two geldings), with an average body weight of 555 ± 60·8 kg, were used in the current study. The horses' ages ranged from 13 to 17 years.

The horses received meadow hay (12·0 kg/day) and crushed oats-barley mix (1 : 1) dependent upon individual rates of exercise (0·9 ± 0·38 kg/day). The chemical composition of offered feedstuffs is provided in Table 1. The nutritive value of the diets was assessed on the basis of metabolizable energy (ME) according to Kienzle & Zeyner (2010), as well as of pre-caecal digestible crude protein (pcdCP) and pre-caecal digestible amino acids (pcdAA) lysine (pcdLYS), methionine + cysteine (pcdMET + CYS) and threonine (pcdTHR) according to Zeyner *et al.* (2015). Feeding levels, expressed as a manifold of maintenance, were 1·32 ± 0·045 (ME), 1·30 ± 0·079 (pcdCP), 1·07 ± 0·063 (pcdLYS), 1·29 ± 0·073 (pcdMET + CYS) and 0·86 ± 0·052 (pcdTHR) following official recommendations of GfE (2014). The horses had free access to tap water. Meals were offered three times a day (at 07.00, 15.00 and 21.30 h) in equal amounts and were consumed entirely.

The concentration of plant alkanes in diet components and quantities of synthetic alkanes in boluses are shown in Table 2.

Bolus preparation

Throughout the experimental period, horses were offered a bolus containing similar amounts of *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) by hand twice a day (07.00 and 21.30 h). Dosages were as described in Smith *et al.* (2007). For the preparation of boluses, crystalline alkanes (Sigma Aldrich Chemie GmbH, Steinheim, Germany) were weighed into gelatine capsules separately, then embedded into a pastry made of oat flakes, wheat flour, sugar beet syrup (mixed by weight:

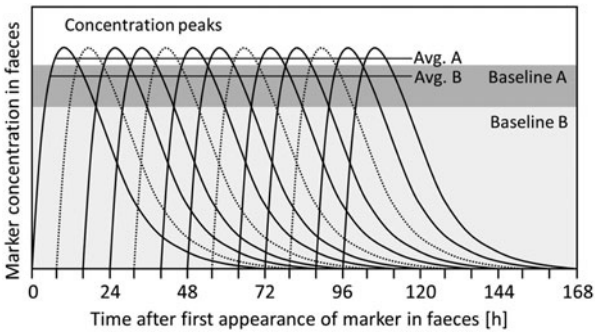

Fig. 1. Hypothetical explanation of marker concentration dynamics in faeces after repeated administration by feed (3 times a day) or bolus (2 times a day). Each curve follows a mealtime or bolus administration in consideration of the marker's transit time; however, dashed curves do not appear for bolus administration. The baselines A and B indicate the basic concentration of feed markers (A) or bolus markers (B). The horizontal lines for average (Avg.) A and B indicate the average concentration of A or B after repeated administration.

1 : 0·6 : 1) and water, and were subsequently baked for 20 min at 180 °C. All horses consumed the boluses completely.

Animal management and experimental design

Horses from the Reitgestüt Knauthain GmbH, Leipzig (Saxony, Germany) were used in the current experiment and were kept and cared for in accordance with the Federation of Animal Science Societies' Animal Care Guidelines (FASS 2010). During the experiment in October and November 2012, horses were housed in single boxes fitted with rubber mats. To ensure a hygienic setting, excreted urine was removed immediately. The horses had free exercise in a 'paddock' with a concrete floor, or by being ridden, for approximately 1 h/day.

The animals were adapted to test conditions 5 days prior to quantitative collection of faeces. Samples of feedstuffs were obtained once a day and combined to bulk samples specific for diet components. Over 3 days, every defecation was collected, a sample taken and stored at –20 °C. Additionally, aliquot sub-samples (0·05) were combined to bulk samples for individual horses.

Alkane analysis

Lyophilized samples of feedstuffs and faeces were ground to pass through a 0·5 mm sieve in a standard

Table 1. *Analysed chemical composition of feedstuffs offered during the experiment*

	Hay	Concentrate
Dry matter	932	908
Organic matter*	925	976
Crude protein (CP)	73	111
Pre-caecal digestible CP*†	36	60
Lysine (LYS)	2·4	4·4
Pre-caecal digestible LYS*†	1·2	2·4
Methionine + cysteine (MET + CYS)	1·9	5·2
Pre-caecal digestible MET + CYS*†	0·9	2·8
Threonine (THR)	2·5	4·0
Pre-caecal digestible THR*†	1·2	2·1
Acid ether extract	10	31
Nitrogen-free extract*	482	733
Crude fibre	360	100
Neutral detergent fibre	664	397
Acid detergent fibre	419	135
Acid detergent lignin	69	30
Cellulose*	350	105
Hemicellulose*	245	263
Gross energy	17·5	18·3
Metabolizable energy*‡	6·1	12·6

Nutrient values are given in g/kg dry matter (DM), energy contents in MJ/kg DM.
* Calculated on the basis of analysed nutrient or energy contents.
† Calculations refer to: Zeyner *et al.* (2015).
‡ Calculations refer to: Kienzle & Zeyner (2010).

laboratory sample mill. To reduce alkane loss during sample preparation, sub-samples of boluses ($n = 10$) had been pre-milled with water-cooling, then parted and milled with the aid of liquid nitrogen at a constant – 196 °C using a Retsch® CryoMill (Retsch® GmbH, Haan, Germany; settings: 25 ml cup, one 14 mm steel ball, 2 min for each iteration). All samples were dried to constant weight to determine total DM content. Alkane extraction and gas-chromatographic analysis (GCA) were conducted as described in Elwert *et al.* (2004). Briefly, samples of feedstuffs, boluses and faeces underwent saponification in ethanolic potassium hydroxide (4 h at 90 °C), hot extraction of lipophilic compounds (at 75 °C), and purification through silica-gel columns. The GCA was performed using a Shimadzu GC-2010 FID unit (Shimadzu Corp., Kyoto, Japan) with on-column injection onto an Rtx®-1 w/Integra-Guard™ column (Restek Corp., Bellefonte, PA, USA). Alkane concentrations were calculated from peak area

Table 2. *Analysed concentration of plant alkanes in diet components, and quantities of synthetic alkanes in boluses*

	Hay	Concentrate	Bolus
n-heptacosane	13	4	n.d.
n-nonacosane	54	7	n.d.
n-hentriacontane	92	12	n.d.
n-tritriacontane	21	n.d.	n.d.
n-octacosane	n.d.	n.d.	134
n-dotriacontane	n.d.	n.d.	143
n-hexatriacontane	n.d.	n.d.	155

n.d., not detectable.
Alkane concentrations are given in mg/kg dry matter (DM), alkane quantities in boluses in mg (in DM).

ratios of target alkanes and internal standards *n*-docosane and *n*-tetratriacontane, which had been added to each sample tube before chemical preparation. The retention times of alkane peaks, as well as any device-specific discrimination, were determined using a standard solution of homologous alkanes between *n*-docosane and *n*-octatriacontane. The peak areas had additionally been corrected for any discrimination of higher-molecular alkanes that might have occurred during solvent extraction (Oliván & Osoro 1999).

Additional chemical analyses

The gross energy content of feedstuffs and faeces bulk samples was obtained by bomb calorimetry (C7000 Oxygen Bomb Calorimeter, IKA® Werke, Staufen, Germany). Dry matter, ash, crude protein (CP), acid ether extract, crude fibre, neutral detergent fibre, acid detergent fibre and acid detergent lignin were determined according to official methods (VDLUFA 2012, methods no. 3·1, 4·1·1, 5·1·1 B, 6·1·1, 6·5·1, 6·5·2, 6·5·3 and 8·1). Amino acids were analysed using ion exchange chromatography (Biochrom 30, Biochrom Ltd., Cambridge, UK) according to the protocol of VDLUFA (2012, method no. 4·11·1). Contents of organic matter (OM), cellulose, hemicellulose and nitrogen-free extract were calculated upon that basis. Neutral detergent insoluble CP was determined according to Licitra *et al.* (1996), the Cornell Net Carbohydrate Protein Model, and used to calculate quantities of pcdCP and pcdAA according to Zeyner *et al.* (2015).

Calculations

Each day of the collection period was divided into 12 equal timeframes beginning with the first meal, or bolus administration, at 07.00 h. Feeding periods, or administration periods, were defined as the period between a meal or marker dose and the following meal or dose, respectively.

Alkane faecal recovery was calculated according to Eqn (1) and balanced over 3 days of sampling:

$$\mathrm{AFR}_{i,j} = \frac{\sum \mathrm{SFQ} \times F_{i,j}}{H \times H_i + C \times C_i + D_j} \quad (1)$$

where SFQ is single faeces quantity, H and C are total received quantities of hay and concentrate (kg DM), F_i, H_i and C_i are concentrations of plant alkane i in faeces, hay and concentrate (mg/kg DM), F_j is the concentration of synthetic alkane j in faeces (mg/kg DM) and D_j is the total quantity of j offered by boluses (kg).

Dry matter intake and faecal DM output (DMO) were recorded during adaption and quantitative collection, respectively. This was used to calculate DMD and was again balanced over 3 days of sampling.

Dry matter intake of diet (Eqn (2)) and hay (Eqn (3)) was estimated using the alkane-pair technique proposed by Mayes *et al.* (1986). Twelve possible combinations between plant alkanes *n*-heptacosane (C27), *n*-nonacosane (C29), *n*-hentriacontane (C31) and *n*-tritriacontane (C33), and synthetic alkanes (C28, C32 and C36) were tested. For estimation of hay DMI, concentrate DMI was assumed to be known.

$$\mathrm{DMI(kg/d)} = \frac{D_j}{(F_j/F_i) \times (h \times H_i + c \times C_i)} \quad (2)$$

$$\mathrm{DMI(kg/d)} = \frac{(F_i/F_j) \times D_j - I_c \times C_i}{H_i} \quad (3)$$

where F_i, H_i and C_i are concentrations of plant alkane i in faeces, hay and concentrate (mg/kg DM), h and c are relative proportions of hay and concentrate in the diet, I_c is the known concentrate intake (kg DM/day), F_j is the concentration of synthetic alkane j in faeces (mg/kg DM), and D_j is the quantity of j offered by boluses (mg/day).

Dry matter intake of the diet was also calculated (Eqn (4)) from separate estimates of DMD and DMO, using the product of SFQ and the daily defecation frequency (DDF) for the latter:

$$\mathrm{DMI(kg/d)} = \frac{\mathrm{SFQ} \times \mathrm{DDF}}{1 - \mathrm{DMD}} \quad (4)$$

Additionally, DMO was estimated using the quotient of dose rate (mg/day) and faecal concentration (mg/kg DM) of synthetic alkanes.

Apparent digestibility of dietary DM, energy, OM (OMD) and proximate nutrients was estimated using individual plant alkanes as given in Eqns (5) and (6), respectively. For the latter calculation, only samples of timeframe one (07.00 to 08.59 h) were used.

$$\mathrm{DMD} = 1 - \frac{h \times H_i + c \times C_i}{F_i} \qquad (5)$$

$$\mathrm{OMD} = 1 - \frac{h \times H_i + c \times C_i}{F_i} \times \frac{F_n}{h \times H_n + c \times C_n} \qquad (6)$$

where F_i, H_i and C_i are concentrations of plant alkane i in faeces, hay and concentrate (mg/kg DM), h and c are relative proportions of hay and concentrate in the diet, and F_n, H_n and C_n are concentrations of energy, OM or nutrients in faeces, hay and concentrate (mg/kg DM).

Statistical analyses

Statistical analyses were performed with SAS 9·4 analytical software (SAS Institute Inc., Cary, NC, USA) using the MIXED procedure. Least squares means (LSM) of SFQ, daily faeces quantity (DFQ) and DDF were established regarding differences between animals, days and feeding periods within the days (Model 1). Subsequently, LSM of SFQ and zootechnical variables DMI, DMO and DMD were estimated for defined timeframes (Model 2), and the latter were compared with corresponding measured counterparts. In Model 2, the effect of timeframe was substituted by a fixed marker effect in the case of estimates of OMD, energy and nutrient digestibility because it was estimated only for one selected timeframe. Differences with $P < 0{\cdot}05$ were considered to be significant.

For statistical analysis, the following models were used:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + a \times \mathrm{covariable}_{ijk} + \underline{e}_{ijk} \qquad (\textit{Model}\ 1)$$

where y_{ijk} are measurements of SFQ, DFQ and DDF as a function of factors i, j and k; μ is the general mean; α_i is the fixed effect of animal i ($i = 1,\ldots, 5$); β_j is the fixed effect of day j ($j = 1, 2, 3$); γ_k is the fixed effect of feeding period k (k = A, B, C for plant alkanes: A: 07.00–14.59 h, B: 15.00–20.59 h and C: 21.00–06.59 h, and k = A, B for synthetic alkanes: A: 07.00–20.59 h and B: 21.00–06.59 h); $\mathrm{covariable}_{ijk}$ is the linear effect of either DDF (for SFQ and DFQ analysis) or SFQ (for DDF analysis) with regression coefficient a; $\underline{e}_{ijk}$ is the random residual effect.

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \underline{a}_k + \underline{e}_{ijk} \qquad (\textit{Model}\ 2)$$

where y_{ijk} are measurements of SFQ or estimates of zootechnical variables as a function of factors i, j and k; μ is the general mean; α_i is the fixed effect of day i ($i = 1, 2, 3$); β_j is the fixed effect of timeframe or marker j ($j = 1,\ldots$, 13: 1: 07.00–08.59 h, 2: 09.00–10.59 h, 3: 11.00–12.59 h, 4: 13.00–14.59 h, 5: 15.00–16.59 h, 6: 17.00–18.59 h, 7: 19.00–20.59 h, 8: 21.00–22.59 h, 9: 23.00–00.59 h, 10: 01.00–02.59 h, 11: 03.00–04.59 h, 12: 05.00–06.59 h, 13 denotes the measured values for DMI, DMO and DMD, respectively, or $j = 1,\ldots$, 5: 1–4: plant alkanes, 5 denotes the measured values for OMD, energy and nutrient digestibility, respectively); $\alpha\beta_{ij}$ is the interaction between α_i and β_j; $\underline{a}_k$ is the random effect of animal k ($k = 1,\ldots, 5$) with consideration of repeated records; $\underline{e}_{ijk}$ is the random residual effect.

RESULTS

Patterns of faecal quantity and faecal alkane concentrations

In the current study, 5·9 ± 0·44 kg DM faeces was defecated daily, ranging from 5·0 to 6·6 kg DM. The SFQ was 0·39 ± 0·120 kg DM, and ranged from 0·08 to 0·80 kg DM. The SFQ and DFQ did not differ significantly between days or horses. The former was similar in feeding periods A and B, but differed in comparison with period C ($P < 0{\cdot}01$), as mealtimes were distributed unevenly throughout the days. The DDF was 16 ± 3·3 times on average, in a range between 11 and 22 times. It differed between days ($P = 0{\cdot}016$) and horses ($P = 0{\cdot}058$) by tendency, and significantly between feeding periods ($P < 0{\cdot}001$). The SFQ and the DDF were negatively correlated, as shown in Fig. 2, and had therefore been used to calculate DMO.

Alkane faecal recovery was 82 ± 4·1 (C36), 91 ± 10·5 (C27), 98 ± 10·1 (C33), 99 ± 6·3 (C32), 101 ± 9·8 (C29), 105 ± 8·8 (C31) and 108 ± 11·1% (C28).

Throughout the day, specific patterns of SFQ (Fig. 3(*a*)) and faecal concentrations of plant (Fig. 4(*a*)) and synthetic alkanes (Fig. 5(*a*)) were observed. These patterns repeated from day to day, which is shown

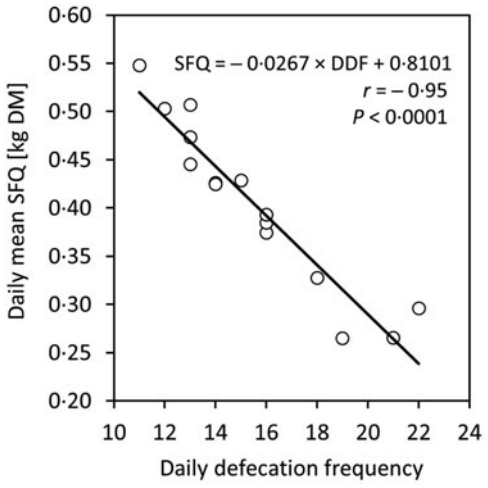

Fig. 2. Relationship between daily mean single faeces quantity (SFQ) and the individual daily defecation frequency.

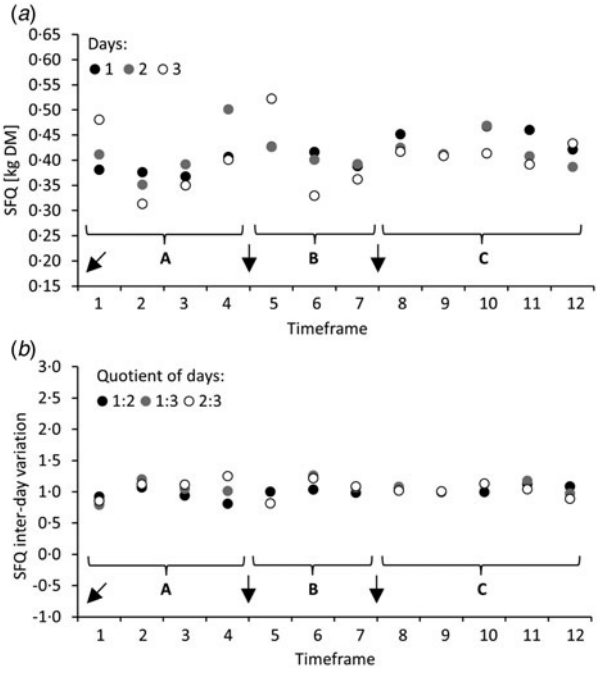

Fig. 3. (*a*) Intra-day and (*b*) inter-day variation of least squares means of single faeces quantity (SFQ): A, B and C denote feeding periods following meals. Arrows indicate the time of meal presentation.

in Figs 3(*b*), 4(*b*) and 5(*b*) by inter-day quotients that in most timeframes did not differ markedly from one (one indicates no difference). For illustration purposes, patterns of C29 and C32 concentrations in faeces are shown as examples. Least squares means of SFQ showed pronounced dynamics within and lesser dynamics between the days, characterized by

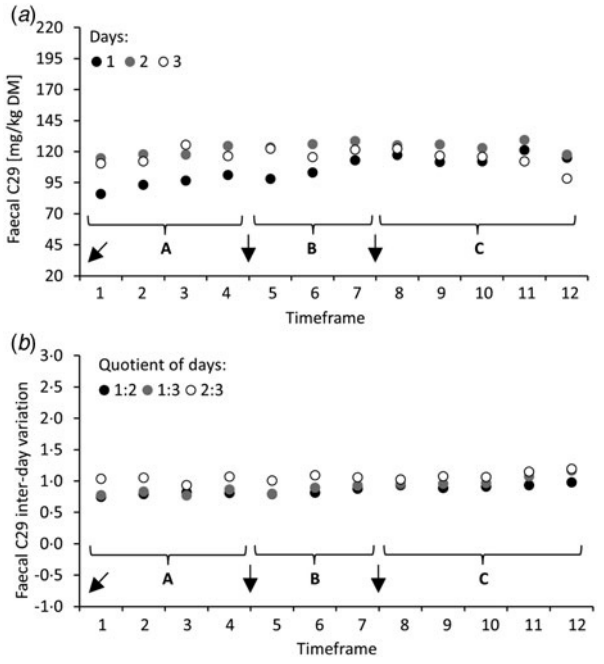

Fig. 4. (*a*) Intra-day and (*b*) inter-day variation of least squares means of faecal *n*-nonacosane (C29) concentration: A, B and C denote feeding periods following meals. Arrows indicate the time of meal presentation.

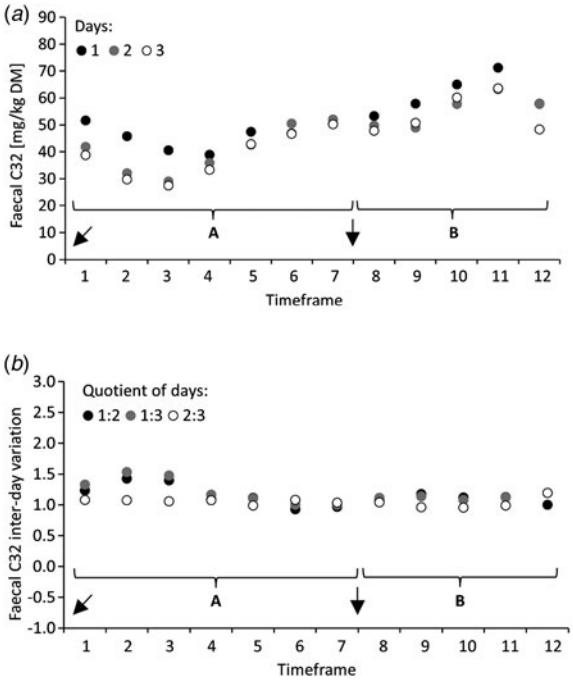

Fig. 5. (*a*) Intra-day and (*b*) inter-day variation of least squares means of faecal *n*-dotriacontane (C32) concentration: A and B denote periods following bolus administration. Arrows indicate the administration time.

decreasing quantities up to approximately 3–4 h after mealtime, and subsequently increasing quantities until the next mealtime. This pattern was similar between feeding periods A and B, but much less pronounced during evening and night hours (period C).

Average rates of C27, C29, C31 and C33 concentrations in faeces were 25 ± 3·9, 115 ± 14·7, 204 ± 38·5 and 45 ± 8·5 mg/kg DM, respectively. The faecal concentration of plant alkanes consistently showed little diurnal variability. However, differences were distinct between days, with the lowest concentrations generally on day one (Fig. 4(*b*)).

In contrast, clear intra-day dynamics of the faecal concentration of synthetic alkanes were detected. The average faecal concentration was 49 ± 13·2 (C28), 48 ± 12·9 (C32) and 43 ± 12·7 mg/kg DM (C36). It was reached approximately at the beginning of day three of treatment after the 5th repeated dose. Within a day, concentrations in faeces initially decreased after the 1st dose in the morning, with minimal concentration in timeframe three, and then increased gradually beginning in timeframe four. In the evening hours, between timeframes six to eight, concentrations remained unaltered. After the 2nd dose, concentrations increased again, and reached a maxima in the night always approximately 7–8 h later (timeframe 11). Generally, faecal concentrations of synthetic alkanes differed notably between consecutive days only during timeframes one to three, whereby highest concentrations were found on day one (Fig. 5(*b*)).

Dry matter intake

To estimate daily DMI, all possible combinations between plant C27, C29, C31 and C33, and synthetic C28, C32 and C36 alkanes were tested. It became clear that most estimates obtained using pairings to C36 (except C27:C36) differed significantly in comparison with the measured DMI ($P < 0·05$). Only in timeframes 10 and 11, approximately 5–8 h after the second dose of C36, did estimates not differ significantly. Least squares means of estimates from selected alkane pairs are shown in Table 3. Estimates were mostly unbiased in timeframes one, five to nine, 10 and 12, regardless of what alkane combination was used. During 3–8 h after the morning meal (timeframes 2–4), measured DMI was over-estimated ($P < 0·001$), but under-estimated numerically between 5 and 6 h (timeframe 10), and significantly between 7 and 8 h after the 3rd meal ($P < 0·05$, timeframe 11). The most accurate estimates of DMI were obtained using C29:C28 and C33:C28 pairs in timeframe one, and C31:C32 in timeframe 12.

Selected LSM of alternatively calculated DMI (Eqn (4)) are given in Table 3. Measured DMI was underestimated when faeces samples were taken between 3 and 4 h after the morning meal (timeframe 2), and over-estimated during any other time of the day. The most accurate estimates were made with C29 and C33 as digestibility markers between 5 and 6 h after the morning meal (timeframe 3).

It was evident that the estimation of daily DMI was similar between day two and three of quantitative collection, but differed markedly from day one (Supplementary Fig. S1, available from http://journals.cambridge.org/AGS).

Faecal dry matter output

Using synthetic alkanes, measured and estimated DMO differed particularly during timeframes one to four, and 10–12, respectively, while reliable estimates were obtained from faeces samples taken between timeframes five and nine, approximately 6 h before to 4 h after the 2nd dose of synthetic alkanes (Table 4). Estimates based upon C36 mostly exceeded their measured counterpart, particularly between the timeframes one and five ($P < 0·01$).

Calculation of DMO upon the basis of SFQ × DDF was unbiased during timeframes six to seven, nine and 11 (Table 4). Despite deviations up to 1·1 kg/day ($P < 0·05$), these estimates were generally more reliable and consistent in comparison with those obtained using synthetic alkanes.

Estimates of DMO were inconsistent across the days, which was less obvious in the 2nd approach and was limited to individual timeframes (timeframes 1, 4, 5, 11 and 12, Supplementary Fig. S2, available from http://journals.cambridge.org/AGS). Again, marker-based estimates of DMO differed distinctly between day one and the other 2 days of quantitative collection.

Apparent digestibility of dry matter, organic matter, energy and nutrients

Least squares means of estimates of DMD are shown in Table 5. Estimates made using C27 were the lowest. Thus, measured DMD was under-estimated in timeframes one and two, while estimates were largely unbiased throughout the remainder of the

Table 3. *Least squares means* (*LSM*) *of estimates of daily dry matter* (*DM*) *intake* (*DMI, kg/day*) *of diet and hay in a sequence of equal timeframes throughout the day* (*1,…, 12*) *using selected pairs of plant and synthetic alkanes or separate estimates of DM output and digestibility, compared with LSM of measured DMI of the diet* (*12·0 kg/day*) *and hay* (*11·2 kg/day*)

	Alkane pair technique				Alternative method	
Timeframe	C29:C28	C31:C32	C33:C28	C33:C32	C29	C33
1 (07.00–08.59 h)	12·3/11·4	14·7/13·6	12·1/11·3	13·8/12·8	13·1	13·2
2 (09.00–10.59 h)	16·2/15·1	19·3/18·0	16·4/15·2	18·5/17·2	10·9	10·9
3 (11.00–12.59 h)	19·1/17·8	22·2/20·8	19·4/18·0	21·6/20·1	12·2	12·4
4 (13.00–14.59 h)	16·7/15·6	19·3/18·0	16·9/15·7	18·4/17·1	14·5	14·7
5 (15.00–16.59 h)	14·1/13·2	15·8/14·7	14·3/13·3	15·1/14·0	15·6	15·9
6 (17.00–18.59 h)	12·5/11·6	14·2/13·3	12·5/11·7	13·5/12·6	12·9	12·8
7 (19.00–20.59 h)	12·2/11·4	14·2/13·2	12·4/11·5	13·5/12·6	13·2	13·2
8 (21.00–22.59 h)	12·6/11·7	14·4/13·5	12·9/12·0	13·9/12·9	15·3	15·4
9 (23.00–00.59 h)	12·0/11·1	13·7/12·7	12·3/11·4	13·2/12·2	14·1	14·3
10 (01.00–02.59 h)	10·6/9·8	11·5/10·7	10·7/10·0	11·0/10·3	15·3	15·5
11 (03.00–04.59 h)	9·4/8·7	10·9/10·2	9·4/8·8	10·4/9·7	14·5	14·4
12 (05.00–06.59 h)	10·8/10·1	12·1/11·2	11·2/10·4	11·8/11·0	13·6	14·1
Range of S.E.	0·74–0·79	0·94–0·99	0·96–1·01	0·94–0·97	0·85–0·91	0·97–1·03
Range of D.F.	37·3–46·5	36·4–43·7	19·0–22·4	30·2–34·3	44·6–55·1	38·2–47·2

C27, *n*-heptacosane; C28, *n*-octacosane; C29, *n*-nonacosane; C31, *n*-hentriacontane; C32, *n*-dotriacontane; C33, *n*-tritriacontane; D.F., degree of freedom; S.E., standard error.
Significant differences of LSM between estimated and measured DMI are highlighted, where light grey means $P < 0{\cdot}05$, grey means $P < 0{\cdot}01$ and dark grey means $P < 0{\cdot}001$. Estimates for diet DMI are given before the solidus, estimates for hay DMI thereafter.

Table 4. *Least squares means* (*LSM*) *of estimates of daily faecal dry matter output* (*DMO, kg/day*) *in a sequence of equal timeframes throughout a day* (*1,…, 12*) *using synthetic alkanes or the product of single faeces quantity* (*SFQ*) *and daily defecation frequency* (*DDF*), *compared with LSM of measured DMO* (*5·9 kg/day*)

Timeframe	C28	C32	C36	SFQ × DDF
1 (07.00–08.59 h)	6·1	6·8	7·8	6·4
2 (09.00–10.59 h)	7·6	8·6	10·2	5·2
3 (11.00–12.59 h)	8·4	9·4	11·6	5·5
4 (13.00–14.59 h)	7·4	8·1	10·0	6·5
5 (15.00–16.59 h)	6·3	6·6	8·4	7·0
6 (17.00–18.59 h)	5·6	6·1	7·1	5·7
7 (19.00–20.59 h)	5·2	5·7	6·8	5·6
8 (21.00–22.59 h)	5·3	5·8	7·0	6·5
9 (23.00–00.59 h)	5·3	5·6	7·0	6·1
10 (01.00–02.59 h)	4·7	4·8	5·7	6·7
11 (03.00–04.59 h)	3·9	4·4	5·1	6·2
12 (05.00–06.59 h)	5·1	5·4	6·8	6·3
Range of S.E.	0·41–0·43	0·43–0·44	0·60–0·62	0·34–0·37
Range of D.F.	15·9–19·1	33·1–37·2	32·8–38·0	44·2–56·0

C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; D.F., degree of freedom; S.E., standard error.
Significant differences of LSM between estimated and measured DMO are highlighted, where light grey means $P < 0{\cdot}05$, grey means $P < 0{\cdot}01$ and dark grey means $P < 0{\cdot}001$.

Table 5. *Least squares means (LSM) of estimates of apparent dry matter (DM) digestibility coefficients (DMD) of the diet in a sequence of equal timeframes throughout a day (1,…, 12) obtained by means of plant alkanes, compared with LSM of DMD (0·51) calculated from measured DM intake and output*

Timeframe	C27	C29	C31	C33
1 (07.00–08.59 h)	0·43	0·49	0·50	0·47
2 (09.00–10.59 h)	0·48	0·52	0·52	0·51
3 (11.00–12.59 h)	0·51	0·54	0·54	0·54
4 (13.00–14.59 h)	0·50	0·55	0·55	0·54
5 (15.00–16.59 h)	0·50	0·55	0·56	0·54
6 (17.00–18.59 h)	0·49	0·55	0·56	0·54
7 (19.00–20.59 h)	0·53	0·58	0·59	0·57
8 (21.00–22.59 h)	0·53	0·58	0·60	0·58
9 (23.00–00.59 h)	0·52	0·56	0·58	0·57
10 (01.00–02.59 h)	0·51	0·56	0·58	0·55
11 (03.00–04.59 h)	0·54	0·58	0·59	0·57
12 (05.00–06.59 h)	0·49	0·53	0·55	0·54
Range of S.E.	0·026–0·027	0·014–0·015	0·012–0·013	0·021–0·022
Range of D.F.	7·9–8·6	14·9–17·4	22·3–28·3	13·0–14·1

C27, *n*-heptacosane; C29, *n*-nonacosane; C31, *n*-hentriacontane; C33, *n*-tritriacontane; D.F., degree of freedom; S.E., standard error.

Significant differences of LSM between estimated and measured DMD are highlighted, where light grey means $P < 0·05$, grey means $P < 0·01$ and dark grey means $P < 0·001$.

day. Estimates based upon C29, C31 and C33 were unbiased in timeframe two, approximately 3–4 h after the morning meal, and then consistently over-estimated measured DMD.

At each timeframe, DMD estimates were widely similar between days two and three of quantitative collection, but again differed in comparison with day one (Supplementary Fig. S3, available from http://journals.cambridge.org/AGS).

During timeframe one, OMD and digestibility of dietary energy and proximate nutrients was generally under-estimated. Particularly estimates of C27 and C33 differed significantly from the measured counterparts ($P < 0·05$). Most accurate estimates were obtained using C31 despite numerical differences of up to 6·5% (Table 6).

DISCUSSION

The present study was conducted under practical husbandry and feeding conditions. Plant and synthetic alkanes were used to estimate DMI, DMO and apparent DMD simultaneously with one set of markers. Even-chain alkanes are mostly unavailable in feed plants (Dove *et al.* 1996). As synthetics, they are easy to combine with plant alkanes, which is advantageous because analytical efforts can be significantly reduced (Dove & Mayes 2006).

The type of bolus used to administer synthetic alkanes is accepted well by horses (Bachmann *et al.* 2016*b*). Dose rate and frequency were based upon a validation study by Smith *et al.* (2007). On average, C28 and C32 were 16 (11%) and 8 mg (5%) beneath, and C36 5 mg (3%) above the target dose. The variation between bolus samples was high, with a coefficient of variation of 9·7 (C28), 9·8 (C32) and 9·9% (C36), and it was probably high between boluses administered to the horses as well. Variation of alkane doses may have occurred during the filling of gelatine capsules and subsequent baking because exposure to persistently high temperature resulted in losses of up to 5·2 (C28), 2·2 (C32) and 0·7% (C36) of initial sample weight (Bachmann *et al.* 2016*a*). The capsules offered no protection because bovine gelatine itself melts at 58 °C (Zhang *et al.* 2006). The variation of alkane quantities offered via boluses might have influenced the calculation of AFR and estimation of DMO. Thus, accidental over-dosing of a synthetic alkane have led to an incorrectly depressed AFR (e.g. in C36), while under-dosing may have led to an incorrectly increased AFR (e.g. in C28). Even if estimation of DMI has been affected similarly, this could be compensated by the selection of suitable alkane pairs as discussed below.

Table 6. *Least squares means (LSM) of estimates of apparent digestibility coefficients of dietary energy and proximate nutrients obtained by means of plant alkanes, compared with LSM of digestibility calculated from measured energy and nutrient intake and output*

	Measured	C27	C29	C31	C33
Organic matter	0·54	0·45	0·51	0·52	0·48
Crude protein	0·62	0·54	0·59	0·59	0·57
Acid ether extract	0·32	0·14	0·24	0·26	0·21
Crude fibre	0·43	0·30	0·38	0·39	0·34
Neutral detergent fibre	0·47	0·36	0·43	0·43	0·39
Acid detergent fibre	0·44	0·34	0·41	0·42	0·38
Cellulose	0·48	0·36	0·43	0·44	0·40
Hemicellulose	0·52	0·39	0·45	0·45	0·41
Nitrogen-free extract	0·62	0·55	0·60	0·60	0·57
Gross energy	0·50	0·41	0·48	0·48	0·45
Range of S.E.	0·013–0·070	0·013–0·070	0·013–0·070	0·013–0·070	0·013–0·070
Range of D.F.	7·2–16·7	7·2–16·7	7·2–16·7	7·2–16·7	7·2–16·7

C27, *n*-heptacosane; C29, *n*-nonacosane; C31, *n*-hentriacontane; C33, *n*-tritriacontane; D.F., degree of freedom; S.E., standard error.

Significant differences of LSM between estimated and measured digestibility coefficients are highlighted, where light grey means $P < 0{\cdot}05$, grey means $P < 0{\cdot}01$ and dark grey means $P < 0{\cdot}001$.

Estimation of digestibility was unaffected because synthetic alkanes had not been used.

The quantitative collection of faeces is necessary to quantify faecal output and directly calculate digestibility or recovery rates required for correction of faecal alkane concentrations. During treatment, apparently increasing stress was observed in at least one examined horse through increased unrest resulting in more frequent defecation (up to 22 times a day). The horses' accustomed rate of exercise was restricted. This might also have lowered water intake, decelerated digesta passage, and biased calculation of digestibility (Pagan *et al.* 1998).

The adaption period was set to 5 days, allowing synthetic alkanes to reach equilibrium in faeces following Ferreira *et al.* (2007). Duration of quantitative collection was set to 3 consecutive days according to Goachet *et al.* (2009), who did not note an altered digestibility of DM, OM and fibres compared with a collection period prolonged up to 5 days. In contrast, Smith *et al.* (2007) found that the accuracy of intake estimation increased progressively as the number of sampling days was increased. Three days of faeces collection were sufficient to show specific intra-day and inter-day dynamics of SFQ and faecal alkane concentrations. However, the impact of prolonged collection on DMI, DMO and DMD estimation has not been studied in the current trial.

Alkane faecal recovery decisively influences intake and digestibility estimates. The present AFR were in the range given by Sales (2012) for plant alkanes. According to Sales (2012), AFR of alkanes was generally high but not consistently complete. Incomplete AFR may be explained by the partial absorption of alkane molecules in the small intestine (Kolattukudy & Hankin 1966; Mayes *et al.* 1988) and probably not by microbial fermentation (Keli *et al.* 2008), as digestion does not occur over the time of passage. In horses, AFR is highly variable, probably due to animal individuality and diet composition effects (Ferreira *et al.* 2009). Faecal recovery of plant alkanes was not influenced by molecular chain length, which confirms some previous studies (Ordakowski *et al.* 2001; Peiretti *et al.* 2006) but contradicts others (Gudmundsson & Thorhallsdottir 1998; Ferreira *et al.* 2009). Faecal recovery of synthetic alkanes seemed to decrease with increasing chain length, congruent with results of Gudmundsson & Thorhallsdottir (1998), who offered synthetic alkanes mixed into feed pellets. However, this might be erroneous, and was probably a result of accidental overdosing (C36) and chain-length dependent losses of parts of alkanes incurred during the production of boluses (C28) or pellets under high processing temperature. Because reliable literature data are rare or do not exist, AFR is recommended to be determined

individually for each trial conducted (Ferreira *et al.* 2009) and specific for respective diets (Lin *et al.* 2007). Therefore, the current study tested whether uncorrected faecal alkane concentrations also enable reliable estimates.

Specific intra-day and inter-day dynamics of SFQ and faecal concentrations of plant and synthetic alkanes in horses were shown, which to the authors' knowledge has not thus far been given elsewhere. Diurnal patterns were shown in horses for faecal concentrations of hydrochloric acid-insoluble ash (AIA) and chromium (Cr) from Cr-mordanted hay by Cuddeford & Hughes (1990), and for dosed chromic oxide by Haenlein *et al.* (1966) and Takagi *et al.* (2002), in ruminants for synthetic alkanes by Giráldez *et al.* (2004) and Molina *et al.* (2004), and for ratios between plant and synthetic alkanes by Molina *et al.* (2004) and Oliván *et al.* (2007). Intra-day dynamics of SFQ were more pronounced than those of faecal concentrations of plant alkanes. Patterns of DM concentration in faeces were quite similar to those of plant alkane concentrations and, in turn, those of absolute alkane and faeces quantity were similar too. Thus, intra-day dynamics for SFQ and plant alkane faecal concentrations following three mealtimes daily are similar. In contrast, intra-day dynamics were much more pronounced for concentrations of synthetic alkanes in faeces following twice daily administration. This suggests that frequency of mealtimes or bolus administration has a primary influence on characteristics of intra-day patterns, which confirms previous reports (Haenlein *et al.* 1966; Smith *et al.* 2007). Diurnal patterns of SFQ and faecal alkane concentrations were similar between individual horses, and repeated from day to day. Nevertheless, individual shifts that were either quantitative or temporal have also been noticed. Haenlein *et al.* (1966) mentioned that diet composition affects digesta passage, altering measurable patterns. In ponies, Drogoul *et al.* (2001) reported increasing mean retention time as the proportion of concentrate in the diet increased. Because alkane concentrations differed distinctly between hay and concentrate in the current study, and individual proportions of concentrate were fed, this might have contributed to individual characteristics of those patterns. However, this probably had little effect. It is expected that the time taken by individual horses to consume a single meal and bound plant alkanes both increase with increasing proportions of roughage in the diet. Mealtimes were generally much longer than the ingestion of boluses, which might have contributed to less pronounced diurnal patterns of plant alkanes in faeces. The method of application affects duration of alkane exposition in the gut (Giráldez *et al.* 2004). This, and particularly the association with the solid or liquid phase of digesta, may influence gut passage of synthetic alkanes and the resulting diurnal patterns. Mayes *et al.* (1988) reported amounts of 30–40% of synthetic alkanes found in the liquid phase. Gut passage of liquid-phase markers is hastened compared with that of markers fully bound to coarse plant particles (Udén *et al.* 1982). As mentioned above, restriction of exercise might also have affected measured intra-day and inter-day dynamics of SFQ and alkanes.

Estimation of DMI using the alkane-pair technique (Mayes *et al.* 1986) was particularly affected by pronounced intra-day dynamics of synthetic alkanes. Faecal concentrations decreasing below the average resulted in over-estimated DMO, and thus DMI. Where faecal concentrations increased above the average, DMO and DMI were generally under-estimated. Despite being non-significant, specific differences between estimated and measured DMI could differ by >2 kg/day, which would be unacceptable in practical implementation. The most accurate estimates of DMI were made using the C31:C32 alkane pair within 2 h prior (timeframe 12), as well as using C29:C28 and C33:C28 pairs within 2 h after the morning meal (timeframe 1), where the measured counterpart was over-estimated by 0·8 (C31:C32), 2·5 (C29:C28) and 0·8% (C33:C28), respectively (0·1–0·3 kg/day). The C31:C32 pair has also been used in previous studies that estimated DMI within a similar magnitude (Stevens *et al.* 2002; Ferreira *et al.* 2007; Smith *et al.* 2007). Estimates obtained using C36 as output marker did not display measured DMI accurately, which was probably linked to bolus preparation as discussed above.

The accuracy of DMI calculated from previously estimated DMO and DMD was affected by dynamics of both SFQ and faecal concentrations of plant alkanes, which led to an increasing bias. Even though non-significant, measured DMI was partly under- or over-estimated by >2 kg/day. Estimation of DMI was suitable in timeframe three using C29 (+1·6% deviation) or C33 (+3·3% deviation) as a digestibility marker. The C29 marker was also recommended by Peiretti *et al.* (2006), who calculated DMI from estimations of DMD and DMO measured via total collection with a lowest bias of –3·5% deviation.

Measured DFQ was higher than that recorded previously in thoroughbreds or in quarter horse yearlings (Ordakowski *et al.* 2001; Patterson *et al.* 2002; Takagi *et al.* 2002). Presumably, this was due to comparatively large proportions of roughage in the current diets and perhaps also an effect of body weight, but less so an effect of breed. The high range of defecation frequency, up to 22 times daily, might be a result of higher stress during treatment. However, this was only obvious in one horse. Golonka (2009) reported a mean frequency of six defecations throughout a day in grazing Konik ponies. As far as the authors know, directly comparable data are not available.

As mentioned above, declining faecal concentrations of synthetic alkanes led to over-estimation of measured DMO, while increasing concentrations under-estimated it. In timeframes five to nine, a period between 6 h before and 4 h after the 2nd dose of synthetic alkanes, faecal concentrations remained constant near the average. Differences between measured and estimated DMO were admittedly not significant but numerically within the –13·5 and +10·6% deviation (±0·7 kg/day). The accuracy of estimation was lower than some given in the literature (Castelán-Ortega *et al.* 2007). Again, estimates obtained using the C36 marker were more biased and were only reliable with faeces sampled during the night hours (timeframes 10 and 11) with –3·5 to –15·7% deviation (0·2–0·8 kg/day).

When DMO has been estimated by the product of SFQ and DDF, these estimates were minimally variable, as diurnal fluctuation of SFQ was also minimal. Nevertheless, if SFQ decreases beneath its average, this results in under-estimation, while an SFQ which exceeds this average results in over-estimation of measured DMO. Reliability of DMO estimates was highest when faecal samples were taken in timeframes six and seven, between 3 and 6 h after the 2nd meal, as well as nine and 11, between 3–4 and 7–8 h after the 3rd meal, with –5·4 to +5·1% (±0·3 kg/day) deviation. This calculation requires known DDF. For this, video monitoring may be a helpful tool. To the authors' knowledge, this approach has so far not been reported elsewhere.

Apparent digestibility of DM, OM, dietary energy and proximate nutrients, based upon measured data, were within the wide range of comparable data given in the literature (Pagan *et al.* 1998; Takagi *et al.* 2002; Zeyner & Kienzle 2002; Goachet *et al.* 2009; Jensen *et al.* 2010).

Although intra-day variation of plant alkanes' faecal concentration was less pronounced, it was evident that decreasing concentrations resulted in the under-estimation of measured DMD, such as during timeframe one using C29, C31 and C33; analogously increasing concentrations resulted in over-estimation (timeframes 2–12 using C29, C31 and C33). Sampling of faeces in timeframe two, between 3 and 4 h after the morning meal, led to most accurate estimates when C29 or C33 markers were used (+0·8 or +0·2% deviation from measured DMD, respectively). These alkanes can therefore be recommended as digestibility markers, as noted previously by Peiretti *et al.* (2006). The application of C29 and C33 for the estimation of DMD led to estimates in a similar range (Ordakowski *et al.* 2001) or a wider range of deviation (Peiretti *et al.* 2006; Ferreira *et al.* 2007) compared with the current results. The C27 marker was mostly suitable, but DMD estimation may be biased by analytical errors that might occur due to low dietary and faecal concentrations (Brosh *et al.* 2003).

The selection of morning spot sampling of faeces for estimating OMD, energy and nutrient digestibility using plant alkanes was based upon recommendations by Fuchs *et al.* (1987), who used 4 N AIA. In the current study, the digestibility of dietary energy and nutrients was under-estimated. After further analysis of alkane faecal concentration dynamics, it seemed likely that accuracy of estimation would have been increased with later sampling (timeframe 2). This shows that selection of spot samples, or even a small temporal shift in diurnal patterns of alkane faecal concentrations, may noticeably affect the resulting estimates.

CONCLUSIONS

Intra-day and inter-day dynamics of SFQ and of the faecal concentrations of plant alkanes and synthetic bolus alkanes were mainly influenced by the mealtime or the administration interval. Knowledge of these defecation dynamics allows the selection of faecal spot samples where estimates of DMI, DMO and DMD are mostly unbiased. Additionally, incomplete faecal recovery of the marker alkanes can be compensated by sampling. In the current study, the most accurate estimates of DMI were obtained from 2 h before (C31:C32) until 2 h after the morning meal (C29:C28 and C33:C28), as well as between 5 and 6 h after the morning meal using SFQ × DDF and C29 and C33 as digestibility markers. The estimation of DMO using synthetic alkanes had a larger bias than the estimation using SFQ × DDF, which was most

reliable based upon samples taken between 3 and 6 h after the midday meal. For DMI estimation, the latter approach provides the opportunity to omit the administration of synthetic alkanes in addition, but DDF has to be known. Using C29 or C33, DMD was largely unbiased during 3–4 h after the morning meal. In this respect, taking two to three spot samples of faeces evenly distributed between 2 h before and 6 h after the 1st meal or bolus administration per day for at least 3 consecutive days is recommended. Repeated administration of synthetic alkanes twice a day did not prevent fluctuation of faecal concentrations. It may therefore be useful to increase or substitute the administration of synthetic alkanes. It is suggested that the diurnal fluctuation of SFQ and of faecal alkane concentrations becomes lower when the feeding interval decreases and may be absent with *ad libitum* feeding. In this way, any sampling time would be applicable, provided that the used markers have high or complete faecal recovery. Consequently, this approach needs to be validated with a larger quantity of horses and different feeding regimes.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0021859616000344.

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REFERENCES

Bachmann, M., Wensch-Dorendorf, M., Mäder, K., Bulang, M. & Zeyner, A. (2016*a*). Preparation of synthetic alkane waxes and investigations on their suitability for application as dietary markers in farm animals. *Livestock Science* **185**, 110–116.

Bachmann, M., Wensch-Dorendorf, M., Wulf, M., Glatter, M., Siebmann, M., Bierögel, C., Schumann, E., Bulang, M., Aurich, C. & Zeyner, A. (2016*b*). Bolus matrix for administration of dietary markers in horses. *Livestock Science* **185**, 43–49.

Brosh, A., Henkin, Z., Rothman, S. J., Aharoni, Y., Orlov, A. & Arieli, A. (2003). Effects of faecal *n*-alkane recovery in estimates of diet composition. *Journal of Agricultural Science, Cambridge* **140**, 93–100.

Castelán-Ortega, O. A., Estrada-Flores, J. G., Smith, D. G., Colunga, B., Solís-Méndez, A., Avilés-Nova, F., Ramirez, A. & García, I. (2007). Use of *n*-alkanes for estimation of voluntary intake and digestibility in donkeys (*Equus asinus*). *Journal of Animal and Feed Sciences* **16** (Suppl. 2), 307–312.

Cuddeford, D. & Hughes, D. (1990). A comparison between chromium-mordanted hay and acid-insoluble ash to determine apparent digestibility of a chaffed, molassed hay/straw mixture. *Equine Veterinary Journal* **22**, 122–125.

Dove, H. & Mayes, R. W. (1991). The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. *Australian Journal of Agricultural Research* **42**, 913–952.

Dove, H. & Mayes, R. W. (2006). Protocol for the analysis of *n*-alkanes and other plant-wax compounds and for their use as markers for quantifying the nutrient supply of large mammalian herbivores. *Nature Protocols* **1**, 1680–1697.

Dove, H., Mayes, R. W. & Freer, M. (1996). Effects of species, plant part, and plant age on the *n*-alkane concentrations in the cuticular wax of pasture plants. *Australian Journal of Agricultural Research* **47**, 1333–1347.

Drogoul, C., de Fombelle, A. & Julliand, V. (2001). Feeding and microbial disorders in horses: 2: effect of three hay: grain ratios on digesta passage rate and digestibility in ponies. *Journal of Equine Veterinary Science* **21**, 487–491.

Elwert, C., Kluth, H. & Rodehutscord, M. (2004). Effect of variable intake of alfalfa and wheat on faecal alkane recoveries and estimates of roughage intake in sheep. *Journal of Agricultural Science, Cambridge* **142**, 213–223.

FASS (2010). *Guide for the Care and Use of Agricultural Animals in Research and Teaching*, 3rd edn. Champaign, IL, USA: Federation of Animal Science Societies. Available from: http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf (verified 25 February 2016).

Ferreira, L. M. M., Garcia, U., Rodrigues, M. A. M., Celaya, R., Dias-da-Silva, A. & Osoro, K. (2007). Estimation of feed intake and apparent digestibility of equines and cattle grazing on heathland vegetation communities using the *n*-alkane markers. *Livestock Science* **110**, 46–56.

Ferreira, L. M. M., Celaya, R., García, U., Rodrigues, M. A. M. & Osoro, K. (2009). Differences between domestic herbivores species in alkane faecal recoveries and the accuracy of subsequent estimates of diet composition. *Animal Feed Science and Technology* **151**, 128–142.

Fuchs, R., Militz, H. & Hoffmann, M. (1987). Untersuchungen zur Verdaulichkeit der Rohnährstoffe bei Pferden. 1. Mitteilung. Methoden zur Bestimmung

der Verdaulichkeit. *Archives of Animal Nutrition* **37**, 235–246.

GfE (2014). *Empfehlungen zur Energie- und Nährstoffversorgung von Pferden*. Frankfurt, Germany: DLG-Verlag.

Giráldez, F. J., Lamb, C. S., López, S. & Mayes, R. W. (2004). Effects of carrier matrix and dosing frequency on digestive kinetics of even-chain alkanes and implications on herbage intake and rate of passage studies. *Journal of the Science of Food and Agriculture* **84**, 1562–1570.

Goachet, A. G., Philippeau, C., Varloud, M. & Julliand, V. (2009). Adaptations to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. *Animal Feed Science and Technology* **152**, 141–151.

Golonka, M. (2009). Analysis of chosen behavioral forms of Konik Polski horses from the Popielno Reserve. *Annals of Warsaw University of Life Science – SGGW, Animal Science* **46**, 255–259.

Gudmundsson, O. & Thorhallsdottir, A. G. (1998). Evaluation of *n*-alkanes for intake and digestibility determination in horses. In *Proceedings of the 9th European Intake Workshop* (Eds M. J. Gibb & S. M. Rutter), pp. 49–52. Aberystwyth, UK: IGER.

Haenlein, G. F. W., Smith, R. C. & Yoon, Y. M. (1966). Determination of the fecal excretion rate of horses with chromic oxide. *Journal of Animal Science* **25**, 1091–1095.

Hyslop, J. J. (2003). Partitioning degradation of feeds between different segments of the equine digestive tract. In *Emerging Equine Science* (Ed. J. Alliston), pp. 97–111. Occasional Publication of the British Society of Animal Science No. 32. Nottingham: Nottingham University Press.

Jensen, R. B., Brøkner, C., Bach Knudsen, K. E. & Tauson, A. H. (2010). Comparative study of the apparent total tract digestibility of fibre in Icelandic and Danish Warmblood horses. In *The Impact of Nutrition on the Health and Welfare of Horses* (Eds A. D. Ellis, A. C. Longland, M. Coenen & N. Miraglia), pp. 207–209. EAAP publication No. 128. Wageningen: Wageningen Academic Publishers.

Keli, A., Mayes, R. W. & de Vega, A. (2008). In vitro studies of the metabolism of [^{14}C]-*n*-alkanes using ruminal fluid of sheep as substrate. *Animal* **2**, 1748–1752.

Kienzle, E. & Zeyner, A. (2010). The development of a metabolizable energy system for horses. *Journal of Animal Physiology and Animal Nutrition* **94**, 231–240.

Koch, K. & Ensikat, H.-J. (2008). The hydrophobic coatings of plant surfaces: epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. *Micron* **39**, 759–772.

Kolattukudy, P. E. & Hankin, L. (1966). Metabolism of a plant wax paraffin (*n*-nonacosane) in the rat. *Journal of Nutrition* **90**, 167–174.

Licitra, G., Hernandez, T. M. & Van Soest, P. J. (1996). Standardization of procedures for nitrogen fractionation of ruminant feeds. *Animal Feed Science and Technology* **57**, 347–358.

Lin, L.-J., Luo, H.-L., Zhang, Y.-J. & Shu, B. (2007). The effects, in sheep, of dietary plant species and animal live weight on the faecal recovery rates of alkanes and the accuracy of intake and diet composition estimates obtained using alkanes as faecal markers. *Journal of Agricultural Science, Cambridge* **145**, 87–94.

Mayes, R. W., Lamb, C. S. & Colgrove, P. M. (1986). The use of dosed and herbage *n*-alkanes as markers for the determination of herbage intake. *Journal of Agricultural Science, Cambridge* **107**, 161–170.

Mayes, R. W., Lamb, C. S. & Colgrove, P. M. (1988). Digestion and metabolism of dosed even-chain and herbage odd-chain *n*-alkanes in sheep. In *Proceedings of the 12th General Meeting of the European Grassland Federation* (Ed. European Grassland Federation), pp. 159–163. Wicklow, Ireland: Wicklow Press.

Molina, D. O., Matamoros, I. & Pell, A. N. (2004). Accuracy of estimates of herbage intake of lactating cows using alkanes: comparison of two types of capsules. *Animal Feed Science and Technology* **114**, 241–260.

Oliván, M. & Osoro, K. (1999). Effect of temperature on alkane extraction from faeces and herbage. *Journal of Agricultural Science, Cambridge* **132**, 305–312.

Oliván, M., Ferreira, L. M. M., Celaya, R. & Osoro, K. (2007). Accuracy of the *n*-alkane technique for intake estimates in beef cattle using different sampling procedures and feeding levels. *Livestock Science* **106**, 28–40.

Ordakowski, A. L., Kronfeld, D. S., Holland, J. L., Hargreaves, B. J., Gay, L. S., Harris, P. A., Dove, H. & Sklan, D. (2001). Alkanes as internal markers to estimate digestibility of hay or hay plus concentrate diets in horses. *Journal of Animal Science* **79**, 1516–1522.

Pagan, J. D., Harris, P. A., Brewster-Barnes, T., Duren, S. E. & Jackson, S. G. (1998). Exercise affects digestibility and rate of passage of all-forage and mixed diets in thoroughbred horses. *Journal of Nutrition* **128**, 2704S–2707S.

Patterson, D. P., Cooper, S. R., Freeman, D. W. & Teeter, R. G. (2002). Technical note: estimation of fecal output and dry matter digestibility using various chromic oxide marker methods in the horse. *Professional Animal Scientist* **18**, 176–179.

Peiretti, P. G., Meineri, G., Miraglia, N., Mucciarelli, M. & Bergero, D. (2006). Intake and apparent digestibility of hay or hay plus concentrate diets determined in horses by the total collection of feces and *n*-alkanes as internal markers. *Livestock Science* **100**, 189–194.

Rosenfeld, I., Austbø, D. & Volden, H. (2006). Models for estimating digesta passage kinetics in the gastrointestinal tract of the horse. *Journal of Animal Science* **84**, 3321–3328.

Sales, J. (2012). A review on the use of indigestible dietary markers to determine total tract apparent digestibility of nutrients in horses. *Animal Feed Science and Technology* **174**, 119–130.

Smith, D. G., Mayes, R. W., Hollands, T., Cuddeford, D., Yule, H. H., Malo Ladrero, C. M. & Gillen, E. (2007). Validating the alkane pair technique to estimate dry matter intake in equids. *Journal of Agricultural Science, Cambridge* **145**, 273–281.

Stevens, D. M., van Ryssen, J. B. J. & Marais, J. P. (2002). The use of *n*-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. *South African Journal of Animal Science* **32**, 50–56.

Takagi, H., Hashimoto, Y., Yonemochi, C., Asai, Y., Yoshida, T., Ohta, Y., Ishibashi, T. & Watanabe, R. (2002). Usefulness of chromic oxide index method for determination of digestibility of nutrients in feeds for thoroughbreds. *Journal of Equine Science* **13**, 19–22.

Udén, P., Rounsaville, T. R., Wiggans, G. R. & Van Soest, P. J. (1982). The measurement of liquid and solid digesta retention in ruminants, equines and rabbits given timothy (*Phleum pratense*) hay. *British Journal of Nutrition* **48**, 329–339.

Van Weyenberg, S., Sales, J. & Janssens, G. P. J. (2006). Passage rate of digesta through the equine gastrointestinal tract: a review. *Livestock Science* **99**, 3–12.

VDLUFA (2012). *Methodenbuch, Vol. 3, Die Chemische Untersuchung von Futtermitteln*. Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten. Darmstadt: VDLUFA-Verlag.

Zeyner, A. & Kienzle, E. (2002). A method to estimate digestible energy in horse feed. *Journal of Nutrition* **132**, 1771S–1773S.

Zeyner, A., Kirchhof, S., Susenbeth, A., Südekum, K.-H. & Kienzle, E. (2015). A new protein evaluation system for horse feed from literature data. *Journal of Nutritional Science* **4**, e4. DOI: http://dx.doi.org/10.1017/jns.2014.66.

Zhang, Z., Li, G. & Shi, B. (2006). Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather Technologists and Chemists* **90**, 23–28.

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Bolus matrix for administration of dietary markers in horses

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ABSTRACT

External markers are useful or required to estimate forage intake, digestibility, faecal output and passage rate in horses. Oral administration is challenging because of horses' high sensitivity and selection skills. A suitable bolus matrix should ensure high and consistent acceptance. Boluses were investigated with and without labelling. Synthetic alkane wax, embedded in filter paper or contained in hypromellose capsules, was used as a test marker. Boluses were baked (100 °C, 30 min) or freeze-dried. The freeze-dried boluses varied in size (1.5, 2, or 3 cm Ø) and drying time (6, 12, 24, or 48 h). In two tests (AT1 and AT2), acceptance by the horses was assessed with scores between 1 (complete intake) and 4 (refusal). In AT1, marginal rejection of the marker was recorded, whereas the following tests were performed with placebos only. In two bending tests (BT1 and BT2), the force required to break the boluses (FL, flexural load) was determined because this may affect acceptance. Pre-selected variants were stored for 4 weeks in closed boxes under controlled conditions (20 °C and light for 16 h/d, 16 °C and night for 8 h/d, and 65% relative humidity) and were subsequently analysed for residual moisture (RM) and spoilage-indicating microbes. In baked boluses, the alkanes were partly found outside of the inner matrices. This was not evident in the freeze-dried variants. Acceptance of the labelled boluses (scores $\leq 1.7 \pm 0.18$, AT1), baked placebos (scores $\leq 2.2 \pm 0.35$, AT1) and the freeze-dried placebos (scores $\leq 1.1 \pm 0.31$, AT2) was consistently high. This was explained by the BT, with a mean FL of 202 ± 16.5 N for the baked (BT1) and up to 257 ± 22.5 N for the freeze-dried placebos (BT2) being obtained, which was close to the masticatory forces in horses. However, when the boluses distinctly exceeded a size of 3 cm Ø, this size probably led to increased FL (BT1), thereby depressing the acceptance of the dried placebos (AT1). The results indicated that the adaption to suitable boluses can lead to increased acceptance. Limiting the drying time to at most 24 h seemed justified, especially for the smaller boluses. After 6 and 12 h, the RM was $7.5 \pm 0.52\%$ (1.5 cm Ø) and $5.7 \pm 0.52\%$ (2 cm Ø), which make the risk of microbial spoilage appear low. Tested boluses were unspoiled for up to 1 month after preparation. It was suggested that the variable sizes of the boluses may enable the use of various marker dosages and, incidentally, also use in different target animals. A freeze-dried matrix is likewise open to use with other thermolabile markers or substances. We recommend the pre-administration of placebos prior to marker administration to ensure high acceptance of the labelled boluses.

1. Introduction

External markers such as chromium oxide (Takagi et al., 2002), lanthanides (Miyaji et al., 2014) or wax and synthetic alkanes (Elwert and Dove, 2006; Ferreira et al., 2007) can be used as indicators in nutrition studies with ruminant and monogastric livestock. If direct measurement is impossible, markers provide important information for the estimation of feed intake when coupled with plant-inert markers (Mayes et al., 1986), feed and nutrient digestibility (Takagi et al., 2002), faecal output (Giráldez et al., 2004) and digesta kinetics (Bulang et al., 2008). Markers that are incompletely bound to plant material have the disadvantage that they do not completely label the solid phase of digesta (Bulang et al., 2008). This can predominantly affect estimates of passage parameters. This problem can be overcome by a single administration of real plant markers such as alkanes or hydrochloric acid-insoluble ash. Then, intake and digestibility can be estimated

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simultaneously (Giráldez et al., 2004). However, plant marker concentrations are commonly too low for a single administration. So far, the application of boluses with unbound or mordanted markers is therefore still the best method of choice.

Marker administration in horses is challenging because of horses' particularly high sensitivity and selection skills. Markers offered via bread pieces (Kuntz et al., 2006), biscuits (Castelán-Ortega et al., 2007; Smith et al., 2007), pellets (Stevens et al., 2002) and capsules may easily be regurgitated, separated, or ingested incompletely, which is likewise reported for markers mixed into concentrated feed (Smith et al., 2007). Invasive methods, such as controlled release devices (Dove et al., 1991) and compulsory marker administration (Marais et al., 1996; Friend et al., 2004), are either not useable in horses, undesirable, or unfeasible with free-ranging animals.

We hypothesized that, for application in horses, a suitable bolus matrix ensures broadly consistent and high acceptance for ingestion and resists microbial spoilage over a sufficient period of time.

The size of the boluses, preparation and dry matter (DM) content may influence the required strain for mastication. This is surmised to be one main factor affecting acceptance in horses. This study was carried out stepwise to test acceptance of the different types of boluses, which varied in size (1.5, 2 and 3 cm diameter, Ø) and preparation (baking or freeze-drying). Both unlabelled boluses (placebo, P) and those labelled with wax of mixed synthetic alkanes as the test markers (verum, V) were investigated in horses in at least one of two acceptance tests (AT). In addition, unlabelled bolus types were subjected to bending tests (BT) to obtain an indication of the horses' required masticatory strain. The most suitable variants were subsequently stored under standardized conditions and tested with regard to resistance to microbial spoilage.

2. Materials and methods

2.1. Bolus preparation

The outer matrix shell of all types of boluses was made of oat flakes (fine grade), sugar beet syrup and wheat flour of food quality, mixed by weight at 1:0.8:0.6, and a small quantity of water. The analysed chemical composition is given in Table 1. Test markers were made of synthetic *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) alkanes (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The tertiary mixtures were melted at 80 °C, re-crystallized to wax at ambient temperature and subsequently chopped by hand. Alkane wax fragments were weighed either into smooth laboratory filter paper (LFP, 1 piece per bolus, grade 388, 7 cm Ø, Munktell & Filtrak GmbH, Bärenstein, Germany) or hypromellose (HPMC) capsules (1 piece per bolus, size 000, Silvaco A/S, Dah Feng, Taiwan) as the inner matrix shell using a dosage that was recommended for administration in horses 2 times a day (in total: 450 mg per bolus; 150 mg of each alkane per bolus; Smith et al., 2007). The parts and ingredients of the matrix did not contain measurable quantities of target alkanes. The boluses were prepared by baking (30 min at 100 °C) or freeze-drying for a defined duration (specified below). The following bolus types were formed: V1: V with filter paper, 3 cm Ø, baked; V2: V with filter paper, 3 cm Ø, freeze-dried (48 h); V3: V with HPMC capsule, 3 cm Ø, baked; V4: V with HPMC capsule, 3 cm Ø, freeze-dried (48 h); P1: P, 3 cm Ø, baked; and P2: P, 3 cm Ø, freeze-dried (48 h). In addition, the freeze-dried P boluses were graded by size (1.5, 2 and 3 cm Ø) and drying time (6, 12, 24 and 48 h), which led to 12 possible combinations. After pre-selection (explained below), the P boluses were graded as follows: P3: 1.5 cm Ø, freeze-dried (24 h); P4: 2 cm Ø, freeze-dried (24 h); and P5: 3 cm Ø, freeze-dried (24 h).

Table 1
Analysed chemical composition of the outer matrix shell of boluses.

	P1	P2
Dry matter (DM) [g/kg]	882	937
Crude ash [g/kg DM]	13	12
Crude protein [g/kg DM]	100	98
Acid ether extract [g/kg DM]	30	37
Acid detergent fibre [g/kg DM]	35	39
Starch [g/kg DM]	433	427
Sugar [g/kg DM]	248	233
Gross energy [MJ/kg DM]	18.5	18.1

P1-bulk sample: 5 × 20%-aliquots, P2-bulk sample: 5 × 20%-aliquots.
P1, placebo, baked (30 min, 100 °C), 3 cm Ø; P2, placebo, freeze-dried (48 h), 3 cm Ø.

2.2. Chemical analyses

The freeze-dried boluses were pre-chopped by hand. Before analysis, the baked boluses were freeze-dried (48 h) to achieve a pulverisable condition. The inner shell of the matrix in the V-type boluses, which included an alkane dosage, was separated manually from the outer one. The outer shell of the matrix was pre-milled with water-cooling, separated into parts and milled under liquid nitrogen at a constant −196 °C using a Retsch® CryoMill (Retsch® GmbH, Haan, Germany; settings: 25 ml cup, 14 mm steel ball, and 5 min per iteration). This allowed sufficient homogenization of the remaining alkanes and the matrix but avoided the high processing temperature, which is known to affect alkane recovery in sample material. Dried P samples and matrix pastry ingredients were ground to pass through a 0.5 mm sieve in a standard laboratory sample mill.

Dry matter determination and crude nutrient analyses of the outer matrix shell were performed according to official methods (VDLUFA, 2012, method no. 3.1: DM, 4.1.1: crude protein, 5.1.1 B: acid ether extract, 6.5.2: acid detergent fibre, 7.1.2: sugar and 8.1: crude ash) using a FOSS 2300 Kjeltec™ Analyser Unit for nitrogen determination and a FOSS Tecator™ Soxtec™ 1047 Hydrolysing Unit and a Soxtec™ HT 1043 Extraction Unit for acid ether extract analysis (FOSS GmbH, Rellingen, Germany). The gross energy was ascertained by bomb calorimetry using a C7000 Oxygen Bomb Calorimeter (IKA® Werke, Staufen, Germany). Starch was determined enzymatically referring to the amyloglucosidase method (VDLUFA, 2012, method no. 7.2.5).

Alkane extraction from the matrices of the V samples and gas chromatographic analysis were performed according to Elwert et al. (2004). A Shimadzu GC-2010 FID unit (Shimadzu Corporation, Kyoto, Japan) with an on-column injection and an Rtx®-1 w/ Integra-Guard™-column (Restek Corporation, Bellefonte, PA, USA) were used.

2.3. Testing procedures

The tests were carried out in the order AT1, BT1, BT2, AT2 and storage test (ST). In the AT1, the P (P1 and P2) and V-type boluses (V1, V2 and V4) were tested simultaneously. The V3-type boluses had already been excluded during the preparation process because baking led to visible losses of the marker alkanes at the bolus surface. Rejection of the marker dosage itself has rarely been recorded in AT1. Therefore, acceptance was assumed to be mainly influenced by properties of the bolus matrix. Consequently, only P variants were subjected to the following tests. Bending test 1 was performed to obtain an explanation for the poor acceptance of P2 compared with P1 in AT1. Upon the basis of the results of AT1 and

BT1, the P1 was assessed to be suitable for application but limited to thermostable markers only. Thus, for further examination, the freeze-dried P type was graded by the size and drying time (specified above). Using these variants, BT2 was performed to estimate the horses' masticatory force need to crush the bolus in question. In addition, the residual moisture (RM) content of the various freeze-dried P types was determined. After analysis of alterations of the flexural load (FL, explained below) and the RM content in relation to the size gradation and drying time of the boluses, the variants P3, P4 and P5 were pre-selected and then investigated for acceptance (AT2) and storage stability (ST).

2.3.1. Acceptance tests

All horses that were used in this study were kept and cared for in accordance with the Federation of Animal Science Societies' Animal Care Guidelines (FASS, 2010).

Acceptance test 1 was carried out in July and August 2014 using 26 Warmblood horses, one Rhenish German Coldblood and one Haflinger (12 stallions and 16 geldings), between 4 and 18 years of age, with a mean bodyweight (BW) of 588 ± 69.6 kg. The horses were kept by the Brandenburg State Stud, Neustadt (Dosse), Germany. The horses were housed in single boxes with daily access to a sand paddock and/or controlled exercise. They received individually composed hay-based and mineral-supplemented diets according to their performance levels and had free access to tap water. Six mares (4 Trotter, 1 Trakehner and 1 Mecklenburger) with a mean BW of 532 ± 40.1 kg and mean body condition score (BCS) of 5.3 ± 0.36 (in a range from 1 to 9 according to Kienzle and Schramme, 2004) were involved in AT2, which was performed in June 2015. The mares' ages ranged from 7 to 14 years. The mares were kept by the Research Centre for Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, in Merbitz, Wettin/Löbejün (Saxony-Anhalt, Germany). During the investigation period, they were maintained exclusively on pasture with free access to tap water and did not receive any additional feed. All horses were under regular veterinary supervision. Abnormalities with respect to dental health have not been detected.

Acceptance test 1 was divided into (a) and (b) sub-tests to eliminate a possible effect of pre-administration of the V boluses. Using a cross-over design, the horses were randomly allocated to six groups, with three individuals each in AT1 (a) and two groups with five individuals each in AT1 (b). In AT2, the mares were allocated randomly to the six different sequences of administration periods. The experimental designs of these tests are given in Table 2. Each administration period consisted of 5 days. In AT2, the administration of P1 was used as the reference and shortened to 3 days each before and after the trial. To determine acceptance after repeated administration, the type of bolus offered did not change within but changed between the periods (Table 2). No breaks occurred between the individual administration periods. Every day, one bolus was offered to each horse by hand, and the acceptance was assessed by the examiner using the scores (AS): (1) for the complete intake of the bolus without visible crumb losses of the outer and/or inner matrix shell or marker wax, (2) for the occurrence of visible crumb losses of the outer matrix shell, (3) for the occurrence of visible losses of the inner matrix shell or marker wax and (4) for the refusal of the bolus in AT1, and analogously, (1) for the complete intake of the bolus, (2) for the occurrence of crumb losses and (3) for the refusal of bolus in AT2. The time of bolus administration varied from day to day. The boluses were fed individually, independent from mealtimes and the administration of other feedstuffs.

2.3.2. Bending tests

The various bolus matrices were subjected to BT to determine the force specifically required for breaking (FL). Placebo variants P1 ($n=20$) and P2 ($n=9$) were tested in BT1, and all freeze-dried variants (12 combinations) were tested in BT2 ($n=5$ per variant), respectively. The samples were loaded up to breakage in a three-point bending configuration (short-beam test) using a ZWICKI (2.5 kN) material testing machine (Zwick GmbH & Co. KG, Ulm, Germany). Settings were equal for both tests: 1 N preload, 500 mm/min testing velocity, and 10 mm supporting width. Load and deflection were recorded simultaneously so that stiffness and strength could be evaluated.

Table 2
Schedule of bolus acceptance tests.

Test	Horses	Sequence of bolus administration
AT1 (a)	Group 1 (3 horses)	V1 – V2 – V4 – P1 – P2
	Group 2 (3 horses)	V1 – V4 – V2 – P1 – P2
	Group 3 (3 horses)	V2 – V1 – V4 – P2 – P1
	Group 4 (3 horses)	V2 – V4 – V1 – P2 – P1
	Group 5 (3 horses)	V4 – V1 – V2 – P2 – P1
	Group 6 (3 horses)	V4 – V2 – V1 – P1 – P2
AT1 (b)	Group 1 (5 horses)	P1 – P2
	Group 2 (5 horses)	P2 – P1
AT2	Horse 1	P1 – P3 – P4 – P5 – P1
	Horse 2	P1 – P3 – P5 – P4 – P1
	Horse 3	P1 – P4 – P3 – P5 – P1
	Horse 4	P1 – P4 – P5 – P3 – P1
	Horse 5	P1 – P5 – P3 – P4 – P1
	Horse 6	P1 – P5 – P4 – P3 – P1

Boluses were offered once a day to individual horses with repetition for 5 consecutive days in AT1 (a), AT1 (b), and for P3, P4 and P5 in AT2, and for 3 consecutive days for P1 in AT2 (administration periods).
AT, acceptance test; P1, placebo, baked (30 min, 100 °C), 3 cm Ø; P2, placebo, freeze-dried (48 h), 3 cm Ø; P3, placebo, freeze-dried (24 h), 1.5 cm Ø; P4, placebo, freeze-dried (24 h), 2 cm Ø; P5, placebo, freeze-dried (24 h), 3 cm Ø; V1, verum with filter paper, baked (30 min, 100 °C), 3 cm Ø; V2, verum with filter paper, freeze-dried (48 h), 3 cm Ø; V4, verum with HPMC capsule, freeze-dried (48 h), 3 cm Ø.

2.3.3. Storage test

Samples ($n=10$) of each of the pre-selected freeze-dried boluses (P3, P4 and P5) were stored for 4 weeks in a Percival climatic chamber with an Intellus control system (type: AR-95HIL.X, CLF Plant Climatics GmbH, Wertingen, Germany). Afterwards, subsamples ($n=3$) of each variant were taken randomly, and their storage stabilities were assessed microbiologically via culture methods according to VDLUFA (2012, method no. 28.1.1 to 28.1.4). The climatic settings were as follows: alternation of day and night with 16 and 8 h, respectively; temperatures of 20 °C (day) and 16 °C (night); light intensity of ~630 µmol/m^2/s; and relative humidity of 65%. The climatic settings aimed to simulate an approximate regional yearly average. During storage, each sample was placed in a closed transparent plastic box.

2.4. Statistical analysis

Statistical analysis was performed with the SAS 9.4 software package (SAS Institute Inc., Cary, NC, USA).

The deviations of FL measured during BT1 were assessed with an unpaired *t*-test using the Satterthwaite algorithm for inequality of the sample variances.

The RM content of the variously prepared matrices and the FL obtained from BT2 were analysed using the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \underline{e}_{ijk},$$

in which, y_{ijk} is the RM of the tested variants or the FL required for breaking these; μ is the general mean; α_i is the fixed effect of the bolus size i ($i=1, 2, 3$, where 1 = 1.5 cm Ø, 2 = 2 cm Ø, 3 = 3 cm Ø); β_j is the fixed effect of the drying time j ($j=1, \ldots, 4$, where 1 = 6 h, 2 = 12 h, 3 = 24 h, 4 = 48 h); $\alpha\beta_{ij}$ is the interaction between α_i and

β_j; and $\underline{e}_{ijk}$ is the random residual effect.

Records of acceptance were analysed according to Jones and Kenward (2003) using the following model:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\gamma_{ik} + \beta\gamma_{jk} + \underline{a}_l + \underline{e}_{ijkl},$$

in which, y_{ijkl} is the AS (1, …, 4 in AT1 and 1, …, 3 in AT2, where 1=the bolus ingested completely, 2=the bolus ingested with crumb losses, 3=the bolus ingested with marker losses and 4=the bolus denied in AT1, and 1=the bolus ingested completely, 2=the bolus ingested with crumb losses and 3=the bolus denied in AT2, defined in more detail in Section 2.3.1.); μ is the general mean; α_i is the fixed effect of administration period i (i=1, …, 5 in AT1 (a) and AT2, and i=1, 2 in AT1 (b)); β_j is the fixed effect of bolus variant j (j=1, …, 5 in AT1, where 1=P1, 2=P2, 3=V1, 4=V2 and 5=V4, and j=1, …, 4 in AT2, where 1=P1, 2=P3, 3=P4 and 4=P5); γ_k is the fixed effect of day k (k=1, …, 5 per administration period, except P1 in AT2, where k=1, 2, 3); $\alpha\gamma_{ik}$ is the interaction between α_i and γ_k; $\beta\gamma_{jk}$ is the interaction between β_j and γ_k; $\underline{a}_l$ is the random effect of animal l (l=1, …, 18 in AT1 (a), 1, …, 10 in AT1 (b) and 1, …, 6 in AT2), with consideration to repeated records per animal within an administration period; and $\underline{e}_{ijkl}$ is the random residual effect.

Using the two linear models explained above and the MIXED procedure, the least squares means (LSM) were estimated for each of the respective bolus types and compared with regard to the parameters FL, RM and AS. The level of significance was $P<0.05$. The values given for alkane quantities, BW, BCS and FL (BT1) are means ± standard deviation; those for FL (BT2), RM and AS are LSM ± standard error.

3. Results and discussion

3.1. Bolus preparation

The LFP used as the inner shell of the bolus matrix is usually not intended for consumption. Although it has been used in very small quantities, an application of HPMC capsules is thought to be safer (Burdock, 2007), which probably extends to its application in horses. Hypromellose capsules are commonly used for carrying drugs or additives for human consumption in the food industry (Al-Tabakha, 2010).

The outer shell of the matrix was expected to be rapidly dispersed in the stomach, whereas the inner filter paper may partly prolong marker exposure. However, this was neither investigated in the current, or to our knowledge, previous experiments. The HPMC capsules are known to be readily soluble when in contact with human gastrointestinal fluid (Chiwele et al., 2000). We surmised that this is similar in the equine gastrointestinal tract, which would lead to rapid marker release. *In vitro* shell dissolution of the empty HPMC capsules (size 0) with exposure to human artificial gastric juice under a temperature similar to that measured in the gastrointestinal tract of horses (38 °C, Green et al., 2005) was reported to be ~250 s (Chiwele et al., 2000).

Mean quantities of 23.9 ± 11.9 mg C28, 19.9 ± 10.9 mg C32 and 21.0 ± 13.4 mg C36 were found in the outer shell of V1; 0.4 ± 0.4 mg C28, 0.4 ± 0.5 mg C32 and 0.5 ± 0.5 mg C36 in that of V2; and 0.1 ± 0.09 mg C28, 0.2 ± 0.2 mg C32 and 0.3 ± 0.1 mg C36 in that of V4-type boluses. These results suggest that the alkanes had melted during baking, whereupon some alkanes soaked into the outer matrix shell. Neither the LFP nor HPMC capsules were able to delay molten alkanes completely. In practical application, crumb losses are not always avoidable, so marker losses would occur in V1-type boluses. The crossing of markers was very low in freeze-dried variants V2 and V4, which suggests that these types are more suitable for the administration of thermolabile markers such as alkanes.

Table 3
Acceptance of boluses in horses.

	AT1 (a)		AT1 (b)	AT2
Acceptance score				
V1	1.5 ± 0.18			
V2	1.7 ± 0.18			
V4	1.6 ± 0.18			
P1	1.2 ± 0.15		2.2 ± 0.35	1.0 ± 0.00
P2	2.0 ± 0.15		3.6 ± 0.35	
P3				1.1 ± 0.31
P4				1.1 ± 0.26
P5				1.0 ± 0.00
Effects (*P*, *F*-test)	V	P	P	P
Variant	0.153	< 0.0001	< 0.01	0.131
Period	< 0.05	0.503	0.885	0.246
Day	< 0.05	< 0.001	0.337	0.642
Variant × day	0.769	< 0.0001	0.121	0.428
Period × day	< 0.05	< 0.0001	0.087	0.612

Acceptance scores: AT1: 1, bolus ingested completely; 2, bolus ingested with crumb losses; 3, bolus ingested with marker losses; 4, bolus denied. AT2: 1, bolus ingested completely; 2, bolus ingested with crumb losses; 3, bolus denied (AT2).
AT, acceptance test; P, placebo; P1, P, baked (30 min, 100 °C), 3 cm Ø; P2, P, freeze-dried (48 h), 3 cm Ø; P3, P, freeze-dried (24 h), 1.5 cm Ø; P4, P, freeze-dried (24 h), 2 cm Ø; P5, P, freeze-dried (24 h), 3 cm Ø; V, verum; V1, V with filter paper, baked (30 min, 100 °C), 3 cm Ø; V2, V with filter paper, freeze-dried (48 h), 3 cm Ø; V4, V with HPMC capsule, freeze-dried (48 h), 3 cm Ø.

3.2. Acceptance tests

During the AT, all horses were healthy, and none showed behavioural syndromes. The AS are summarized in Table 3.

Acceptance test 1: In both parts of AT1, the acceptance of P1 was significantly better than that of P2 ($P<0.01$). In AT1 (a), the acceptance of P1 and P2 was similar in periods 4 and 5. This acceptance remained unaltered or became slightly better with progression through the periods. The acceptance was unaltered (P1) or increased across the days (P2). In AT1 (b), the acceptance of P1 and P2 was unaffected by previous administration periods and was worse than in (a). This acceptance remained unaltered or slightly decreased within the administration periods. The acceptance was unaltered across the days for P1 but tended to decrease for P2 due to negative experience. The results probably indicated an adaption effect in horses depending on previous experience. Similar results had been reported by LaCasha et al. (1999) in Quarter Horse yearlings, where a preference for or aversion to a previously fed hay type affected subsequent selection. In AT1 (a), no significant differences were noticed between the AS of V1, V2 and V4. The marker dosage was rejected 3 times in V1- and 4 times in V4-type boluses, which was in 1.3% of all total applications. The acceptance of those boluses clearly increased in period 1 and towards period 2 ($P<0.01$) and slightly declined towards period 3, which again indicated an adaption effect. In periods 2 and 3, the acceptance remained unaltered. Across consecutive days, acceptance tended to increase. Before marker administration, horses should be adapted with P boluses for at least 3 days to avoid bolus rejection. This may be especially important in passage rate studies using a single application of marker.

Acceptance test 2: In general, the acceptance was high during AT2 and did not differ between the tested P variants. Only one of the horses ingested the boluses incompletely, regardless of the type; none of the horses completely rejected the boluses.

Conclusively, the mastication of V types was expected to be

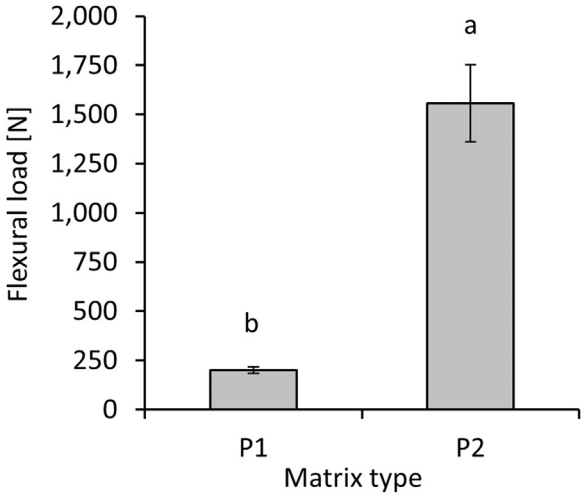

Fig. 1. Bending test 1: Means of the flexural load that is required to break the baked (P1, 3 cm Ø, $n=20$) and freeze-dried placebo type (P2, 3 cm Ø, 48 h drying, $n=9$): different superscripts indicate a significant difference, ^{ab} $P<0.0001$.

easier than of P types because the shell thickness was lower. However, this has not been verified by BT.

3.3. Bending tests

Bending test 1: This test revealed that the mean FL required for breaking P1 was 8 times less than for P2 ($P<0.0001$, Fig. 1). The measured FL was similar to the masticatory forces measured (Staszyk et al., 2006) or calculated (Huthmann et al., 2009) in horses. According to this, P1 must be chewed during the closing stroke with 248 to 554 N on average (Huthmann et al., 2009). In contrast, the P2 boluses caused a considerable drag when closing the jaw and were at most chewed during the power stroke with 875 to 1956 N on average (Huthmann et al., 2009). The P2 boluses partly exceeded the target size (3 cm Ø). Additionally, the P2 boluses were approximately 9% higher in the DM than the P1 boluses (P1: 854 ± 10.9 g/kg, P2: 943 ± 8.42 g/kg). This probably led to increased masticatory strain and might be a reason for the lower acceptance of P2 in AT1.

Bending test 2: Fig. 2 shows the alterations of the FL that were required for breaking bolus matrices depending on their size and their drying time. No significant differences were found between the drying times in either the boluses with 1.5 cm Ø or those with 2 cm Ø. For boluses with a size of 3 cm Ø, the required FL increased from 6 to 24 h ($P<0.05$) but was similar between 12 and 48 h drying time. No significant differences were observed within 6 h of drying, but some differences were observed in the subsequent drying steps ($P<0.05$). Generally, a gradation appeared depending on the size of boluses. The required FL of all types tested in BT2 fell within or beneath the magnitude of masticatory force given for the closing stroke by Huthmann et al. (2009). Conclusively, BT2 gave no additional indication of an optimal choice of the drying time specifically required for the freeze-dried boluses.

The alteration of RM as a function of drying time and the specific sizes of the boluses are shown in Fig. 3. Boluses with 1.5 cm Ø reached a consistent weight after 6 h of drying, so there were no significant differences between the drying steps. The RM of the boluses with 2 cm Ø decreased significantly ($P<0.0001$) from 6 to 12 h of drying and then remained constant. The RM of boluses with 3 cm Ø was not constant until 24 h of drying. Therefore, significant differences existed between 6, 12 and 24 h drying time ($P<0.0001$). In most cases, the content of the RM of the variously sized boluses differed significantly within the drying steps ($P<0.05$, Fig. 3) with the exception of those with 1.5 and 2 cm Ø after 24 h of drying. The boluses with 3 cm Ø lost most of their moisture content between 12 and 24 h of drying. To reduce time and energy costs, the drying time may be limited to a maximum of 24 h. With this drying time, the selected samples had a mean RM content of $7.0 \pm 0.52\%$ (P3), $6.2 \pm 0.52\%$ (P4) and $3.2 \pm 0.52\%$ (P5), which was generally less than that required to promote spoilage (Weißbach, 1993). The drying time of boluses with 2 (P4) and 1.5 cm Ø (P3) may even be reduced up to 12 and 6 h, respectively. At these drying durations, the RM was $5.7 \pm 0.52\%$ (P4) and $7.5 \pm 0.52\%$ (P3), which is also suggested to be safe.

3.4. Storage test

After the selected P variants P3, P4 and P5 had been stored under defined conditions (see Section 2.3.3.), the aerobic mesophilic bacteria, mould and sooty-mould fungi, and yeast contents were determined. Indicator organisms are defined in detail by VDLUFA (2012, method no. 28.1.3). Yeasts, mould and sooty-mould fungi and spoilage-indicating bacteria were not detectable. So called Yellow germs of the genera *Pantoea*, *Enterobacter*, *Stenotrophomonas* and others were ascertained with 0.01×10^6 colony

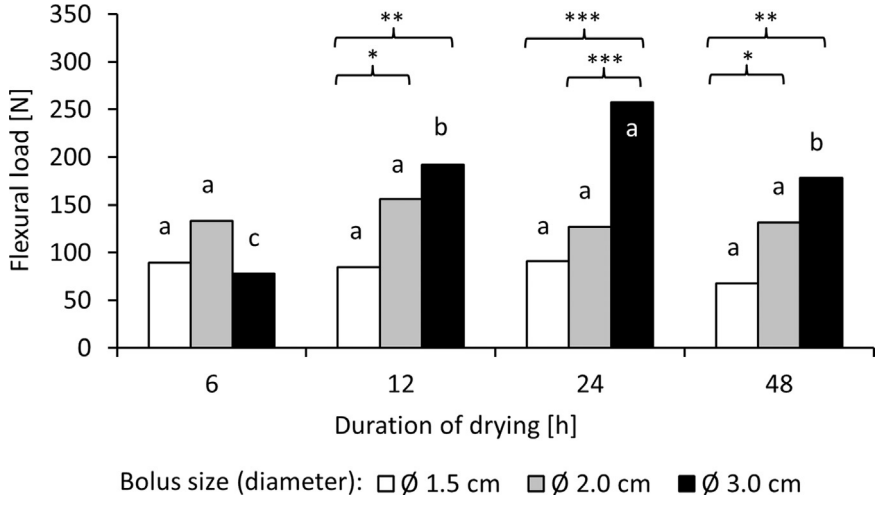

Fig. 2. Bending test 2: Least squares means of the flexural load that is required to break the freeze-dried placebo type depending on the size and drying time ($n=5$ per combination): different superscripts indicate significant difference within size classes, asterisks within the steps of drying time, ^{ab} $P<0.05$, ^{ac} $P<0.0001$, ^{bc} $P<0.001$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

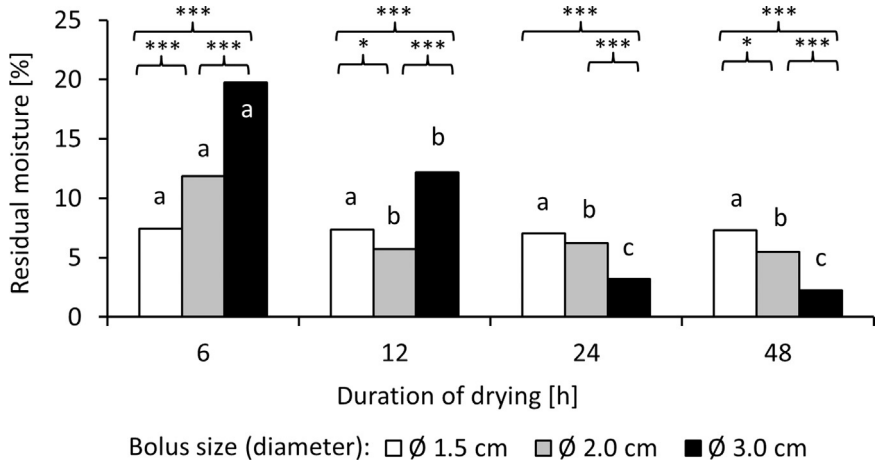

Fig. 3. Least squares means of the residual moisture of the freeze-dried placebo type after preparation depending on the size and drying time ($n=5$ per combination): different superscripts indicate significant difference within size classes, asterisks within the steps of drying time, $^{ab,\ bc}$ $P<0.0001$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

forming units per g, a level below the accepted value that is given for mixed feedstuffs in horses (0.5×10^6 colony forming units per g, VDLUFA, 2012, method no. 28.1.4). Yellow germs are typically found in feedstuffs and do not indicate spoilage. Conclusively, the tested matrices were microbially unspoiled and could be used safely for at least up to 1 month after preparation, provided the boluses were stored in a closed box to prevent absorption of humidity. The microbiological quality with regard to the freshness of the boluses is probably influenced by the hygienic quality and the DM content of the raw materials, which should therefore be used only in guaranteed unspoiled and hygienically perfect condition.

4. Conclusions

In this study, a bolus matrix that is suitable for the administration of dietary markers in horses has been introduced and was tested in multiple variants. Synthetic alkane waxes were used as test markers. Tests were performed in the order AT1, BT1, BT2, AT2 and ST.

Baking of the boluses (30 min at 100 °C) led to consistently high acceptance by horses with LSM of AS less than 2.2 ± 0.35 (in a range from 1 to 4, AT1). However, baking seemed to be unsuitable for the use of thermolabile marker types, such as alkanes, because distinct quantities of alkanes entered the outer matrix shell during baking. The acceptance of freeze-dried matrices was high with labelling (AT1), and after taking into consideration the size (1.5, 2 and 3 cm Ø) and the drying time (6, 12, 24 and 48 h), also the acceptance of the unlabelled boluses was high (AT2). At this, the LSM of AS were less than 1.7 ± 0.18. However, acceptance seemed to be depressed in the freeze-dried boluses exceeding 3 cm Ø in size, which became evident in AT1 and BT1, where the LSM of AS were up to 3.6 ± 0.36 and FL was 8 times higher than that of baked boluses. The markers showed almost no rejection by horses in AT1, which is the reason further tests were performed with P only. With defined sizes and drying time, the FL of the boluses was similar to the usual masticatory force reported in horses (67 to 257 N, BT2). Boluses with 1.5, 2 and 3 cm Ø may enable the use of various marker dosages and thus also to be used in different target animals. However, this has not been investigated in this study. To reduce the preparation time and energy costs, the drying time may be limited to 24 h or less, depending on the size of the boluses. Boluses that had been freeze-dried for 24 h were tested for storage stability, and nonspoilage from microbes for up to 1 month after preparation was confirmed, provided that they were stored in closed boxes (ST). For further application, freeze-drying of the bolus matrix is preferable to baking and will offer the possibility to use every type of marker or other substances, including thermolabile ones or ones with thermolabile coatings. An adaption effect was observed, which led to increased or depressed acceptance depending on the horses' experience. We therefore recommend the pre-administration of P boluses for at least 3 days prior to marker administration to ensure a high acceptance of the labelled boluses.

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References

Al-Tabakha, M.M., 2010. HPMC capsules: current Status and future prospects. J. Pharm. Pharm. Sci. 13, 428–442.
Bulang, M., Elwert, C., Spilke, J., Rodehutscord, M., 2008. Suitability of synthetic alkanes as markers for the estimation of passage rate in sheep. Livest. Sci. 115, 42–52.
Burdock, G.A., 2007. Safety assessment of hydroxypropyl methylcellulose as a food ingredient. Food Chem. Toxicol. 45, 2341–2351.
Castelán-Ortega, O.A., Estrada-Flores, J.G., Smith, D.G., Colunga, B., Solís-Méndez, A., Avilés-Nova, F., Ramirez, A., García, I., 2007. Use of n-alkanes for estimation of voluntary intake and digestibility in donkeys (Equus asinus). J. Anim. Feed. Sci. 16 (Suppl. 2), 307–312.
Chiwele, I., Jones, B.E., Podczeck, F., 2000. The shell dissolution of various empty hard capsules. Chem. Pharm. Bull. 48, 951–956.
Dove, H., Mayes, R.W., Lamb, C.S., Ellis, K.J., 1991. Evaluation of an intra-ruminal controlled-release device for estimating herbage intake using synthetic and plant cuticular wax alkanes. In: Proceedings of the 3rd International Symposium on the Nutrition of Herbivores, p. 82.
Elwert, C., Dove, H., 2006. Estimation of roughage intake in sheep using a known daily intake of a labelled supplement. Anim. Sci. 81, 47–56.
Elwert, C., Kluth, H., Rodehutscord, M., 2004. Effect of variable intake of alfalfa and wheat on faecal alkane recoveries and estimates of roughage intake in sheep. J. Agric. Sci. 142, 213–223.
FASS, 2010. Guide for the Care and Use of Agricultural Animals in Research and Teaching. Federation of Animal Science Societies, Champaign, IL, USA, ⟨http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf⟩.
Ferreira, L.M.M., García, U., Rodrigues, M.A.M., Celaya, R., Dias-da-Silva, A., Osoro, K., 2007. Estimation of feed intake and apparent digestibility of equines and cattle grazing on heathland vegetation communities using the *n*-alkane markers.

Livest. Sci. 110, 46–56.
Friend, M.A., Avery, A., Nash, D., 2004. Intake of improved and unimproved pastures in two seasons by grazing weanling horses. Anim. Prod. Aust. 1, 61–64.
Giráldez, F.J., Lamb, C.S., López, S., Mayes, R.W., 2004. Effects of carrier matrix and dosing frequency on digestive kinetics of even-chain alkanes and implications on herbage intake and rate of passage studies. J. Sci. Food Agric. 84, 1562–1570.
Green, A.R., Gates, R.S., Lawrence, L.M., 2005. Measurement of horse core body temperature. J. Therm. Biol. 30, 370–377.
Huthmann, S., Staszyk, C., Jacob, H.-G., Rohn, K., Gasse, H., 2009. Biomechanical evaluation of the equine masticatory action: Calculation of the masticatory forces occurring on the cheek tooth battery. J. Biomech. 42, 67–70.
Jones, B., Kenward, M.G., 2003. Design and Analysis of Cross-Over Trials, second ed. Chapman & Hall/CRC, Boca Raton, FL, USA.
Kienzle, E., Schramme, S., 2004. Beurteilung des Ernährungszustandes mittels Body Condition Scores und Gewichtsschätzung beim adulten Warmblutpferd. Pferdeheilkunde 20, 517–524.
Kuntz, R., Kubalek, C., Ruf, T., Tataruch, F., Arnold, W., 2006. Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*). J. Exp. Biol. 209, 4557–4565.
LaCasha, P.A., Brady, H.A., Allen, V.G., Richardson, C.R., Pond, K.R., 1999. Voluntary Intake, Digestibility, and Subsequent Selection of Matua Bromegrass, Coastal Bermudagrass, and Alfalfa Hays by Yearling Horses. J. Anim. Sci. 77, 2766–2773.
Marais, J.P., Figenschou, D.L., Escott-Watson, P.L., Webber, L.N., 1996. Administration in suspension-form of n-alkane external markers for dry matter intake and diet selection studies. J. Agric. Sci. 126, 207–210.
Mayes, R.W., Lamb, C.S., Colgrove, P.M., 1986. The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. J. Agric. Sci. 107, 161–170.
Miyaji, M., Ueda, K., Hata, H., Kondo, S., 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. J. Anim. Sci. 92, 1574–1581.
Smith, D.G., Mayes, R.W., Hollands, T., Cuddeford, D., Yule, H.H., Malo Ladrero, C.M., Gillen, E., 2007. Validating the alkane pair technique to estimate dry matter intake in equids. J. Agric. Sci. 145, 273–281.
Staszyk, C., Lehmann, F., Bienert, A., Ludwig, K., Gasse, H., 2006. Measurement of masticatory forces in the horse. Pferdeheilkunde 22, 12–16.
Stevens, D.M., van Ryssen, J.B.J., Marais, J.P., 2002. The use of n-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. S. Afr. J. Anim. Sci. 32, 50–56.
Takagi, H., Hashimoto, Y., Yonemochi, C., Asai, Y., Yoshida, T., Ohta, Y., Ishibashi, T., Watanabe, R., 2002. Usefulness of chromic oxide index method for determination of digestibility of nutrients in feeds for thoroughbreds. J. Equine Sci. 13, 19–22.
VDLUFA, 2012. Methodenbuch, Vol. 3, Die chemische Untersuchung von Futtermitteln. Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA-Verlag, Darmstadt, Germany.
Weißbach, F., 1993. Grünfutter und Grünfutterkonservate. In: Jeroch, H., Flachowsky, F., Weißbach, F. (Eds.), Futtermittelkunde. Gustav Fischer Verlag, Jena, pp. 74–154.

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Preparation of synthetic alkane waxes and investigations on their suitability for application as dietary markers in farm animals

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ABSTRACT

Synthetic alkanes can be applied as external markers to estimate faecal output, digestibility and passage kinetics and are furthermore easy to combine with plant alkanes for the estimation of feed intake. Successful application requires an accurate and uniform labelling of boluses or feedstuffs, which is in turn supported through simplified handling of the markers during preparation. In this study, it was tested whether melting of synthetic alkanes to wax is able to enhance the accuracy and uniformity of subsequent bolus labelling and further simplifies it. The preparation of alkane waxes was performed on model scale using a portion in a ratio of approximately 1:300 to a dosage, which is recommended for administration in large livestock 2 times a day. Additionally, the temperature sensitivity of a range of synthetic alkanes was studied to clarify so far non-explained losses of alkanes, which were observed frequently during the labelling of boluses and feedstuffs or the processing of samples for analysis. Using *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) synthetic alkanes, three single-component waxes (of C28, C32 and C36, respectively), three binary waxes (C28:C32, C28:C36 and C32:C36) and one tertiary mixed wax (C28:C32:C36) were produced with 30 repetitions each. To assess the impact of melting and re-crystallization, the quantity of individual alkanes was determined by gas chromatographic analysis (GCA) in untreated crystals, crystalline mixtures (GCA1) and the finished model waxes (GCA2). Additionally, sub-samples of the waxes were heated for 30 min at 100 °C or freeze-dried for 48 h, respectively, to simulate baking of boluses or freeze-drying as an alternative method for preparation (GCA3). The temperature sensitivity of *n*-tetracosane (C24) to *n*-octatriacontane (C38) even-chain alkanes was studied by thermogravimetric analysis (TGA) with consistently increasing temperature (20 to maximal 600 °C at 10 K/min, TGA1) and under isothermal conditions (180 °C for 20 min, TGA2), respectively. Depending on chain length and thus molecular weight of alkanes, weight reduction by emergence of soot during heating-up started between 176 °C (C24) and 227 °C (C38) and further increased rapidly. Throughout isothermal treatment, weight loss from alkanes was lowest with highest chain length (0.0% for C38) and *vice versa* (23.8% for C24). The originally weighed and via GCA measured quantities of crystalline alkanes did not differ ($P > 0.05$), except for single C36, where the measured quantities were always higher than the weighed ones ($P = 0.019$). The weighed and measured quantities of individual alkanes in single-component and of total alkanes in multi-component waxes were similar with a maximal relative difference of $6.6 \pm 5.5\%$, given as the mean $\pm$ standard deviation among the repetitions of a sample variant. The relative difference between weighed and measured quantities of individual alkanes in multi-component waxes was maximal $47.4 \pm 25.7\%$ and was highly variable. Unexpectedly, the relative difference between weighed and measured quantities was low for C28 ($5.9 \pm 5.8\%$) and C32 ($5.7 \pm 4.3\%$) in their combined binary waxes. The additional treatment (baking or freeze-drying) did not alter the recovery of alkanes from the model waxes. Synthetic alkanes are thermolabile why exposure to high temperature during preparation of boluses or labelling of feedstuffs needs to be assessed critically. Reasons for that might be complex disorders of the conformation of alkane molecules particularly during the melting of alkane mixtures and the apparently incomplete separation following re-crystallization from the melt. This may lead to displacements within waxes, which cannot

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be foreseen or quantified, and thus to the loss of their suitability as dietary markers. Alkane recovery from binary waxes of C28 and C32 was unbiased on model scale and whether this can be confirmed on original scale needs to be validated further. For practical use, alkane waxes might nevertheless be beneficial because the handling is easier than that of alkane crystals.

1. Introduction

Saturated aliphatic hydrocarbons (*n*-alkanes) can be found in the cuticular and epicuticular wax of conventional feed plants and also in other natural waxes. Additionally, they are available as synthetics. In livestock nutrition studies, synthetic alkanes can be applied to estimate the faecal output (Dove and Mayes, 2006), digestibility (Hatt et al., 2001) and passage kinetics (Giráldez et al., 2004; Bulang et al., 2008). Furthermore, they are easy to combine with plant alkanes for a more sophisticated investigation of the intake of feedstuffs of distinct biological origin (Mayes et al., 1986). The suitability of synthetic alkanes to estimate the passage rate of digesta is limited, because they can pass the digestive tract in the solid phase of digesta and likewise in the liquid one (Bulang et al., 2008). The use of markers that are chemically (e.g. plant alkanes and hydrochloric acid-insoluble ash) or at least physically bound to the feed plants (mordants) could overcome this problem but they are usually in very low concentration for a single administration. However, the single administration of a synthetic alkane via bolus followed by repeated sampling of faeces can be applied to determine the faecal excretion curve of this marker, the faecal output and the forage intake of the animal simultaneously (Giráldez et al., 2004). It might be that this approach can have advantage towards bolus administration once or at multiple times per day, which is known to induce diurnal variation of the marker concentration in faeces (Giráldez et al., 2004; Molina et al., 2004). Further work is needed to clarify that issue.

The accurate and uniform labelling of boluses is a basic requirement, regardless of the chosen methodical approach. Using repeated administration of boluses throughout consecutive days, a high uniformity of consecutive dosages may reduce the risk of an additional variation of faecal alkane concentrations. Using the single administration of a bolus, a high uniformity of labelling is required among boluses intended to be applied in a number of animals, because the marker dose rate can be determined from a representative sample of the boluses but not for each animal individually.

Administration methods that can be used to supply external alkanes in ruminant and monogastric livestock involve labelled paper pellets, filters or capsules, labelled feedstuffs (roughages or concentrates) or feed pellets, alkane suspensions or oil-in-water emulsion that need to be offered compulsorily in liquid form and controlled-release devices that are specifically used in ruminants (summarized by Dove and Mayes (2006)). In pigs, alkane-labelled cakes were used as boluses (Mowat et al., 2001). In poultry, synthetic alkanes were mixed into a ground seed mixture and subsequently pelleted (Hatt et al., 2001). For administration in equids specifically, also bread-pieces and other types of biscuits have been used as boluses (Table 1). In most cases, the techniques for preparation of boluses or the labelling of feedstuffs used alkanes that are dissolved to be spread onto the bolus matrix or substrate. According to our experience, this may be accompanied by a higher risk of precipitation and losses of the alkanes in part. Some of the techniques for the labelling of boluses and feedstuffs also involved the exposure to high processing temperatures with the aim to fix the alkanes onto the bolus matrix or substrate (Kuntz et al., 2006; Smith et al., 2007). It is known that plant and other natural alkanes are thermolabile. For example, temperature-dependent losses occurred during oven-drying of faeces samples from sheep that were previously fed with unlabelled or beeswax-labelled diets at 105 °C and 24 h drying time (Elwert et al., 2006). Data of the temperature sensitivity of synthetic alkanes are not available but it is likely that there exists a link to so far non-explained losses of such alkanes during the preparation of boluses and labelled feedstuffs and the further processing of samples for analysis.

We hypothesized that melting synthetic alkanes to wax might enhance the accuracy and the uniformity of subsequent bolus labelling and further simplify it. This is particularly required when multiple alkanes are combined or large quantities of boluses are needed. The aim of the study was to test a procedure of preparing alkane waxes on model scale at a ratio of approximately 1:300 basing upon a dosage of synthetic alkanes, which is, in large livestock, recommended for a daily twice administration (Smith et al., 2007). Additionally, the study was conducted to assess the temperature sensitivity of synthetic alkanes and to clarify how they act when exposed to a processing temperature of 180 °C for a timeframe of 20 min.

Table 1
Methods for the preparation of synthetic alkanes to be applied in equids – literature review.

Preparation method	Alkane	Dosage [mg/d]	Reference
Absorbed into shredded paper and mixed with concentrate (B)	C32, C36	627, 492 (repeated)	Stefanon et al. (1999)
Mixed into concentrate (LF)	C32	n.g. (onetime)	Stevens et al. (2002)
Coated on roughage fibre and compressed into pellets (B)	C32	600 (repeated)	Stevens et al. (2002)
Coated on cellulose, dissolved and mixed with xanthan gum (S)	C32	500 (repeated)	Friend et al. (2004)
Spread onto bread-pieces and microwaved (B)	C32	4000 (two times)	Kuntz et al. (2006)
Dissolved and spread onto biscuits (B)	C32	150 (repeated)	Castelán-Ortega et al. (2007)
Dissolved and sprayed onto shredded paper (B)	C24, C32, C36	914.3–1033.7 (repeated)	Ferreira et al. (2007)
Dissolved, sprayed onto roughage fibre, mixed with gelatine and compressed (B)	C36	308.3 (repeated)	Smith et al. (2007)
Dissolved, pipetted onto Weetabix®-pieces, dried and baked (B)	C32	221.4–454.2 (repeated)	Smith et al. (2007)
Dissolved and sprayed onto concentrate (LF)	C36	337.5 (repeated)	Chavez et al. (2014)

Administration form: B, bolus; LF, labelled feed; S, suspension.
C24, *n*-tetracosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; n.g., not given.

2. Materials and methods

2.1. Preparation of single- and multi-component waxes

Using the *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) synthetic alkanes (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), three single-component waxes (W1: C28; W2: C32; and W3: C36), three binary waxes (W4: C28:C32; W5: C28:C36; and W6: C32:C36) and one tertiary wax (W7: C28: C32:C36) were produced with 30 repetitions each. According to the manufacturer, the specific purity of C28, C32 and C36 was 99%, 97% and 98%, respectively, which was considered for the composition of the mixtures. The preparation of waxes involved the following steps: (A) the weighing of the crystalline alkanes into a suitable vessel; (B) the complete melting of the crystalline alkanes or their mixtures in a water bath using a temperature that was suitable to induce melting of the individual alkane or melting of the highest-molecular alkane in the mixture; the onset and maximal temperatures of the melting areas of the tested synthetic alkanes are provided in Table 2; (C) the cooling of the molten alkanes at room temperature until wax formation; additional refrigeration may improve the fracture strength, which is required to crush the wax into fragments; and (D) the crushing of the wax into small fragments. The Supplementary Figs. S1–S4 provide further illustration of the preparation process.

2.2. Alkane analyses

In trial 1 of gas chromatographic analysis (GCA1), individual crystalline alkanes (C28, C32 and C36) and their binary (C28:C32, C28:C36 and C32:C36) and tertiary (C28:C32:C36) mixtures were directly weighed into sample vials ($n=5$ per variant, 1.02 ± 0.475 mg per vial) and subjected to GCA to assess the measuring accuracy that could maximally be achieved using these sample types and dosage.

In trial 2 of GCA (GCA2), waxes of individual alkanes, binary waxes and tertiary waxes (specified above) were weighed into sample vials ($n=30$ per variant, 0.52 ± 0.146 mg per vial) and measured by gas chromatography to study the impact of melting and re-crystallization on the recovery of synthetic alkanes after wax preparation.

In trial 3 of GCA (GCA3), sub-samples of the same waxes were exposed to 100 °C for 30 min ($n=5$ per variant) or were freeze-dried for 48 h ($n=5$ per variant) to simulate the baking of boluses or freeze-drying as an alternative method of bolus preparation, respectively. The treated samples were subsequently analysed by GCA to determine alterations of alkane recovery from the waxes after additional treatment. All samples were weighed using a Sartorius CP2P microbalance (Sartorius AG, Göttingen, Germany).

The GCA was carried out according to Elwert et al. (2004). After individual portions of crystalline alkanes or alkane waxes had been weighed into the sample vials, the internal standards *n*-docosane (C22) and *n*-tetratriacontane (C34) with a specific purity higher than 98% were added to each vial. The GCA was performed on a Shimadzu GC-2010 FID unit (Shimadzu Corporation, Kyoto, Japan) that was fitted with an Rtx®-1 w/Integra-Guard™-column (Restek Corporation, Bellefonte, PA, USA) using on-column injection. A standard solution, which contained a homologous sequence of long-chain alkanes between C22 and *n*-octatriacontane (C38), was measured regularly to calibrate the evaluation method for alkane retention times and to determine a device-internal discrimination of alkanes with higher molecular weight. Alkane concentrations were quantified on peak area basis relative to the sample-internal standards C22 and C34.

The impact of temperature on alterations of the mass (weight reduction) of synthetic alkanes and thus on the retrievable amount of alkanes in different target samples, were studied using *n*-tetracosane (C24), *n*-hexacosane (C26), C28, C32, C36 and C38 as test alkanes. Previously, the onset and maximum temperatures of the respective melting areas were verified via differential scanning calorimetry (DSC) using a Netzsch DSC 200 unit (Netzsch Gerätebau GmbH, Selb, Germany; temperature program: 25–400 °C at 10 K/min) and are presented in Table 2. Two trials of a thermogravimetric analysis (TGA) were performed using a Netzsch TG 209 unit (Netzsch Gerätebau GmbH, Selb, Germany). Firstly (TGA1), the crystalline alkanes were heated separately using a temperature that was increased from 20 to maximal 600 °C at a constant rate of 10 K/min. The weight reduction of each test alkane was recorded 20 times per min. Secondly (TGA2), after the temperature was increased from 20 to 180 °C at a constant rate of 20 K/min, the crystalline alkanes were heated separately for 20 min with a constant 180 °C to simulate exposure to high treating temperature over a timeframe, which is a sufficient baking time for bolus matrices and also surmised to be sufficient for fixing synthetic alkanes onto substrates of distinct origin. The weight reduction of each test alkane was recorded 50 times per min.

Table 2
Measured onset and maximum temperatures of the melting temperature area of long-chain synthetic alkanes.

	Onset [°C]	Maximum [°C]
n-tetracosane (C24)	47.2	55.1
n-hexacosane (C26)	52.1	62.9
n-octacosane (C28)	57.1	65.0
n-dotriacontane (C32)	64.6	72.0
n-hexatriacontane (C36)	72.3	77.4
n-octatriacontane (C38)	77.5	80.9

2.3. Statistical analysis

Statistical analysis was performed with the SAS 9.4 software package (SAS Institute Inc., Cary, NC, USA). Using the REG procedure, a linear regression analysis was performed to study the relationship between alkane quantities in crystalline or wax samples that were originally weighed with those measured by GCA and to determine the specific difference of the slopes of respective regression lines from one (GCA1 and GCA2). Subsequently, the GLM procedure was used to test whether an additional treatment (baking or freeze-drying) affects the recovery of synthetic alkanes from the single- (3 types) or multi-component waxes (4 types). Fourteen variants were tested. The specific deviation of the slopes of general and variant-specific regression lines from zero was determined. Deviations were considered significant when $P<0.05$. The relative difference between the weighed and measured alkane quantities is given as the mean ± standard deviation among the repetitions of each sample variant.

3. Results and discussion

3.1. Gas-chromatographic analyses

The even-chain alkanes C28, C32 and C36 are commonly little available or absent in feed plants (Dove and Mayes, 1991). They can be applied as external markers to estimate the faecal output (Dove and Mayes, 2006), digestibility (Hatt et al., 2001) and passage kinetics (Giráldez et al., 2004; Bulang et al., 2008) and are furthermore predestined to be used in combination with primarily odd-chain plant alkanes to estimate the intake of feedstuffs (Mayes et al., 1986). In this study, waxes were made of these alkanes on model scale by use of an individual portion of approximately

Table 3
Deviations between the originally weighed and via GC measured quantities of individual crystalline alkanes, individual crystalline alkanes in multi-component mixtures and total alkanes in multi-component mixtures.

Sample	Total			C28			C32			C36		
	RD	*P*	R^2	RD	*P*	R^2	RD	*P*	R^2	RD	*P*	R^2
C28				2.1 ± 0.29	0.943	0.993						
C32							3.4 ± 1.9	0.466	0.972			
C36										9.7 ± 5.3	0.019	0.994
C28:C32	5.6 ± 4.3	0.374	0.520	3.6 ± 1.2	0.291	0.987	8.2 ± 9.8	0.772	0.851			
C28:C36	5.6 ± 1.5	0.191	0.993	2.2 ± 2.1	0.091	0.997				10.1 ± 1.82	0.512	0.979
C32:C36	5.9 ± 2.8	0.153	0.994				5.7 ± 3.3	0.124	0.994	9.0 ± 9.2	0.672	0.954
C28:C32:C36	3.9 ± 2.9	0.375	0.963	3.6 ± 1.6	0.188	0.998	4.4 ± 3.5	0.313	0.977	4.5 ± 3.9	0.356	0.984

C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; GC, gas chromatography; RD, relative difference, given in % as mean ± standard deviation among the repetitions of each variant.

1:300 basing upon 150 mg per bolus, which is a dosage recommended to be applied in large livestock (horses) for an administration 2 times a day (Smith et al., 2007). According to our experience, the measurement of alkane dosages on original scale is not possible without the use of several steps of dilution (see e.g. Giráldez et al., 2004), because this would overload the GC-column and bias the determination of alkane peak areas and alkane concentrations. The model scale was chosen to examine the recovery of crystalline alkanes after GCA separately or in mixture and the recovery of individual alkanes in single- or multi-component waxes without the influence of bolus preparation and the subsequent processing of samples.

In GCA1, the alkane crystals and their various mixtures were analysed untreated. The results of the linear regression analysis of the originally weighed and subsequently measured quantities of crystalline alkanes are shown in Table 3. In general, the slopes of the regression lines for the individual alkanes separately, for total alkanes in a mixture and for individual alkanes in a mixture did not differ from one, so no differences ($P > 0.05$) were detected between the weighed quantities of alkanes and the measured ones. However, in those samples that contained C36 exclusively, the measured quantities were consistently higher ($P = 0.019$) than those weighed out. In binary mixed samples, higher deviations were found infrequently for C32 and particularly for C36. A consistently high variance was detected between the repetitions of each test variant. This particularly became obvious for the C32 and C36 alkanes. Nevertheless, the R^2 value was consistently high indicating a close linear relationship between the weighed and measured alkane quantities.

Throughout the preparation of waxes, some observations were made that might be relevant for an easy handling of such synthetic waxes in practice. Those waxes that were made of individual alkanes (W1 to W3) were very stiff and brittle, which seemed to increase with an increasing number of carbon atoms in the molecules (chain length). These waxes were therefore much harder to crush. The strength of binary mixed waxes differed depending on what alkanes had been merged together. The binary mixed waxes with a chain length difference of four carbon atoms between the alkanes (W4 and W6) were less stiff and thus easier to crush but started to paste already at room temperature. The binary mixed waxes became stiffer and more brittle in the order W4, W6 and W5. The tertiary mixed waxes (W7) were stiffer than the binary ones but were easier to crush than those consisting of only one alkane.

The results of the linear regression analysis of the originally weighed and subsequently measured quantities of alkanes in single- and multi-component waxes (GCA2) are shown in Table 4 and the Fig. 1a–d, respectively. For total alkanes in the binary (W4 to W6) and tertiary mixed waxes (W7), the mean relative difference between the weighed and measured quantities was below 4%. The slopes of the respective regression lines did not differ from one ($P > 0.05$), except in the W5 waxes, where the differences were significant ($P < 0.05$). Also individual alkanes were almost well recovered from the single-component waxes (W1 to W3). The alkanes C28 and C32 were found to be recovered from their combined binary waxes (W4) with a low bias. The slopes of their regression lines did not differ from one ($P > 0.05$). By contrast, the measured quantities of individual alkanes in the other binary (W5 and W6) and tertiary mixed waxes (W7) differed from the respective weighed portions up to 47.4 ± 25.7% (C36 in W7). The deviation between the weighed and measured quantities of total alkanes in binary mixed waxes did not follow any clear direction. Although this was similar in the tertiary mixed waxes, there was a slight trend of the measured quantities to be lower than the respective weighed portions. Also individual alkanes in the single- and multi-component waxes did not show consistency in the deviation of measured quantities to be lower or higher than the respective weighed portions. However, the quantities of C28 tended to be increased after preparation of the tertiary mixed waxes (W7) and

Table 4
Deviations between the originally weighed and via GC measured quantities of individual alkanes after wax preparation, individual alkanes in multi-component waxes and total alkanes in multi-component waxes.

Sample	Total			C28			C32			C36		
	RD	*P*	R^2	RD	*P*	R^2	RD	*P*	R^2	RD	*P*	R^2
W1 (C28)				4.7 ± 4.7	0.424	0.935						
W2 (C32)							6.6 ± 5.5	0.054	0.931			
W3 (C36)										5.9 ± 5.1	0.687	0.949
W4 (C28:C32)	3.9 ± 2.3	0.659	0.981	5.9 ± 5.8	0.412	0.932	5.7 ± 4.3	0.707	0.920			
W5 (C28:C36)	3.4 ± 2.2	0.026	0.984	19.2 ± 11.5	0.206	0.594				21.1 ± 14.4	0.060	0.733
W6 (C32:C36)	3.8 ± 1.9	0.346	0.985				43.7 ± 27.3	0.658	0.296	46.4 ± 29.5	0.764	0.194
W7 (C28:C32:C36)	3.5 ± 2.2	0.471	0.994	27.9 ± 16.7	0.344	0.603	43.9 ± 32.2	0.229	0.093	47.4 ± 25.7	0.448	0.305

C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; GC, gas chromatography; RD, relative difference, given in % as mean ± standard deviation among the repetitions of each variant.

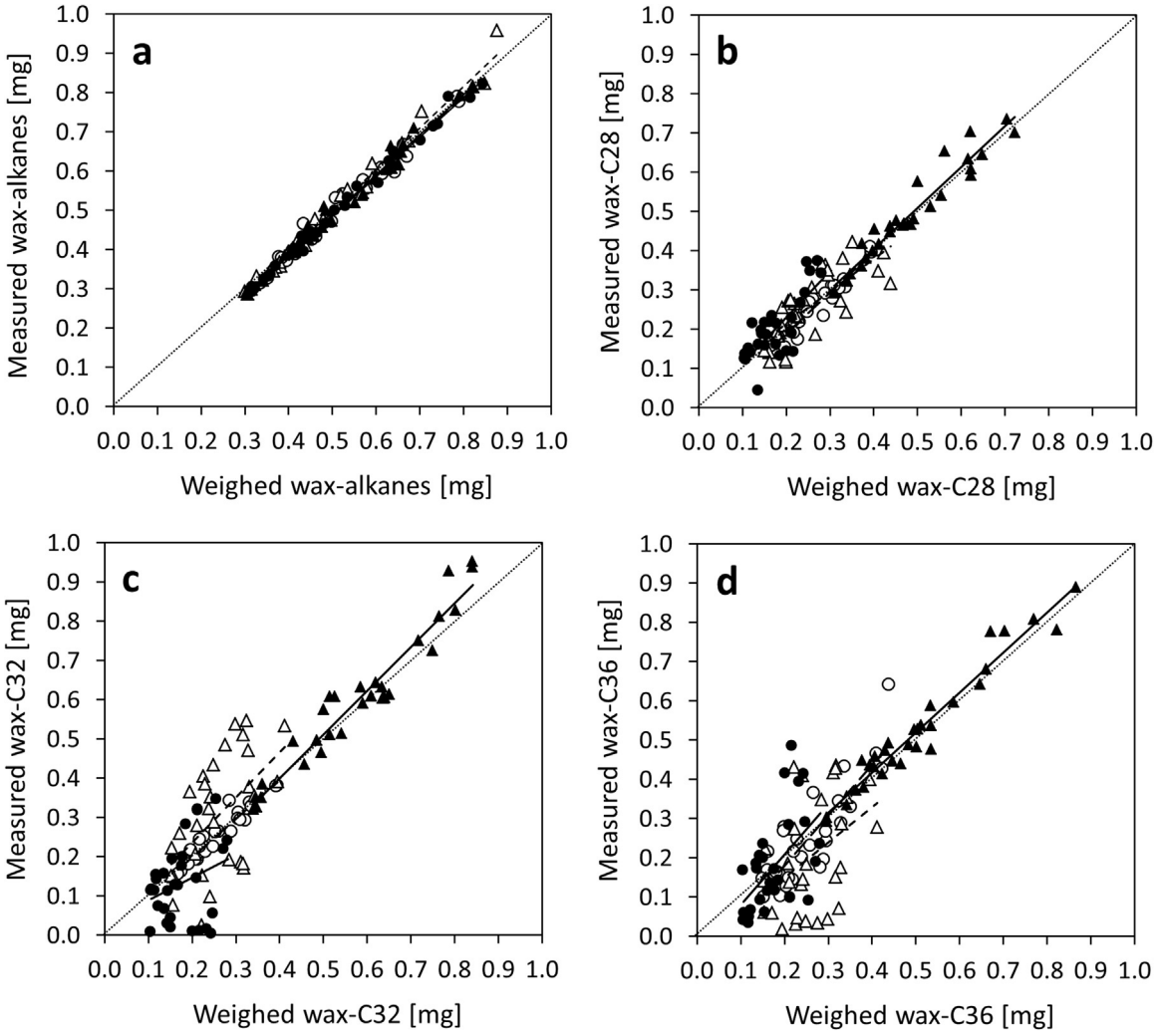

Fig. 1. Relationship between the originally weighed and via gas chromatography measured quantities of (a) total alkanes in multi-component waxes, (b) C28 in single- and multi-component waxes, (c) C32 in single- and multi-component waxes and (d) C36 in single- and multi-component waxes: the dashed diagonal line indicates the reference line, where $y=x$; the symbols indicate the type of wax: (a) ○ W4, Δ W5, ▲ W6, ● W7; (b) ○ W4, Δ W5, ▲ W1, ● W7; (c) ○ W4, Δ W6, ▲ W2, ● W7; and (d) ○ W5, Δ W6, ▲ W3, ● W7; C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; W1, waxes of C28; W2, waxes of C32; W3, waxes of C36; W4, waxes of C28:C32; W5, waxes of C28:C36; W6, waxes of C32:C36; and W7, waxes of C28:C32:C36.

the quantities of C32 tended to be decreased after preparation of the W4 binary mixed waxes. Again, a consistently high variance was detected between the repetitions of each test variant. This may explain the lack of statistical significance even when the weighed and measured alkane quantities differed a lot and might also be the reason for the very low R^2 values, which were found particularly for individual alkanes in W5 to W7 waxes (Table 4).

The additional treatment of the waxes by heating (simulating the baking of boluses) or freeze-drying did not influence the recovery of alkanes in waxes in the model test (GCA3, data not shown). The slope of the general regression line and the slopes of the regression lines specific for the variants with no exposure to an additional treatment and with exposure to either heating or freeze-drying did not differ from zero ($P > 0.05$). However, previous work has shown that notable quantities of molten alkanes entered the matrix of boluses from inside, although a moderate temperature (100 °C) and baking time (30 min) had been used for bolus preparation (Bachmann et al., 2016). In practical application, crumb losses of the bolus matrix are not avoidable in every animal. Marker losses would be the result. Marginal to none residuals of alkanes were found in the matrix when the boluses were freeze-dried for 48 h (Bachmann et al., 2016). Thus, freeze-drying of boluses may be more suitable to prevent marker losses when applied in farm animals.

Melting leads to the homogenization of alkanes in a mixture (Chazhengina et al., 2003). During this process, alkane crystals experience some reversible structural transformations while they undergo transitions across various rotator phases (Chazhengina et al., 2003). When the molten mixtures of crystalline alkanes are cooled down, alkane molecules re-crystallize, where their chain-packing can be disordered for a time. This is particularly evident in the interlayer regions of the crystals due to a chain length mismatch (Snyder et al., 2007). At this, infrequent vacancies in the crystals' interlayer regions may facilitate chain migration via reptation (Snyder et al., 1992) in trying to compensate the conformational disorder (Clavell-Grunbaum et al., 1997). As reported by Snyder et al. (2007), the rate of demixing during cooling down a melt of various alkanes is largely affected by the chain length difference of the mixed compounds, where complete separation is expected not until a chain length difference above 10 carbon atoms (Snyder et al., 2007). It remains unclear, whether and how far individual alkanes distribute homogeneously when waxes have cooled down from the melt. The magnitude of displacements of alkanes within the waxes in part and the time, which is required to achieve the complete separation, cannot be foreseen or quantified (Snyder et al., 2007) and this significantly limits the suitability of synthetic alkane waxes to be applied as dietary markers.

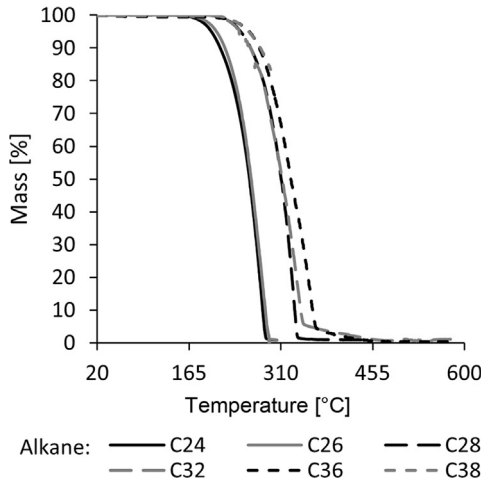

Fig. 2. Weight reduction of crystalline synthetic alkanes during thermogravimetric analysis using a continuously increasing temperature from 20 to maximal 600 °C at 10 K/min; C24, *n*-tetracosane; C26, *n*-hexacosane; C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; C38, *n*-octatriacontane.

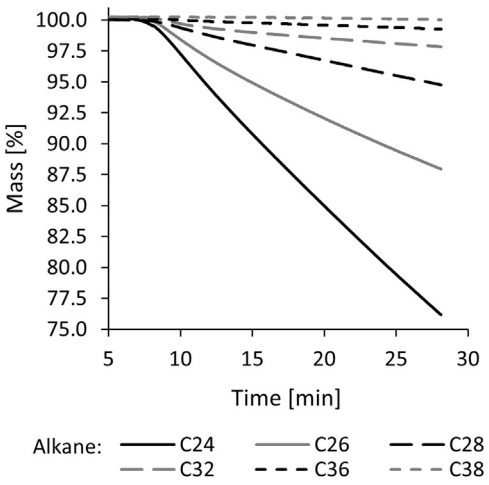

Fig. 3. Weight reduction of crystalline synthetic alkanes during thermogravimetric analysis using isothermality (180 °C for 20 min) after heating-up from 20 to 180 °C at 20 K/min; C24, *n*-tetracosane; C26, *n*-hexacosane; C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; C38, *n*-octatriacontane.

3.2. Thermogravimetric analyses

During the preparation of boluses for administration of synthetic alkanes or labelling of feedstuffs, high temperature (around 100 °C) was commonly used to fix the alkanes onto the substrate, where the time of exposure to that temperature ranged from several minutes (Kuntz et al., 2006) to hours (Smith et al., 2007). Using TGA, we investigated whether mass losses of crystalline even-chain alkanes (C24, C26, C28, C32, C36 and 38) occur after they were treated with either consistently increasing (TGA1), or constant temperature (180 °C) over 20 min after heating-up to that temperature (TGA2). Depending on the chain length and thus molecular weight of alkanes, weight reduction by emergence of soot started at a temperature of 175.5 (C24), 183.1 (C26), 219.6 (C28), 220.8 (C32), 218.7 (C36) and 226.6 °C (C38) and further increased rapidly (TGA1, Fig. 2). As shown in Fig. 2, it seemed that weight reduction under consistently increasing temperature proceeds in a similar way in C24 and C26, in C28 and C32, and in C36 and C38 alkanes, respectively. However, the thermogravimetric measurement of C38 was broken off at a temperature of 293.8 °C because of an intense soot deposition in the measuring unit. Throughout isothermal treatment (TGA2), weight loss from alkanes was lowest with highest chain length (0.0% for C38) and *vice versa* (23.8% for C24; Fig. 3). As a consequence, the resistance of synthetic alkanes to a high temperature depends on the temperature itself, the alkanes' chain length and the time of exposure to that temperature (see Figs. 2 and 4). Fig. 4 shows a curvilinear association between these variables. The mass of alkanes remaining after isothermal treatment seemed to approximate a plateau, which means no mass loss, with increasing chain length. In the alkanes with higher molecular weight (starting from C36), almost no loss of mass was found after isothermal heating at 180 °C. Previous work has shown that plant and natural wax (beeswax) alkanes are thermolabile (Elwert et al., 2006). The results of this study now confirm this for the synthetic alkanes. The thermolability of alkanes needs to be assessed critically when they are applied as dietary markers, particularly during the preparation of boluses or the labelling of feedstuffs and also during the processing of boluses, feedstuffs and faeces samples for a subsequent analysis, e.g. in drying (Elwert et al., 2006) and milling of the samples.

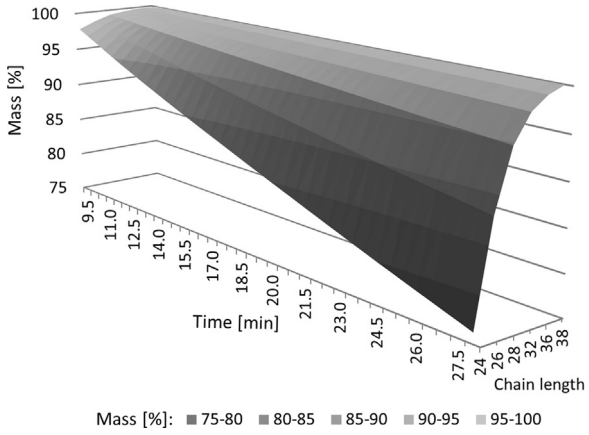

Fig. 4. Relationship between the weight reduction of crystalline synthetic alkanes, the number of carbon atoms (chain length) of the molecules and the duration of isothermal treatment (180 °C for 20 min) after heating-up from 20 to 180 °C at 20 K/min using thermogravimetric analysis. For a better clarity, the records were reduced and are plotted at intervals of 0.5 min: C24, *n*-tetracosane; C26, *n*-hexacosane; C28, *n*-octacosane; C32, *n*-dotriacontane; C36, *n*-hexatriacontane; C38, *n*-octatriacontane.

4. Conclusions

The current study has shown that synthetic alkanes are thermolabile, which may explain losses of alkanes that were observed frequently in the past, especially during the preparation of boluses or the labelling of feedstuffs. The thermolability of synthetic alkanes may limit the suitability of alkane waxes as markers for the estimation of faecal output, digestibility, passage kinetics and the feed intake in farm animals. Reasons might be that complex disorders of the conformation of alkane molecules occur particularly during the melting of alkane mixtures and that individual alkanes separate incompletely during re-crystallization from the melt and the formation of wax. This may lead to displacements of individual alkanes within waxes in part, which cannot be foreseen or quantified. In this study, the recovery of C28 and C32 alkanes from their combined binary waxes was unbiased on model scale and whether this can be confirmed on original scale needs to be validated further. Nevertheless, for practical use, the preparation of waxes might be beneficial because the handling is easier than that of alkane crystals.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.livsci.2016.01.018.

References

Bachmann, M., Wensch-Dorendorf, M., Wulf, M., Glatter, M., Siebmann, M., Bierögel, C., Schumann, E., Bulang, M., Aurich, C., Zeyner, A., 2016. Bolus matrix for administration of dietary markers in horses. Livest. Sci. 185, 43–49.

Bulang, M., Elwert, C., Spilke, J., Rodehutscord, M., 2008. Suitability of synthetic alkanes as markers for the estimation of passage rate in sheep. Livest. Sci. 115, 42–52.

Castelán-Ortega, O.A., Estrada-Flores, J.G., Smith, D.G., Colunga, B., Solís-Méndez, A., Avilés-Nova, F., Ramirez, A., García, I., 2007. Use of n-alkanes for estimation of voluntary intake and digestibility in donkeys (*Equus asinus*). J. Anim. Feed Sci. 16, 307–312.

Chavez, S.J., Siciliano, P.D., Huntington, G.B., 2014. Intake estimation of horses grazing tall fescue (*Lolium arundinaceum*) or fed tall fescue hay. J. Anim. Sci. 92, 2304–2308.

Chazhengina, S.Y., Kotelnikova, E.N., Filippova, I.V., Filatov, S.K., 2003. Phase transitions of *n*-alkanes as rotator crystals. J. Mol. Struct. 647, 243–257.

Clavell-Grunbaum, D., Strauss, H.L., Snyder, R.G., 1997. Structure of model waxes: conformational disorder and chain packing in crystalline multicomponent *n*-alkane solid solutions. J. Phys. Chem. 101, 335–343.

Dove, H., Mayes, R.W., 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Aust. J. Agric. Res. 42, 913–952.

Dove, H., Mayes, R.W., 2006. Protocol for the analysis of *n*-alkanes and other plant-wax compounds and for their use as markers for quantifying the nutrient supply of large mammalian herbivores. Nat. Protoc. 1, 1680–1697.

Elwert, C., Kluth, H., Rodehutscord, M., 2004. Effect of variable intake of alfalfa and wheat on faecal alkane recoveries and estimates of roughage intake in sheep. J. Agric. Sci. Cambridge 142, 213–223.

Elwert, C., Dove, H., Rodehutscord, M., 2006. Effect of roughage species consumed on fecal alkane recovery in sheep, and effect of sample drying treatment on alkane concentrations. Aust. J. Exp. Agric. 46, 771–776.

Ferreira, L.M.M., García, U., Rodrigues, M.A.M., Celaya, R., Dias-da-Silva, A., Osoro, K., 2007. Estimation of feed intake and apparent digestibility of equines and cattle grazing on heathland vegetation communities using the *n*-alkane markers. Livest. Sci. 110, 46–56.

Friend, M.A., Avery, A., Nash, D., 2004. Intake of improved and unimproved pastures in two seasons by grazing weanling horses. Anim. Prod. Aust. 1, 61–64.

Giráldez, F.J., Lamb, C.S., López, S., Mayes, R.W., 2004. Effects of carrier matrix and dosing frequency on digestive kinetics of even-chain alkanes and implications on herbage intake and rate of passage studies. J. Sci. Food Agric. 84, 1562–1570.

Hatt, J.-M., Mayes, R.W., Clauss, M., Lechner-Doll, M., 2001. Use of artificially applied *n*-alkanes as markers for the estimation of digestibility, food selection and intake in pigeons (*Columba livia*). Anim. Feed Sci. Technol. 94, 65–76.

Kuntz, R., Kubalek, C., Ruf, T., Tataruch, F., Arnold, W., 2006. Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*). I. Energy intake. J. Exp. Biol. 209, 4557–4565.

Mayes, R.W., Lamb, C.S., Colgrove, P.M., 1986. The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. J. Agric. Sci. Cambridge 107, 161–170.

Molina, D.O., Matamoros, I., Pell, A.N., 2004. Accuracy of estimates of herbage intake of lactating cows using alkanes: comparison of two types of capsules. Anim. Feed Sci. Technol. 114, 241–260.

Mowat, D., Watson, C.A., Mayes, R.W., Kelly, H., Browning, H., Edwards, S.A., 2001. Herbage intake of growing pigs in an outdoor organic production system. In: Proceedings of the Annual Meeting of the British Society of Animal Science, p. 169.

Smith, D.G., Mayes, R.W., Hollands, T., Cuddeford, D., Yule, H.H., Malo Ladrero, C.M., Gillen, E., 2007. Validating the alkane pair technique to estimate dry matter intake in equids. J. Agric. Sci. Cambridge 145, 273–281.

Snyder, R.G., Clavell-Grunbaum, D., Strauss, H.L., 2007. Spinodal phase separation of unstable solid-state binary n-alkane mixtures. J. Phys. Chem. 111, 13957–13966.

Snyder, R.G., Goh, M.C., Srivatsavoy, V.J.P., Strauss, H.L., Dorset, D.L., 1992. Measurement of the growth kinetics of microdomains in binary n-alkane solid solutions by infrared spectroscopy. J. Phys. Chem. 96, 10008–10019.

Stefanon, B., Volpelli, L., Bovolenta, S., Pinosa, M., 1999. Estimation of fescue hay intake in horses using the n-alkanes method. Zootec. Nutr. Anim. 25, 243–248.

Stevens, D.M., van Ryssen, J.B.J., Marais, J.P., 2002. The use of n-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. S. Afr. J. Anim. Sci. 32, 50–56.

5. General discussion and conclusions

5.1. Effects on the suitability of alkanes as dietary markers

5.1.1. Major factors and their interdependency

Major and additional factors affecting the suitability of alkane markers for the estimation of feed intake, feed selection, digestibility and passage kinetics, and their interdependency that can be identified from literature review, are summarized in Fig. 6. Detailed explanations can be found in the Sections 2.5.5. to 2.5.7. Main influencing factors are discussed in the following in relation to the current methodical investigations.

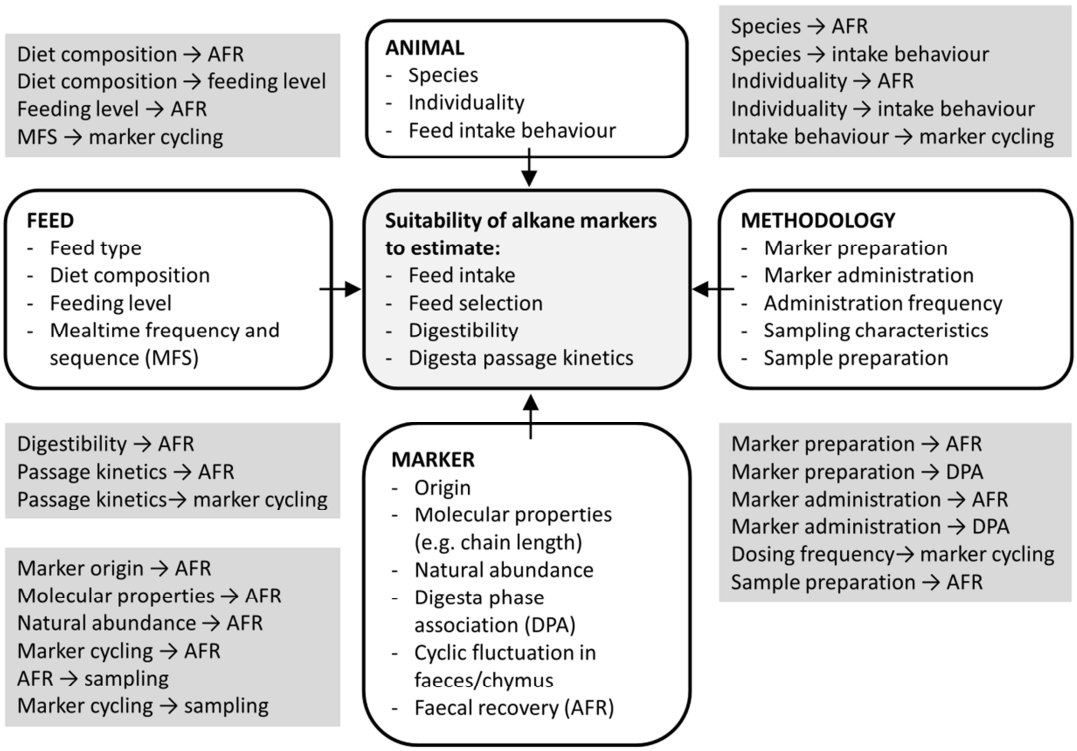

Fig. 6. Interdependency among key factors affecting the suitability of long-chain alkanes as markers for the estimation of feed intake, feed selection, digestibility and digesta passage kinetics in farm animals directly or indirectly: arrows indicate direct influence; dependencies among individual factors that have been documented in literature are specified in grey-highlighted fields.

5.1.2. Effects of alkane preparation

The main objectives of the preparation of boluses or the labelling of substrates for administration of external alkanes (either as synthetics or in form of natural waxes) are to reduce the variation among a batch of boluses or labelled substrates and to ensure a homogenous distribution within them. This allows for a widely consistent dosing of the animals under test and throughout several days (Owens and Hanson, 1992; Molina *et al.*, 2004). It has been described in the literature review (Section 2.5.4.) that the majority of studies used techniques involving the dissolution of synthetic alkanes prior to the application onto a carrying matrix. Own attempts had shown that the labelling of a matrix with an alkane solution can lead to a rapid precipitation of the alkane depending on its dosage and hence this results in losses of the alkane in part. This is why it might be better to use the alkanes undissolved but the pure crystalline form is hard to handle under preparation due to distinct adhesive properties and an accurate weighting into a matrix is difficult. When large quantities of boluses are required, this method is extremely laborious. This has been observed during preparation of boluses for the first study, where synthetic alkanes were weighted into gelatine capsules (see Article 1). As a consequence, we hypothesized that the properties of alkanes can be modified by melting them to waxes, which are much easier to handle and to apply to bolus matrices. Furthermore, this method was hypothesized to enable producing homogenous mixtures of synthetic alkanes in form of multi-component waxes, which significantly increase the achievable accuracy and uniformity of labelling.

Waxes that were made of individual alkanes were very stiff and brittle, which seemed to increase with an increasing CL. These waxes were therefore much harder to crush. The strength of binary mixed waxes differed depending on what alkanes had been merged together. The binary mixed waxes became stiffer and more brittle in the order W4, W6 and W5. The tertiary mixed waxes (W7) were stiffer than the binary ones but were easier to crush than those consisting of only one alkane (see Article 3 for wax specifications). Apart from such specific properties of single wax types, a significant simplification has been achieved all in all when compared to the handling of pure crystals under preparation. Linear regression analysis of model waxes before and after GCA showed a mostly reliable recovery of total alkanes in the binary (W4 to W6) and tertiary mixed waxes (W7), which was similar for the individual alkanes in the single-component waxes (W1

to W3). Also the C28 and C32 alkanes were recovered from their combined binary waxes (W4) with a low bias, which was unexpected since the measured quantities of individual alkanes in the other binary (W5 and W6) and tertiary mixed waxes (W7) differed from the respective weighed portions up to 47.4 ± 25.7 % (C36 in W7). These deviations did not follow any clear direction but showed a distinct variation. As intended, melting leads to the homogenization of alkanes in a mixture (Chazhengina *et al.*, 2003). During this process, the van der Waals forces between molecules become increasingly weakened (Reynhardt and Riederer, 1994) and alkane crystals experience some reversible structural transformations while they undergo transitions across various rotator phases (Chazhengina *et al.*, 2003). Melting is completed when the entire mixture is present in the mobile amorphous zone (Reynhardt and Riederer, 1994). However, what we failed to regard is that when molten mixtures of alkanes are cooled down, the alkane molecules in mixture re-crystallize, where their chain-packing can be disordered for a time. This is particularly evident in the interlayer regions of the crystals due to a chain length mismatch, where infrequent vacancies may facilitate chain migration *via* reptation (Snyder *et al.*, 1992) in trying to compensate the conformational disorder (Clavell-Grunbaum *et al.*, 1997). It consequently remains unclear, whether and how far individual alkanes distribute homogeneously when the waxes have cooled down from the melt. The magnitude of displacements of alkanes within the waxes and the time, which is required to achieve the complete separation, cannot be foreseen or quantified (Snyder *et al.*, 2007). Therefore, it has to be stressed that the suitability of such mixed waxes as dietary markers is significantly limited.

Labelling techniques that omit the dissolution of alkanes usually used high processing temperatures with the aim to fix the alkanes onto the bolus matrix or the substrate (Kuntz *et al.*, 2006; Smith *et al.*, 2007). In study 3, we had investigated whether mass losses of the crystalline synthetic alkanes C24, C26, C28, C32, C36 and 38 occur after they were treated with either consistently increasing, or constant temperature (180 °C) over 20 min after heating-up to that temperature through TGA (see Article 3 for specifications). At a certain temperature above 175.5 °C, at which weight reduction started in C24, weight reduction decreased with an increasing CL of the alkanes. As a result, the isothermal treatment revealed that resistance of synthetic alkanes to a high temperature depends on the temperature itself, alkane CL, and the time of temperature exposure, for which a curvilinear association has been found (Article 3 – Fig. 4). With an increasing CL,

the mass of alkanes remaining after isothermal treatment seemed to approximate a plateau, which means no mass loss. However, in order to confirm that observation, a wider range of alkanes would be required in an equal analysis. Consequently, the thermolability of synthetic alkanes and of plant or other natural alkanes as well (see Elwert *et al.*, 2006) thus needs to be assessed critically when they are applied as dietary markers. This is particularly important during the preparation of boluses or the labelling of feedstuffs, and during the processing of boluses, feeds and faeces samples for a subsequent analysis.

In this context, an issue of concern is the AFR of the C36 alkane. There is a number of studies that reported unexpectedly low AFR of C36, which did not fit with the AFR of adjacent alkanes, neither in a linear nor a curvilinear relation, and for which actually no clear explanation exists (see Vulich *et al.*, 1991; Unal and Garnsworthy, 1999; Molina *et al.*, 2004; Lin *et al.*, 2007; Elwert *et al.*, 2008). We observed the same in the first of the current studies, where with 82 ± 4.1 % C36 had the lowest AFR (Article 1). Concurrently, C36 had the highest concentrations in boluses, which has consistently been observed across all our investigations, and has finally been confirmed through the initial GCA in the third study. Therein, the crystalline alkanes and their various mixtures were analysed completely untreated (see Article 3 for specifications). An overestimated dosage of C36 that does not match its faecal concentration thus leads to an underestimated AFR. However, it still remains unclear what exactly occurs with C36 during GCA leading to an overestimation, and whether this is contemplable to be one reason for the underestimation of C36 AFR.

In large livestock such as horses, there is a considerable diluting effect by digesta resulting in large quantities of synthetic alkanes (ranging from 150 to 4000 mg/d in horses; see Article 3 – Table 1) that need to be applied to achieve measurable marker concentrations in faeces. Hence, the dosage given per individual bolus or mealtime is high, which makes the assessment of dosing accuracy and uniformity by means of a representative sample difficult. High sample representativeness is doubtful since it requires a very uniform distribution of the marker substance within the matrix. In case of boluses, analysis of the total dosage per bolus would be beneficial. However, the GCA of such dosages is, to my experience, not possible without several steps of dilution avoiding biased determination of alkane peak areas and concentrations due to a GC column overload. An appropriate sample preparation method was proposed by Giráldez *et al.* (2004). The

milling of alkane-labelled samples during sample preparation is surmised to provoke losses of alkanes through heat development, which is why we used cryo-milling with success (see Articles 1 and 2).

5.1.3. Effects of bolus matrix and alkane administration

Irrespective of the animal species, the matrix type is supposed to affect the site and the rate of alkane release in the gut. Then, it consequently should affect the diurnal variation of alkane concentration in faeces (see Giráldez *et al.*, 2004; Dove and Mayes, 2006). In goats, Giráldez *et al.* (2004) reported different TMRT of synthetic alkanes administered *via* varying bolus matrices in accordance to their breakdown rate, *i.e.* particle size reduction rate, in the gut. Large particles as *e.g.* shredded paper, filters and paper bungs need longer time to be broken down before having the condition to leave the rumen, which might explain shorter TMRT of cellulose powder (Giráldez *et al.*, 2004). Matrix differences in water absorbency may additionally hasten or retard the particle breakdown (Giráldez *et al.*, 2004). Also in monogastric animals, differences in the matrix breakdown rate should influence the site of marker exposition. To me, there is no study known, which reported respective test results. In study 2 of the current thesis, we compared laboratory filter papers and hypromellose capsules regarding their matrix properties. Although this was not specifically investigated, it seems to be likely that the filter papers move marker exposition to a more caudal site of the gut and prolong it, whereas the capsules should rapidly be dispersed within in the stomach. It has been reported that hypromellose capsules are readily soluble in contact with artificial human gastrointestinal fluid (~ 250 s; Chiwele *et al.*, 2000). A retarded matrix breakdown probably causes an incomplete mixing of the marker and gut contents, and increase diurnal variation of faecal marker concentrations (Giráldez *et al.*, 2004).

Especially in horses, it is likely that matrix properties affect the acceptance of boluses because of the distinct sensitivity and selection skills typical for that animal species. It was reported that alkanes administered *via* labelled feed are prone to easily be regurgitated, separated, or ingested incompletely (Smith *et al.*, 2007). Therefore, one objective of the current investigations was to test and develop a bolus matrix for the administration of external markers, which is consistently accepted and stable towards microbial spoilage over a sufficient period of time (see

Article 2). Baked boluses (30 min at 100 °C) had a consistently high acceptance by horses, but baking is unsuitable for the use of thermolabile markers such as alkanes. Distinct quantities of alkanes were observed to enter the outer matrix shell during baking and thus getting lost. The acceptance of freeze-dried boluses with a defined size (1.5, 2 and 3 cm Ø) and duration of drying (6, 12, 24 and 48 h) was high and consistent as well. Such boluses are therefore suitable for oral administration of thermolabile markers or other thermolabile substances. The horses mostly did not separate and reject the markers specifically. The variable sizing of boluses is supposed to enable the use of various marker dosages and thus also the use in different target animals, which needs, however, to be confirmed through future studies. To reduce the preparation time and energy costs, the drying time of such boluses can be limited to 24 h or less, depending on their size. The tested freeze-dried boluses were stable towards spoilage from microbes for at least 1 month after preparation, provided that they were stored in closed boxes.

Besides the type of the bolus matrix itself, the application interval of boluses or feedstuffs became obvious to be the major factor affecting the cycling variation of faecal marker concentrations within and across test days when markers are applied in repetition. This relationship seems to be independent from the marker type and the animal species (see Haenlein *et al.*, 1966; Cuddeford and Hughes, 1990; Dove and Mayes, 1991; Giráldez *et al.*, 2004; Molina *et al.*, 2004; Smith *et al.*, 2007). It was found that, in plant alkanes offered 3 times a day *via* feed and synthetic alkanes administered 2 times a day *via* bolus, the diurnal fluctuations were the more pronounced the less often alkanes were administered (see Article 1 for specific results). This relationship might hypothetically be explained by the succession and overlap of individual excretion curves that result from individual meals or bolus administrations. This results in the specific diurnal patterns of alkane concentrations that can be observed in faeces. The study confirmed similar diurnal patterns of faecal alkane concentrations between individual horses, which repeated from day to day. Nevertheless, individual shifts that were either quantitative or temporal have been noticed contributing to the individuality of the faecal patterns. Here, all factors that directly or indirectly affect digesta or marker passage kinetics are potential contributors. They might involve: (1) diet composition effects (Haenlein *et al.*, 1966; Drogoul *et al.*, 2001); (2) effects of the matrix type affecting the site and duration of alkane exposition in the gut (Giráldez *et al.*, 2004); (3) effects of the labelling technique leading to a more pronounced associa-

tion of artificially bonded alkanes to the liquid digesta phase because of an increased rate of particle size reduction during feed labelling and the favoured presence of small particles in gut liquids (Owens and Hanson, 1992; Giráldez *et al.*, 2006); and (4) the effects of marker – digesta phase association, or marker migration effects, respectively (Mayes *et al.*, 1988, 1997; Bulang *et al.*, 2008). Gut passage of liquid-phase markers is hastened compared to that of markers fully bound to coarse plant particles (Udén *et al.*, 1982). In horses, the restriction of exercise possibly contributes to shifts in faecal marker concentration patterns as well, because restricted exercise is associated with reduced water intake and reduced voluntary feed intake, which might reduce flow speed of coarse particulate digesta (Orton *et al.*, 1985; Pagan *et al.*, 1998). On the contrary, the retention of liquid digesta and fine particles was increased in exercised horses, which might have increased the digestibility of such higher digestible components (Orton *et al.*, 1985). Because of a considerable time delay of the ingestion of plant alkanes during mealtime compared to the ingestion of synthetic alkanes *via* bolus, plant alkanes are less prone to diurnal fluctuation in faeces. Increasing the number of mealtimes per day is nevertheless recommended as it better matches horses' ingestive behaviour (Mayes and Duncan, 1986). Apparent digestibility is not altered through mealtime frequency (van Weyenberg *et al.*, 2007).

5.1.4. Effects of faeces sampling

Taking faeces samples, which provide representative data of the mean concentration of alkanes, is important since it helps to reduce the concentration variability within and across days. It decisively influences the estimation reliability of feed intake, feed selection, digestibility and passage kinetics. Major variables regarding the faeces sampling procedure are: (1) the consideration of individual animals or the sampling on group scale; (2) the number of consecutive sampling days; (3) the quantity of samples per day and animal; (4) the use of spot or bulk samples; and (5) the selection of sampling timeframes. In previous studies, individual animals were consistently considered for estimation purposes or the validation of the alkane technique. It was found that, using the same dosing schedule, faecal concentrations of synthetic alkanes had lower variation among individuals than those of the plant alkanes, being less affected by individual feeding patterns or digestion efficiency (Oliván *et al.*, 2007a). However, regarding pronounced individuality ef-

fects, the alkane method is thought to be more valid on group scale (C. Scharch, personal communication). Regarding the duration of quantitative collection of faeces in horses, Goachet *et al.* (2009) did not note any time effect on digestibility determination and alteration between 3, 4 or 5 sampling days, which was equal for the estimation of digestibility using dietary lignin as the marker. However, digestibility estimation was significantly affected using AIA as the marker, pronouncing an overestimation over time (Goachet *et al.*, 2009). Sutton *et al.* (1977) found no differences between 1 day and up to 5 days of faeces sampling in horses, determining digestibility of nitrogen and energy *via* total faecal collection or estimating it through AIA dietary and faecal concentrations. No difference in the estimated TMRT of Cr-EDTA and lanthanides was detected between 1.5 and up to 5 days of sampling, regardless of the sampling method, *i.e.* total faeces collection *vs.* spot sampling, but it differed significantly compared to only 1 day of sampling (Goachet *et al.*, 2009). Using alkanes in horses, Smith *et al.* (2007) found that the accuracy of intake estimation increased progressively as the number of sampling days was increased. This confirmed the study of Malossini *et al.* (1994), who stated that it is preferable to increase the number of sampling days rather than the quantity of samples per day. In cows, the authors did not find a significant difference in faecal concentrations of continuously ingested C31 and C33 plant alkanes and C32 offered once a day between 1 sample and the means of up to 4 samples per day (Malossini *et al.*, 1994). However, this cannot be confirmed since a relevant diurnal variation of alkane faecal concentrations was found to influence the estimates (see Giráldez *et al.*, 2004; Molina *et al.*, 2004; and own results shown in Article 1). Bulk samples of faeces have widely been used rather than spot samples to reduce procedural and analytical effort. Moreover, bulk samples may compensate missing individual samples to some extent (Vulich and Hanrahan, 1995), provided that they occur unsystematically. Even if marker defecation is not uniform but predictable, then this can be used to eliminate total faeces collection by periodical spot sampling (Kotb and Luckey, 1972) and the bias of estimates can be reduced through an appropriate timing (Vulich and Hanrahan, 1995). We used the knowledge of type and dependency of defecation dynamics of plant and synthetic alkanes to select faeces spot samples allowing for uncorrected and unbiased estimation of DMI, DMO and DMD in horses (see Article 1 for specific results). Accurate estimates of DMI were obtained from 2 h before (C31:C32) until 2 h after the morning meal (C29:C28 and C33:C28), as well as between 5 and 6 h after the

morning meal using SFQ × DDF and C29 and C33 as digestibility markers. As this in part confirms the results of Oliván *et al.* (2007a) obtained from studying beef cattle, it might be indicative for the surmised general independency of faecal alkane dynamics from animal species. The estimation of DMO using synthetic alkanes had a larger bias than the estimation using SFQ × DDF, which was most reliable based on samples taken between 3 and 6 h after the midday meal. Using C29 or C33, DMD was largely unbiased during 3 to 4 h after the morning meal. Conclusively, taking two to three spot samples of faeces evenly distributed between 2 h before and 6 h after the first meal or bolus administration per day is recommended for at least 3 consecutive days.

5.2. Conclusions

The application of alkanes and congeneric markers is a beneficial advancement in estimating feed intake, selection, digestibility and digesta passage kinetics although necessity for further basic research and validation remains. The results of the current investigations may contribute to the development of a method that is easily applicable for scientists and practitioners alike, as a helpful tool for feed evaluation and the assessment of animal welfare in livestock rearing, feeding and husbandry systems. Replies to the hypotheses, the main conclusions are:

(1) The use of synthetic waxes instead of crystalline alkanes as markers simplifies the handling under preparation and thus may contribute to a more accurate and uniform labelling of boluses or feedstuffs, but the suitability of mixed waxes can significantly be limited through unpredictable displacements of individual alkanes occurring after the waxes have cooled down from the melt.

(2) Synthetic alkanes are thermolabile substances as it is similarly known for plant and other natural alkanes, which is why any high temperature exposure of alkane markers during preparation of boluses or the labelling of feedstuffs, and during the processing of boluses, feedstuffs and faeces samples for subsequent analyses, needs to be assessed critically. The critical temperature and duration of exposure, at which or from which losses of alkanes must be awaited, respectively, essentially depends on the alkanes' CL.

(3) The bolus matrix that has been proposed was consistently well accepted in horses. The freeze-dried variants additionally enable the application of ther-

molabile markers such as alkanes or other thermolabile substances, and are stable towards microbial spoilage for at least 1 month when stored in closed boxes. A flexible variation of the bolus size is supposed to enable the use of various marker dosages and thus also the use in different target animals.

(4) Synthetic alkanes and other external markers are recommended to be administered onetime prior to faeces collection enabling the estimation of marker passage kinetics, curve characteristics, faecal output, and, in combined use of plant markers, the estimation of feed intake and digestibility simultaneously. Excessive diurnal variation of synthetic alkane concentrations in faeces can alternatively be reduced through a more frequent administration with shortened application interval. Plant alkanes are less prone to diurnal fluctuation in defecation but we nevertheless recommend also increasing the number of mealtimes per day.

(5) A total faeces collection can be eliminated by periodical spot sampling, and the bias of estimates can be reduced through an appropriate timing, which is, basing on the current results and depending on the target parameter for estimation, a timeframe of 2 h before to 6 h after the first meal or bolus administration. Within this timeframe two to three spot samples of faeces should be taken evenly distributed. The collection period should last for at least 3 consecutive days to compensate interday differences of faecal marker concentrations.

6. Summary, Zusammenfassung

6.1. Summary

In livestock nutrition studies, the combined application of long-chain plant and synthetic *n*-alkanes enables the simultaneous estimation of feed intake, feed selection, faecal output, digestibility and passage kinetics. However, the main limitations for a successful practical application are inconsistent faecal recovery of alkanes and a cyclic fluctuation of alkane concentrations in faeces. The preparation of synthetic alkanes and administration might become sources of bias, when unpredicted losses of alkanes are provoked and alkanes are ingested incompletely. The presented studies investigated the suitability of alkane markers for the estimation of feed intake and digestibility in horses, focussed on synthetic alkane preparation, administration and the representative sampling of faeces in particular. The thesis comprises the following three consecutive studies:

Study 1: "Impact of dynamics of faecal concentrations of plant and synthetic *n*-alkanes on their suitability for the estimation of dry matter intake and apparent digestibility in horses." In a quantitative faeces collection trial using 5 horses, faecal concentration dynamics of plant *n*-heptacosane, *n*-nonacosane (C29), *n*-hentriacontane (C31) and *n*-tritriacontane (C33), offered 3 times a day *via* feed, and of *n*-octacosane (C28), *n*-dotriacontane (C32) and *n*-hexatriacontane (C36) synthetic alkanes, offered 2 times a day *via* bolus, were compared. Dry matter intake (DMI), output (DMO) and digestibility (DMD) were determined from the total collection trial and additionally estimated for each of 12 equal timeframes throughout the day. The diurnal patterns of the single faeces quantity (SFQ) and faecal alkane concentrations were similar between horses and were repeated from day to day. The intraday dynamic of SFQ was pronounced. The dynamic of the faecal concentration was much more pronounced when the alkane was administered twice instead of three times a day. Measured DMI was 12.0 kg/d, measured DMO was 5.9 kg/d and measured DMD was 0.51. Reliable estimates were obtained for DMI with 12.3 ± 0.79 kg/d for the combination of C29 and C28 and 12.1 ± 1.01 kg/d for the combination of C33 and C28 at 2 h after administration, and 12.1 ± 0.96 kg/d for the combination of C31 and C32 at 2 h prior to the morning meal, which included the first bolus administration. When calculated from DMO and DMD, DMI was 12.2 ± 0.89 kg/d for C29 and 12 ± 1.0 kg/d for C33 between 5 and 6 h after the morning meal. Estimates of DMD were unbiased between the

third and fourth hour after the morning meal with 0.52 ± 0.014 for C29 and 0.51 ± 0.021 for C33, respectively. The DMO was 5.7 ± 0.34 kg/d and 6.1 ± 0.43 kg/d when estimated 3 to 4 h after the second meal, or prior to the second bolus administration, using the product of SFQ and the daily defecation frequency or synthetic alkanes, respectively. Knowledge of defecation dynamics might be helpful for simplifying experimental trials. They followed intake dynamics, which can prospectively be used to select sampling timeframes. The selection of two to three spot samples of faeces, evenly distributed between 2 h before and 6 h after the morning meal, which was the time of bolus administration, allows for greatest reliability.

Study 2: "Bolus matrix for administration of dietary markers in horses." Oral administration of external markers is challenging because of horses' high sensitivity and selection skills. It was tested whether a suitable bolus matrix ensures high and consistent acceptance. Boluses were investigated with and without labelling. Synthetic alkane wax was used as the test marker. Boluses were baked (100 °C, 30 min) or freeze-dried. The freeze-dried boluses varied in size (1.5, 2, or 3 cm Ø) and drying time (6, 12, 24, or 48 h). In two tests (AT1 and AT2), acceptance by the horses was assessed with scores between 1 (complete intake) and 4 (refusal). In AT1, marginal rejection of the marker was recorded, whereas the following tests were performed with placebos only. In two bending tests (BT1 and BT2), the force required to break the boluses (FL, flexural load) was determined because this may affect acceptance. Preselected variants were stored for 4 weeks in a climatic chamber under controlled conditions and were subsequently analysed for residual moisture (RM) and spoilage-indicating microbes. In baked boluses, the alkanes were partly found outside of the inner matrices. This was not evident in the freeze-dried variants. Acceptance of labelled boluses (scores ≤ 1.7 ± 0.18, AT1), baked placebos (scores ≤ 2.2 ± 0.35, AT1) and freeze-dried placebos (scores ≤ 1.1 ± 0.31, AT2) was consistently high. This was explained by the BT, with a mean FL of 202 ± 16.5 N for the baked (BT1) and up to 257 ± 22.5 N for the freeze-dried placebos (BT2) being obtained, which was close to the masticatory forces in horses. The results indicated that the adaption to suitable boluses (placebos) can lead to increased acceptance. Limiting the drying time to at most 24 h seemed justified, especially for the smaller boluses. After 6 and 12 h, the RM was 7.5 ± 0.52 % (1.5 cm Ø) and 5.7 ± 0.52 % (2 cm Ø), which make the risk of microbial spoilage appear low. Tested boluses were unspoiled for 1 month after preparation. It was suggested that variable sizes of the boluses may enable the use of various marker dosages

and, incidentally, also for different target animals. A freeze-dried matrix is likewise open to use with other thermolabile markers or substances.

Study 3: "Preparation of synthetic alkane waxes and investigations on their suitability for application as dietary markers in farm animals." Successful application of synthetic alkanes requires an accurate and uniform labelling of boluses or feeds, which is in turn supported through simplified handling of the markers during preparation. In this study, it was tested whether melting of synthetic alkanes to wax is able to enhance the accuracy and uniformity of subsequent bolus labelling and further simplifies it. Additionally, the temperature sensitivity of a range of synthetic alkanes was studied by thermogravimetry (either with constant heating-up or isothermality) to clarify so far non-explained losses of alkanes, which were observed frequently during the labelling of boluses and feedstuffs or the processing of samples for analysis. Depending on chain length, weight reduction in alkanes by emergence of soot during heating-up started between 176 °C (*n*-tetracosane, C24) and 227 °C (*n*-octatriacontane, C38). Throughout isothermal treatment, weight loss from alkanes was lowest with highest chain length (0.0 % for C38) and *vice versa* (23.8 % for C24). The weighed and *via* gas chromatography measured quantities of individual alkanes in single-component and of total alkanes in multi-component waxes were similar with a maximal relative difference of 6.6 ± 5.5 %. The relative difference between weighed and measured quantities of individual alkanes in multi-component waxes was maximal 47.4 ± 25.7 %, but was unexpectedly low for C28 (5.9 ± 5.8 %) and C32 (5.7 ± 4.3 %) in their combined binary waxes. Synthetic alkanes are thermolabile. This is why exposure to high temperature during preparation of boluses or labelling of feedstuffs needs to be assessed critically. Complex conformational disorders can occur in alkane molecules particularly during the melting of alkane mixtures. This, and apparent incomplete separation of individual alkanes after re-crystallization from the melt, may lead to displacements within waxes, which cannot be foreseen or quantified, and thus to the loss of their suitability as dietary markers. For practical use, alkane waxes might nevertheless be beneficial because the handling is easier than that of alkane crystals.

6.2. Zusammenfassung

Die kombinierte Anwendung pflanzlicher und synthetischer *n*-Alkane in ernährungswissenschaftlichen Studien mit landwirtschaftlichen Nutztieren ermöglicht die simultane Schätzung von Futteraufnahme, Futterselektion, Kotausscheidung, Verdaulichkeit und Kinetik der Darmpassage. Wesentliche Hindernisse für eine erfolgreiche Anwendung der Methode in der Praxis sind die unbeständige fäkale Wiederfindung der Alkane und deren zyklische Fluktuation bei fäkaler Ausscheidung. Die Aufbereitung synthetischer Alkane und deren Verabreichung können zu Fehlerquellen werden, wenn nicht bestimmbare Verluste der Marker hervorgerufen, bzw. diese nicht vollständig vom Tier aufgenommen werden. Die vorgestellten Studien untersuchten die Eignung von Alkanen zur Schätzung von Futteraufnahme und Verdaulichkeit (VK) bei Pferden, speziell Aufbereitung und Verabreichung synthetischer Alkane und die Entnahme repräsentativer Kotproben.
Studie 1: „Auswirkung der Dynamik fäkaler Konzentrationen pflanzlicher und synthetischer *n*-Alkane auf deren Eignung zur Schätzung von Trockenmasseaufnahme und scheinbarer Verdaulichkeit bei Pferden." Nach quantitativer Kotsammlung bei 5 adulten Pferden, wurde die Dynamik der fäkalen Konzentrationen der pflanzlichen Alkane *n*-Heptacosan, *n*-Nonacosan (C29), *n*-Hentriacontan (C31) und *n*-Tritriacontan (C33), die dreimal täglich über dem Futter verabreicht wurden, mit denen von synthetischem *n*-Octacosan (C28), *n*-Dotriacontan (C32) und *n*-Hexatriacontan (C36), zweimal täglich über einen Bolus verabreicht, verglichen. Die Aufnahme von Trockenmasse (TM), deren Ausscheidung und VK wurden mittels Gesamtkotsammlung bestimmt und zusätzlich über den Tag hinweg für 12 gleiche Zeiträume geschätzt. Die diurnalen Muster von Einzelkotmenge und fäkaler Alkankonzentrationen waren zwischen den Tieren ähnlich und wiederholten sich von Tag zu Tag. Die diurnale Dynamik von Einzelkotmenge und fäkaler Konzentrationen synthetischer Alkane waren ausgeprägt, wohingegen die der frequenter verabreichten pflanzlichen Alkane weniger deutlich war. Trockenmasseaufnahme wurde mit 12,0 kg/d, TM-Ausscheidung mit 5,9 kg/d und VK mit 0,51 bestimmt. Zuverlässige Schätzungen der TM-Aufnahme wurden mit 12,3 ± 0,79 kg/d durch die Kombination von C29 und C28 und mit 12,1 ± 1,01 kg/d durch die Kombination von C33 und C28 innerhalb von 2 h nach Markergabe und mit 12,1 ± 0,96 kg/d durch die Kombination von C31 und C32 innerhalb von 2 h vor morgendlicher Fütterung, bzw. Markergabe, erzielt. Die Berechnung der TM-

Aufnahme aus geschätzter TM-Ausscheidung und VK ergab 12,2 ± 0,89 kg/d für C29 und 12,0 ± 1,0 kg/d für C33 zwischen 5 und 6 h nach der morgendlichen Fütterung. Schätzungen von VK waren mit 0,52 ± 0,014 durch C29 bzw. 0,51 ± 0,021 durch C33 innerhalb von 3 bis 4 h nach der morgendlichen Fütterung unverfälscht. Die TM-Ausscheidung wurde mit 5,7 ± 0,34 kg/d bzw. 6,1 ± 0,43 kg/d innerhalb von 3 bis 4 h nach der zweiten Mahlzeit bzw. vor der zweiten Markergabe durch Einzelkotmenge × Tagesausscheidungsfrequenz oder synthetische Alkane zuverlässig geschätzt. Da die Dynamik von Ausscheidung und Aufnahme eines Markers eng miteinander verbunden sind, können gezielt Probenentnahmezeiträume ausgewählt werden. Zwei bis drei Kotproben, je Entnahmetag gleichverteilt zwischen 2 h vor und 6 h nach der ersten Markergabe, können zuverlässige Schätzungen ermöglichen.

Studie 2: „Bolusmatrix zur Verabreichung von Futtermarkern bei Pferden." Die orale Gabe externer Marker ist aufgrund der ausgeprägten Empfindlichkeit und des Selektionsvermögens besonders bei Pferden herausfordernd. Es wurde untersucht, inwieweit eine geeignete Bolusmatrix hohe und durchgängige Akzeptanz ermöglicht. Boli sind sowohl markiert, als auch unmarkiert geprüft worden. Als Testmarker dienten synthetische Alkanwachse. Boli wurden entweder gebacken (100 °C, 30 Min.) oder gefriergetrocknet (gft.). Letztere wurden in Größe (1,5, 2 oder 3 cm Ø) und Trocknungsdauer (TD; 6, 12, 24 oder 48 h) variiert. In 2 Tests (AT1 und AT2) wurde die Akzeptanz bei Pferden geprüft und mit Noten zwischen 1 (vollständige Aufnahme) und 4 (Ablehnung) bewertet. In AT1 wurden allenfalls marginale Markerverluste verzeichnet, weshalb alle weiteren Tests mit Placebos (P) durchgeführt worden sind. In 2 Biegetests (BT1 und BT2) wurde die Biegekraft (BK) gemessen, die benötigt wird um die Boli zu zerbrechen, da diese die Akzeptanz beim Tier maßgeblich beeinflusst. Auf diese Weise vorselektierte Varianten wurden für 4 Wochen unter konstanten Bedingungen in einer Klimakammer gelagert und anschließend hinsichtlich Restfeuchtegehalt (RFG) und verderbsanzeigenden Keimen untersucht. In gebackenen Boli wurden Alkane teilweise auf und innerhalb der äußeren Schicht der Matrix gefunden, was bei gft. Varianten nicht auftrat. Die Akzeptanz der markierten Boli (Noten ≤ 1,7 ± 0,18, AT1), gebackenen P (Noten ≤ 2,2 ± 0,35, AT1) und gft. P (Noten ≤ 1,1 ± 0,31, AT2) war durchgehend hoch, was durch mittlere BK von 202 ± 16,5 N für gebackene (BT1) und bis zu 257 ± 22,5 N für gft. P (BT2), ähnlich der Kaukräfte beim Pferd, erklärt werden kann. Eine Adaptation an P kann die Bolusakzeptanz verbessern. Die Beschränkung der

TD bei gft. Boli auf maximal 24 h scheint vor allem für kleinere Varianten gerechtfertigt. Nach 6 bzw. 12 h Trocknung betrug der RFG 7,5 ± 0,52 % (1,5 cm Ø) bzw. 5,7 ± 0,52 % (2 cm Ø), wobei das Risiko mikrobiellen Verderbs gering ist. Geprüfte P waren nach 1 Monat Lagerung unverdorben. Variable Bolusgrößen könnten verschiedene Markerdosierungen bei unterschiedlichen Zieltierarten ermöglichen, eine gft. Matrix zudem die Verwendung thermolabiler Marker und Substanzen.
Studie 3: „Herstellung synthetischer Alkanwachse und Untersuchungen zu deren Eignung für die Anwendung als Futtermarker bei Nutztieren." Die erfolgreiche Anwendung synthetischer Alkane erfordert die genaue und einheitliche Markierung von Boli oder Futtermitteln, was wiederum durch eine vereinfachte Handhabung der Marker während der Aufbereitung unterstützt wird. In dieser Studie wurde geprüft, inwieweit das Schmelzen synthetischer Alkane zu Wachs die Genauigkeit und Einheitlichkeit der Bolusmarkierung verbessert und diese vereinfacht. Zusätzlich wurde die Temperaturempfindlichkeit synthetischer Alkane mittels Thermogravimetrie (durch konstante Erhitzung bzw. Isothermie) untersucht, um bislang unerklärte Verluste an Alkanen zu klären, die während der Markierung von Boli und Futter oder Probenaufbereitung wiederholt beobachtet worden sind. Abhängig von der Kettenlänge wurde ein Masseverlust unter Rußfreisetzung während des Erhitzens ab 176 °C (*n*-Tetracosan, C24) bzw. 227 °C (*n*-Octatriacontan, C38) festgestellt. Im Verlauf isothermer Behandlung war der Masseverlust am Geringsten bei höchster Kettenlänge (0,0 % bei C38) und *vice versa* (23,8 % bei C24). Die eingewogenen und mittels Gaschromatografie gemessenen Gehalte einzelner Alkane in Einzel- und der Gesamtheit der Alkane in Mischwachsen unterschieden sich mit einer maximalen relativen Abweichung von 6,6 ± 5,5 % kaum voneinander. Diese lag im Vergleich eingewogener und gemessener Gehalte einzelner Alkane in Mischwachsen hingegen bei maximal 47,4 ± 25,7 %. Sie war unerwartet gering bei C28 (5,9 ± 5,8 %) und C32 (5,7 ± 4,3 %) in gemeinsamen binären Mischwachs. Da synthetische Alkane thermolabil sind, muss jede Einwirkung höherer Temperaturen während der Markierung von Boli und Futter kritisch bewertet werden. Unvorhersehbare, nicht quantifizierbare Verschiebungen der molekularen Konformation können beim Schmelzen vor allem von Alkanmischungen auftreten, die nach Abkühlen nicht vollständig reversibel sind. Solche Wachse sind als Marker nicht geeignet. Bei der praktischen Handhabung zeigten Wachse dennoch deutliche Vorteile gegenüber kristallinen Alkanen.

7. References

The following list refers to the references cited in the introduction, the background sections and the general discussion. The used journal title abbreviations refer, insofar as given, to the Caltech Library available at https://library.caltech.edu/reference/abbreviations/.

Aharoni, Y., Brosh, A., Holzer, Z., 1999. Comparison of models estimating digesta kinetics and fecal output in cattle from fecal concentrations of single-dosed markers of particles and solutes. J. Anim. Sci. 77, 2291-2304.

Ali, H. A. M., Mayes, R. W., Lamb, C. S., Hector, B. L., Verma, A. K., Ørskov, E. R., 2004. The potential of long-chain fatty alcohols and long-chain fatty acids as diet composition markers: development of methods for quantitative analysis and faecal recoveries of these compounds in sheep fed mixed diets. J. Agr. Sci. 142, 71-78.

Ali, H. A. M., Mayes, R. W., Hector, B. L., Verma, A. K., Ørskov, E. R., 2005. The possible use of *n*-alkanes, long-chain fatty alcohols and long-chain fatty acids as markers in studies of the botanical composition of the diet of free-ranging herbivores. J. Agr. Sci. 143, 85-95.

Argenzio, R. A., Lowe, J. E., Pickard, D. W., Stevens, C. E., 1974. Digesta passage and water exchange in the equine large intestine. Am. J. Physiol. 226, 1035-1042.

Austreng, E., Storebakken, T., Thomassen, M. S., Refstie, S., Thomassen, Y., 2000. Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. Aquaculture 188, 65-78.

Baker, G., Jones, L. H. P., Milne, A. A., 1961a. Opal uroliths from a ram. Aust. J. Agr. Res. 12, 473-482.

Baker, G., Jones, L. H. P., Wardrop, I. D., 1961b. Opal phytoliths and mineral particles in the rumen of the sheep. Aust. J. Agr. Res. 12, 462-472.

Barcia, P., Bugalho, M. N., Campagnolo, M. L., Cerdeira, J. O., 2007. Using *n*-alkanes to estimate diet composition of herbivores: a novel mathematical approach. Animal 1, 141-149.

Barnes, D. L., 1976. A review of plant-based methods of estimating food consumption, percentage utilisation, species preferences and feeding patterns of grazing and browsing animals. P. Grassland Soc. S. Afr. 11, 65-71.

Barroso, F. G., Alados, C. L., Boza, J., 1995. Food selection by domestic goats in Mediterranean arid shrublands. J. Arid Environ. 31, 205-217.

Bartley, E. E., Helmer, L. G., Meyer, R. M., 1971. Metabolism of ^{14}C-labeled octadecane by cattle. J. Anim. Sci. 33, 1351-1355.

Benjamin, R. W., Chen, M., Degen, A., Aziz, A., Al Hadad, M. J., 1977. Estimation of the dry and organic matter intake of young sheep grazing a dry Mediterranean pasture and their maintenance requirements. J. Agr. Sci. 88, 313-320.

Bergero, D., Préfontaine, C., Miraglia, N., Peiretti, P. G., 2009. A comparison between the 2N and 4N HCl acid-insoluble ash methods for digestibility trials in horses. Animal 3, 1728-1732.

Berry, N. R., Scheeder, M. R. L., Sutter, F., Kröber, T. F., Kreuzer, M., 2000. The accuracy of intake estimation based on the use of alkane controlled-release capsules and faeces grab sampling in cows. Ann. Zootech. 49, 3-13.

Berry, N. R., Jewell, P. L., Sutter, F., Edwards, P. J., Kreuzer, M., 2002. Selection, intake and excretion of nutrients by Scottish Highland suckler beef cows and calves, and Brown Swiss dairy cows in contrasting alpine grazing systems. J. Agr. Sci. 139, 437-453.

Berthe-Corti, L., Fetzner, S., 2002. Bacterial metabolism of *n*-alkanes and ammonia under oxic, suboxic and anoxic conditions. Acta Biotechnol. 22, 299-336.

Bezabih, M., Pellikaan, W. F., Hendriks, W. H., 2011a. Using *n*-alkanes and their carbon isotope enrichments ($\delta^{13}C$) to estimate the botanical composition of pasture mixes from the Mid Rift Valley grasslands of Ethiopia. Livest. Sci. 142, 298-304.

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

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

Bi, X., Sheng, G., Liu, X., Li, C., Fu, J., 2005. Molecular and carbon and hydrogen isotopic composition of *n*-alkanes in plant leaf waxes. Org. Geochem. 36, 1405-1417.

Blaxter, K. L., Graham, N. McC., Wainman, F. W., 1956. Some observations on the digestibility of feed by sheep and on related problems. Brit. J. Nutr. 10, 69-91.

Bochnia, M., Schäfer, S., Simroth, K., Glatter, M., Goetz, F., Boesel, M., Bachmann, M., Zeyner, A., 2016. The daytime chewing frequencies and activity patterns of permanently grazing warmblood type horses, in: Proceedings of the 20th Congress of the European Society of Veterinary and Comparative Nutrition (in press).

Bowers, C. L., Friend, T. H., Grissom, K. K., Lay, Jr., D. C., 1993. Confinement of lambs (*Ovis aries*) in metabolism stalls increased adrenal function, thyroxine and motivation for movement. Appl. Anim. Behav. Sci. 36, 149-158.

Brøkner, C., Nørgaard, P., Søland, T. M., 2006. The effect of grain type and processing on equine chewing time. Pferdeheilkunde 22, 453-460.

Brøkner, C., Nørgaard, P., Hansen, H. H., 2008. Effect of feed type and essential oil product on equine chewing activity. J. Anim. Physiol. An. N. 92, 621-630.

Brosh, A., Henkin, Z., Rothman, S. J., Aharoni, Y., Orlov, A., Arieli, A., 2003. Effects of faecal *n*-alkane recovery in estimates of diet composition. J. Agr. Sci. 140, 93-100.

Brüschweiler, H., Felber, H., Schwager, F., 1989. Bienenwachs – Zusammensetzung und Beurteilung der Reinheit durch gaschromatographische Analyse. Fat Sci. Technol. 91, 73-79.

Bugalho, M. N., Dove, H., Kelman, W., Wood, J. T., Mayes, R. W., 2004. Plant wax alkanes and alcohols as herbivore diet composition markers. Rangeland Ecol. Manag. 57, 259-268.

Buijs, N. A., Zhou, Y. J., Siewers, V., Nielsen, J., 2015. Long-chain alkane production by the yeast *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 112, 1275-1279.

Bulang, M., 2005. Untersuchungen zur Bewertung von Luzernesilage als Rationskomponente für Milchkühe. Dissertation, Halle-Wittenberg.

Bulang, M., Elwert, C., Spilke, J., Rodehutscord, M., 2008. Suitability of synthetic alkanes as markers for the estimation of passage rate in sheep. Livest. Sci. 115, 42-52.

Bulang, M., Boguhn, J., Rodehutscord, M., 2010. Enrichment of ^{13}C in *Medicago sativa* and *Lolium multiflorum n*-alkanes, in: Proceedings of the 23th General Meeting of the European Grassland Federation, pp. 437-439.

Büsing, K., Harmeyer, J., Markuske, K. D., Zeyner, A., 2011. Microscopic evidence for the uptake of orally given humic acids by the intestinal mucosa in piglets. Anim. Prod. Sci. 51, 967-974.

Carlson, D. A., Langley, P. A., Huyton, P., 1978. Sex pheromone of the tsetse fly: isolation, identification, and synthesis of contact aphrodisiacs. Science 201, 750-753.

Castelán-Ortega, O. A., Estrada-Flores, J. G., Smith, D. G., Colunga, B., Solís-Méndez, A., Avilés-Nova, F., Ramirez, A., García, I., 2007. Use of *n*-alkanes for estimation of voluntary intake and digestibility in donkeys (*Equus asinus*). J. Anim. Feed Sci. 16, Suppl. 2, 307-312.

Castle, E. J., 1956. The rate of passage of foodstuffs through the alimentary tract of the goat. Brit. J. Nutr. 10, 15-23.

Cayley, J. W. D., Bird, P. R., 1996. Techniques for Measuring Pastures, 2nd ed. Pastoral and Veterinary Institute, Hamilton.

Celaya, R., Ferreira, L. M. M., García, U., García, R. R., Osoro, K., 2011. Diet selection and performance of cattle and horses grazing in heathlands. Animal 5, 1467-1473.

Charmley, E., Dove, H., 2007. Using plant wax markers to estimate diet composition and intakes of mixed forages in sheep by feeding a known amount of alkane-labelled supplement. Aust. J. Agr. Res. 58, 1215-1225.

Chavez, S. J., Siciliano, P. D., Huntington, G. B., 2014. Intake estimation of horses grazing tall fescue (*Lolium arundinaceum*) or fed tall fescue hay. J. Anim. Sci. 92, 2304-2308.

Chazhengina, S. Y., Kotelnikova, E. N., Filippova, I. V., Filatov, S. K., 2003. Phase transitions of *n*-alkanes as rotator crystals. J. Mol. Struct. 647, 243-257.

Chikaraishi, Y., Kaneko, M., Ohkouchi, N., 2013. Reprint of "Stable hydrogen and carbon isotopic compositions of long-chain (C_{21}-C_{33}) *n*-alkanes and *n*-alkenes in insects". Geochim. Cosmochim Ac. 111, 78-87.

Chiwele, I., Jones, B. E., Podczeck, F., 2000. The shell dissolution of various empty hard capsules. Chem. Pharm. Bull. 48, 951-956.

Clauss, M., Schiele, K., Ortmann, S., Fritz, J., Codron, D., Hummel, J., Kienzle, E., 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J. Anim. Physiol. An. N. 98, 107-118.

Clavell-Grunbaum, D., Strauss, H. L., Snyder, R. G., 1997. Structure of model waxes: conformational disorder and chain packing in crystalline multicomponent *n*-alkane solid solutions. J. Phys. Chem. 101, 335-343.

Cochran, R. C., Adams, D. C., Wallace, J. D., Galyean, M. L., 1986. Predicting digestibility of different diets with internal markers: evaluation of four potential markers. J. Anim. Sci. 63, 1476-1483.

Cottle, D. J., Romero, C., 2014. Improving pasture intake predictions by variable weighting of plant marker concentrations. Anim. Feed Sci. Technol. 187, 30-43.

Cottle, D. J., 2016. Estimation of the pasture intake of individual yearlings by controlled supplementation with natural ^{13}C or alkanes and alcohols. Livest. Sci. 184, 13-20.

Crooker, B. A., Clark, J. H., Shanks, R. D., 1982. Rare earth elements as markers for rate of passage measurements of individual feedstuffs through the digestive tract of ruminants. J. Nutr. 112, 1353-1361.

Cuddeford, D., Hughes, D., 1990. A comparison between chromium-mordanted hay and acid-insoluble ash to determine apparent digestibility of a chaffed, molassed hay/straw mixture. Equine Vet. J. 22, 122-125.

Cuddeford, D., Pearson, R. A., Archibald, R. F., Muirhead, R. H., 1995. Digestibility and gastro-intestinal transit time of diets containing different proportions of alfalfa and oat straw given to Thoroughbreds, Shetland ponies, Highland ponies and donkeys. Anim. Sci. 61, 407-417.

Cummings, J. H., Wiggins, H. S., 1976. Transit through the gut measured by analysis of a single stool. Gut 17, 219-223.

Davies, J. B., 1964. Microbial incorporation of fatty acids derived from *n*-alkanes into glycerides and waxes. Appl. Microbiol. 12, 210-214.

Dhanoa, M. S., Siddons, R. C., France, J., Gale, D. L., 1985. A multicompartmental model to describe marker excretion patterns in ruminant faeces. Brit. J. Nutr. 53, 663-671.

Dove, H., Mayes, R. W., 1991. The use of plant wax alkanes as marker substances in studies of the nutrition of herbivores: a review. Aust. J. Agr. Res. 42, 913-952.

Dove, H., Mayes, R. W., Lamb, C. S., Ellis, K. J., 1991. Evaluation of an intra-ruminal controlled-release device for estimating herbage intake using synthetic and plant cuticular wax alkanes, in: Proceedings of the 3rd International Symposium on the Nutrition of Herbivores, p. 82.

Dove, H., Coombe, J. B., 1992. A comparison of methods for estimating supplement intake and diet digestibility in sheep. P. Aus. S. Ani. 19, 239-241.

Dove, H., Moore, A. D., 1995. Using a least-squares optimization procedure to estimate botanical composition based on the alkanes of plant cuticular wax. Aust. J. Agr. Res. 46, 1535-1544.

Dove, H., Mayes, R. W., 1996. Plant wax components: a new approach to estimating intake and diet composition in herbivores. J. Nutr. 126, 13-26.

Dove, H., Mayes, R. W., Freer, M., 1996. Effects of species, plant part, and plant age on the *n*-alkane concentrations in the cuticular wax of pasture plants. Aust. J. Agr. Res. 47, 1333-1347.

Dove, H., Oliván, M., 1998. Using synthetic or beeswax alkanes for estimating supplement intake in sheep. Anim. Prod. Aust. 22, 189-192.

Dove, H., Mayes, R. W., Lamb, C. S., Ellis, K. J., 2002. Factors influencing the release rate of alkanes from an intra-ruminal, controlled-release device, and the resultant accuracy of intake estimation in sheep. Aust. J. Agr. Res. 53, 681-696.

Dove, H., Mayes, R. W., 2006. Protocol for the analysis of *n*-alkanes and other plant-wax compounds and for their use as markers for quantifying the nutrient supply of large mammalian herbivores. Nat. Protoc. 1, 1680-1697.

Dove, H., Oliván, M., 2006. The possible use of the alkenes (unsaturated hydrocarbons) of plant cuticular wax as diet composition markers in sheep, in: Sandoval-Castro, C. A., Hovell, D., Torres-Acosta, J. F. J., Ayala-Burgos, A. (Eds.), Herbivores: assessment of intake, digestibility and the roles of secondary compounds. Nottingham University Press, Nottingham, pp. 1-7.

Dove, H., Charmley, E., 2008. Using the alkanes and long-chain alcohols of plant cuticular wax to estimate diet composition and the intakes of mixed forages in sheep consuming a known amount of alkane-labelled supplement. Animal 2, 1474-1485.

Dowler, L. E., Siciliano, P. D., Pratt-Phillips, S. E., Poore, M., 2012. Determination of pasture dry matter intake rates in different seasons and their application in grazing management. J. Equine Vet. Sci. 32, 85-92.

Drogoul, C., Poncet, C., Tisserand, J. L., 2000. Feeding ground and pelleted hay rather than chopped hay to ponies. 1. Consequences for *in vivo* digestibility and rate of passage of digesta. Anim. Feed Sci. Technol. 87, 117-130.

Drogoul, C., de Fombelle, A., Julliand, V., 2001. Feeding and microbial disorders in horses: 2: effect of three hay:grain ratios on digesta passage rate and digestibility in ponies. J. Equine Vet. Sci. 21, 487-490.

Duncan, A. J., Mayes, R. W., Lamb, C. S., Young, S. A., Castillo, I., 1999. The use of naturally occurring and artificially applied *n*-alkanes as markers for estimation of short-term diet composition and intake in sheep. J. Agr. Sci. 132, 233-246.

Duren, S. E., Dougherty, C. T., Jackson, S. G., Baker, J. P., 1989. Modification of ingestive behavior due to exercise in yearling horses grazing orchardgrass. Appl. Anim. Behav. Sci. 22, 335-345.

Edouard, N., Fleurance, G., Martin-Rosset, W., Duncan, P., Dulphy, J. P., Grange, S., Baumont, R., Dubroeucq, H., Pérez-Barbería, F. J., Gordon, I. J., 2008. Voluntary intake and digestibility in horses: effect of forage quality with emphasis on individual variability. Animal 2, 1526-1533.

Edouard, N., Duncan, P., Dumont, B., Baumont, R., Fleurance, G., 2010. Foraging in a heterogeneous environment – an experimental study of the trade-off between intake rate and diet quality. Appl. Anim. Behav. Sci. 126, 27-36.

Eglinton, G., Hamilton, R. J., 1967. Leaf epicuticular waxes. Science 156, 1322-1335.

Ehle, F. R., 1984. Influence of feed particle density on particulate passage from rumen of Holstein cow. J. Dairy Sci. 67, 693-697.

Ellis, A. D., 2010. Biological basis of behaviour in relation to nutrition and feed intake in horses, in: Ellis, A. D., Longland, A. C., Coenen, M., Miraglia, N. (Eds.), The impact of nutrition on the health and welfare of horses, EAAP Publ. 128. Wageningen Academic Publishers, Wageningen, pp. 53-74.

Ellis, W. C., Lascano, C., Teeter, R. G., Owens, F. N., 1982. Solute and particulate flow markers, in: Owens, F. N. (Ed.), Protein Requirements for Cattle, Symposium, Misc. Publ. 109. Oklahoma Agricultural Experiment Station, Stillwater, pp. 37-56.

Elwert, C., Kluth, H., Rodehutscord, M., 2004. Effect of variable intake of alfalfa and wheat on faecal alkane recoveries and estimates of roughage intake in sheep. J. Agr. Sci. 142, 213-223.

Elwert, C., Rodehutscord, M., 2004. Notes on the use of beeswax as a source of alkanes for the alkane technique, in: Proceedings of the 20th General Meeting of the European Grassland Federation, pp. 1052-1054.

Elwert, C., Dove, H., 2005. Estimation of roughage intake in sheep using a known daily intake of a labelled supplement. Anim. Sci. 81, 47-56.

Elwert, C., Dove, H., Rodehutscord, M., 2006. Effect of roughage species consumed on fecal alkane recovery in sheep, and effect of sample drying treatment on alkane concentrations. Aust. J. Exp. Agr. 46, 771-776.

Elwert, C., Dove, H., Rodehutscord, M., 2008. Faecal alkane recoveries from multicomponent diets and effects on estimates of diet composition in sheep. Animal 2, 125-134.

Fahey, Jr., G. C., Jung, H. G., 1983. Lignin as a marker in digestion studies: a review. J. Anim. Sci. 57, 220-225.

Faichney, G. J., 1975. The use of markers to partition digestion within the gastro-intestinal tract of ruminants, in: Proceedings of the 4th International Symposium on Ruminant Physiology, pp. 277-291.

Ferreira, L. M. M., Oliván, M., García, U., Rodrigues, M. A. M., Osoro, K., 2005. Validation of the alkane technique to estimate diet selection of goats grazing heather-gorse vegetation communities. J. Sci. Food Agr. 85, 1636-1646.

Ferreira, L. M. M., García, U., Rodrigues, M. A. M., Celaya, R., Dias-da-Silva, A., Osoro, K., 2007a. Estimation of feed intake and apparent digestibility of equines and cattle grazing on heathland vegetation communities using the *n*-alkane markers. Livest. Sci. 110, 46-56.

Ferreira, L. M. M., García, U., Rodrigues, M. A. M., Celaya, R., Dias-da-Silva, A., Osoro, K., 2007b. The application of the *n*-alkane technique for estimating the composition of diets consumed by equines and cattle feeding on upland vegetation communities. Anim. Feed Sci. Technol. 138, 47-60.

Ferreira, L. M. M., Oliván, M., Celaya, R., García, U., Rodrigues, M. A. M., Osoro, K., 2007c. The use of *n*-alkanes to estimate diet composition of ruminants grazing on species diverse plant communities – effect of feeding selectivity on diet composition estimates. Livest. Sci. 111, 114-123.

Ferreira, L. M. M., Oliván, M., Celaya, R., García, U., Rodrigues, M. A. M., Osoro, K., 2007d. The use of the alkane technique to estimate diet selection of sheep grazing grass-clover/heather-gorse vegetation communities. J. Sci. Food Agr. 87, 274-285.

Ferreira, L. M. M., Carvalho, S., Falco, V., Celaya, R., García, U., Santos, A. S., Rodrigues, M. A. M., Osoro, K., 2009a. Assessment of very long-chain fatty acids as complementary or alternative natural fecal markers to *n*-alkanes for estimating diet composition of goats feeding on mixed diets. J. Anim. Sci. 87, 2732-2745.

Ferreira, L. M. M., Celaya, R., García, U., Rodrigues, M. A. M., Osoro, K., 2009b. Differences between domestic herbivores species in alkane faecal recoveries and the accuracy of subsequent estimates of diet composition. Anim. Feed Sci. Technol. 151, 128-142.

Ferreira, L. M. M., Celaya, R., Santos, A. S., Falco, V., Guedes, C., Rodrigues, M. A. M., Osoro, K., 2010. Comparison of long-chain fatty acids and alkanes as markers to estimate diet composition of equines and cattle consuming heathland vegetation species. Livest. Sci. 131, 260-271.

Ferreira, L. M. M., Celaya, R., Benavides, R., Jáuregui, B. M., García, U., Santos, A. S., García, R. R., Rodrigues, M. A. M., Osoro, K., 2013. Foraging behaviour of domestic herbivore species grazing on heathlands associated with improved pasture areas. Livest. Sci. 155, 373-383.

Ferreira, L. M. M., Daniel, J. B., Celaya, R., Santos, A. S., Osoro, K., Rodrigues, M. A. M., Pellikaan, W. F., 2014. Utilization of carbon isotope enrichments ($\delta^{13}C$) of alkanes as faecal markers to estimate diet composition of goats fed with heathland vegetation. Anim. Feed Sci. Technol. 191, 26-38.

Ferreira, L. M. M., Celaya, R., Santos, A. S., Mayes, R. W., Rodrigues, M. A. M., Osoro, K., 2015. Application of long-chain alcohols as diet composition markers in sheep fed on grass-white clover and heather-gorse plant species. Grass Forage Sci. 70, 30-43.

Fleurance, G., Duncan, P., Mallevaud, B., 2001. Daily intake and the selection of feeding sites by horses in heterogeneous wet grasslands. Anim. Res. 50, 149-156.

Forbes, J. M., 1995. Voluntary Food Intake and Diet Selection in Farm Animals. CAB International, Wallingford.

France, J., Dhanoa, M. S., Siddons, R. C., Thornley, J. H. M., Poppi, D. P., 1988. Estimating the production of faeces by ruminants from faecal marker concentration curves. J. Theor. Biol. 135, 383-391.

Frape, D. L., Tuck, M. G., Sutcliffe, N. H., Jones, D. B., 1982. The use of inert markers in the measurement of the digestibility of cubed concentrates and of hay given in several proportions to the pony, horse and white rhinoceros (*Diceros simus*). Comp. Biochem. Physiol. 72A, 77-83.

Fraser, M. D., Theobald, V. J., Moorby, J. M., 2006. Determining diet composition on complex swards using *n*-alkanes and long-chain fatty alcohols. Ecol. Appl. 16, 1901-1910.

Friedemann, K., Kunert, G., Gorb, E., Gorb, S. N., Beutel, R. G., 2015. Attachment forces of pea aphids (*Acyrthosiphon pisum*) on different legume species. Ecol. Entomol., http://dx.doi.org/10.1111/een.12249.

Friend, M. A., Avery, A., Nash, D., 2004. Intake of improved and unimproved pastures in two seasons by grazing weanling horses. Anim. Prod. Aust. 1, 61-64.

Fuchs, R., Militz, H., Hoffmann, M., 1987. Untersuchungen zur Verdaulichkeit der Rohnährstoffe bei Pferden. 1. Mitteilung. Methoden zur Bestimmung der Verdaulichkeit. Arch. Anim. Nutr. 37, 235-246.

Galyean, M. L., 1993. Technical note: an algebraic method for calculating fecal output from a pulse dose of an external marker. J. Anim. Sci. 71, 3466-3469.

Gamarra, B., Kahmen, A., 2015. Concentrations and δ^2H values of cuticular *n*-alkanes vary significantly among plant organs, species and habitats in grasses from an alpine and a temperate European grassland. Oecologia 178, 981-998.

Garcia, E. E., Nogueira, A. R. A., Nóbrega, J. A., 2001. Matrix effects on the determination of dysprosium, europium and ytterbium used as animal faecal markers by inductively coupled plasma optical emission spectrometry with axially- and radially-viewed configurations. J. Anal. At. Spectrom. 16, 825-830.

Gesellschaft für Ernährungsphysiologie (GfE), 2003. Communications of the Committee for Requirement Standards of the Society of Nutrition Physiology. Prediction of digestible energy (DE) in horse feed, in: Proceedings of the 57th Conference of the Society of Nutrition Physiology, pp. 123-126.

Gesellschaft für Ernährungsphysiologie (GfE), 2014. Energie- und Nährstoffbedarf landwirtschaftlicher Nutztiere. Empfehlungen zur Energie- und Nährstoffversorgung von Pferden, Nr. 11. DLG-Verlag, Frankfurt am Main.

Ginzel, M. D., Blomquist, G. J., Millar, J. G., Hanks, L. M., 2003. Role of contact pheromones in mate recognition in *Xylotrechus colonus*. J. Chem. Ecol. 29, 533-545.

Giráldez, F. J., Lamb, C. S., López, S., Mayes, R. W., 2004. Effects of carrier matrix and dosing frequency on digestive kinetics of even-chain alkanes and implications on herbage intake and rate of passage studies. J. Sci. Food Agr. 84, 1562-1570.

Giráldez, F. J., López, S., Lamb, C. S., Mayes, R. W., 2006. The use of even-chain alkanes sprayed onto herbage as rate of passage markers in goats. Livest. Sci. 100, 195-202.

Glindemann, T., Tas, B. M., Wang, C., Alvers, S., Susenbeth, A., 2009. Evaluation of titanium dioxide as an inert marker for estimating faecal excretion in grazing sheep. Anim. Feed Sci. Technol. 152, 186-197.

Glunk, E. C., Pratt-Phillips, S. E., Siciliano, P. D., 2013. Effect of restricted pasture access on pasture dry matter intake rate, dietary energy intake, and fecal pH in horses. J. Equine Vet. Sci. 33, 421-426.

Goachet, A. G., Philippeau, C., Varloud, M., Julliand, V., 2009. Adaptions to standard approaches for measuring total tract apparent digestibility and gastro-intestinal retention time in horses in training. Anim. Feed Sci. Technol. 152, 141-151.

Grovum, W. L., Williams, V. J., 1973. Rate of passage of digesta in sheep. 4. Passage of marker through the alimentary tract and the biological relevance of rate-constants derived from the changes of concentration of marker in faeces. Brit. J. Nutr. 30, 313-329.

Gudmundsson, O., Thorhallsdottir, A. G., 1998. Evaluation of *n*-alkanes for intake and digestibility determination in horses, in: Proceedings of the 9th European Intake Workshop, pp. 49-52.

Guo, N., Gao, J., He, Y., Zhang, Z., Guo, Y., 2014. Variations in leaf epicuticular *n*-alkanes in some *Broussonetia*, *Ficus* and *Humulus* species. Biochem. Syst. Ecol. 54, 150-156.

Hadjigeorgiou, I. E., Gordon, I. J., Milne, J. A., 2003. Intake, digestion and selection of roughage with different staple lengths by sheep and goats. Small Ruminant Res. 47, 117-132.

Haenlein, G. F. W., Smith, R. C., Yoon, Y. M., 1966. Determination of the fecal excretion rate of horses with chromic oxide. J. Anim. Sci. 25, 1091-1095.

Hallas, L. E., Vestal, J. R., 1978. The growth of *Mycobacterium convolutum* on solid *n*-alkane substrates: effect on cellular lipid composition. Can. J. Microbiol. 24, 1197-1203.

Hamilton, R. J., Long, M., Raie, M. Y., 1972. Sperm whale oil. Part 3: alkanes and alcohols. J. Am. Oil Chem. Soc. 49, 307-310.

Hankin, L., Kolattukudy, P. E., 1968. Metabolism of a plant wax paraffin (*n*-nonacosane) by a soil bacterium (*Micrococcus cerificans*). J. Gen. Microbiol. 51, 457-463.

Harger, M., Zheng, L., Moon, A., Ager, C., An, J. H., Choe, C., Lai, Y.-L., Mo, B., Zong, D., Smith, M. D., Egbert, R. G., Mills, J. H., Baker, D., Swanson Pultz, I., Siegel, J. B., 2013. Expanding the product profile of a microbial alkane biosynthetic pathway. ACS Synth. Biol. 2, 59-62.

Hatt, J.-M., Mayes, R. W., Clauss, M., Lechner-Doll, M., 2001. Use of artificially applied *n*-alkanes as markers for the estimation of digestibility, food selection and intake in pigeons (*Columba livia*). Anim. Feed Sci. Technol. 94, 65-76.

Hatt, J.-M., Gisler, R., Mayes, R. W., Lechner-Doll, M., Clauss, M., Liesegang, A., Wanner, M., 2002. The use of dosed and herbage *n*-alkanes as markers for the determination of intake, digestibility, mean retention time and diet selection in Galapagos tortoises (*Geochelone nigra*). Herpetol. J. 12, 45-54.

Holland, J. L., Kronfeld, D. S., Sklan, D., Harris, P. A., 1998. Calculation of fecal kinetics in horses fed hay or hay and concentrate. J. Anim. Sci. 76, 1937-1944.

Holleman, D. F., White, R. G., 1989. Determination of digesta fill and passage rate from nonabsorbed particulate phase markers using the single dosing method. Can. J. Zool. 67, 488-494.

Holloway, P. J., 1969. Chemistry of leaf waxes in relation to wetting. J. Sci. Food Agr. 20, 124-128.

Huber, G. W., Dumesic, J. A., 2006. An overview of aqueous-phase catalytic processes for production of hydrogen and alkanes in a biorefinery. Catal. Today 111, 119-132.

Huhtanen, P., Kaustell, K., Jaakkola, S., 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48, 211-227.

Hummel, J., Scheurich, F., Gerken, M., Ortmann, S., Clauss, M., 2016. Size-dependent particle retention in the gastrointestinal tract of equids? A non-invasive approach, in: Proceedings of the 70th Conference of the Society of Nutrition Physiology, p. 63.

Hyslop, J. J., 2003. Partitioning degradation of feeds between different segments of the equine digestive tract, in: Alliston, J. (Ed.), Emerging Equine Science, Occasional Publication of the British Society of Animal Science, No. 32. Nottingham University Press, Nottingham, pp. 97-111.

Ince, J., Longland, A. C., Newbold, J. C., Harris, P. A., 2011. Changes in proportions of dry matter intakes by ponies with access to pasture and haylage for 3 and 20 hours per day respectively, for six weeks. J. Equine Vet. Sci. 31, p. 283.

Jagger, S., Wiseman, J., Cole, D. J. A., Craigon, J., 1992. Evaluation of inert markers for the determination of ileal and faecal apparent digestibility values in the pig. Brit. J. Nutr. 68, 729-739.

Jetter, R., Schäffer, S., 2001. Chemical composition of the *Prunus laurocerasus* leaf surface. Dynamic changes of the epicuticular wax film during leaf development. Plant Physiol. 126, 1725-1737.

Jones, R. J., Ludlow, M. M., Troughton, J. H., Blunt, C. G., 1979. Estimation of the proportion of C_3 and C_4 plant species in the diet of animals from the ratio of natural ^{12}C and ^{13}C isotopes in the faeces. J. Agr. Sci. 92, 91-100.

Jurjanz, S., Germain, K., Dziurla, M. A., Juin, H., Jondreville, C., 2014. Use of acid-insoluble ash and *n*-alkanes as markers of soil and plant ingestion by chickens. Anim. Feed Sci. Technol. 188, 92-101.

Kaske, M., von Engelhardt, W., 1990. The effect of size and density on mean retention time of particles in the gastrointestinal tract of sheep. Brit. J. Nutr. 63, 457-465.

Keli, A., Andueza, D., de Vega, A., Guada, J. A., 2008a. Validation of the *n*-alkane and NIRS techniques to estimate intake, digestibility and diet composition in sheep fed mixed lucerne:ryegrass diets. Livest. Sci. 119, 42-54.

Keli, A., Mayes, R. W., de Vega, A., 2008b. *In vitro* studies of the metabolism of [^{14}C]-*n*-alkanes using ruminal fluid of sheep as substrate. Animal 2, 1748-1752.

Keli, A., Olmos, G., de Vega, A., Guada, J. A., 2013. Net transfer of nutrients to the duodenum and disappearance of *n*-alkanes in the reticulo-rumen and the hindgut of sheep fed grass/legume combinations. Brit. J. Nutr. 109, 1765-1778.

Kerr, B. J., Weber, T. E., Ziemer, C. J., 2015. Dietary marker effects on fecal microbial ecology, fecal VFA, nutrient digestibility coefficients, and growth performance in finishing pigs. J. Anim. Sci. 93, 2183-2190.

Kienzle, E., Zeyner, A., 2010. The development of a metabolizable energy system for horses. J. Anim. Physiol. An. N. 94, e231-e240.

Koch, K., Ensikat, H.-J., 2008. The hydrophobic coatings of plant surfaces: epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. Micron 39, 759-772.

Kolattukudy, P. E., Hankin, L., 1966. Metabolism of a plant wax paraffin (*n*-nonacosane) in the rat. J. Nutr. 90, 167-174.

Kotb, A. R., Luckey, T. D., 1972. Markers in nutrition. Nutr. Abstr. Rev. 42, 813-845.

Kozloski, G. V., Stefanello, C. M., Mesquita, F. R., Alves, T. P., Ribeiro Filho, H. M. N., Almeida, J. G. R., Moraes Genro, T. C., 2014. Technical note: evaluation of markers for estimating duodenal digesta flow and ruminal digestibility: acid detergent fibre, sulfuric acid detergent lignin, and *n*-alkanes. J. Dairy Sci. 97, 1730-1735.

Krull, H.-D., 1984. Untersuchungen über Aufnahme und Verdaulichkeit von Grünfutter beim Pferd. Dissertation, Stuttgart-Hohenheim/Hannover.

Kunst, L., Samuels, A. L., 2003. Biosynthesis and secretion of plant cuticular wax. Prog. Lipid Res. 42, 51-80.

Kuntz, R., Kubalek, C., Ruf, T., Tataruch, F., Arnold, W., 2006. Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*). I. Energy intake. J. Exp. Biol. 209, 4557-4565.

Labinger, J. A., Bercaw, J. E., 2002. Understanding and exploiting C-H bond activation. Nature 417, 507-514.

LaCasha, P. A., Brady, H. A., Allen, V. G., Richardson, C. R., Pond, K. R., 1999. Voluntary intake, digestibility, and subsequent selection of Matua Bromegrass, Coastal Bermudagrass, and alfalfa hays by yearling horses. J. Anim. Sci. 77, 2766-2773.

Ladygina, N., Dedyukhina, E. G., Vainshtein, M. B., 2006. A review on microbial synthesis of hydrocarbons. Process Biochem. 41, 1001-1014.

Lechowicz, M. J., 1982. The sampling characteristics of electivity indices. Oecologia 52, 22-30.

Leiber, F., Kreuzer, M., Leuenberger, H., Wettstein, H.-R., 2006. Contribution of diet type and pasture conditions to the influence of high altitude grazing on intake, performance and composition and renneting properties of the milk of cows. Anim. Res. 55, 37-53.

Lennen, R. M., Braden, D. J., West, R. M., Dumesic, J. A., Pfleger, B. F., 2010. A process for microbial hydrocarbon synthesis: overproduction of fatty acids in *Escherichia coli* and catalytic conversion to alkanes. Biotechnol. Bioeng. 106, 193-202.

Lin, L. J., Luo, H. L., Zhang, Y. J., Shu, B., 2007. The effects, in sheep, of dietary plant species and animal live weight on the faecal recovery rates of alkanes and the accuracy of intake and diet composition estimates obtained using alkanes as faecal markers. J. Agr. Sci. 145, 87-94.

Lin, L. J., Luo, H. L., Zhang, Y. J., Wang, H., Shu, B., Hong, F. Z., 2009. The potential use of long-chain alcohols and fatty acids as diet composition markers: factors influencing faecal recovery rates and diet composition estimates in sheep. Animal 3, 1605-1612.

Lin, L. J., Zhu, X. Y., Jiang, C., Luo, H. L., Wang, H., Zhang, Y. J., Hong, F. Z., 2012. The potential use of *n*-alkanes, long-chain alcohols and long-chain fatty acids as diet composition markers: indoor validation with sheep and herbage species from the rangeland of Inner Mongolia of China. Animal 6, 449-458.

Linskens, H. F., Heinen, W., Stoffers, A. L., 1965. Cuticula of leaves and the residual problem, in: Gunther, F. A. (Ed.), Residue Reviews, Vol. 8. Springer Verlag, Berlin, pp. 136-178.

Longland, A. C., Ince, J., Harris, P. A., 2011. Estimation of pasture intake by ponies from liveweight change during six weeks at pasture. J. Equine Vet. Sci. 31, 275-276.

López, C. L., Celaya, R., Santos, A. S., Rodrigues, M. A. M., Osoro, K., Ferreira, L. M. M., 2016. Combination of long-chain alcohols and fatty acids with alkanes as faecal markers to estimate feed intake and digestibility in horses and cattle fed on grass-heathland vegetation communities. Can. J. Anim. Sci. 96, 221-231.

Lucas, Jr., H. L., Smart, Jr., W. W. G., Cipolloni, M. A., Gross, H. D., 1961. Relations between digestibility and composition of feeds and foods, Report no. S-45. North Carolina State University, Raleigh.

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., Mahajan, A., 2013. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol. 6, 666-677.

Mader, T. L., Teeter, R. G., Horn, G. W., 1984. Comparison of forage labeling techniques for conducting passage rate studies. J. Anim. Sci. 58, 208-212.

Malossini, F., Piasentier, E., Bovolenta, S., 1990. *n*-Alkane content of some forages. J. Sci. Food Agr. 53, 405-409.

Malossini, F., Bovolenta, S., Piasentier, E., Valentinotti, M., 1994. Variability of *n*-alkane content in a natural pasture and in faeces of grazing dairy cows. Anim. Feed Sci. Technol. 50, 113-122.

Marais, J. P., Figenschou, D. L., Escott-Watson, P. L., Webber, L. N., 1996. Administration in suspension-form of *n*-alkane external markers for dry matter intake and diet selection studies. J. Agr. Sci. 126, 207-210.

Marais, J. P., 2000. Use of markers, in: D'Mello, J. P. F. (Ed.), Farm Animal Metabolism and Nutrition. CABI Publishing, New York, pp. 255-277.

Martins, H., Elston, D. A., Mayes, R. W., Milne, J. A., 2002. Assessment of the use of *n*-alkanes as markers to describe the complex diets of herbivores. J. Agr. Sci. 138, 425-434.

Matis, J. H., 1972. Gamma time-dependency in Blaxter's compartmental model. Biometrics 28, 597-602.

Mayer, A. C., Stöckli, V., Huovinen, C., Konold, W., Estermann, B. L., Kreuzer, M., 2003. Herbage selection by cattle on sub-alpine wood pastures. Forest Ecol. Manag. 181, 39-50.

Mayes, E., Duncan, P., 1986. Temporal patterns of feeding behaviour in free-ranging horses. Behaviour 96, 105-129.

Mayes, R. W., Lamb, C. S., Colgrove, P. M., 1986. The use of dosed and herbage *n*-alkanes as markers for the determination of herbage intake. J. Agr. Sci. 107, 161-170.

Mayes, R. W., Lamb, C. S., Colgrove, P. M., 1988. Digestion and metabolism of dosed even-chain and herbage odd-chain *n*-alkanes in sheep, in: Proceedings of the 12th General Meeting of the European Grassland Federation, pp. 159-163.

Mayes, R. W., Giráldez, F. J., Lamb, C. S., 1997. Estimation of gastrointestinal passage rates of different plant components in ruminants using isotopically-labelled plant wax hydrocarbons or sprayed even-chain alkanes. P. Nutr. Soc. 56, p. 187.

Mayes, R. W., Dove, H., 2000. Measurement of dietary nutrient intake in free-ranging mammalian herbivores. Nutr. Res. Rev. 13, 107-138.

Mayland, H. F., Florence, A. R., Rosenau, R. C., Lazar, V. A., Turner, H. A., 1975. Soil ingestion by cattle on semi-arid range as reflected by titanium analysis of feces. J. Range Manage. 28, 448-452.

Maynard, L. A., Loosli, J. K., 1962. Animal Nutrition, 5th ed. McGraw-Hill Book Company, Inc., New York.

McCarthy, R. D., 1964. Mammalian metabolism of straight-chain saturated hydrocarbons. Biochim. Biophys. Acta 84, 74-79.

McGreevy, P. D., Webster, A. J. F., Nicol, C. J., 2001. Study of the behaviour, digestive efficiency and gut transit times of crib-biting horses. Vet. Rec. 148, 592-596.

McManus, W. R., Dudzinski, M. L., Arnold, G. W., 1967. Estimation of herbage intake from nitrogen, copper, magnesium and silicon concentrations in faeces. J. Agr. Sci. 69, 263-268.

Meier-Augenstein, W., 2002. Stable isotope analysis of fatty acids by gas chromatography-isotope ratio mass spectrometry. Anal. Chim. Acta 465, 63-79.

Menard, C., Duncan, P., Fleurance, G., Georges, J.-Y., Lila, M., 2002. Comparative foraging and nutrition of horses and cattle in European wetlands. J. Appl. Ecol. 39, 120-133.

Miller, R. M., Bartha, R., 1989. Evidence from liposome encapsulation for transport-limited microbial metabolism of solid alkanes. Appl. Environ. Microb. 55, 269-274.

Miyaji, M., Ueda, K., Hata, H., Kondo, S., 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. J. Anim. Sci. 92, 1574-1581.

Mohanta, J., Dey, D. G., Mohanty, N., 2014. Studies on lac insect (*Kerria lacca*) for conservation of biodiversity in Similipal Biosphere Reserve, Odisha, India. J. Entomol. Zool. Stud. 2, 1-5.

Molina, D. O., Matamoros, I., Pell, A. N., 2004. Accuracy of estimates of herbage intake of lactating cows using alkanes: comparison of two types of capsules. Anim. Feed Sci. Technol. 114, 241-260.

Moore-Colyer, M. J. S., Morrow, H. J., Longland, A. C., 2003. Mathematical modelling of digesta passage rate, mean retention time and *in vivo* apparent digestibility of two different lengths of hay and big-bale grass silage in ponies. Brit. J. Nutr. 90, 109-118.

Morais, J. A. S., Berchielli, T. T., de Vega, A., Queiroz, M. F. S., Keli, A., Reis, R. A., Bertipaglia, L. M. A., Souza, S. F., 2011. The validity of *n*-alkanes to estimate intake and digestibility in Nellore beef cattle fed a tropical grass (*Brachiaria brizantha* cv. Marandu). Livest. Sci. 135, 184-192.

Morikawa, M., Kanemoto, M., Imanaka, T., 1996. Biological oxidation of alkane to alkene under anaerobic conditions. J. Ferment. Bioeng. 82, 309-311.

Mortimer, C. E., Müller, U., 2007. Chemie. Das Basiswissen der Chemie, 9th ed. Georg Thieme Verlag, Stuttgart.

Moshtaghi Nia, S. A., Wittenberg, K. M., 2002. Evaluation of *n*-alkanes as markers for estimation of dry matter intake and digestibility in steers consuming all-forage or forage-concentrate diets. Can. J. Anim. Sci. 82, 419-425.

Motiuk, K., 1980. Wool wax hydrocarbons: a review. J. Am. Oil Chem. Soc. 57, 145-146.

Mowat, D., Watson, C. A., Mayes, R. W., Kelly, H., Browning, H., Edwards, S. A., 2001. Herbage intake of growing pigs in an outdoor organic production system, in: Proceedings of the Annual Meeting of the British Society of Animal Science, p. 169.

Myers, W. D., Ludden, P. A., Nayigihugu, V., Hess, B. W., 2006. Excretion patterns of titanium dioxide and chromic oxide in duodenal digesta and feces of ewes. Small Ruminant Res. 63, 135-141.

National Research Council (NRC), 2007. Nutrient Requirements of Horses. National Academy Press, Washington, DC.

Newman, J. A., Thompson, W. A., Penning, P. D., Mayes, R. W., 1995. Least-squares estimation of diet composition from *n*-alkanes in herbage and faeces using matrix mathematics. Aust. J. Agr. Res. 46, 793-805.

Newman, J. A., Cribari-Neto, F., Jensen, M. J., 1998. The sensitivity of *n*-alkane analysis to measurement error: implications for use in the study of diet composition. J. Agr. Sci. 131, 465-476.

Norbury, G. L., Sanson, G. D., 1992. Problems with measuring diet selection of terrestrial, mammalian herbivores. Aust. J. Ecol. 17, 1-7.

Nottle, M. C., Armstrong, J. M., 1966. Urinary excretion of silica by grazing sheep. Aust. J. Agr. Res. 17, 165-173.

Ohajuruka, O. A., Palmquist, D. L., 1991. Evaluation of *n*-alkanes as digesta markers in dairy cows. J. Anim. Sci. 69, 1726-1732.

O'Keefe, N. M., McMeniman, N. P., 1998. The recovery of natural and dosed *n*-alkanes from the horse. Anim. Prod. Aust. 22, p. 337.

Oliván, M., Ferreira, L. M. M., Celaya, R., Osoro, K., 2007a. Accuracy of the *n*-alkane technique for intake estimates in beef cattle using different sampling procedures and feeding levels. Livest. Sci. 106, 28-40.

Oliván, M., Ferreira, L. M. M., García, U., Celaya, R., Osoro, K., 2007b. Application of *n*-alkanes as diet composition markers in grazing/browsing goats and sheep: effect of using different faecal recovery corrections and plant species grouping approaches. Aust. J. Agr. Res. 58, 1013-1022.

Ordakowski, A. L., Kronfeld, D. S., Holland, J. L., Hargreaves, B. J., Gay, L. S., Harris, P. A., Dove, H., Sklan, D., 2001. Alkanes as internal markers to estimate digestibility of hay or hay plus concentrate diets in horses. J. Anim. Sci. 79, 1516-1522.

Orton, R. K., Hume, I. D., Leng, R. A., 1985. Effects of exercise and level of dietary protein on digestive function in horses. Equine Vet. J. 17, 386-390.

Owens, F. N., Hanson, C. F., 1992. External and internal markers for appraising site and extent of digestion in ruminants. J. Dairy Sci. 75, 2605-2617.

Pagan, J. D., Harris, P. A., Brewster-Barnes, T., Duren, S. E., Jackson, S. G., 1998. Exercise affects digestibility and rate of passage of all-forage and mixed diets in Thoroughbred horses. J. Nutr. 128, 2704S-2707S.

Palmgren Karlsson, C., Lindberg, J. E., Rundgren, M., 2000. Associative effects on total tract digestibility in horses fed different ratios of grass hay and whole oats. Livest. Prod. Sci. 65, 143-153.

Parkins, J. J., Snow, D. H., Adams, S., 1982. The apparent digestibility of "complete diet" cubes given to Thoroughbreds horses and the use of chromic oxide as an inert faecal marker. Brit. Vet. J. 138, 350-355.

Patterson, D. P., Cooper, S. R., Freeman, D. W., Teeter, R. G., 2002. Technical note: estimation of fecal output and dry matter digestibility using various chromic oxide marker methods in the horse. Prof. Anim. Sci. 18, 176-179.

Peiretti, P. G., Meineri, G., Miraglia, N., Mucciarelli, M., Bergero, D., 2006. Intake and apparent digestibility of hay or hay plus concentrate diets determined in horses by the total collection of feces and *n*-alkanes as internal markers. Livest. Sci. 100, 189-194.

Piasentier, E., Bovolenta, S., Malossini, F., Susmel, P., 1995. Comparison of *n*-alkanes or chromium oxide methods for estimation of herbage intake by sheep. Small Ruminant Res. 18, 27-32.

Playne, M. J., Megarrity, R. G., LeFeuvre, R. P., 1978. Estimation by calcium analysis of the proportion of grass in oesophageal fistula samples from cattle grazing grass-stylo pastures. Aust. J. Exp. Agr. 18, 329-334.

Pond, K. R., Ellis, W. C., Matis, J. H., Ferreiro, H. M., Sutton, J. D., 1988. Compartment models for estimating attributes of digesta flow in cattle. Brit. J. Nutr. 60, 571-595.

Ratledge, C., 1984. Microbial conversions of alkanes and fatty acids. J. Am. Oil Chem. Soc. 61, 447-453.

Rayburn, E. B., Rayburn, S. B., 1998. A standardized plate meter for estimating pasture mass in on-farm research trials. Agron. J. 90, 238-241.

Reynhardt, E. C., Riederer, M., 1994. Structures and molecular dynamics of plant waxes. II. Cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L. Eur. Biophys. J. 23, 59-70.

Rogers, J. A., Odwongo, W. O., Conrad, H. R., 1985. Estimation of forage dry-matter intake in lactating dairy cows using a deuterium oxide dilution technique. J. Dairy Sci. 68, 2596-2601.

Romney, D. L., Sendalo, D. S. C., Owen, E., Mtenga, L. A., Penning, P. D., Mayes, R. W., Hendy, C. R. C., 1996. Effects of tethering management on feed intake and behaviour of Tanzanian goats. Small Ruminant Res. 19, 113-120.

Rosenfeld, I., Austbø, D., Volden, H., 2006. Models for estimating digesta passage kinetics in the gastrointestinal tract of the horse. J. Anim. Sci. 84, 3321-3328.

Rosenfeld, I., Austbø, D., 2009. Effect of type of grain and feed processing on gastrointestinal retention times in horses. J. Anim. Sci. 87, 3991-3996.

Sagalowicz, L., Leser, M. E., 2010. Delivery systems for liquid food products. Curr. Opin. Colloid In. 15, 61-72.

Sales, J., Janssens, G. P. J., 2003. The use of markers to determine energy metabolizability and nutrient digestibility in avian species. World Poultry Sci. J. 59, 314-327.

Sales, J., 2012. A review on the use of indigestible dietary markers to determine total tract apparent digestibility of nutrients in horses. Anim. Feed Sci. Technol. 174, 119-130.

Schäfers, S., Bulang, M., Dänicke, S., Zeyner, A., 2015. Effects of type and concentration of dietary fat on alkane recovery in broilers, in: Proceedings of the 69th Conference of the Society of Nutrition Physiology, p. 73.

Schiestl, F. P., Ayasse, M., Paulus, H. F., Löfstedt, C., Hansson, B. S., Ibarra, F., Francke, W., 1999. Orchid pollination by sexual swindle. Nature 399, 421-422.

Schirmer, A., Rude, M. A., Li, X., Popova, E., del Cardayre, S. B., 2010. Microbial biosynthesis of alkanes. Science 329, 559-562.

Schloerb, P. R., Friis-Hansen, B. J., Edelman, I. S., Solomon, A. K., Moore, F. D., 1950. The measurement of total body water in the human subject by deuterium oxide dilution. J. Clin. Invest. 29, 1296-1310.

Schmidt, L., Weißbach, F., Hoppe, T., Kuhla, S., 1999. Untersuchungen zur Verwendung der Kotstickstoff-Methode für die Schätzung des energetischen Futterwertes von Weidegras und zum Nachweis der selektiven Futteraufnahme auf der Weide. Landbauforsch. Volk. 49, 123-135.

Schmidt, L., Hoppe, T., Weissbach, F., 2004. Anwendung der Kotstickstoff- und der *n*-Alkan-Methode zur Ermittlung der Futter- und Energieaufnahme von Jungrindern auf der Weide. Landbauforsch. Volk. 54, 35-44.

Siciliano, P. D., 2012. Estimation of pasture dry matter intake and its practical application in grazing management for horses, in: Proceedings of the 10th Mid-Atlantic Nutrition Conference, pp. 9-12.

Silanikove, N., Holzer, Z., Cohen, D., Benjamin, R., Gutman, M., Meltzer, A., 1987. Interrelationship between metabolism of tritiated water, ^{22}sodium and dry matter intake by beef cattle consuming wheat straw and poultry litter in free choice. Comp. Biochem. Phys. A 88, 113-118.

Smith, D. G., Mayes, R. W., Raats, J. G., 2001. Effect of species, plant part, and season of harvest on *n*-alkane concentrations in the cuticular wax of common rangeland grasses from southern Africa. Aust. J. Agr. Res. 52, 875-882.

Smith, D. G., Mayes, R. W., Hollands, T., Cuddeford, D., Yule, H. H., Malo Ladrero, C. M., Gillen, E., 2007. Validating the alkane pair technique to estimate dry matter intake in equids. J. Agr. Sci. 145, 273-281.

Snyder, R. G., Goh, M. C., Srivatsavoy, V. J. P., Strauss, H. L., Dorset, D. L., 1992. Measurement of the growth kinetics of microdomains in binary *n*-alkane solid solutions by infrared spectroscopy. J. Phys. Chem.-US 96, 10008-10019.

Snyder, R. G., Clavell-Grunbaum, D., Strauss, H. L., 2007. Spinodal phase separation of unstable solid-state binary *n*-alkane mixtures. J. Phys. Chem. B 111, 13957-13966.

Stefanon, B., Volpelli, L., Bovolenta, S., Pinosa, M., 1999. Estimation of fescue hay intake in horses using the *n*-alkanes method. Zootec. Nutr. Anim. 25, 243-248.

Stevens, D. M., van Ryssen, J. B. J., Marais, J. P., 2002. The use of *n*-alkane markers to estimate the intake and apparent digestibility of ryegrass and Kikuyu by horses. S. Afr. J. Anim. Sci. 32, 50-56.

Sun, Z., Zhou, D., Ferreira, L. M. M., Zhong, Q., Lou, Y., 2008. Diet composition, herbage intake and digestibility in Inner Mongolian Cashmere goats grazing on native *Leymus chinensis* plant communities. Livest. Sci. 116, 146-155.

Sunvold, G. D., Cochran, R. C., 1991. Technical note: evaluation of acid detergent lignin, alkaline peroxide lignin, acid insoluble ash, and indigestible acid detergent fibre as internal markers for prediction of alfalfa, bromegrass, and prairie hay digestibility by beef steers. J. Anim. Sci. 69, 4951-4955.

Sutton, E. I., Bowland, J. P., McCarthy, J. F., 1977. Studies with horses comparing 4N-HCl insoluble ash as an index material with total fecal collection in the determination of apparent digestibilities. Can. J. Anim. Sci. 57, 543-549.

Svejcar, T. J., Judkins, M. B., Boutton, T. W., 1993. Technical note: labeling of forages with ^{13}C for nutrition and metabolism studies. J. Anim. Sci. 71, 1320-1325.

Takagi, H., Hashimoto, Y., Yonemochi, C., Asai, Y., Yoshida, T., Ohta, Y., Ishibashi, T., Watanabe, R., 2002a. Usefullness of chromic oxide index method for determination of digestibility of nutrients in feeds for Thoroughbreds. J. Equine Sci. 13, 19-22.

Takagi, H., Hashimoto, Y., Yonemochi, C., Asai, Y., Yoshida, T., Ohta, Y., Ishibashi, T., Watanabe, R., 2002b. Digestibility of nutrients of roughages determined by total feces collection method in Thoroughbreds. J. Equine Sci. 13, 23-27.

Thielemans, M. F., Francois, E., Bodart, C., Thewis, A., 1978. Mesure du transit gastrointestinal chez le porc à l'aide des radiolanthanides. Comparaison avec le mouton. Ann. Biol. Anim. Bioch. 18, 237-247.

Thomas, D. T., Rintoul, A. J., Masters, D. G., 2007. Sheep select combinations of high and low sodium chloride, energy and crude protein feed that improve their diet. Appl. Anim. Behav. Sci. 105, 140-153.

Titgemeyer, E. C., Armendariz, C. K., Bindel, D. J., Greenwood, R. H., Löest, C. A., 2001. Evaluation of titanium dioxide as a digestibility marker for cattle. J. Anim. Sci. 79, 1059-1063.

Todd, L. K., Sauer, W. C., Christopherson, R. J., Coleman, R. J., Caine, W. R., 1995. The effect of level of feed intake on nutrient and energy digestibilities and rate of feed passage in horses. J. Anim. Physiol. An. N. 73, 138-148.

Tulloch, A. P., 1970. The composition of beeswax and other waxes secreted by insects. Lipids 5, 247-258.

Turchiuli, C., Jimenez Munguia, M. T., Hernandez Sanchez, M., Cortes Ferre, H., Dumoulin, E., 2014. Use of different supports for oil encapsulation in powder by spray drying. Powder Technol. 255, 103-108.

Udén, P., Colucci, P. E., Van Soest, P. J., 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agr. 31, 625-632.

Udén, P., Rounsaville, T. R., Wiggans, G. R., Van Soest, P. J., 1982. The measurement of liquid and solid digesta retention in ruminants, equines and rabbits given timothy (*Phleum pratense*) hay. Brit. J. Nutr. 48, 329-339.

Unal, Y., Garnsworthy, P. C., 1999. Estimation of intake and digestibility of forage-based diets in group-fed dairy cows using alkanes as markers. J. Agr. Sci. 133, 419-425.

Valiente, O. L., Delgado, P., de Vega, A., Guada, J. A., 2003. Validation of the *n*-alkane technique to estimate intake, digestibility, and diet composition in sheep consuming mixed grain:roughage diets. Aust. J. Agr. Res. 54, 693-702.

Van Soest, P. J., 1967. Development of a comprehensive system of feed analyses and its application to forages. J. Anim. Sci. 26, 119-128.

van Weyenberg, S., Sales, J., Janssens, G. P. J., 2006. Passage rate of digesta through the equine gastrointestinal tract: a review. Livest. Sci. 99, 3-12.

van Weyenberg, S., Buyse, J., Janssens, G. P. J., 2007. Digestibility of a complete ration in horses fed once or three times a day and correlation with key blood parameters. Vet. J. 173, 311-316.

Vogg, G., Fischer, S., Leide, J., Emmanuel, E., Jetter, R., Levy, A. A., Riederer, M., 2004. Tomatoe fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. J. Exp. Bot. 55, 1401-1410.

Vulich, S. A., O'Riordan, E. G., Hanrahan, J. P., 1991. Use of *n*-alkanes for the estimation of herbage intake in sheep: accuracy and precision of the estimates. J. Agr. Sci. 116, 319-323.

Vulich, S. A., Hanrahan, J. P., 1995. Faecal sampling for the estimation of herbage intake using *n*-alkanes: evaluation of sample pooling and the use of rectal grab samples. J. Agr. Sci. 124, 79-86.

Warner, D., Ferreira, L. M. M., Breuer, M. J. H., Dijkstra, J., Pellikaan, W. F., 2013. Stable isotope labeled *n*-alkanes to assess digesta passage kinetics through the digestive tract of ruminants. PloS One 8, http://dx.doi.org/10.1371/journal.pone.0075496.

Wentzel, A., Ellingsen, T. E., Kotlar, H.-K., Zotchev, S. B., Throne-Holst, M., 2007. Bacterial metabolism of long-chain *n*-alkanes. Appl. Microbiol. Biot. 76, 1209-1221.

Werner, J., Umstatter, C., Zehner, N., Niederhauser, J. J., Schick, M., 2016. Validation of a sensor-based automatic measurement system for monitoring chewing activity in horses. Livest. Sci. 186, 53-58.

Wilson, H., Sinclair, A. G., Hovell, F. D., Mayes, R. W., Edwards, S. A., 1999. Validation of the *n*-alkane technique for measuring herbage intake in sows, in: Proceedings of the Annual Meeting of the British Society of Animal Science, p. 177.

Winstel, R., Rentschler, I., 1975. Elektronenmikroskopische Untersuchungen der Blattoberfläche verschiedener Futterpflanzen. Micron 6, 1-9.

Wood, S. J., Smith, D. G., Morriss, C. J., Cuddeford, D., 2012. The effect of pasture restriction on dry matter intake of foraging donkeys in the United Kingdom, in: Saastamoinen, M., Fradinho, M. J., Santos, A. S., Miraglia, N. (Eds.), Forages and graz-

ing in horse nutrition, EAAP Scientific Series, Vol. 132. Wageningen Academic Publishers, Wageningen, pp. 163-176.

Zengler, K., Richnow, H. H., Rosselló-Mora, R., Michaelis, W., Widdel, F., 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401, 266-269.

Zeyner, A., Kienzle, E., 2002. A method to estimate digestible energy in horse feed. J. Nutr. 132, 1771S-1773S.

Zeyner, A., 2005. Methodology to studying digestibility *in vivo*. Pferdeheilkunde 21, 27-28.

Zeyner, A., Kirchhof, S., Susenbeth, A., Südekum, K.-H., Kienzle, E., 2010. Protein evaluation of horse feed: a novel concept, in: Ellis, A. D., Longland, A. C., Coenen, M., Miraglia, N. (Eds.), The impact of nutrition on the health and welfare of horses, EAAP Publ. 128. Wageningen Academic Publishers, Wageningen, pp. 40-42.

Zeyner, A., Kirchhof, S., Susenbeth, A., Südekum, K.-H., Kienzle, E., 2015. A new protein evaluation system for horse feed from literature data. J. Nutr. Sci. 4, http://dx.doi.org/10.1017/jns.2014.66.

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9. Appendix

9.1. Formulary

This formulary contains a brief selection of equations that are commonly used in relation to feed intake, digestibility, diet composition and passage rate estimation procedures.

$$I = \frac{FO}{1-AD} \tag{1}$$

where, I is intake; FO is faecal output; and AD is apparent digestibility of DM, OM, energy or proximate nutrients (Dove and Mayes, 2006)

$$I_f = \frac{\frac{F_i}{F_j}(D_j + I_c \times C_j) - I_c \times C_i}{H_i - \frac{F_i}{F_j} \times H_j} \tag{2}$$

where, I_f is forage intake [kg DM/d] assuming that concentrate intake is known; H_i, C_i and F_i are concentrations of the plant marker (alkane) i in herbage, concentrate and faeces, respectively [mg/kg DM]; H_j, C_j and F_j are concentrations of the external marker (synthetic alkane) j in herbage, concentrate and faeces, respectively [mg/kg DM]; I_c is the known concentrate intake [kg DM/d]; and D_j is the dosage of j that is externally administered [mg/d] (Mayes *et al.*, 1986).

$$I_f = I_s \times \frac{P_f}{P_s} \tag{3}$$

where, I_f is forage intake [kg DM/d]; I_s is the known supplement intake [kg DM/d]; and P_f and P_s are the respective proportions of forage and supplement in the diet that are estimated using specific marker profiles of the diet components and non-negative least-squares optimization (Equation 11) of marker concentrations in feed and faeces (Dove and Charmley, 2008)

According to Giráldez *et al.* (2004), intake can be estimated using multi-compartmental model (Dhanoa *et al.*, 1985) parameters obtained from fitting to marker faecal excretion curves (or parameters obtained from fits of alternative models; see Moore-Colyer *et al.*, 2003; Rosenfeld *et al.*, 2006):

$$FO = \frac{24 \times K \times D_j}{A} \tag{4}$$

where, FO is faecal output [kg DM/d]; D_j is the single dose of an external marker [mg]; A is a scale parameter (obtained from the multi-compartmental model of Dhanoa *et al.*, 1985); and K is calculated as follows (Giráldez *et al.*, 2004)

$$K = \mathrm{K1} \times \mathrm{K2} \times \frac{\langle \prod_{i=3}^{N-1} (1 + \mathrm{K2}) / ((i-2) \times (\mathrm{K2} - \mathrm{K1})) \rangle}{(N-2) \times (\mathrm{K2} - \mathrm{K1})} \tag{5}$$

where, K1 and K2 are estimates of slow and fast fractional outflow rates of digesta; and N is the quantity of compartments (parameters are obtained from the multi-compartmental model of Dhanoa *et al.*, 1985, or alternative models) (Giráldez *et al.*, 2004)

$$I = \frac{F_j}{H_i / F_i} \tag{6}$$

where, I is intake [kg DM/d]; F_j is faecal output [kg DM/d] estimated from a single dose of external marker j; and H_i and F_i are internal marker concentrations [mg/kg DM] in forage and faeces, respectively (Giráldez *et al.*, 2004)

$$AD = \frac{I - FO}{I} \tag{7}$$

where, AD is apparent digestibility; I is intake; and FO is the faecal output of DM, OM, energy or proximate nutrients (Dove and Mayes, 2006)

$$AD = 1 - \frac{D_i}{F_i} \tag{8}$$

where, AD is apparent digestibility; and D_i and F_i are concentrations of internal marker i in the diet and faeces, respectively (Bergero *et al.*, 2009)

$$AD = 1 - \frac{D_i}{F_i} \times \frac{F_n}{D_n} \tag{9}$$

where, AD is apparent digestibility; D_i and F_i are concentrations of internal marker i in the diet and faeces, respectively; and D_n and F_n are concentrations of OM, energy or a nutrient n in the diet and faeces, respectively (Bergero *et al.*, 2009)

$$AD = \frac{(a + \ldots + n) - 1}{a + \ldots + n} \tag{10}$$

where, a, …, n are the components of a diet; the proportion of these components can be estimated by least-squares optimization as described in Equation 11 (Dove and Mayes, 2006)

$$\sum_{i=1}^{n} (M-E)^2 = \sum_{i=1}^{n} \left(\frac{F_i}{F_t} - \frac{a \times C1_i + b \times C2_i + c \times C3_i}{a \times C1_t + b \times C2_t + c \times C3_t} \right)^2 \min \quad (11)$$

where, M is the measured faecal proportion of marker i; E is the estimated dietary proportion of i ($E = i / t$); F_i is the faecal concentration of i; F_t is the concentration of total markers t in faeces; $C1_i$ to $C3_i$, and $C1_t$ to $C3t$ are concentrations of marker i, respectively total marker concentration t in diet components $C1$ to $C3$; and a, b and c are hypothetical intakes of diet components $C1$ to $C3$ (Ferreira *et al.*, 2007c)

$$\text{TMRT [h]} = \frac{\sum t_i \times c_i \times \Delta t_i}{\sum c_i \times \Delta t_i} \quad (12)$$

where, TMRT is total mean retention time [h] of marker/digesta within the digestive tract; t_i is the time delay [h] between administration of the marker and the midpoint of the ith collection interval; c_i is the marker concentration in the ith faeces sample; and Δt_i is the time interval [h] between two consecutive sampling times (Thielemans *et al.*, 1978)

$$\text{TT [h]} = \frac{\sum x_i \times t_i}{\sum x_i} \quad (13)$$

where, TT is the transit time of a marker [h] throughout the digestive tract; t_i is the time delay [h] between administration of the marker and the time of faeces/chymus collection; and x_i is the marker quantity in each faeces sample (McGreevy *et al.*, 2001; based on Cummings and Wiggins, 1976)

9.2. Supplementary material

Supplementary material of Article 1

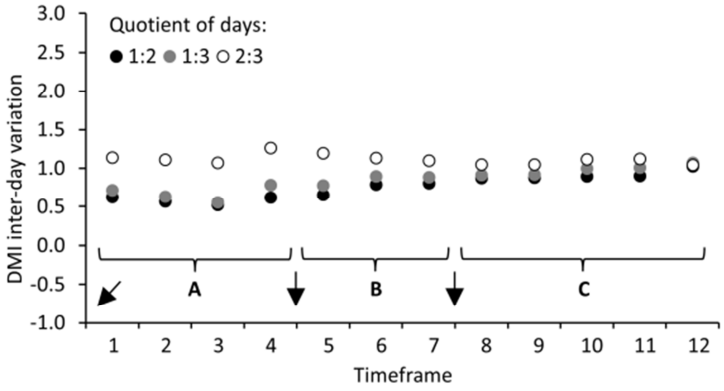

Fig. S1. Interday variation of least squares means of dry matter intake (DMI) estimates based upon the *n*-nonacosane : *n*-octacosane alkane pair: A, B and C denote periods following meals or bolus administration. Arrows indicate the time of meal presentation or bolus administration.

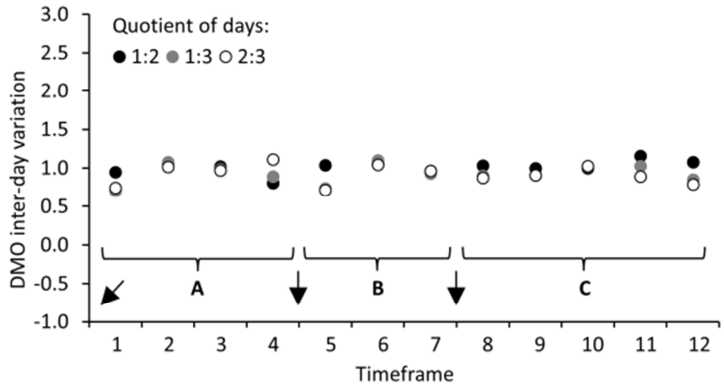

Fig. S2. Interday variation of least squares means of dry matter output (DMO) estimates based upon the product of single faeces quantity and daily defecation frequency: A, B and C denote feeding periods following meals. Arrows indicate the time of meal presentation.

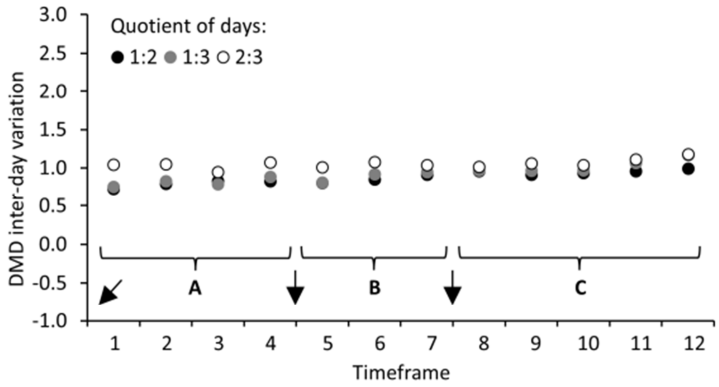

Fig. S3. Interday variation of least squares means of dry matter digestibility (DMD) estimates based upon *n*-nonacosane (C29): A, B and C denote feeding periods following meals. Arrows indicate the time of meal presentation.

Supplementary material of Article 3

Fig. S1. Step A of wax preparation: The weighing of one or multiple crystalline alkanes into a vessel. For a better visualization of the wax, the lipophilic pigment Sudan III (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added.

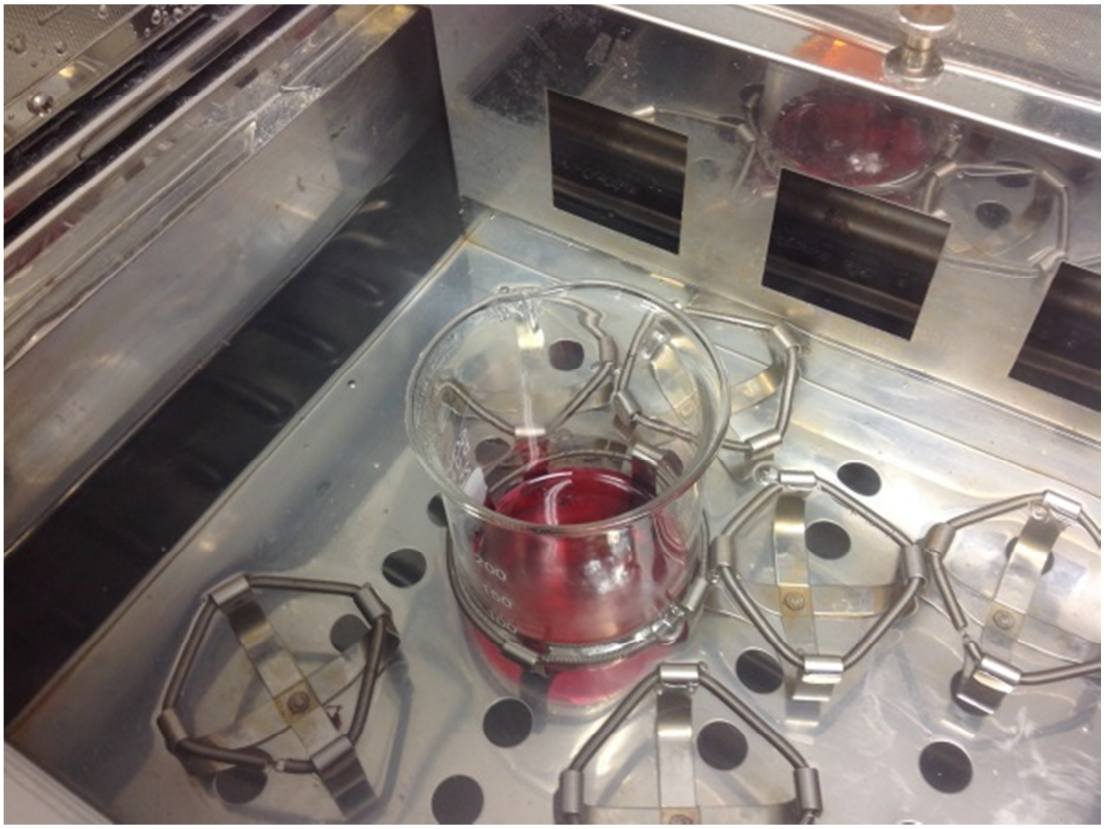

Fig. S2. Step B of wax preparation: The melting of the alkane or the mixture of multiple alkanes in a water bath.

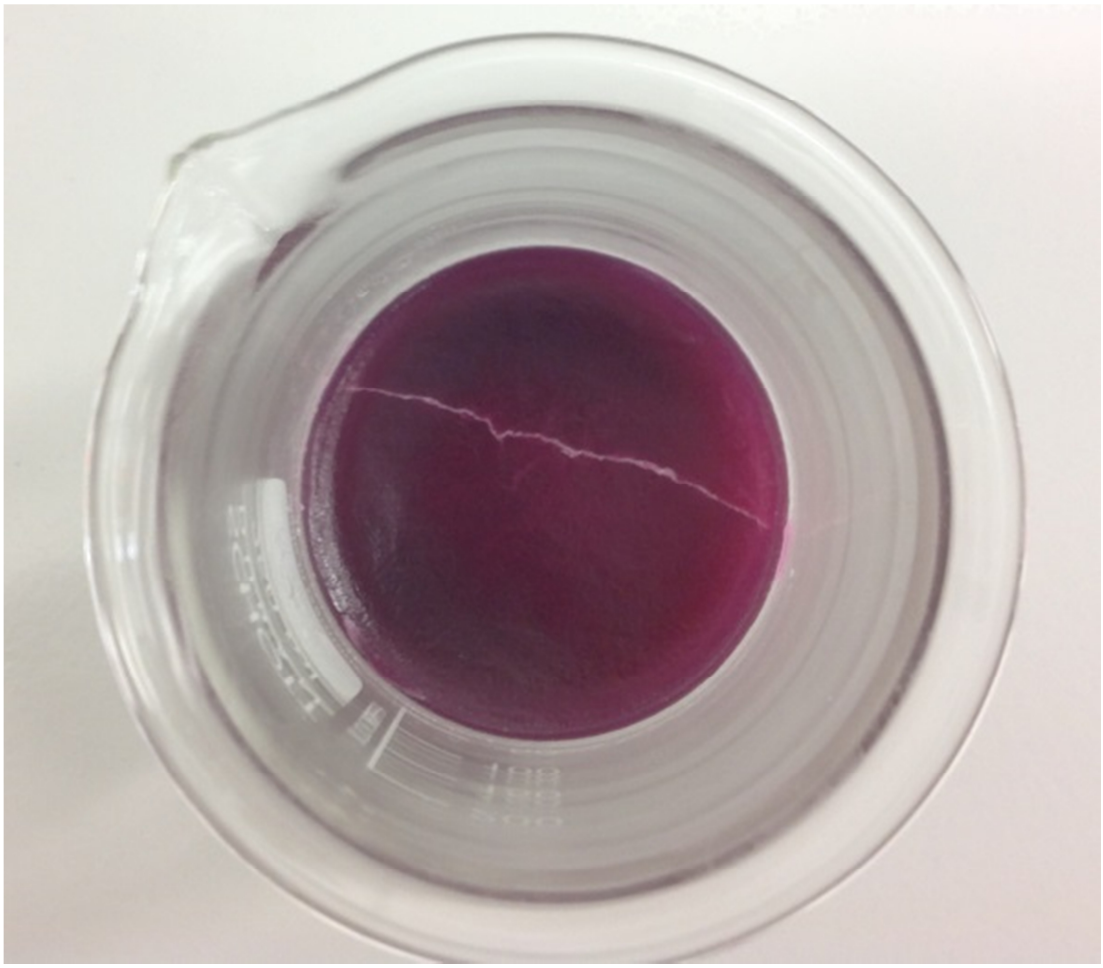

Fig. S3. Step C of wax preparation: The cooling of the molten alkanes at room temperature until formation and hardening of the wax.

Fig. S4. Step D of wax preparation: The crushing of the hardened wax to small fragments.

Eidesstattliche Erklärung / Declaration under oath

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Bachmann, M., Wensch-Dorendorf, M., Hoffmann, G., Steinhöfel, I., Bothendorf, S., Kemper, N., 2014. Pedometers as supervision tools for mares in the prepartal period. Appl. Anim. Behav. Sci. 151, 51-60.

Bachmann, M., Wensch-Dorendorf, M., Wulf, M., Glatter, M., Siebmann, M., Bierögel, C., Schumann, E., Bulang, M., Aurich, C., Zeyner, A., 2016. Bolus matrix for administration of dietary markers in horses. Livest. Sci. 185, 43-49.

Bachmann, M., Wensch-Dorendorf, M., Mäder, K., Bulang, M., Zeyner, A., 2016. Preparation of synthetic alkane waxes and investigations on their suitability for application as dietary markers in farm animals. Livest. Sci. 185, 110-116.

Bachmann, M., Wensch-Dorendorf, M., Bulang, M., Zeyner, A., 2016. Impact of dynamics of faecal concentrations of plant and synthetic *n*-alkanes on their suitability for the estimation of dry matter intake and apparent digestibility in horses. J. Agr. Sci. 154, 1291-1305.

Glatter, M., Wiedner, K., Hirche, F., Mielenz, N., Hillegeist, D., Bochnia, M., Cehak, A., Bachmann, M., Greef, J. M., Glaser, B., Wolf, P., Breves, G., Zeyner, A., 2016. Fermentation characteristics along the gastrointestinal tract after feeding of Jerusalem artichoke meal to adult healthy Warmblood horses. J. Anim. Nutr. 1, available at http://animal-nutrition.imedpub.com/.

Conference contributions

Bachmann, M., Wensch-Dorendorf, M., Hoffmann, G., Steinhöfel, I., Bothendorf, S., Kemper, N., 2012. Analyse des Einsatzes von Pedometern zur Überwachung von Kühen und Stuten im präpartalen Zeitraum, in: Proceedings of the Deutsche Gesellschaft für Züchtungskunde/Gesellschaft für Tierzucht Gemeinschaftstagung, D21. Conference held in Halle (Saale), Germany, 12 – 13 Sep 2012.

Bachmann, M., Wensch-Dorendorf, M., Hoffmann, G., Steinhöfel, I., Bothendorf, S., Kemper, N., 2013. The use of pedometers as supervision tools for cows and mares in the prepartal period, in: Proceedings of the 16th International Congress in Animal Hygiene, pp. 411-413. Conference held in Nanjing, China, 05 – 09 May 2013.

Bachmann, M., Wensch-Dorendorf, M., Bulang, M., Zeyner, A., 2015. Suitability of plantal *n*-alkanes for the estimation of dry matter intake and apparent digestibility in horses – pilot study, in: Proceedings of the 69th Conference of the Society of Nutrition Physiology, p. 25. Conference held in Göttingen, Germany, 10 – 12 Mar 2015.

Glatter, M., Bochnia, M., Bachmann, M., Wiedner, K., Mielenz, N., Breves, G., Zeyner, A., 2015. Einfluss von Topinamburmehl auf das equine gastrointestinale Milieu, in: Proceedings of the Göttinger Pferdetage, p. 131. Conference held in Göttingen, Germany, 10 – 11 Mar 2015.

Glatter, M., Bochnia, M., Bachmann, M., Wiedner, K., Breves, G., Zeyner, A., 2015. Impact of Jerusalem artichoke meal on metabolites of microbial fermentation in the equine gastrointestinal tract, in: Proceedings of the International Scientific Conference on Probiotics and Prebiotics, p. 74. Conference held in Budapest, Hungary, 23 – 25 Jun 2015.

Glatter, M., Borewicz, K., Wiedner, K., Mielenz, N., Bochnia, M., Bachmann, M., Smidt, H., Glaser, B., Breves, G., Zeyner, A., 2015. Feeding of Jerusalem artichoke meal alters the

microbial community and fermentation products in the foregut of horses, in: Proceedings of the 19th Congress of the European Society of Veterinary and Comparative Nutrition, p. 73. Conference held in Toulouse, France, 17 – 19 Sep 2015.

Glatter, M., Borewicz, K., Wiedner, K., Mielenz, N., Bochnia, M., Bachmann, M., Smidt, H., Glaser, B., Breves, G., Zeyner, A., 2015. Feeding of Jerusalem artichoke meal alters the microbial community and fermentation products in the hindgut of horses, in: Proceedings of the 19th Congress of the European Society of Veterinary and Comparative Nutrition, p. 138. Conference held in Toulouse, France, 17 – 19 Sep 2015.

Bachmann, M., Wensch-Dorendorf, M., Bulang, M., Zeyner, A., 2015. Impact of dynamics of faecal concentrations of natural and synthetic *n*-alkanes on estimation of dry matter intake and apparent digestibility in horses, in: Proceedings of the 19th Congress of the European Society of Veterinary and Comparative Nutrition, p. 36. Conference held in Toulouse, France, 17 – 19 Sep 2015.

Bachmann, M., Wensch-Dorendorf, M., Wulf, M., Glatter, M., Siebmann, M., Bierögel, C., Schumann, E., Bulang, M., Aurich, C., Zeyner, A., 2016. Preparation of boluses for administration of dietary markers to horses and investigations on their suitability, in: Proceedings of the 70th Conference of the Society of Nutrition Physiology, p. 64. Conference held in Hannover, Germany, 08 – 10 Mar 2016.

Bachmann, M., Wensch-Dorendorf, M., Mäder, K., Bulang, M., Zeyner, A., 2016. Use of synthetic alkane waxes as single- or multi-component dietary markers in farm animals, in: Proceedings of the 70th Conference of the Society of Nutrition Physiology, p. 65. Conference held in Hannover, Germany, 08 – 10 Mar 2016.

Bochnia, M., Schaefer, S., Simroth, K., Glatter, M., Goetz, F., Boesel, M., Bachmann, M., Zeyner, A., 2016. Chewing frequency and moved distances of warmblood-type horses all-day grazing on an extensive pasture, in: Proceedings of the 70th Conference of the Society of Nutrition Physiology, p. 122. Conference held in Hannover, Germany, 08 – 10 Mar 2016.

Bachmann, M., Bochnia, M., Mielenz, N., Spilke, J., Souffrant, W. B., Azem, E., Schliffka, W., Zeyner, A., 2016. Impact of alpha-amylase supplementation on energy balance and performance of high-yielding dairy cows on moderate starch feeding, in: Proceedings of the 20th Congress of the European Society of Veterinary and Comparative Nutrition. Conference in Berlin, Germany, 15 – 17 Sep 2016.

Bochnia, M., Schaefer, S., Simroth, K., Glatter, M., Goetz, F., Boesel, M., Bachmann, M., Zeyner, A., 2016. The daytime chewing frequencies and activity patterns of permanently grazing warmblood type horses, in: Proceedings of the 20th Congress of the European Society of Veterinary and Comparative Nutrition. Conference in Berlin, Germany, 15 – 17 Sep 2016.

Glatter, M., Bochnia, M., Cehak, A., Bachmann, M., Breves, G., Zeyner, A., 2016. Compliance between fermentation characteristics in different segments of the terminal tract of horses fed a hay-oats diet alone or added with Jerusalem artichoke meal, in: Proceedings of the 20th Congress of the European Society of Veterinary and Comparative Nutrition. Conference in Berlin, Germany, 15 – 17 Sep 2016.

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Datum / Date Unterschrift / Signature