What happens in the bag?

Development and evaluation of a modified *in situ* protocol to estimate degradation of nitrogen and starch in the rumen

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To my father,

Evert de Jonge

(1927 – 2004)

Abstract

The most widely used method to estimate the rumen degradation of dietary components in feedstuffs is the *in situ* or *in sacco* method. This method is based on rumen incubation of substrate (feed) in nylon or dacron bags followed by rinsing and analysis of the residue. Small pores in the bag allow microbes to enter the bag whilst a variable portion of the feed is retained in the bag. The results are used to estimate the ruminal effective degradation (ED) that is used in several protein evaluation systems. The weaknesses of the *in situ* method are its low precision, the lack of standardization, and its inaccuracy. The accuracy can be divided in a bias with respect to the *in situ* method itself, and the difference between the *in situ* and *in vivo* degradation. The bias of the *in situ* method itself is related to several assumptions regarding the size and degradation rate of the washable fraction, secondary particle loss, and microbial contamination. The aim of this thesis was to examine possibilities to reduce this bias by modification of the *in situ* methodology.

The bias related to the assumptions regarding the washout fractionwas successfully reduced by developing and using a modified rinsing method that involves less vigorously shaking conditions and a solvent which mimics the rumen pH and osmolality. This modified rinsing method markedly reduced the soluble (S) fraction of N, especially for legume seeds, and the non-soluble washout (W-S) fraction, especially for starch, compared to the conventional method. Consequently, the estimation of the ED became less dependent on the assumptions regarding the degradation of the S and W-S fraction. *In vitro* results did not support the assumption of a much faster degradation of the W-S fraction of starch than that of the non-washout fraction of starch. The modified rinsing method also allowed measuring the *in situ* degradation of products that contain mainly small particles such as wheat yeast concentrates.

The bias related to the breakdown of particles was also successfully reduced by development and application of a combination of the modified rinsing method and an *in vitro* method that simulates particulate matter loss during incubation. This *in vitro* method was based on in situ results obtained with an inert marker (*i.e.*, silica gel) which showed that particulate matter loss during incubation was moderate and limited to particles smaller than approximately 40 µm. Correction for these losses decreased the estimated ED of feed ingredients used. This study also showed that the fractional degradation rate of starch in

grains was strongly affected by the process of secondary particulate loss when using the conventional method, and applying the modified rinsing method markedly reduced the error due to secondary particulate loss.

In comparison to the conventional method, the modified method resulted in an increase of the bias related to microbial contamination of the residues. The ratio between diaminopimelic acid (DAPA), a marker for bacterial protein, and N in the residues was higher when using the modified method than that in the conventional method. The results obtained for the modified method also indicated lysis of bacterial cells during rinsing. This bias led to a lower ED when using the modified method with the impact greatly depending on the degree of lysis of bacterial cells.

In summary, the modified method increased the non-washout fraction of N and starch of various feed ingredients, which offers the possibility to use it for a larger range of feed ingredients, and reduced the bias related to assumptions on the washout fraction and the breakdown of particles compared to the conventional method. On the other hand, the modified method increased the bias related to microbial contamination and enlarged the difference between the *in situ* and *in vivo* degradation.

Table of Content

Chapter 1

Introduction

Dairy production

The growth of the world population and improvement in welfare has led to a strong increase in global demand for and subsequent production of dairy products, from 482 million tonnes in 1982 to 754 million tonnes in 2012 (FAO, 2015). This increase in production was realized by both an increase in number of cattle and a higher productivity per animal. The increased number of cattle results in the use of more resources such as feed ingredients, water and soil resulting in an increased ecological impact (Steinfeld *et al*., 2006). Therefore, the dairy sector is focussing on increasing the efficiency and sustainability of the production system. At an animal level, the efficiency of production is mainly determined by the feed conversion efficiency. Also economically this is most relevant as feed costs are a major contribution to the total production costs. In addition to forage, economically optimal dairy diets often contain feed ingredients with a high protein and starch content, which in part can be consumed by humans. With the expected increase in global population and the consequential increase in demand for both dairy products and feed ingredients, the challenge is to increase dairy production, while minimizing the input of (human edible) feed ingredients, thus optimizing the efficiency of dairy production.

An efficient and profitable dairy production requires an accurate and precise match between the animal's requirements for nutrients and nutrient supply from the feed, so that over- and undersupply of nutrients are avoided. For this purpose, much research work has been and is conducted that focuses on the digestion and utilization of feed ingredients in dairy cows. This research has been the basis for the development of several feed evaluation systems such as the French PDI system (Verite *et al.*, 1979), the Dutch DVE/OEB system (Tamminga *et al.*, 1994; van Duinkerken *et al.*, 2011), the Scandinavian Norfor system (Volden, 2011), the American NRC (NRC, 2001) and the British FIM system (Thomas, 2004). These models estimate the nutritional value of feed ingredients which assist the farmer or nutritionist to compose an optimum diet based on the required animal production.

Feed evaluation systems

In the Netherlands, since 1991 the DVE/OEB system (Tamminga *et al.*, 1994) is the national protein evaluation system for dairy cows. For each ingredient, this system predicts the amount of protein available for absorption in the small intestine (DVE). The

requirements of a cow with a given body weight to produce a specific amount of milk and milk protein are expressed in grams of DVE per day.

The calculation of the amount of DVE for each feed ingredient is mainly based on a rumen model that describes the processes of microbial protein production, as well as passage and microbial degradation of feed components in the rumen of the dairy cow and the subsequent digestion in the small intestines. The rumen is an anaerobic compartment containing microbes which enables the cow to digest cell walls and produce volatile fatty acids and microbial protein. The consequence is that only a part of the dietary feed components will escape ruminal degradation and become available in the small intestine. The total amount of DVE is the sum of digestible dietary protein that escapes rumen degradation (DVBE) and the digestible microbial protein (DVME) after correction for metabolic nitrogen losses (DVMFE) (equation 1).

$$DVE = DVBE + DVME – DVMFE \quad (1)$$

Degradation of dietary components in the rumen depends on the rate of degradation by the microbes and the passage rate. The fractional degradation rate is usually estimated by the *in situ* method, and this method is subject of the present thesis. The passage rate describes the outflow of rumen content and therefore determines the time substrate is available for ruminal degradation. The effective rumen degradation (ED) of dietary components is positively related to their fractional degradation rate and negatively to their fractional passage rate. The fractional passage rate is usually measured by feeding, or introduction into the rumen, of labelled feed or feed components, mostly with a trace-element like cobalt or chromium, with subsequent measurement of its appearance in faeces (Arroyo and González, 2013) and estimation of the fractional passage rate based on these data using a mathematical model (for example the model of Dhanoa *et al.* (1985)). In more recent studies, the fractional passage rates of specific components in forages and concentrates were measured by an alternative technique, the stable isotope ratio (^{13}C:^{12}C) technique (Warner, 2013). Although the fractional passage rate is a crucial factor in the estimation of the ED, it is not a subject of this thesis.

Optimal production of microbial protein (DVME) depends on the balance between N and energy available for microbial synthesis. In the DVE/OEB system, this balance is

expressed as OEB which is calculated as the difference between microbial protein synthesised in the rumen based on available N (MREN) and microbial protein synthesised in the rumen based on available energy (MREE) (equation 2).

$$OEB = MREN - MREE \quad (2)$$

In situ method

The most widely used method to estimate the rumen degradation of dietary components in feedstuffs is the *in situ* or *in sacco* method which was first described by Quin *et al*. (1938). The method is based on rumen incubation of substrate (feed) in porous (nylon or dacron) bags which allows the influx of microbes leading to degradation of the feed components. After rumen incubation, nylon bags are routinely rinsed either by hand or washing machine. The objective of this rinsing step is to stop microbial activity and to remove rumen fluid and particularly rumen microorganisms (Michalet-Doreau and Ould-Bah, 1992). The effect of rinsing on the disappearance of substrate for the nylon bag is estimated by rinsing of non-incubated nylon bags, called zero-hour incubation. The fraction lost during rinsing is termed the W (*i.e.* washout) fraction and contains soluble components and small particles. Disappearance of feed components from the bag at incubation time t (D(t)) is often modelled as a first order equation as introduced by Ørskov and McDonald (1979) (equation 3).

$$D(t) = W + D \times (1 - \exp(-k_d \times t)) \quad (3)$$

where W is the washout fraction, D is the potential degradable fraction, and k_d is the fractional degradation rate of the D fraction. In general, the disappearance curve approaches an asymptote called the U (*i.e.* non-degradable) fraction at infinite incubation time (Figure 1). The U fraction is regarded to be the fraction of the nutrient which is non-degradable under rumen conditions.

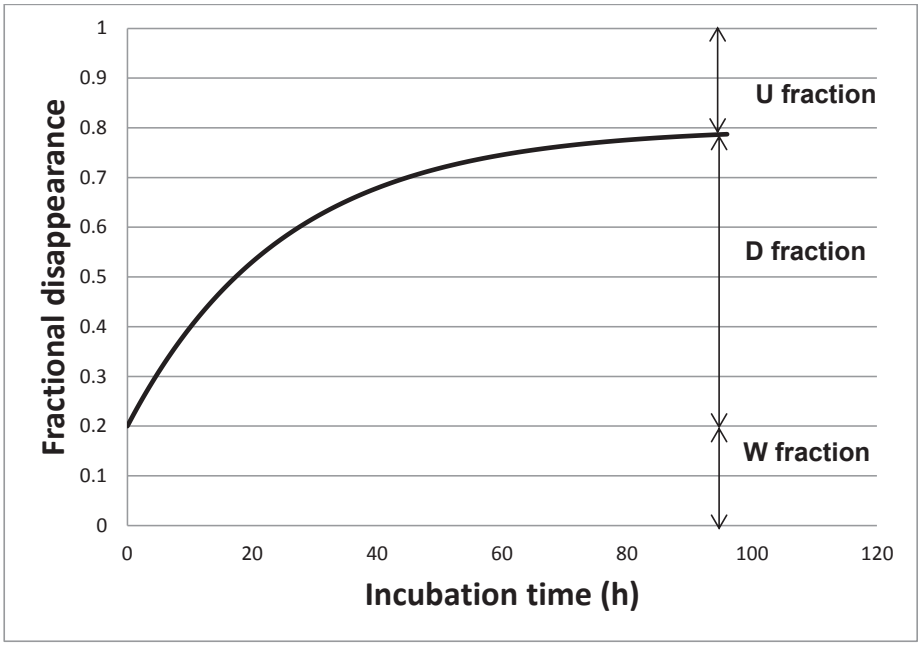

Figure 1 Example of *in situ* disappearance curve

It is important to emphasise that the *in situ* method only estimates the fractional degradation rate (k_d) of the D fraction. For protein, the W fraction may be distinguished into a fraction containing soluble components (*i.e.* S fraction) and a fraction containing small particles (*i.e.* W-S fraction) whereas for starch and NDF, the complete W fraction is regarded as insoluble (*i.e.* S fraction is zero and W fraction equal to W-S fraction). The *in situ* method is mostly used to estimate the ruminal degradation of organic matter (OM) and N (*i.e.* protein), but also to estimate the ruminal degradation of starch and NDF when applicable.

Estimation of effective rumen degradation

The effective rumen degradation (ED) of each nutrient in a feed is the sum of the ED of the different degradable fractions (*e.g.* S, W-S, and D). In the DVE/OEB system 2007 (van Duinkerken *et al.*, 2011), the following equation is used:

$$ED(nutrient) = S \times [k_d(S) / (k_p(S) + k_d(S))] + (W\text{-}S) \times [k_d(W\text{-}S) / (k_p(W\text{-}S) + k_d(W\text{-}S))] + D \times [k_d(D) / (k_p(D) + k_d(D))] \quad (4)$$

where $k_d(x)$ and $k_p(x)$ are the fractional degradation rate and fractional passage rate, respectively, of fraction x. As mentioned previously, the *in situ* method only estimates $k_d(D)$ which means that for the fractional degradation rates of both the S and W-S fraction (*i.e.* $k_d(S)$ and $k_d(W\text{-}S)$) assumptions have to be made to calculate total ED. The S fraction is only relevant for protein, and for starch and NDF this term is removed from equation 4. The fraction of bypass protein, *i.e.* protein that escapes ruminal degradation, is estimated as 1 – ED(protein). In the Dutch DVE/OEB system, this value is used to calculate the amount of dietary protein which is available for absorption in the small intestine (DVE in equation 1). The ruminal degradation of all relevant nutrients is used to calculate ruminal microbial protein production (DVME in equation 1).

Evaluation of the *in situ* method

The *in situ* method is widely used to estimate rumen degradation of nutrients and its results are used mainly in protein evaluation systems to calculate nutritive value. Despite its large scale use and acceptance, the *in situ* method has a number of methodological weaknesses that negatively affects the quality of its results. According to Vanzant *et al.* (1998), the greatest problem is low precision and lack of standardization, which negatively affects the comparability of the results obtained by various laboratories. This problem was clearly observed in several collaborative trials (Madsen and Hvelplund, 1994; Wilkerson *et al.*, 1995; Mathis *et al.*, 2001). In general, the quality of a method is judged by its precision and its accuracy. Basically, the precision is the variation between the repeated measurements, while the accuracy is the difference between the measured and the true value.

Precision

The *in situ* method contains several steps such as preparation of nylon bags, incubation in the rumen of different cows, rinsing of bags, and chemical analyses of the residue which all contribute to the variation in the final result. The variation caused by the incubation of the nylon bags in the rumen is largest compared to the other steps (Vanzant *et al.*, 1998). This variation comprises differences between individual bags, between animals, and between days. Based on several studies, Vanzant *et al.* (1998) showed that variation

between animals, days and bags contributed 40, 30, and 30% to the total variation, respectively. To reduce this variation, Nocek (1988) and Huntington and Givens (1995) emphasized the importance to minimize differences between animals regarding their physiological state and to standardize husbandry and environmental conditions. To investigate large numbers of feeds, the "complete exchange" method (Paine *et al.*, 1982) or "all-in all-out" method in which each single incubation time is performed at different days, is more efficient than "all-in" or "all-out" methods, but also negatively affects the precision as day to day variation is confounded with incubation time, increasing the variation for the estimate of k_d.

Accuracy

The aim of the *in situ* method is to estimate the true *in vivo* ruminal degradation of feed components. The accuracy of the *in situ* method can be divided into two aspects: the bias with respect to the *in situ* degradation itself and the difference between the *in situ* and *in vivo* degradation. In this thesis, the bias is the structural of systematic distortion of the method due to procedural matters. The *in situ* method estimates the in situ degradation within the nylon bag which is potentially biased by assumptions on the S and W-S fraction, secondary particulate loss and microbial contamination. Differences between the degradation conditions within and outside the nylon bag, such as pH and availability of macro- and microminerals, may lead to a difference between the *in situ* and *in vivo* degradation.

Assumptions on S and W-S fractions

In the past, S and W-S fractions were generally assumed to be very rapidly degradable and therefore fully contributing to the ED (Tamminga *et al.*, 1994). This general assumption, however, was a topic of discussion and the subject of further research work which led to modifications with respect to the k_d used for both fractions. The S fraction of N is still regarded as very fast degradable (DVE/OEB: 2.00 h^{-1}; FiM: 0.90 h^{-1}; Norfor: 1.50 h^{-1}), despite indications (*e.g.* Volden *et al.*, 2002) that a significant amount of soluble dietary N in grass and grass silage can escape rumen degradation. Resistance of soluble proteins to rumen fermentation was also observed *in vitro* (Broderick, 1987; Broderick and Graig, 1989; Messman *et al.*, 1994; Hedqvist and Udén, 2006). Degradation of the W-S fraction of N is

generally assumed to be equal to that of the D fraction which was partly based on similarity between the *in vitro* degradation of both fractions for several feeds (Gierus *et al.*, 2005). The W-S fraction of starch is still regarded as very fast degradable (DVE/OEB: $2 \times k_d(D) + 0.375$ h^{-1}; Norfor; 1.50 h^{-1}) partly based on the differences found between *in situ* and *in vivo* ED (van Duinkerken *et al.*, 2011). The very fast degradation of the W-S fraction, however, is not supported by *in vitro* gas production results of the different fractions (Yang *et al.,* 2005; Cone *et al.,* 2006; Stevnebø *et al.,* 2009).

The effect of these assumptions on accuracy depends on the presence of soluble components and small particles and, therefore, varies between feed ingredients. In general, a large S fraction for N can be found in fermented forages, such as grass and maize silages (Ali, 2013), and untreated legume seeds (Azarfar *et al.*, 2007). The W-S fraction is related to the presence of small particles, *i.e.* < 50 µm, in feedstuffs (Michalet-Doreau and Ould-Bah, 1992). In grains and legume seeds, starch is the dominant component in these small particles (Azarfar *et al.*, 2007), whereas for by-products from ethanol fermentation, such as wheat yeast concentrates, a large fraction of N is located in small particles. The size of the S and W-S fraction depends also on sample preparation, pore size of the nylon bag, and rinsing method. Reduction of the pore size of the sieve during grinding affects the particle size distribution and can increase the washout fraction as was observed for roughage (Lindberg and Knutsson, 1981), soybean meal (Nocek, 1985; Weakley *et al.*, 1983), distiller's grains (Weakley *et al.*, 1983) and for legume seeds, grains and hay (Michalet-Doreau and Cerneau, 1991). For fresh forages and silages, cutting or chopping fresh material instead of drying and grinding it, strongly reduces the washout fraction (Merwe *et al.*, 2005). Furthermore, the washout fraction obtained from different feed ingredients was positively related to the pore size of the nylon bags (Lindberg and Knutsson, 1981; Weakley *et al.*, 1983; Nocek, 1985). A higher severity of rinsing by using a washing machine leads to higher a washout fraction compared to a manual method, as shown for orchard grass hay and corn (Cherney *et al.*, 1990) as well as grass silage and maize silage (Cockburn *et al.*, 1994). This difference can also be observed for grains, as the washout fraction of starch from barley was 0.267 (Batajoo and Shaver, 1998) and 0.517 $g \cdot g^{-1}$ (Offner *et al.*, 2003) using manual and washing machine rinsing, respectively. More intensive rinsing, *e.g.* by repeated manual or washing machine rinsing, further increases the disappearance of substrate as observed by Coblentz *et al.* (1997) and Nguyen and Nguyen (1999).

In summary, major assumptions with respect to size and degradation of the soluble and non-soluble, washout fractions need to be made. The impact of these assumptions varies between feed ingredients and depends on experimental conditions such as sample preparation, pore size, and rinsing method.

Secondary particulate loss

Secondary particulate loss is the formation of new small particles (*e.g.* < 50 µm) by the breakdown of substrate that may be lost during the incubation or rinsing as described by Huhtanen and Sveinbjörnsson (2006) and Krämer *et al.* (2013). This loss leads to an overestimation of the true degradation and was observed for starch in different products (Huhtanen and Sveinbjörnsson, 2006). The effect of this secondary particulate loss on the accuracy is difficult to estimate because of the limited number of studies on this specific issue. The effect of the pore size on the k_d of the D fraction could be an indication of the impact of secondary particulate loss. A larger pore size increases loss of newly formed small particles leading to an increase of the k_d as observed for N in soybean meal (Nocek, 1985), and starch in maize and barley (Tothi *et al.*, 2003). The k_d of NDF in forages and concentrates was less affected by pore size (Krämer *et al.,* 2013) compared to the effects found for starch (Tothi *et al.*, 2003).

The impact of secondary particulate loss on the bias varies between feed ingredients and depends on the pore size of the nylon bag.

Microbial contamination

Microbial contamination is the consequence of the attachment of microbes to the substrate during the incubation in the rumen, leading to an underestimation of the *in situ* degradation of the substrate. Different studies with specific markers, *e.g.* diaminopimelic acid (DAPA), D-alanine, and purines, or ^{15}N labelling have shown the presence of microbial contamination in the substrate even after vigorous rinsing (Broderick and Merchen, 1992). This contamination concerns mostly solid associate bacteria (SAB) with high protein content (González *et al.*, 1998; González *et al.*, 2006) and, therefore, mainly affects the estimated degradation rate of N. A correction for the presence of microbial starch is suggested by McAllen and Smith (1994) but has not been adopted in *in situ* studies. The average fraction

of starch in rumen bacteria is generally lower than the average fraction of protein, and estimated to be around 0.05 $g \cdot g^{-1}$ bacteria DM (Volden, 2011).

The largest effect of microbial contamination on the accuracy is observed for feedstuffs with a low protein and a high fibre content (Michalet-Doreau and Ould-Bath, 1992; Vanzant *et al.*, 1996; Rodríguez and González, 2006). Arroya *et al.* (2013) found an increase of the ED of 0.271 $g \cdot g^{-1}$ for N in Italian ryegrass after correction for microbial contamination whereas for sunflower seed meal this increase was limited to 0.003 $g \cdot g^{-1}$. With respect to experimental conditions, a larger pore size of the nylon bag facilitates the influx of microbes, especially protozoa (Kayouli *et al.*, 1984; Lindberg *et al.*, 1984) whereas a vigorous rinsing method will remove these microbes more effectively.

The impact of microbial contamination on the bias varies between feed ingredients, is likely to be most pronounced for N in feed ingredients.

Difference between *in situ* and *in vivo* conditions

The *in situ* degradation is assumed to be equal to *in vivo* degradation. However, different studies as reviewed by López (2005) describe the conditions in the nylon bag within the rumen as less optimal for degradation compared to the true rumen environment. The nylon bag itself is a physical barrier between the substrate and rumen, which leads to differences in the type and number of microorganisms inside and outside the nylon bag, and an accumulation of end products such as volatile fatty acids and ammonia which consequently decrease the pH within the nylon bag and negatively affects the *in situ* degradation compared to the *in vivo* situation (Nozière and Michalet-Doreau, 2000). Pore size < 10 µm seems to limit the influx of microbes, especially protozoa, and therefore differs from the *in vivo* conditions (Kayouli *et al.*, 1984; Lindberg *et al.*, 1984). Carro *et al*. (1995) showed *in vitro* that the influx of protozoa was less when using the standard pore size (*i.e.* approximately 50 µm) compared to the use of a larger pore size (*i.e.* 100 µm), which negatively affected the degradation of NDF. The ratio between sample size and internal bag surface is regarded to be negatively related to degradation as was observed for cell wall in guinea grass by Udén and van Soest (1984). A ratio of 10 $mg \cdot cm^{-2}$ is a good simulation of the *in vivo* situation according to Vanzant *et al.* (1998). The diet consumed by the fistulated animals directly affects their microbial population in the rumen (Nocek, 1985). Weakley *et al.* (1983) observed a difference between the degradation of crude protein in soybean meal

caused by the ratio between roughage and concentrate in the diet. A larger amount of grain in the diet is related to lower estimates of rate and extent of *in situ* disappearance of forages (Nocek, 1988; Weiss, 1994). Rodríguez *et al.* (2000) found small effects of the feed intake on microbial composition in the rumen of sheep while Martin *et al.* (1994) showed a difference in the chemical composition of ruminal microbes after feeding with only hay compared to feeding a mixture of hay+barley (65:35).

The difference between *in vivo* and *in situ* degradation seems to be a general problem which concerns all feed ingredients. The study of Offner and Sauvant (2004) showed the difference between the degradation found by the *in situ* and *in vivo* method for starch in a large number of feedstuffs. This difference varied between feedstuffs and was pronounced for maize as shown by Hindle *et al.* (2005). Regarding to experimental conditions, the pore size and substrate to area ratio seems to affect the difference between *in vivo* and *in situ*.

The last two issues, *i.e.* microbial contamination and *in vivo vs. in situ* conditions, will lead to an underestimate of *in vivo* degradation when using the *in situ* method for all feedstuffs. This underestimation may, however, be compensated by the first two issues, *i.e.* current assumptions on fractional degradation rates of the S and W-S fraction and secondary particulate loss, all leading to an overestimate of *in vivo* degradation. The contribution of the first two issues to the bias within the *in situ* method strongly varies between feed ingredients which means a variation in the difference between *in vivo* degradation and *in situ* degradation for these products as was observed for starch in wheat and maize in the study of Hindle *et al.* (2005). For wheat, the presence of a large washout fraction and the assumptions made for kd of this fraction strongly reduced the difference between *in situ* and *in vivo* as compared to maize, which has a small washout fraction. For forages, Vanzant *et al.* (1996) found a higher *in situ* protein degradation than *in vivo*, which could also be related to the presence of a higher washout fraction, as the washout fraction was assumed to be total fermentable in these products. These differences in bias negatively affect the use of the *in situ* method and can potentially influence the ranking of feed ingredients based on their predicted *in vivo* degradation and therefore nutritional value.

Aims and outline of this thesis

The previous sections indicate that the difference between *in vivo* and *in situ* for feed ingredients significantly varies between feed ingredients and between nutrients, and is affected by experimental conditions used. This thesis focuses on factors related to the bias within the *in situ* method itself, in particular assumptions regarding the S and W-S fraction, secondary particulate loss, and microbial contamination. This variation in bias not only negatively affects the comparability of *in situ* results between institutes but can also influence the ranking of the nutritional value of feed ingredients based on *in situ* data. The difference between *in situ* and the *in vivo* degradation is very relevant but outside the direct scope of this thesis.

The aim of this thesis is to examine possibilities to reduce the bias within the *in situ* method itself by modification of the methodology. The first modification concerns the development of a new rinsing method that better reflects the conditions in the rumen and minimizes particulate matter loss. This makes the method less sensitive to assumptions on k_d of the washout fraction and reduces the bias caused by secondary particulate loss. The development and evaluation of this new rinsing method are described in Chapter 2 and 3 of this thesis. The second modification is to quantify particulate matter loss during rumen incubation. For this purpose a laboratory method that simulates particulate matter loss during incubation was developed. This can be used to correct observed disappearance of substrate from the bag, and therefore reduces the contribution of particle loss during incubation to total bias. The process of particulate matter loss during the incubation and the development of the correction method were conducted using an inert marker (silica gel), which is described in Chapter 4. In this Chapter, both modifications are also applied to estimate the *in situ* degradation of wheat yeast concentrates, a feed ingredient that mainly contains small particles (*i.e.* < 50 µm) and has a high washout fraction in the traditional *in situ* method. The effects of this new protocol on the *in situ* degradation of starch and protein in several legume seeds and grains and a comparison of this new protocol with the traditional method using washing machine rinsing are the subjects of Chapter 5 and 6. For starch, the *in situ* results are also compared to *in vitro* results to evaluate the assumptions related to the degradation of the W-S fraction. For protein, the effect of the new approach on microbial contamination compared to the standard *in situ* method was investigated as well. The general discussion (Chapter 7) evaluates the efforts of these modifications to

reduce the variation in the bias and describes a mathematical model to describe the different processes during the *in situ* incubation and evaluates the consequences for feed evaluation.

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Chapter 2

Effects of pH, temperature and osmolality on the level and composition of soluble N in feedstuffs for ruminants

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Abstract

Solubility of N is an important parameter in many protein evaluation systems for ruminants. The influence of different rumen conditions, such as pH, osmolality and temperature of solvents, on solubility of N compounds in various animal feed ingredients was examined in two experiments. In the first experiment, 17 feedstuffs were evaluated including legumes, oilseeds, grains, ryegrass and silages. Solvents were tap water at 18 and 38˚C, borate-phosphate buffer of pH 6.8 and sodium acetate buffer of pH 5.0, both at 38˚C. In the second experiment N, solubility of soybeans, soybean meal, peas, faba beans and lupins was evaluated in buffers at 4 pH values (5.0, 5.6, 6.2 and 6.8) and 2 osmolality values (300 and 400 mOsm/l) at 38˚C.The N solubility in feedstuffs was affected by the pH and osmolality of the solvent used, with the largest effects related to pH. Nitrogen solubility decreased upon reduction in pH from 6.8 to 5.0 for many feeds investigated, except for grass silage, maize silage, formaldehyde treated feeds, rapeseed meal and maize. The largest effect was for untreated legumes, where the average solubility as a fraction of the total N decreased from 0.43 at pH 6.2 to 0.26 at pH 5.6. The pH did not affect the NPN expressed as a fraction of total N, but NPN as a fraction of soluble N increased from 0.35 at pH 6.2 to 0.67 at pH 5.0. SDS-Page electrophoresis of the soluble N fraction of lupins, peas and soybeans showed that the decline in pH increased the ratio between albumins and storage globulins, especially 11S, in this fraction. This decline also decreased the proportion of 11S globulins of the soluble N fraction in faba beans. The effect of osmolality on N solubility was less pronounced than that of pH. Increasing osmolality from 300 to 400 mOsm/l generally increased N solubility. However, a significant pH – osmolality interaction for beans, lupins and peas occurred because osmolality did not affect N solubility at the highest pH level. Results show that pH of the solvent, and to a lesser extent osmolality, affect N solubility, and suggest that evaluation of CP degradation in the rumen requires consideration of rumen fluid pH and osmolality.

Keywords: Soluble nitrogen; rumen degradability; pH levels; osmolality

Introduction

Feed protein ingested by ruminants is subject to extensive degradation in the rumen. Correct estimation of the rate and extent of crude protein (CP) degradation in the rumen is

an essential element in current protein evaluation systems for ruminants. Increasing the efficiency of protein use by ruminants, resulting in a lower N excretion, is becoming an environmental imperative in many countries, which requires proper estimation of CP degradation in the rumen (Dijkstra *et al*., 2007). In current protein evaluation systems, feed protein fractionation systems have been adopted based on rumen *in situ* techniques and on solubility in buffers and detergent solutions. Solubility of CP is an important factor in determining rumen degradable CP (RDP). Some systems, including the DVE/OEB (Tamminga *et al.*, 1994), NRC (2001) and PDI (Verité *et al.*, 1979) systems, assume that the fraction of CP lost from *in situ* bags after washing with water (W fraction) in a washing machine is rapidly degraded in the rumen. This W fraction can be divided into two fractions (Gierus *et al*., 2005). The first fraction (S fraction) contains all soluble true protein (TP) and non-protein N (NPN), the latter including ammonia, amino acids and small peptides. The second fraction (W-S fraction) contains insoluble proteins in small particles, which escape from the *in situ* bag during washing. Although effective degradability using the *in situ* technique is often calculated assuming rapid or complete degradation of the W fraction, the rate of degradation of the S-fraction may be higher than that of the W-S fraction (Gierus *et al*., 2005; Yang *et al*., 2005).

In the Cornell Net Carbohydrate Protein System (CNCPS) (Sniffen *et al*., 1992), solubility of CP is determined by a borate phosphate solution at pH 6.8 and the soluble CP fraction is divided into NPN and true protein, the latter determined as the trichloroacetic acid (TCA) precipitable fraction. The choice for this buffer was based on its stable pH over prolonged storage and the high correlation coefficient (0.92) with insoluble N obtained with autoclaved rumen fluid (Krishnamoorthy *et al*., 1982) However, the characteristics of the solvent influence the type and amount of protein extracted. Kandylis and Nikokyris (1997) investigated the relationship between protein solubility in three solvents (McDougall's buffer; 0.02 M NaOH; 0.15 M NaCl) and *in situ* protein degradability of various feedstuffs. When the feedstuffs were categorised into groups of the same type of feedstuff (*i.e.* cereals, plant protein sources, and animal and fish by products), there was a high correlation (r = 0.64 to 0.91) between ruminal CP degradation and solubility in the three solvents.

Clearly rumen conditions in terms of pH and osmolality are not constant, but are influenced by diet, intake patterns and other feed and animal related characteristics. Consumption of high amounts of rapidly degradable carbohydrates may lower rumen pH to

values considerably lower than 6.8 in dairy cattle (Bach *et al*., 2007) as well as beef cattle (Nagaraja and Titgemeyer, 2007), and can increase the osmotic pressure to 400 $mOsm \cdot l^{-1}$, especially in combination with a high amount of soluble ash (Giger-Reverdin, 2000). This variation in pH and osmolality impacts the solubility of dietary N (Lakemond *et al*., 2000; Chavan *et al*., 2001). Therefore use of a single solvent, like a borate phosphate solution at pH 6.8 or water, and associated assumptions of fermentability of the soluble fraction, appears insufficient to accurately predict N excretion and milk urea content based on variation in dietary soluble CP content (Haig *et al*., 2002).

The aim of this study was to investigate the influence of pH, osmolality and temperature on the amount of N in the S and W fractions of various feedstuffs. For a small group of feedstuffs, the influence of pH and osmolality on the size and composition the S fraction for N was investigated.

Materials and methods

The effects of temperature, osmolality and pH were tested with various feedstuffs in two experiments. In the first experiment, the size of the W fraction and the influence of pH, osmolality and temperature on the solubility of N were determined in 17 ruminant feedstuffs. Based on the results from the first experiment, a number of feedstuffs were selected for a second experiment to investigate the effects of pH and osmolality on N solubility and on protein composition of the soluble fraction into more detail.

Sample preparation and chemical analyses

Feedstuffs in the experiments included legumes including soybeans, soybean meal (solvent extracted), Rumi-S (soybean meal, formaldehyde treated), peas, faba beans, lupins (a mixture of white and spotted lupins), oil seeds including rapeseed meal (solvent extracted), Rumi-Rape (rapeseed meal, formaldehyde treated), sunflower seed meal (solvent extracted), grains including maize, wheat, barley, oats, wet brewers grains, and forages including ryegrass, ryegrass silage, and maize silage. These feedstuffs were supplied by Nutreco B.V. (Boxmeer, The Netherlands). All feeds, except ryegrass, ryegrass silage, maize silage and wet brewers grains, were ground through a 3 mm sieve (Retsch ZM100, Haan, Germany) and stored at approximately 4°C. Grass, grass silage, maize silage and wet brewers

grains were stored at -20°C and were thawed before incubation. Grass, grass silage and maize silage were cut with a paper cutter at a length of approximately 1 cm.

First experiment

All feeds except soybeans were investigated during the first experiment. Nitrogen solubility in various solvents was determined by extracting N from each feedstuff with a solvent in a beaker under constant mechanical stirring during one hour in duplicate. For incubations at 38°C, the beakers were placed in a temperature controlled water bath. Incubations with tap water at 18°C were performed at room temperature. After incubation, the mixture was centrifuged at 3000 × *g* for 10 min at 20°C. The supernatant was removed and the absolute amount of N in the residue determined. The S fraction was calculated as the difference between the amount of N in the original sample and in the residue after incubation, and expressed as a proportion of the N in the original sample. The amount of sample and solvent used was designed to have a fairly constant ratio of feedstuff N to solvent. For feedstuffs with a high N content (*i.e.* legumes and oil seeds), 0.5 g of sample and 25 ml of solvent was used. For other feeds, 50 ml solvent was used in combination with 1 g for grains, 2 g for grass silage and 3 g for grass, grass silage, maize silage and brewers grains. The solvents used during the first experiment were tap water (pH 7.5; 9 $mOsm \cdot l^{-1}$) at 18 and 38˚C, a borate-phosphate buffer pH 6.8 (12.2 g $NaH_2PO_4 \bullet H_2O$ and 8.91 g $Na_2B_4O_7 \bullet 10\ H_2O$ in 1 l; 276 $mOsm \cdot l^{-1}$) at 38°C and a sodium-acetate buffer pH 5.0 (10 g sodium acetate in 1 l adjusted with 37% chloric acid; 286 $mOsm \cdot l^{-1}$) at 38°C.

The W fraction was determined according to the Dutch *in situ* protocol as described by Tas *et al*. (2006). Briefly, nylon bags with an inner size of 10 × 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30, Nybolt, Switzerland) were filled with approximately 5 g DM of feedstuffs in triplicate. The bags were washed in a programmable washing machine (AEG Turnamat, Nuremberg, Germany) with tap water at approximately 18°C using the gentle 'wool wash' program without centrifuging (40 min in approximately 80 l tap water with 3 swing turns). The bags were dried for 48 h at 70°C and weighed. Triplicates were pooled, ground through a 1 mm sieve (Retsch, ZM100) and analysed in duplicate for DM and N. The W fraction was calculated as the difference between the amount of N in the original sample and in the residue after washing.

Second experiment

The second experiment included the legumes soybeans, soybean meal, peas, faba beans and lupins. These feedstuffs were chosen because of the large pH effect on N solubility found in the first experiment. Soybeans were added to this experiment in view of the large effects of pH on N solubility in unprocessed legumes. The solvents used are described in Table 1 and cover 4 pH values (*i.e.* 5.0, 5.6, 6.2, 6.8) and 2 osmolality values (*i.e.* 300 and 400 mOsm·l^{-1}). The ratio between feedstuff and solvent was the same as in the first experiment (*i.e.* 0.5 g and 25 ml). The N solubility of the feedstuffs was measured four times at each pH and osmolality combination.

Chemical analyses

The DM content was determined by drying to a constant weight at 103°C (ISO 6496). Nitrogen was determined using a Kjeldahl method with $CuSO_4$ as the catalyst (ISO 5983).

The amount of NPN was measured by adding 2 ml of 400 g·l^{-1} TCA to the total supernatant (*i.e.* 25 ml) and mixed on a vortex to precipitate proteins. After 10 min, the solution was centrifuged at 3000 × *g* for 10 min at 20°C and the precipitate analysed for N. The NPN fraction in the sample was calculated by subtracting the amount of N in the precipitate from the total amount of soluble N in the sample, and was expressed as proportion of the total amount of N in the feedstuff. Determination of NPN was limited to pH 5.0 and 6.2 at 400 mOsm·l^{-1}, for the feedstuffs in the second experiment.

SDS-PAGE electrophoresis of soluble proteins

The molecular weights of the solubilized proteins at pH 5.0, 5.6, 6.2 and 6.8 at 300 mOsm/l of soybean, peas, faba beans and lupins were determined by SDS-PAGE electrophoresis. Because of the low N solubility, no electrophoresis was completed on soybean meal. Feedstuff, 0.2 g, was dissolved in 5.0 ml of buffer solution (see Table 1) for 1 h at 38°C, followed by centrifugation during 10 min at 3000 × *g* at 20°C. To increase detection of individual proteins, especially at pH 5.0 and 5.6, the ratio between the feedstuff and solvent was decreased compared to that in the first and second experiment. Proteins in the supernatant were analyzed by the Agilent Protein 80 kit (Agilent Technologies, Santa Clara, CA, USA). Briefly, this procedure contained the following steps: 4 µl supernatant was mixed with 2 µl denaturing solution containing 35 g·l^{-1} DDT (Agilent Technologies) and heated for 5

min at 90°C. This solution was diluted with 84 µl water and 6 µl was brought into the well of the protein chip. The chip was fully automatically analyzed by the 2100 Bioanalyzer (Agilent Technologies). The calibration solution included was used for the sizing of the proteins by the software. The relative amount of the different proteins was automatically quantified based on the peak area by the software.

Table 1 Composition and characteristics of solvents used in the second experiment

			Composition ($g \cdot l^{-1}$)			
Solvent	pH	$mOsmol \cdot l^{-1}$	Phosphate[a]	Borate[b]	Acetate[c]	NaCl
1	6.8	297	12.2	8.91		1.03
2	6.8	392	12.2	8.91		4.13
3	6.2[d]	299	12.2	8.91		
4	6.2[d]	397	12.2	8.91		2.94
5	5.6[d]	300			10.0	1.79
6	5.6[d]	394			10.0	4.99
7	5.0[d]	298			10.0	1.19
8	5.0[d]	396			10.0	4.44

[a] 12.2 $g \cdot l^{-1}$ $NaH_2PO_4 \bullet H_2O$

[b] 8.91 $g \cdot l^{-1}$ $Na_2B_4O_7 \bullet 10H_2O$

[c] 10.0 $g \cdot l^{-1}$ sodium acetate

[d] pH adjusted by addition of 37% HCl

Soluble proteins from these legumes were characterised as albumins and storage globulins (7S and 11S) based on data from literature (Carbonaro *et al*., 2005; Freitas *et al*., 2000; Hou and Chang, 2004; Le Gall *et al*., 2005; Magni *et al*., 2007; Spilatro *et al*., 1996).

Statistical analyses

Analysis of variance was conducted using the GLM procedure of SAS (2002). In the first experiment, the effect of treatment (*i.e.* cold water; hot water; Borate-phosphate buffer; sodium acetate buffer) on measured N solubility was analyzed by feedstuff. For the second experiment, the effect of pH, osmolality and their interaction on N solubility, and the effect of pH on the composition of the soluble N fraction were tested. When treatment effects were detected (*i.e.* $P < 0.05$), Tukey's test was used to test for pairwise comparisons between treatments.

Results

First experiment

For most feedstuffs investigated, treatments affected *(*$P < 0.05$) N solubility (Table 2). Only for Rumi-S, Rumi-Rape, brewers grains and maize, did treatments not influence N solubility. For both Rumi-S and Rumi-Rape, the soluble N fraction was especially low (< 0.05) in all treatments. Effects for the other feeds can be separated into an effect of water temperature (*i.e.* water 18°C *vs.* water 38°C), and an effect of buffer (*i.e.* pH and osmolality of solvents being water 38°C *vs.* borate-phosphate buffer *vs.* acetate buffer).

For all feedstuffs (except Rumi-S and sunflower seed meal), the W fraction was, as expected, larger than the S fraction measured with water at 18°C (Table 2). The smallest difference was approximately 0.02 $g \cdot g^{-1}$ of the total N (rapeseed meal) and the largest difference was approximately 0.36 $g \cdot g^{-1}$ of the total N (oats). For untreated legume seeds and grains, the difference was approximately 0.10 of total N.

Increasing the temperature of water from 18 to 38°C led to a numerically higher N-solubility for most feedstuffs (Table 2).

The final pH of the solutions for incubation with water at 18°C varied among feedstuffs (Table 3). As this pH was not influenced by the temperature of the solution (18 or 38°C), only the results for 18°C are reported. In general, the final pH of the solvent was lower than the initial value and, for dried feeds, varied between 5.6 and 6.4 whereas for silages the final pH was below 5.0. This change in pH value did not occur when the buffer solutions were used.

Table 2 Crude protein content, fraction of N washed out from *in situ* bags (W; $g \cdot g^{-1}$), and fraction of soluble N ($g \cdot g^{-1}$) in water of 18°C and 38°C, phosphate-borate buffer (pH 6.8), and acetate buffer (pH 5.0) (n = 2)

			Faction of soluble N				
	CP[1]	W	Water[2] 18°C	Water 38°C	Buffer pH 6.8	Buffer pH 5.0	SEM
SBM[3]	518	0.091	0.068	0.105	0.128	0.068	0.0108
Rumi-S	511	0.010	0.020	0.037	0.020	0.035	0.0096
RSM[4]	378	0.096	0.080^{a}	0.096^{a}	0.140^{b}	0.155^{b}	0.0032
Rumi-Rape	382	0.041	0.033	0.040	0.046	0.050	0.0049
SSM[5]	326	0.115	0.129^{ab}	0.201^{a}	0.177^{ab}	0.076^{b}	0.0208
Lupins	306	0.282	0.195^{ac}	0.271^{a}	0.569^{b}	0.096^{c}	0.0284
Faba beans	312	0.620	0.540^{a}	0.548^{a}	0.492^{b}	0.189^{c}	0.0032
Peas	261	0.667	0.575^{a}	0.586^{a}	0.520^{b}	0.242^{c}	0.0069
Oats	108	0.515	0.160^{a}	0.232^{ab}	0.264^{b}	0.155^{a}	0.0158
Maize	91	0.134	0.088	0.091	0.129	0.087	0.0128
Wheat	112	0.294	0.193^{a}	0.198^{ac}	0.263^{b}	0.229^{c}	0.0056
Barley	134	0.339	0.194^{a}	0.221^{ac}	0.274^{b}	0.232^{c}	0.0051
Brewers Grains	261	0.245	0.098	0.074	0.089	0.059	0.0068
Ryegrass	168	0.266	0.170^{a}	0.215^{a}	0.297^{b}	0.215^{a}	0.0081
Ryegrass silage	201	0.603	0.561^{a}	0.546a	0.485^{b}	0.550^{a}	0.0109
Maize silage	82	0.678	0.525^{ab}	0.529a	0.466^{b}	0.510^{b}	0.0110

N solubility means within a row with different letters differ ($P < 0.05$)

[1] Crude protein ($g \cdot kg^{-1}$ DM)

[2] pH = 7.8

[3] SBM: Soybean meal (solvent extracted)

[4] RSM: Rapeseed meal (solvent extracted)

[5] SSM: Sunflowerseed meal (solvent extracted)

Decreasing pH of the buffer from 6.8 to 5.0 at the same osmolality (about 280 mOsm/l), had a negative effect on the N solubility of most feedstuffs (Table 2). The biggest effect ($P < 0.05$) occurred for untreated legume seeds (*i.e.* faba beans, lupins, and peas), where the average solubility as a fraction of total N decreased from 0.53 $g \cdot g^{-1}$ at pH 6.8 to 0.17 $g \cdot g^{-1}$ at pH 5.0. A trend ($P < 0.10$) occurred for sunflower seed meal and brewers grains. In contrast, in silages the pH decrease from 6.8 to 5.0 increased N solubility.

The results for the N solubility in water (38°C) for the feedstuffs could not be correlated with the values found for any of the buffers (*i.e.* pH 6.8 and 5.0).

Table 3 Final pH after incubation of different raw materials with water of 18°C (pH 7.8) 1 h at room temperature (n = 2)

	Final pH	SE
Soybean meal	5.8	0.10
Rumi-S	6.2	0.15
Rapeseed meal	6.3	0.25
Rumi-R	6.7	0.05
Sunflowerseed meal	6.0	0.10
Lupins	6.3	0.05
Faba beans	5.5	0.05
Peas	6.2	0.15
Oats	5.6	0.05
Maize	6.7	0.10
Wheat	7.0	0.03
Barley	6.7	0.03
Brewers Grains	5.8	0.10
Ryegrass	5.1	0.10
Ryegrass silage	5.0	0.10
Maize silage	3.9	0.05

Second experiment

Nitrogen solubility was affected by pH of the solvent at both osmolality levels (Table 4). For untreated legumes (*i.e.* faba beans, lupins, peas, soybeans), a decline of the pH from 6.8 to 5.0 led to a decrease in solubility from 0.48 to 0.22 $g \cdot g^{-1}$ of total N. Especially between pH 6.2 and 5.6, a pronounced decrease of the N solubility occurred. Although N solubility in soybean meal was lower than in the other feedstuffs evaluated, it had a similar pattern.

The effect of the osmolality was much smaller than the pH effect, and varied among the feedstuffs. For soybeans and soybean meal, increasing the osmolality from 300 to 400 $mOsm \cdot l^{-1}$ had an effect on N solubility while for the other feeds there was an interaction

between osmolality and pH. For all feeds, however, the magnitude of the osmolality effect depended on the pH level of the solvent. Most effects occurred at pH 5.6 and 6.2.

Table 4 Soluble N (as a fraction of total N; $g \cdot g^{-1}$) of raw materials as affected by pH and osmolality of the solvent at 38°C (n = 4)

		pH					*P*		
	Osm/l	5.0	5.6	6.2	6.8	SEM	pH	Osm	pH × osm
Faba beans	300	0.211[a]	0.228[ax]	0.437[bx]	0.516[c]	0.0044	< 0.001	< 0.001	< 0.001
	400	0.213[a]	0.262[by]	0.463[cy]	0.507[d]				
Lupins	300	0.153[ax]	0.227[b]	0.440[c]	0.493[c]	0.0155	< 0.001	< 0.001	0.029
	400	0.237[ay]	0.266[a]	0.456[b]	0.479[b]				
Peas	300	0.261[a]	0.304[bx]	0.437[c]	0.529[d]	0.0051	< 0.001	< 0.001	0.019
	400	0.283[a]	0.334[by]	0.443[c]	0.527[d]				
Soybean[f]	300	0.166	0.208	0.331	0.395	0.0042	< 0.001	< 0.001	0.382[e]
	400	0.178	0.234	0.353	0.413				
SBM[g]	300	0.066	0.072	0.113	0.142[d]	0.0023	< 0.001	< 0.001	0.068[e]
	400	0.071	0.092	0.125	0.144				

[a,b,c,d] Means in the same row with different letters differ ($P < 0.05$)

[x,y] Means in the same column within a feedstuff with different letters differ ($P < 0.05$)

[e] pH 5.0 < 5.6 < 6.2 < 6.8 ($P < 0.05$)

[f] Protein content is 410 $g \cdot kg^{-1}$ DM.

[g] SBM: Soybean meal (solvent extracted)

The relative amount of NPN in the total N fraction of the feedstuff was not affected by decreasing the pH value from 6.2 to 5.0 (Table 5). For peas, a trend ($P < 0.10$) occurred, but the decline was still much smaller than the effect on the relative amount of soluble N in this pH range (Table 4). Consequently, the fraction of NPN relative to the soluble N increased for the feedstuffs investigated from an average of 0.35 $g \cdot g^{-1}$ at pH 6.2 to 0.67 $g \cdot g^{-1}$ at pH 5.0.

Table 5 Fraction of NPN in total soluble N ($g \cdot g^{-1}$) for different raw materials using a solvent with a pH of 5.0 and 6.2 at 400 mOsm/l (n = 2)

Raw material	pH		SE	*P*
	5.0	6.2		
Faba beans	0.168	0.165	0.0032	0.575
Lupins	0.049	0.039	0.0219	0.767
Peas	0.180	0.146	0.0057	0.054
Soybean	0.117	0.112	0.0053	0.612
Soybean meal	0.074	0.073	0.0050	0.950

Results of the electrophoresis analyses showed that the composition of individual proteins in the soluble fraction of soybean, peas, faba beans and lupins was also affected by the pH value of the solvent used (Figures 1 to 4 and Table 6). At pH 6.2 and 6.8, the storage globulins (7S and 11S) were the most abundant soluble proteins in these four feedstuffs. The solubility of these proteins strongly decreased at lower pH levels (*i.e.* 5.0 and 5.6). The proportion of 11S in the soluble N fraction decreased at these lower pH levels, while that of 7S, except for peas, was not affected by the pH. This indicates that the solubility of 11S, within faba beans, lupins and soybean, was relatively more affected than that of 7S. For lupins, peas and soybean, the fraction of 2S albumins increased from 0.07 to 0.22 of total soluble protein at pH 6.8 and pH 5.0, respectively. This effect was most pronounced for peas and soybean, where the 2S albumins (PA2 for peas and Kunitz trypsin inhibitor for soybean) were seen as the highest peaks at pH 5.0 and 5.6. The solubility of these proteins was not affected by pH, as seen from the response of these compounds (FU-units on the y-scale; Figures 3 and 4).

Table 6 Various soluble proteins (fraction of total soluble proteins) based on SDS electrophoresis peak area in selected feedstuffs at several pH values (n = 2)

			pH				
	Protein	Weight (kDa)	5.0	5.6	6.2	6.8	SEM
Faba beans	11S	18; 30	0.23[a]	0.55[b]	0.70[b]	0.71[b]	0.047
	7S	36-49	0.11	0.18	0.20	0.20	0.037
Lupins	11S	19-23; 34-38	0.44[a]	0.54[a]	0.69[b]	0.70[b]	0.027
	7S	25; 32; 47-59	0.27	0.27	0.22	0.24	0.012
	2S	17	0.17[a]	0.09[ab]	0.03[b]	0.02[b]	0.022
Peas	11S	20-21; 34	0.10[a]	0.21[b]	0.33[c]	0.39[c]	0.010
	7S	43-49	<0.01[a]	0.14[b]	0.25[c]	0.28[c]	0.009
	2S	22	0.38[a]	0.25[b]	0.17[b]	0.14[b]	0.034
Soybean	11S	21-22; 38-40	0.31[a]	0.35[a]	0.63[b]	0.70[b]	0.030
	7S	55-57; 80-85	0.20	0.30	0.22	0.18	0.053
	2S	19	0.24[a]	0.19[ab]	0.08[b]	0.06[b]	0.026
	Lectin	28	0.12[a]	0.06[b]	0.03[c]	0.03[c]	0.002

[a,b,c,d] Means in the same row with different letters differ ($P < 0.05$)

Discussion

This study shows that the N solubility of feedstuffs depends on the characteristics of the solvent, especially its pH. For most feeds, N solubility decreases as the pH drops from 6.8 to 5.0, which corresponds with the rumen physiological range. The largest pH effect occurred for untreated legumes (*i.e.* lupins, faba beans, peas, soybean) which contain high amounts of soluble proteins. For these feedstuffs, soluble N as a fraction of total N decreased from an average of 0.48 $g \cdot g^{-1}$ at pH 6.8 to 0.22 $g \cdot g^{-1}$ at pH 5.0.This decrease in solubility is caused by the increased attraction between the polypeptides (*i.e.* isoelectric point) leading to precipitation of proteins.

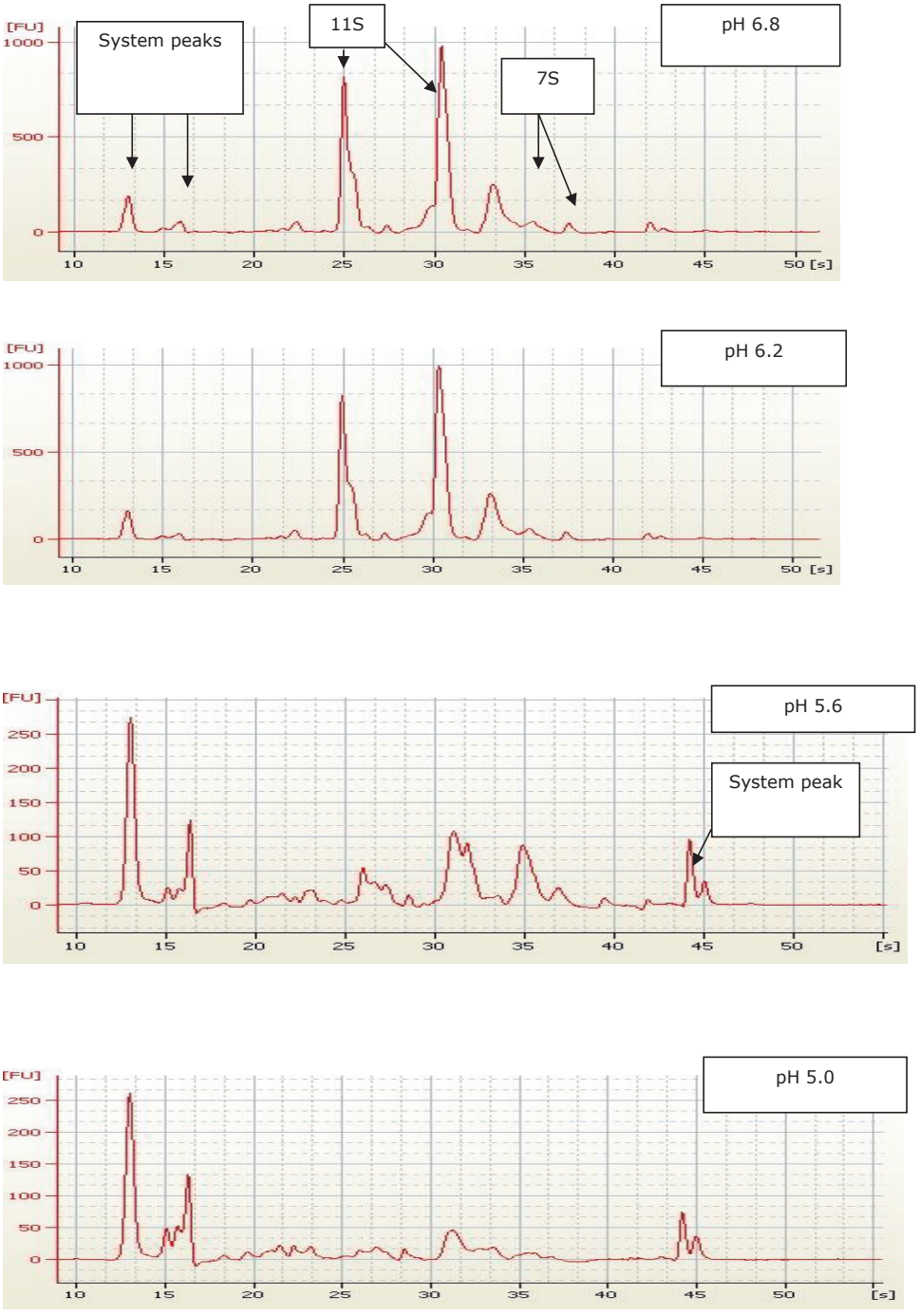

Figure 1 Electrophoresis pattern of soluble proteins of faba beans at pH 6.8, 6.2, 5.6 and 5.0 (from top to bottom)

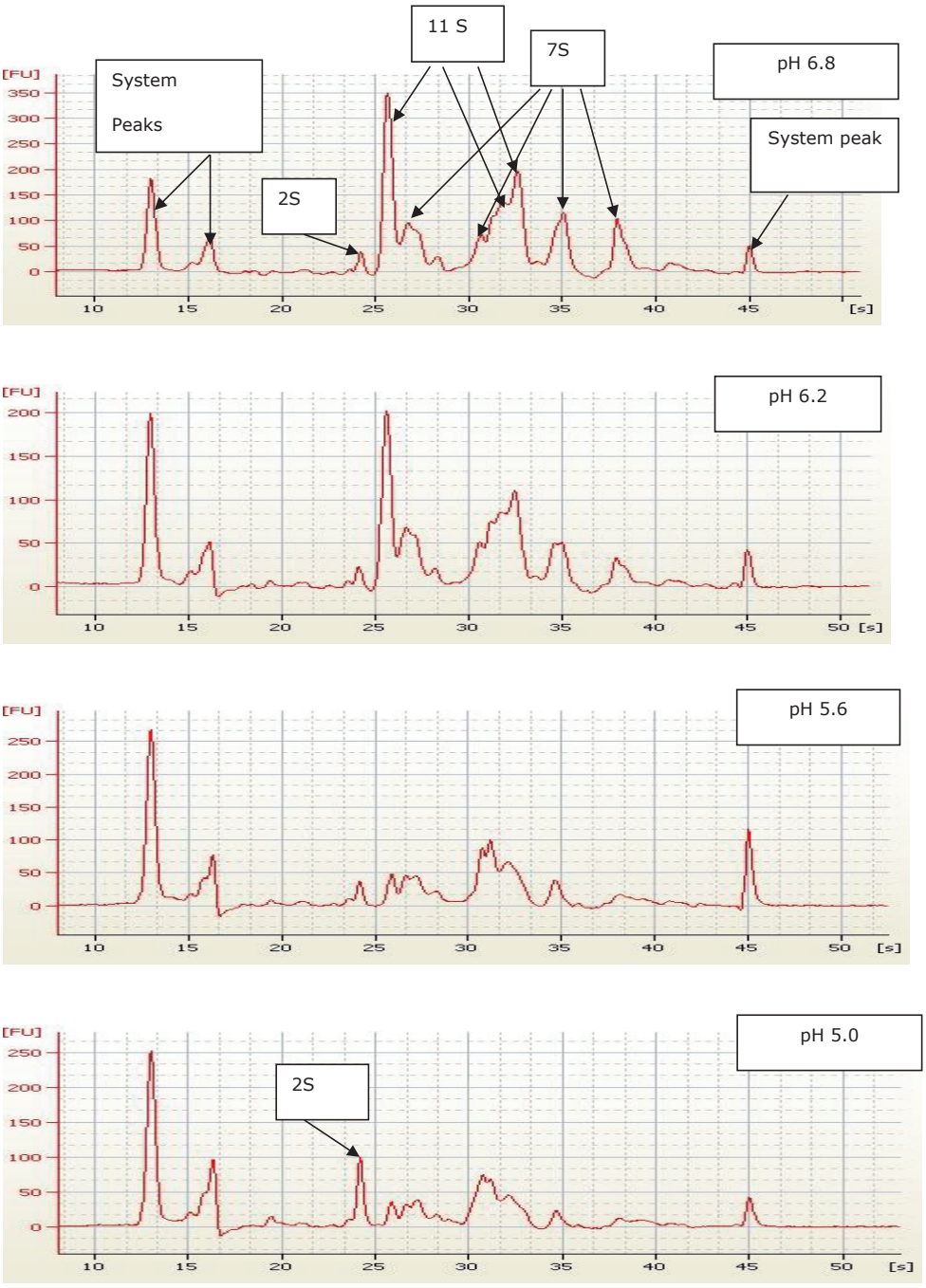

Figure 2 Electrophoresis pattern of soluble proteins of lupins at pH 6.8, 6.2, 5.6 and 5.0 (from top to bottom)

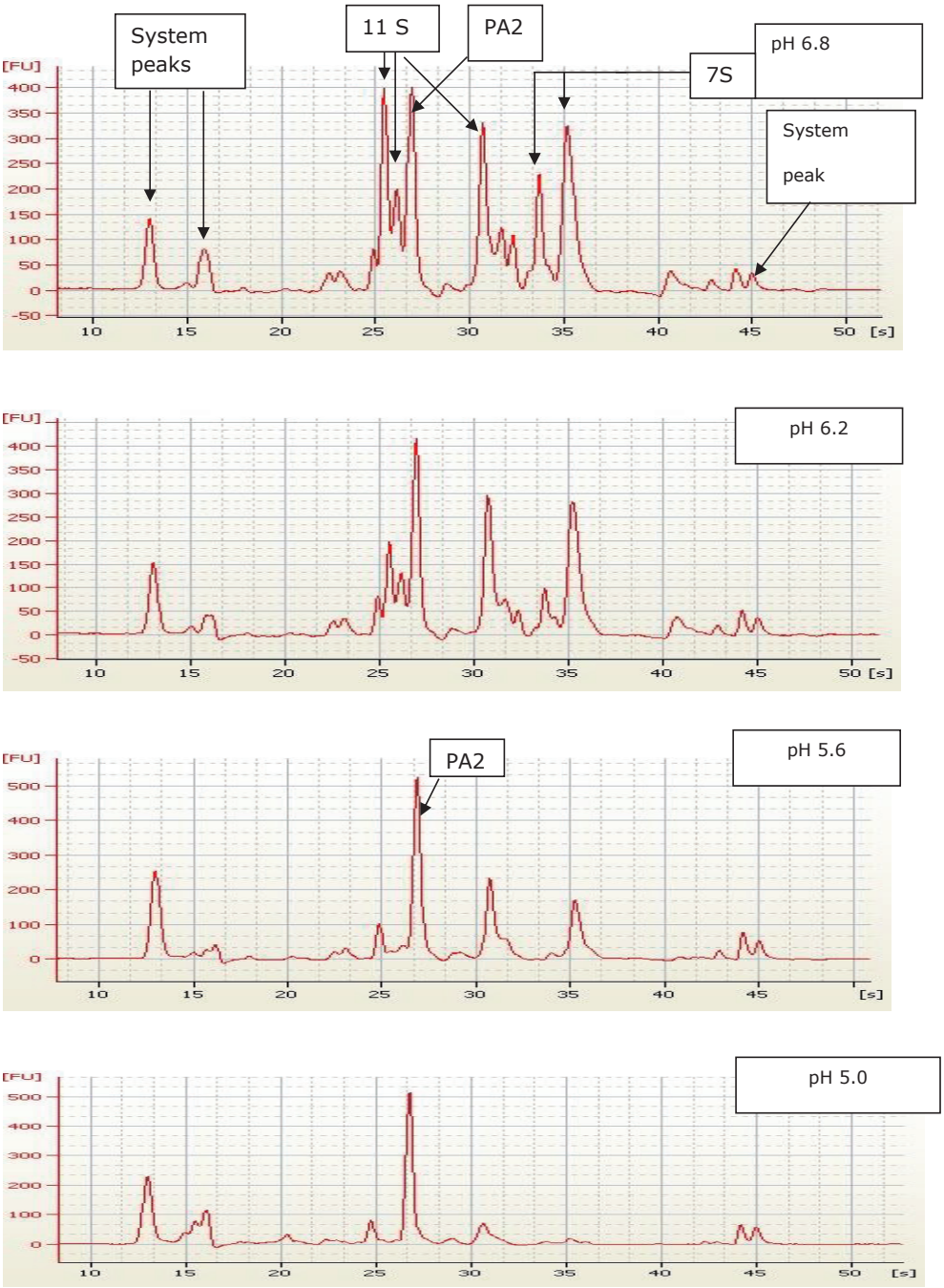

Figure 3 Electrophoresis pattern of soluble proteins of peas at pH 6.8, 6.2, 5.6 and 5.0 (from top to bottom)

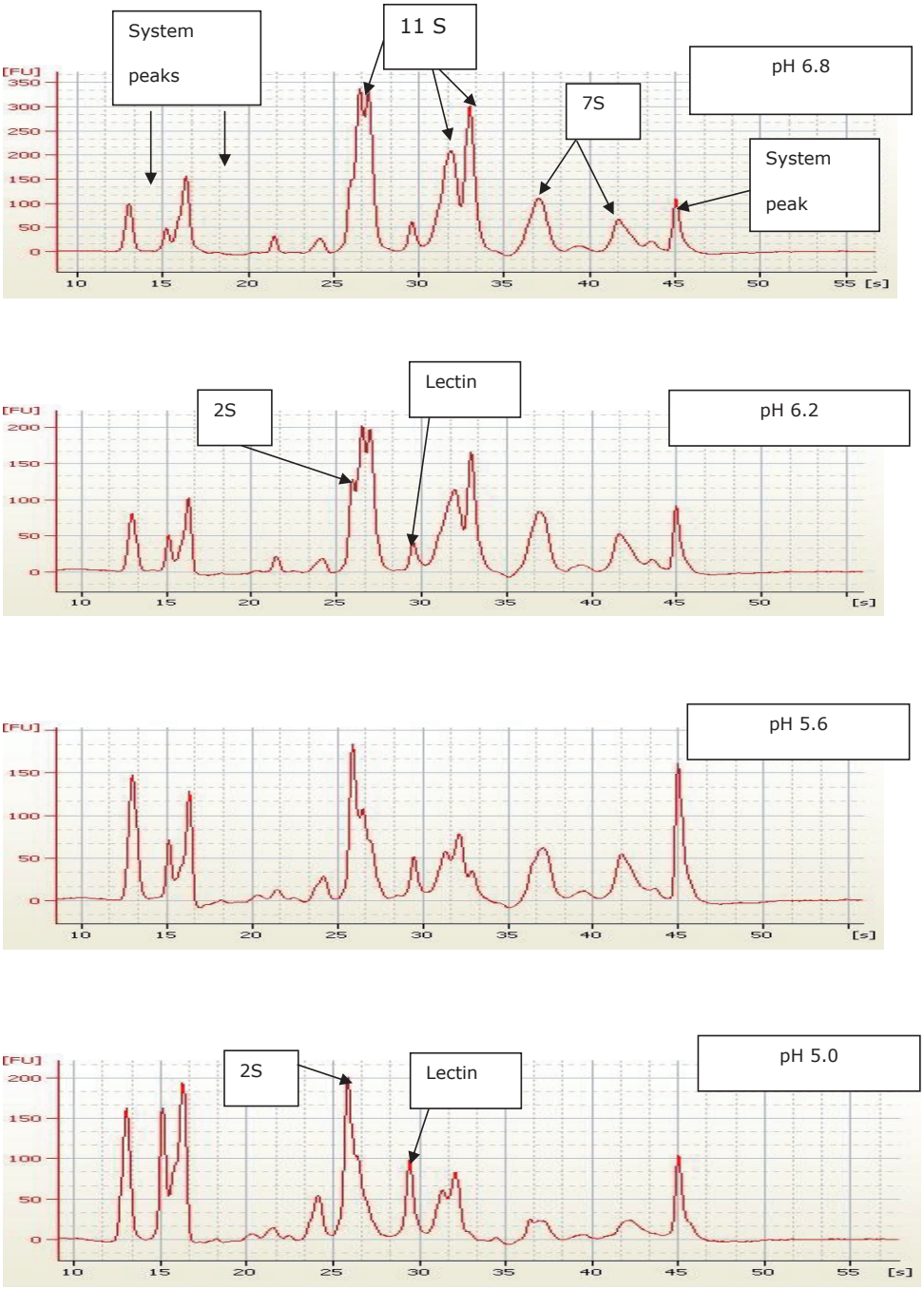

Figure 4 Electrophoresis pattern of soluble proteins of soybean at pH 6.8, 6.2, 5.6 and 5.0 (from top to bottom)

More detailed research with legumes showed that effects on N solubility were most pronounced between pH 5.6 and 6.2, which was caused by the decreased solubility of 7S and 11S globulins. Such a decline is in agreement with findings for faba beans (Rhama, 1988), peas (Chavan *et al*., 2001), lupins (Yoshie-Shark *et al*., 2004) and soybean (Renkema *et al*., 2002). For soybean, 11S (glycinin) was more sensitive to pH change than 7S (ß-conglycinin) which was consistent with changes for these individual proteins (Yuan *et al*., 2002). The decrease in solubility of the globulins consequently led to a different composition of the soluble N fraction. Because the solubility of NPN and 2S albumins seemed not to be affected by pH values, these are the most abundant compounds in the soluble N fraction at pH 5.0 and 5.6, while at pH 6.8 7S and 11S globulins are dominant.

The effect of osmolality on N solubility was less pronounced and also depended on the pH level. For faba beans, at lower pH levels (*i.e.* 5.6 and 6.2) a shift of the osmolality from 300 to 400 $mOsm \cdot l^{-1}$ increased the N solubility, while at pH 6.8 numerically the opposite effect was found. This is comparable to Carbonaro (2006) for 7S globulin, although in that study a bigger difference in osmolality was evaluated. In contrast to the other legumes, no pH – osmolality interaction occurred with soybean and soybean meal, and a rise in osmolality increased N solubility. Yuan *et al*. (2002) showed that the positive effect of osmolality on solubility of 11S globulin (in contrast to 7S globulin) did not depend on pH in the range of 5.0 to 7.0, which is consistent with our responses for soybean and soybean meal.

For silages, in contrast to other feeds, decreasing buffer pH led to an increase of N solubility. This could be caused by differences in composition of the soluble N fraction and sample preparation. As a result of fermentation, the soluble N fraction contains only a small amount of true protein (Gierus *et al*., 2005) and is therefore not as sensitive to pH as other feedstuffs. Silage samples were cut at 1 cm, instead of being ground, leaving more of the cell wall intact than in ground feedstuffs. Therefore, a part of the soluble N components will still be within the cell structure. Further investigation is needed to examine a possible connection between the pH of the solvent and release of soluble N components from within the cell structure.

The N solubility of feed ingredients in water could not be correlated to N solubility in one of the buffers. Such a lack of correlation may be due to the decrease in pH during incubation in water, which was caused by the lack of buffering capacity of water. The

magnitude of this pH shift, however, depends on the chemical composition of the feedstuff and was largest for silages, likely because of the presence of acids, including lactic acid. However, only for forages was there a clear connection between the final pH in water and the solubility measured, compared to the values found in the buffer solutions. This decrease of final pH by addition of feedstuffs was also observed by Giger-Reverdin *et al*. (2002). Differences in the final pH found in their and our study could be caused by differences in solvent (*i.e.* distilled *vs.* tap water) and the ratio between feed and solvent (*i.e.* 1:20 *vs.* 1:50). Giger-Reverdin (2000) showed that the osmolality in water also depends on the feedstuffs examined. Osmolality ranged from 17.8 $mOsm \cdot l^{-1}$ (for sorghum) to 102.5 $mOsm \cdot l^{-1}$ (for alfalfa hay) at a fixed ratio between feed and solvent. Thus if water is used as a solvent the feedstuff will affect both final pH and final osmolality of the solution. This dependency contrasts to the situation in the rumen where the pH and osmolality are mainly regulated by physiological processes such as absorption of VFA and minerals and buffering with bicarbonate. Because both pH and osmolality can affect N solubility, use of water as a solvent for determination of N solubility is not advised. An additional problem is the chemical variation in composition of tap water (such as pH) at different locations, which could also affect N solubility.

Results found in this study may have consequences for development of models and feed evaluation systems estimating rumen degradation of feed proteins. The soluble N fraction is generally considered to be much more rapidly degraded than the non-soluble N fraction (NRC, 2001; Tamminga *et al*., 1994). The proportions of soluble and insoluble N of most feed ingredients, and in particular those for untreated legumes, were markedly affected by rumen pH within the physiological range. If the proportion of soluble N decreases upon a decline in pH a higher amount of rumen escape protein will be obtained when applying the equations in various protein evaluation systems. For a complete nutritional evaluation of this effect, however, the degradation behaviour of the individual proteins should be taken into account. Based on the limited amount of available literature, it can be concluded that the ratio between albumins and globulins in the soluble fraction is higher at a lower pH. In general, albumins are considered to be more resistant to rumen degradation because of their tight structure than one globulins (Spencer *et al*., 1998). This resistance was observed for 2S albumins in peas (Spencer *et al*., 1998; Aufrère *et al*., 2001) and in lupins (Chaudhry and Webster, 2001). Thus, the increase in ratio of albumins to

globulins will decrease the degradation rate of soluble proteins at a lower pH. Electrophoresis results of *in situ* bag residues (Aufrère *et al*., 1999; Aufrère *et al*., 2001; Chiou *et al*., 1999; Sadeghi *et al*., 2006; Spencer *et al*., 1988) indicated that the degradation rate of the globulins in the non-soluble fraction is considerably lower than assumed for soluble proteins. The biggest difference is for the basic subunits of the 11S globulin, which in soybean meal still occurred after 48 h of incubation (Chiou *et al*., 1999). This degradation rate is much lower than that used for soluble proteins discussed previously.

It appears that neglecting the effect of pH on the solubility of N in untreated legumes may lead to overestimation of their rumen degradation. To avoid this problem, the following could be considered. The first is the choice of the solvent used for the determination of N solubility. Use of tap water has a major disadvantage in its lack of buffering capacity which implies that the N solubility of feedstuffs is measured at different pH values and probably also at different osmolality levels. These differences will not be observed by animals, because of the large volume and the buffer capacity of the rumen fluid. Although the use of a buffer solution overcomes this problem, the pH and osmolality of the solvent must be comparable to that of the rumen. In the CNCPS system, a borate phosphate buffer solution of pH 6.8 is used because this buffer maintains a stable pH over prolonged storage and because of its high correlation with insoluble N obtained using autoclaved rumen fluid for five feedstuffs (Krishnamoorthy *et al*., 1982). However, in their study also a difference occurred between the N solubility in both solvents for feedstuffs containing a high amount of soluble TP (*i.e.* peanut meal and oats). Nitrogen solubility was comparable for feedstuffs with a low amount of soluble N (*i.e.* SBM and brewers dried grains) or a high amount of NPN (*i.e.* timothy hay). These results were in line with our observations. In dairy cattle the *in situ* degradation is usually with lactating animals with a rumen pH well below 6.8. This study clearly shows that especially for feedstuffs containing a high amount of soluble TP, N solubility can be affected by this difference in pH level. Therefore, in dairy cattle nutrition, it may be advisable to determine the solubility at common rumen pH values, which implicates a buffer solution with pH in the range of 5.6 to 6.2. In beef cattle, rumen pH will obviously depend largely on the feeding system, such as high forage *vs.* high grain systems. With high forage systems, rumen pH may well be 6.8 or higher, whereas the rumen pH in high grain systems can drop below 5.6 for a considerable period during the feeding cycle (Beauchemin and McGinn, 2005; Nagaraja and Titgemeyer, 2007). In beef cattle nutrition, choice of the

buffer solution to determine solubility may therefore depend on the actual feeding system of interest. In all situations, the buffer should maintain a stable pH (as discussed by Krishnamoorthy *et al*., 1982) to avoid variation in solubility related to fluctuation in pH of the buffer.

The second issue is the treatment of *in situ* bags after incubation to remove contamination from the bag. In the Dutch *in situ* protocol, washing in a washing machine with cold water is used for this purpose. This study, however, showed that the solubility in water can differ from that at normal rumen pH which can lead to dissolved, non-degraded proteins. Again, treatment with a solvent with similar pH and osmolality as the rumen conditions can overcome this problem.

Conclusions

This study showed that decreasing the pH of the solvent, within the rumen physiological range, from 6.8 to 5.0 reduced N solubility of most feedstuffs. The biggest effect was for untreated legumes, where this pH shift also affected the composition of the N soluble fraction by increasing the relative amount of NPN and albumins and decreasing the relative amount of globulins, especially 11S. Solvent osmolality also affected solubility of feed N, but quantitatively the effect was less pronounced than for that of pH. Solubility of N in water differed from that in buffers. With water, the final pH of the solution depended on the feedstuff and this may be an undesirable source of variation among feedstuffs. Because N solubility is used to calculate the amount of degraded protein in various protein evaluation systems, the consequences of pH dependency should be further investigated.

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Chapter 3

A modified rinsing method for the determination of the S, W-S, and D+U fraction of protein and starch in feedstuffs within the *in situ* technique

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Abstract

A modified rinsing method for the *in situ* technique was developed to separate, isolate and characterise the soluble (S), the insoluble washout (W-S) and the non-washout fractions (D+U) within one procedure. For bags that were not incubated in the rumen (t = 0 h), this method was compared with the conventional, Combined Fractionation (CF) method that measures the D+U and S fractions in separate steps and subsequently calculates the W-S fraction. The modified method was based on rinsing of nylon bags in a closed vessel containing a buffer solution (pH 6.2) during 1 h, where shaking speeds of 40, 100, and 160 strokes per min (spm) were evaluated, and tested for 6 dry feed ingredients (faba beans, maize, oats, peas, soybean, and wheat) and 4 forages (2 ryegrass silages and 2 maize silages). The average recoveries as the sum of all fractions were 0.972 $\pm$ 0.041 for N and 0.990 $\pm$ 0.050 for starch (mean $\pm$ SD). The mean W-S fraction increased with increasing shaking speed and varied between 0.017 (N) and 0.083 $g \cdot g^{-1}$ (starch) at 40 spm and 0.078 (N) and 0.303 $g \cdot g^{-1}$ (starch) at 160 spm, respectively. For ryegrass silages, the W-S fraction was absent at all shaking speeds, but was present in the CF method. The modified method, in particular at 40 and 100 spm, reduced the loss of small particles during rinsing resulting in lower W-S and higher D+U fractions for N and starch compared with the CF method. For soybean and ryegrass silage, the modified method reduced the S fraction of N compared with the CF method. The results obtained at 160 spm showed the best comparison to those from the CF method. The W-S fraction of the feedstuffs obtained at 160 spm contained mainly particles smaller than 40 µm (0.908 $\pm$ 0.086). In most feedstuffs, starch was the most abundant chemical component in the W-S fraction and its content (726 $\pm$ 75 $g \cdot kg^{-1}$ DM) was higher than in the D+U fraction (405 $\pm$ 177 $g \cdot kg^{-1}$ DM). Alkaline soluble proteins were the dominant N containing components in the W-S fraction of dry feed ingredients obtained at 160 spm and its relative content (0.79 $\pm$ 0.18 $g \cdot g^{-1}$ of total N in W-S) was higher than that in the D+U fraction (0.59 $\pm$ 0.07 $g \cdot g^{-1}$ of total N in D+U) for all feedstuffs except maize. The molecular weight distribution of the alkaline soluble proteins differed between the W-S and the D+U fractions of all dry feed ingredients, except soybean and wheat.

Keywords: Fractionation; rinsing; *in situ* protocol; proteins; starch.

Implication

A modified rinsing method was developed that improves the accuracy of determining the soluble, washout and non-washout fraction in the *in situ* technique, potentially improving prediction of the nutritional value of feedstuffs. Characteristics of the proteins in the particles normally lost from the nylon bags suggest a more rapid degradation than proteins in the D fraction, which is contrary to assumptions currently used in various protein evaluation systems.

Introduction

The *in situ* technique has been widely used to evaluate the rate and extent of degradation of feed components in the rumen (López, 2005). The technique relies on the assumption that disappearance of substrate from synthetic porous bags incubated in the rumen represents actual substrate degradation by rumen micro-organisms (Ørskov and McDonald, 1979; López, 2005). After ruminal incubation a rinsing step is conducted to remove rumen contamination, such as microbial matter, from the bags. Rinsing of the bags also removes a fraction of the feed (*i.e.* washout or W fraction) containing both soluble components (*i.e.* soluble or S fraction) and small particles (*i.e.* insoluble washout or W-S fraction). The fractional degradation rate of components of the remaining fraction of the feed (*i.e.* non-washout or D+U fraction) can be determined by the *in situ* method. However, there are feed evaluation systems for dairy cows that use W or S and W-S in determining feed value and therefore require a fractional degradation rate for W or S and W-S. Therefore most feed evaluation systems, such as the French PDI (Verité *et al*., 1979), the Dutch DVE (van Duinkerken *et al*., 2011), the British FiM (Thomas, 2004), NRC (NRC, 2001) and the Nordic Norfor (Volden, 2011), use various assumptions on the fractional degradation rate of the S and W-S fraction.

For several feed evaluation systems rinsing of nylon bags by using a washing machine separates the feed in W and D+U fractions. Subsequently for non-incubated nylon bags, the S fraction is determined by additional analysis based on solubility and filtration/centrifugation, after which the W-S fraction is calculated by difference (*e.g.* van Duinkerken *et al*., 2011; Volden, 2011). However, this combined fractionation method (CF-method) has several drawbacks. Firstly, two methods are used that are not equal in solubility conditions, which can lead to systematic differences in determining S and W fractions.

Especially for N, these differences can lead to inaccurate and sometimes even negative values for the W-S fraction (Madsen and Hvelplund, 1994; de Jonge *et al*., 2009). Secondly, the W-S fraction is calculated by difference, which makes it impossible to verify the accuracy of the method based on total recovery. Thirdly, the inability to recover the W-S fraction precludes its further characterization in terms of both chemistry and degradation.

The hypothesis of this study was that by modifying the rinsing method, the modified method could yield similar W fractions compared to the CF method, while enabling direct quantification and characterisation of all the fractions. These modifications involve a closed system and standardised conditions enabling the separation and estimation of all fractions using one rinsing method and the replacement of water by a buffer solution that better mimics the rumen conditions (de Jonge *et al*., 2009). The objectives of this study were to develop and test this modified method and to characterise the isolated W-S fraction. This testing was limited to nylon bags that were not incubated in the rumen (t = 0 h), and focussed on N and starch which are the most important components in the S and W-S fraction (Yang *et al*., 2005), although this modified method potentially can also be used for other components, like organic matter and NDF.

Materials and methods

Materials

Feed ingredients were selected based on a high S fraction (faba beans, peas, soybean) and / or W-S fraction (faba beans, maize, oats, peas, and wheat) as measured with the CF-method. Additionally four forages (two ryegrass silages and two maize silages) were included. Dry feed ingredients were ground to pass a 3 mm sieve (Retsch ZM100, Haan, Germany) and stored at 4˚C. Frozen ryegrass silages were cut with a paper cutter at approximately 1 cm according to the standard Dutch protocol (CVB, 2003), whereas frozen maize silages were cut to below 1 cm using a food cutter type Hobart 84186 (Troy, Ohio, USA). Silages were stored at -20˚C pending analyses.

Methods

Modified method. After acclimatization or thawing, approximately 5 g DM of material was weighed into a nylon bag with an inner size of 10 x 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30, Nybolt, Switzerland). For dry feed ingredients 4 bags and for forages 2 bags

were placed in a glass vessel (Ø 19 cm, 7 cm height) containing 500 ml buffer solution at room temperature. The buffer solution contained 12.2 $g \cdot l^{-1}$ $NaH_2PO_4 \bullet H_2O$ and 8.9 $g \cdot l^{-1}$ $Na_2B_4O_7 \bullet 10H_2O$ (Merck, Darmstadt Germany) and was adjusted to pH 6.2 with HCl (de Jonge *et al*., 2009). The vessels were placed in a mechanical shaker (Julabo SW-20c; Julabo GmhB Seelbach, Germany) for 60 min at a fixed speed. Three speeds (40, 100, and 160 strokes per min (spm)) were investigated, with 40 and 160 spm representing the lowest and highest possible shaking speed. All incubations were performed in duplicate with vessels in different runs.

After 30 min shaking, the nylon bags were turned and after an additional 30 min removed and allowed to drip on a grid above the vessel. After 15 min, the bags were dried for 48 h at 70°C. After weighing, bags from one vessel were pooled and ground to pass a 1 mm sieve (Retsch ZM100, Haan, Germany). This sample corresponded to the D+U fraction. The buffer solution in the vessel was quantitatively centrifuged for 15 min at 20,000 x *g* (to obtain a sharp separation between both the solid and liquid phase), at 25°C and the supernatant was quantitatively collected and weighed (S-fraction).The pellet (W-S fraction) was quantitatively collected, dried for 48 hours at 70°C and ground using a mortar.

The D+U and W-S fractions were analysed for DM, N and starch (the latter not in ryegrass silage and soybean), and the S-fraction for N. The fractions of N and starch were calculated as the absolute amount in a specific fraction divided by the absolute amount in the nylon bags. The recovery of N and starch was calculated as the sum of all fractions (N in S, W-S and D+U fraction; starch in W-S and D+U fraction) relative to the N or starch content in the feed.

The characterisation of the W-S and D+U fractions was limited to the isolates obtained at 160 spm because of its relative similarity to the CF method. This characterisation involves the analyses for particle size distribution, solubility of protein and molecular size distribution of alkali soluble protein.

CF-method. The CF-method was based on the official Dutch protocol (CVB, 2003). The D+U fraction was determined as described by Tas *et al*. (2006) using a programmable washing machine (AEG Turnamat, Nuremberg, Germany) with tap water at approximately 18°C and the gentle 'wool wash' program without centrifuging (40 min in approximately 80 l tap water with 3 swing turns). Two bags per feedstuff were washed in different runs. After drying (70°C for 48 h), bags were weighed, pooled and ground to pass a 1 mm sieve (Retsch ZM 100,

Haan, Germany). The D+U fraction of N and starch was calculated as the remaining absolute amount after rinsing divided by the original amount in the nylon bag. The S fraction for N was determined with duplicates in different runs by extraction of 3 g of feedstuff with 75 ml tap water during 30 min under mechanical stirring at room temperature. The solution was centrifuged for 15 min at 3,000 x *g* and an aliquot of the supernatant was analysed for N. For N and starch the W-S fractions were calculated by difference.

Chemical analyses. Dry feed ingredients were ground to pass a 1 mm sieve before analyses. Fresh forages were air dried at 70˚C during 48 h before grinding. Dry matter (DM) content of feed ingredients and dried residues was determined by drying to a constant weight at 103°C (ISO 6496). Nitrogen was determined using a Kjeldahl method with $CuSO_4$ as the catalyst (ISO 5983-2). Starch was determined by an enzymatic method (ISO 15914).

Determination of particle size distribution. Particle size distribution of the W-S fractions was measured in the buffer solution directly after extraction of nylon bags, by laser diffraction using a Coulter LS 230 particle size analyser (Beckman Coulter Inc., Hialeah, Fl, USA), capable of measuring particle sizes from 0.04 to 2,000 µm. Particle size distribution was expressed as a fraction of the total volume.

Characterization of proteins in the W-S and D+U fractions. For dry feed ingredients, proteins in the W-S fraction and in the D+U fraction were separated into alkaline soluble, acid detergent (AD) soluble and acid detergent insoluble (ADIN). Alkaline soluble proteins were determined by extraction of 0.5 g material with 5 ml 0.1 M sodium hydroxide for 30 min followed by centrifugation at 3,000 x *g* during 10 min and N analysis of the supernatant. ADIN was determined by hydrolysis of 1.0 g material during 1 h with 100 ml AD-reagents (20 g Cetyl trimethylammonium bromide in 1 l 0.5 M sulphuric acid) based on van Soest and Robertson (1985), followed by centrifugation at 3000 *g* during 10 min and determination of N in the residue. All analyses were performed in duplicate. The fraction of AD-soluble protein was calculated as 1 – fraction (alkaline soluble) – fraction (ADIN).

Molecular weight of alkaline soluble protein was determined by vigorously mixing 0.5 ml of the supernatant with 0.25 ml 0.4 M dithiotreitol and 0.25 ml 10% (w/v) sodium dodecyl sulphate (SDS) solution, heating at 95°C for 5 min, and centrifuging at 14,000 × *g* for 2 min. Separation of proteins was conducted by chromatography using a BioSep-SEC-S2000 column (Phenomenex, Utrecht, The Netherlands) on an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA), eluted with a 0.1 M phosphate buffer (pH 6.8) containing 2.5 $g \cdot l^{-1}$ SDS.

Precision Plus protein standard solution of Biorad (Hercules, CA, USA) was used for the identification of the molecular weight. Absorption at 220 nm was used to estimate the ratio between the different groups of proteins.

Statistical analyses

Analysis of variance was conducted using the GLM procedure of SAS (2002) to evaluate the effect of shaking speed (*i.e.* 40, 100, and 160 spm) for the different fractions of each feed ingredient and forage. When treatment effects were detected (*i.e.* $P < 0.05$), Tukey's test was used to test pair wise comparisons between treatments. Comparison between the CF method and the modified method at different shaking speeds for the S and D+U fraction, was conducted using the GLM procedure of SAS (2002) followed by the Dunnett test for pair wise comparison using the CF method as reference. Differences in solubility and molecular size of protein in the W-S *vs.* D+U fraction were evaluated using a t-test.

Results

The DM, N and starch contents of the feed ingredients and forages are presented in Table 1. The N content ranged from 11.4 (maize silage 1) to 65.9 $g \cdot kg^{-1}$ DM (soybean), and the starch content ranged from 314 (maize silage 1) to 687 $g \cdot kg^{-1}$ DM (wheat). Starch content was not determined in soybean and rye grass silages as it is expected to be low or absent in these feeds.

Table 1 Dry matter ($g \cdot kg^{-1}$), N ($g \cdot kg^{-1}$ DM) and starch ($g \cdot kg^{-1}$ DM) content of dry feed ingredients and forages used for the comparison of fractionation methods

Feedstuff	Dry matter	N	Starch
Faba beans	876	50.2	357
Maize	869	17.0	683
Oats	897	17.6	394
Peas	859	37.2	368
Soybean	883	65.9	n.d.
Wheat	878	16.7	687
Maize silage 1	345	11.4	314
Maize silage 2	302	12.0	331
Ryegrass silage 1	554	28.2	n.d.
Ryegrass silage 2	370	23.4	n.d.

n.d.: Not determined

Modified rinsing method and effect of shaking speed

For N (Table 2), the average recovery was 0.972 $\pm$ 0.041 and varied between 0.897 for maize silage 2 at 160 spm and 1.066 for wheat at 100 spm. For faba beans, maize, peas, both maize silages, and ryegrass silage 1, the S fraction was not significantly affected by the shaking speed. For oats and soybean, the S-fraction at 100 spm was significant lower than at 160 spm whereas the S-fraction at 40 spm did not differ from the other shaking speeds. In the case of wheat, the S-fraction at 100 spm was significantly higher than at 40 spm but did not differ with 160 spm. Although the overall effect was significant for the S-fraction of ryegrass silage 2, there were no significant differences between shaking speeds as tested with Tukey pairwise comparison. For dry feed ingredients, except soybean, the W-S fraction increased and the D+U fraction decreased significantly at a higher shaking speed. For soybean and ryegrass silage 1, only the W-S fraction significantly increased yet was very small (for soybean < 0.020 $g \cdot g^{-1}$ and for ryegrass silage 1 < 0.004 $g \cdot g^{-1}$). Shaking speed did not affect the W-S and D+U fractions of maize silages and ryegrass silage 2.

Table 2 Fractionation of N into the soluble (S; $g \cdot g^{-1}$), insoluble washout (W-S; $g \cdot g^{-1}$) and non-washout (D+U; $g \cdot g^{-1}$) fraction in dry feed ingredients and forages using the modified rinsing method at shaking speeds of 40, 100, or 160 spm (n = 2)

		Modified method (spm)				
Feedstuff	Fraction	40	100	160	SE	*P*
Faba beans	S	0.313	0.359	0.409	0.032	0.26
	W-S	0.044[a]	0.114[b]	0.127[b]	0.006	0.003
	D+U	0.551[a]	0.497[ab]	0.439[b]	0.013	0.020
	Recovery	0.909	0.972	0.977		
Maize	S	0.075	0.076	0.074	0.006	0.98
	W-S	0.007[a]	0.018[b]	0.019[b]	0.0006	< 0.001
	D+U	0.916[ab]	0.933[a]	0.892[b]	0.004	0.021
	Recovery	0.998	1.027	0.986		
Oats	S	0.127[ab]	0.094[a]	0.160[b]	0.005	0.009
	W-S	0.032[a]	0.131[b]	0.373[c]	0.009	< 0.001
	D+U	0.763[a]	0.673[b]	0.387[c]	0.012	< 0.001
	Recovery	0.923	0.899	0.920		
Peas	S	0.309	0.302	0.311	0.013	0.88
	W-S	0.034[a]	0.125[b]	0.119[b]	0.009	0.009
	D+U	0.642[a]	0.537[b]	0.576[c]	0.007	< 0.001
	Recovery	0.986	0.965	1.007		
Soybean	S	0.189[ab]	0.163[a]	0.194[b]	0.005	0.044
	W-S	0.005[a]	0.020[b]	0.019[b]	0.002	0.023
	D+U	0.760	0.767	0.753	0.005	0.29
	Recovery	0.955	0.950	0.968		
Wheat	S	0.162[a]	0.196[b]	0.169[ab]	0.005	0.041
	W-S	0.010[a]	0.048[b]	0.052[b]	0.003	0.006
	D+U	0.841[a]	0.821[ab]	0.755[b]	0.013	0.040
	Recovery	1.015	1.066	0.977		
Maize silage 1	S	0.557	0.522	0.519	0.025	0.53
	W-S	0.020	0.034	0.038	0.003	0.070
	D+U	0.430	0.425	0.420	0.012	0.85
	Recovery	1.007	0.982	0.978		
Maize silage 2	S	0.521	0.483	0.519	0.010	0.12
	W-S	0.016	0.022	0.024	0.001	0.080
	D+U	0.394	0.424	0.354	0.019	0.17
	Recovery	0.931	0.929	0.897		
Ryegrass silage 1	S	0.372	0.357	0.370	0.007	0.45
	W-S	< 0.001[a]	< 0.001[a]	0.004[b]	0.0003	0.010
	D+U	0.589	0.615	0.643	0.023	0.39
	Recovery	0.961	0.973	1.018		
Ryegrass silage 2	S	0.551	0.527	0.552	0.004	0.045
	W-S	< 0.001	< 0.001	< 0.001	-	-
	D+U	0.435	0.424	0.482	0.033	0.51
	Recovery	0.986	0.951	1.034		

[a,b,c,d] Means in the same row with different letters differ ($P < 0.05$)

For starch (Table 3), the average recovery was 0.990 ± 0.050 and varied between 0.888 for maize silage 2 at 100 spm and 1.069 for oats at 40 spm. For the dry feed ingredients,

except maize, the W-S fraction increased and the D+U fraction decreased significantly at a higher shaking speed. For maize and maize silage 2, the W-S fraction significantly increased at a higher speed. Shaking speed did not affect the W-S and D+U fractions of maize silage 1.

Comparison between the modified method and the CF-method

For soybean and both ryegrass silages, the S fraction of N obtained by the CF-method was significantly higher than for the modified method at all shaking speeds (Table 4). For oats, the S fraction with the CF method was only higher compared with the S fraction of the modified method at 160 spm. For wheat, the S fraction with the CF-method was significantly lower than with the modified method at 100 and for peas at 40 and 100 spm. The S fraction obtained with the CF-method in maize, both maize silages, and faba beans did not differ from that with the modified method at any shaking speed. The D+U fraction of N for the CF-method was in nearly all cases lower compared to the modified method. For oats, peas, soybean, maize silage 1 and both ryegrass silages, these differences were significant for all shaking speeds. For faba beans, wheat, and maize silage 2, the D+U in the CF-method was significantly lower than for the modified method at 40 and 100 spm. For maize there were no significant differences between the CF-method and the modified method.

Table 3 Fractionation of starch into insoluble washout (W-S; $g \cdot g^{-1}$) and non-washout (D+U; $g \cdot g^{-1}$) fraction in dry feed ingredients and forages using the modified rinsing method at shaking speeds of 40, 100, or 160 spm (n = 2)

		Modified method				
Feedstuff	Fraction	40 spm	100 spm	160 spm	SE	*P*
Faba	W-S	0.075[a]	0.269[b]	0.305[b]	0.016	0.004
beans	D+U	0.976[a]	0.690[b]	0.623[bc]	0.012	< 0.001
	Recovery	1.051	0.959	0.928		
Maize	W-S	0.013[a]	0.018[a]	0.032[b]	0.0016	0.008
	D+U	1.014	0.977	0.936	0.013	0.060
	Recovery	1.027	0.996	0.969		
Oats	W-S	0.069[a]	0.435[b]	0.707[c]	0.031	0.002
	D+U	0.999[a]	0.530[b]	0.317[c]	0.026	< 0.001
	Recovery	1.069	0.967	1.025		
Peas	W-S	0.099[a]	0.259[b]	0.292[b]	0.022	0.016
	D+U	0.943[a]	0.729[b]	0.746[b]	0.021	0.010
	Recovery	1.042	0.988	1.039		
Wheat	W-S	0.047[a]	0.142[b]	0.300[c]	0.016	0.003
	D+U	0.925[a]	0.849[b]	0.631[c]	0.009	< 0.001
	Recovery	0.972	0.991	0.931		
Maize	W-S	0.183	0.238	0.289	0.038	0.29
silage 1	D+U	0.795	0.779	0.726	0.060	0.72
	Recovery	0.978	1.017	1.015		
Maize	W-S	0.096[a]	0.289[b]	0.194[ab]	0.019	0.010
silage 2	D+U	0.804	0.598	0.838	0.063	0.13
	Recovery	0.900	0.888	1.032		

[a, b, c] Means in the same row with different letters differ ($P < 0.05$)

In general, the calculated W-S fractions of N obtained with the CF-method were larger than that measured by the modified method. The relatively greatest differences were found for the two ryegrass silages, where the W-S fraction of N with the modified method was very small but was 0.073 and 0.106 $g \cdot g^{-1}$ with the CF-method.

Table 4 Comparison of the CF method for the soluble (S; $g \cdot g^{-1}$) and non-washout (D+U; $g \cdot g^{-1}$) fraction for N and starch with the modified method at shaking speed 40, 100 or 160 spm (n = 2)

Feedstuff	Fraction (component)	CF - method	Modified method (spm) 40	100	160	SE	*P*
Faba beans	S (N)	0.406	NS	NS	NS	0.028	0.19
	D+U (N)	0.394	**	**	NS	0.012	0.002
	D+U (starch)	0.492	***	**	*	0.019	< 0.001
Maize	S (N)	0.075	NS	NS	NS	0.006	0.99
	D+U (N)	0.881	NS	NS	NS	0.016	0.25
	D+U (starch)	0.914	*	*	NS	0.012	0.016
Oats	S (N)	0.118	NS	NS	*	0.006	0.007
	D+U (N)	0.519	**	*	*	0.024	0.001
	D+U (starch)	0.365	***	*	NS	0.028	< 0.001
Peas	S (N)	0.427	*	*	NS	0.023	0.046
	D+U (N)	0.471	***	**	***	0.005	< 0.001
	D+U (starch)	0.523	***	**	**	0.018	< 0.001
Soybean	S (N)	0.269	***	***	***	0.004	< 0.001
	D+U (N)	0.645	***	***	***	0.006	< 0.001
Wheat	S (N)	0.151	NS	*	NS	0.004	0.010
	D+U (N)	0.744	*	*	NS	0.011	0.010
	D+U (starch)	0.676	***	***	*	0.008	< 0.001
Maize silage 1	S (N)	0.552	NS	NS	NS	0.022	0.53
	D+U (N)	0.350	*	*	*	0.011	0.020
	D+U (starch)	0.702	NS	NS	NS	0.106	0.91
Maize silage 2	S (N)	0.511	NS	NS	NS	0.008	0.10
	D+U (N)	0.303	*	*	NS	0.017	0.029
	D+U (starch)	0.391	*	NS	*	0.068	0.028
Ryegrass silage 1	S (N)	0.469	**	***	**	0.006	< 0.001
	D+U (N)	0.458	*	*	*	0.023	0.017
Ryegrass silage 2	S (N)	0.622	*	*	*	0.013	0.030
	D+U (N)	0.272	*	*	*	0.029	0.026

NS $P > 0.05$

* $0.01 \leq P < 0.05$

** $0.001 \leq P < 0.01$

*** $P < 0.001$

For starch, the D+U fraction for the CF-method was generally lower than that with the modified method. In case of faba beans, peas, and wheat the differences between both methods were significant at all shaking speeds. For maize and oats, the D+U fraction of the CF-method was lower compared with the modified method at shaking speeds of 40 and 100 spm only, and for maize silage 2 at 40 and 160 spm. For maize silage 1 there were no significant differences between the CF-method and the modified method.

In general, the calculated W-S fractions in the CF method were larger than that measured by the modified method. The CF method showed the best comparison with the modified method at 160 spm, although there were significant differences between the results obtained by both methods.

The repeatability of the modified method did not differ from that of the CF-method. For the D+U fraction of N and starch and the S-fraction of N, the average over feedstuffs for the difference between the two runs was 0.03 $g \cdot g^{-1}$ for both methods (results not shown).

Characterization of W-S and D+U fractions

In most feedstuffs, starch was the most abundant chemical component in the W-S fraction obtained at 160 spm and its content (726 $\pm$ 75 $g \cdot kg^{-1}$ DM) was higher than in the D+U fraction (405 $\pm$ 177 $g \cdot kg^{-1}$ DM). The starch content in the W-S fraction ranged from 629 $g \cdot kg^{-1}$ DM in faba beans to 866 $g \cdot kg^{-1}$ DM in wheat and in the D+U fraction from 225 $g \cdot kg^{-1}$ DM in oats to 685 $g \cdot kg^{-1}$ DM in maize (results not shown). Most particles in the W-S fraction with the modified method at 160 spm were smaller than 40 µm, which corresponded to the pore size of the nylon bags (Table 5). The fraction of particles larger than 40 µm varied from 0.014 (maize silage 1) to 0.265 (soybean). The fraction of very small particles (*i.e.* < 10 µm) in grains varied between 0.336 and 0.430, whereas this fraction varied between 0.164 and 0.290 in legume seeds. In maize silage more than 95% of the particles were smaller than 20 µm.

Table 5 Particle size distribution (based on partial volume) of the insoluble washout fraction (W-S) of feedstuffs separated with the modified method at a shaking speed of 160 spm (n = 4)

	Particle size fractions (µm)			
Feedstuff	0 – 10	10 – 20	20 – 40	> 40
Faba beans	0.290 ± 0.027	0.345 ± 0.021	0.310 ± 0.016	0.054 ± 0.065
Maize	0.395 ± 0.023	0.382 ± 0.015	0.186 ± 0.008	0.037 ± 0.048
Oats	0.336 ± 0.031	0.297 ± 0.026	0.211 ± 0.017	0.155 ± 0.068
Peas	0.185 ± 0.012	0.291 ± 0.010	0.400 ± 0.012	0.131 ± 0.024
Soybean	0.164 ± 0.018	0.187 ± 0.021	0.384 ± 0.050	0.265 ± 0.086
Wheat	0.430 ± 0.014	0.228 ± 0.010	0.302 ± 0.015	0.040 ± 0.038
Maize silage 1	0.434 ± 0.019	0.518 ± 0.036	0.035 ± 0.011	0.014 ± 0.030
Maize silage 2	0.516 ± 0.026	0.436 ± 0.021	0.009 ± 0.001	0.039 ± 0.048

Except for maize, protein-N in the W-S fraction of the dry feed ingredients was mainly present as alkaline soluble proteins (Table 6). The relative amount of this type of protein-N in the W-S fraction varied between 0.46 for maize and 0.92 $g \cdot g^{-1}$ for oats, and was higher than the relative amount in the D+U fraction, except for maize and soybean. ADIN was not detectable in the W-S fractions (*i.e.* < 0.01 $g \cdot g^{-1}$), whereas it varied between 0.02 and 0.06 $g \cdot g^{-1}$ in the D+U fractions. The calculated fraction of AD-soluble protein N in W-S fraction was lower than in D+U fraction for faba beans, oats, and peas, but was higher for maize. Alkaline soluble proteins in both W-S and D+U fractions contained mainly large (*i.e.* > 40 kDa) subunits (Table 7). The fraction of alkaline soluble proteins between 60 and 80 kDa was higher in the W-S fraction than in the D+U fraction for faba beans, oats, and peas, but lower for maize. In oats, the D+U fraction had a higher fraction of large sized proteins (> 150 kDa) than the W-S fraction, however, this was the opposite in peas. The W-S fraction contained a lower fraction of small (< 40 kDa) alkali soluble proteins than the D+U fraction for faba beans, peas, and oats, but for maize the opposite was found. For soybean and wheat, no differences in distribution of molecular size of alkaline soluble proteins between both fractions were observed.

Table 6 Fraction of N ($g \cdot g^{-1}$) from the insoluble washout fraction (W-S) and non-washout fraction (D+U) separated with the modified method at a shaking speed of 160 spm and based on alkaline or acid detergent solubility (n = 2)

		Distribution of N		
Feedstuff	Fraction	Alkaline soluble	AD soluble[1]	ADIN[2]
Faba beans	W-S	0.90[a]	0.10[a]	n.d.[3]
	D+U	0.63[b]	0.35[b]	0.02
Maize	W-S	0.46	0.54[a]	n.d.
	D+U	0.52	0.43[b]	0.05
Oats	W-S	0.92[a]	0.08[a]	n.d.
	D+U	0.50[b]	0.44[b]	0.06
Peas	W-S	0.87[a]	0.13[a]	n.d
	D+U	0.62[b]	0.34[b]	0.04
Soybean	W-S	0.71	0.29	n.d.
	D+U	0.57	0.37	0.06
Wheat	W-S	0.86[a]	0.14	n.d.
	D+U	0.70[b]	0.28	0.02

[a, b] Means in the same column for each feedstuff with different letters differ ($P < 0.05$)

[1] AD soluble Acid detergent soluble; calculated as 1 – alkaline soluble – ADIN

[2] ADIN Acid detergent insoluble N

[3] n.d. Not detectable (*i.e.* < 0.01 $g \cdot g^{-1}$)

Discussion

The modified rinsing method enables the separation, isolation, and analysis of the different fractions within one procedure for not incubated feedstuffs (t = 0 h). The high average recovery (0.972 $\pm$ 0.041 and 0.990 $\pm$ 0.050 for N and starch, respectively) indicate that the sum of the fractions represented the total feedstuff quite accurately. To obtain complete recovery, which is needed in most feed evaluation systems, a correction factor should be used for all fractions. The solubility of N in the feedstuffs was not systematically affected by shaking speed. Using a higher shaking speed mostly increased the loss of particles leading to an increase of the W-S and a decrease of the D+U fraction, especially for starch.

In general, the D+U fraction of starch and of N was lower for the CF-method than for the modified method, presumably as a consequence of the more vigorous rinsing conditions, which is in line with Cherney *et al*. (1990) and Cockburn *et al*. (1993). The difference between both methods was smaller for the higher shaking speeds of the modified method. Differences between the S fractions of N for both methods were also observed for several feeds, presumably related to differences in solvent, which is in line with previous observations (de Jonge *et al.*, 2009).

Table 7 Molecular size classes of alkaline soluble proteins from the insoluble washout fraction (W-S) and non-washout fraction (D+U), as a fraction of the total alkaline soluble proteins, separated with the modified method at a shaking speed of 160 spm (n = 2)

		Molecular size (kDa)				
Feedstuff	Fraction	< 40	40 – 60	60 - 80	80 - 150	> 150
Faba beans	W-S	0.134[a]	0.259	0.356[a]	0.177[a]	0.072
	D+U	0.162[b]	0.257	0.309[b]	0.189[b]	0.084
Maize	W-S	0.468[a]	0.202	0.188[a]	0.075[a]	0.065
	D+U	0.165[b]	0.189	0.452[b]	0.103[b]	0.086
Oats	W-S	0.042[a]	0.433	0.392[a]	0.099	0.033[a]
	D+U	0.176[b]	0.273	0.259[b]	0.136	0.154[b]
Peas	W-S	0.051[a]	0.334	0.285[a]	0.157	0.168[a]
	D+U	0.136[b]	0.314	0.275[b]	0.156	0.118[b]
Soybean	W-S	0.084	0.297	0.308	0.217	0.091
	D+U	0.064	0.251	0.304	0.241	0.138
Wheat	W-S	0.104	0.265	0.358	0.129	0.145
	D+U	0.099	0.278	0.376	0.145	0.101

[a, b] Means in the same column for each feedstuff with different letters differ ($P < 0.05$)

Differences between the W-S fractions for N found by both methods are the result of the combination of differences found for the other fractions (*i.e.* S and D+U). The greatest difference was obtained for the W-S fraction in ryegrass silage, which was virtually zero in the modified method, whereas the calculated values for the CF method were 0.073 and 0.106 $g \cdot g^{-1}$. The higher values calculated for the W-S fraction of N in ryegrass silages may be explained

by the use of different protocols, in particular the shaking speed, for the determination of the S and the W fraction as in the CF-method.

The second aim of this study was to characterise the isolated W-S and D+U fraction in terms of particle size, chemical composition, and protein structure for not incubated feedstuffs (t = 0 h). The W-S fraction contained mainly particles smaller than 40 µm, which is in line with the observations made by Michalet-Doreau and Ould-Bah (1992) and is similar to the pore size of the nylon bags used. The presence of a fraction larger than 40 µm could be caused by particles that are not completely spherical, for instance rod shaped, that can escape from the nylon bag but are recorded as larger than 40 µm by the particle size analyser. For most feedstuffs, the W-S fraction contained a high content of starch which was in line with the earlier results from Yang *et al*. (2005). For most dry feeds, the largest part of the N in the W-S fraction was present as alkaline soluble proteins which are a part of the B2 fraction in the system described by Licitra *et al*. (1996). The D+U fraction contained relatively more AD soluble proteins that are part of the B2 or B3 fraction in that system. The molecular weight pattern of the alkaline soluble proteins showed that there are differences between the alkali soluble proteins of the W-S and the D+U fraction. The U (non-degradable) fraction for proteins is according to NRC (2001) equal to ADIN. The ADIN fraction was low (less than 0.06 $g \cdot g^{-1}$ of total N) in the D+U fraction and therefore the characteristics of the D+U fraction are assumed to be largely similar to that of the D-fraction in these feedstuffs. In several feed evaluation systems (Thomas, 2004; van Duinkerken *et al*., 2011; Volden, 2011), proteins of the W-S and of the D (potential degradable) fraction are presumed to have the same fractional degradation rate. The results of the present study, however, indicate that protein characteristics in the W-S and D fraction of the dry feed ingredients do differ.

Only limited information is available to evaluate the effect of the differences in alkaline solubility and molecular size distribution of proteins on ruminal N degradation. Kandylis and Nikokyris (1997) found a positive correlation between alkali solubility and ruminal N degradation for different feedstuffs, which could indicate a higher fractional degradation rate for the W-S fraction of the dry feed ingredients (except maize) than for the D+U fraction. The alkali soluble fraction of faba beans, peas, and soybean contains mostly 7S and 11S globulins and oats contains mainly 3S, 7S and 12S globulins (Chang *et al*., 2011). The 11S globulins are more resistant to rumen degradation compared to the other proteins in faba beans (Chaudhry and Webster, 2001), peas (Spencer *et al*., 1988), and soybean (Aufrère

et al., 1999; Chiou *et al*., 1999). The 12S globulins in oats are structurally similar to 11S globulins in peas (Chang *et al*., 2011), which could indicate that this protein is more resistant compared to the other proteins. Basic and acid subunits of 11S and 12S globulins are smaller than 40 kDa whereas the fraction between 60 and 80 kDa contains mainly 7S subunits. For faba beans, oats, and peas, the significantly higher fraction of proteins in the 60-80 kDa fraction indicates more 7S globulins and less 11S globulins in the W-S fraction than in the D+U fraction, which implies a higher fractional ruminal degradation rate. For maize the fraction of proteins smaller than 40 kDa was significantly higher in the W-S fraction than in the D+U fraction, which may be caused by a higher content of *zein* (20 kDa). Romagnolo *et al*. (1994) found a higher fractional ruminal degradation rate for *zein* compared to other proteins, which could indicate that the fractional ruminal degradation rate of the W-S fraction is also higher than of the D+U fraction. These results suggest that the fractional degradation rate of proteins from the W-S fraction is higher than those from the D+U fraction. Previously, various correction methods for losses of small particles in estimating effective degradability of the substrate have been proposed (Dhanoa *et al*., 1999; Weisbjerg *et al*., 1990). In calculating effective degradability, fractional degradation rate has to be determined or assumptions have to be made for fractions not retained in the bag, as well as assumptions on fractional passage rate for each fraction, and results of the present study may help to obtain proper fractional degradation rates. Further research work is needed to evaluate the effect of the different protein composition in both fractions on the consequences for ruminal degradation and ultimately nutritional value.

A possible additional advantage of the modified method is that it allows the use of different shaking speeds that offers the opportunity to reduce the loss of particles during rinsing compared to the CF method. With the CF method, various feedstuffs are characterised by low D+U fractions which hampers a proper quantification of the fractional degradation rate of the complete feedstuff. The lower W-S and higher D+U fraction for the modified method, especially at reduced shaking speeds offers opportunities to increase the proportion of the feed, especially for starch, for which a fractional degradation rate can be determined, as well as to investigate products and nutrients that cannot be accurately measured with the CF method (Dewhurst *et al*., 1995; Ørskov, 2000). On the other hand, reduced shaking speed could lead to a less efficient removal of rumen contamination, especially particle associated bacteria, leading to an underestimation of the fractional

degradation rate of in particular N. This topic should also be the subject of further investigation. Before the modified method can be used in *in situ* studies a full evaluation of its effects on the rinsing of incubated nylon bags and the consequences on the estimated fractional degradation rate of the D+U fraction should be conducted.

Conclusions

The developed modified method enables the direct quantification and characterisation of all fractions for not incubated feedstuffs (t = 0 h). Compare to the CF method, the modified method does result in different values for the S, W-S and D+U fractions depending on the feedstuff. Differences between the CF and the modified method decreased at higher shaking speed. The W-S fraction of most feedstuffs contained mainly starch and alkali soluble proteins. The proteins in the W-S and D+U fraction showed significant differences in solubility and distribution of their molecular size.

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Chapter 4

A new approach to estimate the *in situ* fractional degradation rate of organic matter and nitrogen in wheat yeast concentrates

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Abstract

In the classic *in situ* method, small particles are removed during rinsing, and their fractional degradation rate cannot be determined. A new approach was developed to estimate the fractional degradation rate of nutrients in small particles. This approach was based on an alternative rinsing method to reduce the particulate matter loss during rinsing and on quantifying the particulate matter loss that occurs during incubation in the rumen itself. To quantify particulate matter loss during incubation, loss of small particles during the *in situ* incubation was studied using non-degradable silica with different particle sizes. Particulate matter loss during incubation was limited to particles smaller than approximately 40 µm with a mean fractional particulate matter loss rate of 0.035 (first experiment) and 0.073 h^{-1} (second experiment) and a non-degradable fraction of 0.001 and 0.050 $g \cdot g^{-1}$, respectively. In the second experiment, the fractional particulate matter loss rate after rinsing in a water bath at 50 strokes per min (spm) (0.215 h^{-1}) and the non-degradable fraction at 20 spm (0.461 $g \cdot g^{-1}$) were significantly larger than that upon incubation in the rumen, whereas the fractional particulate matter loss rate (0.140 and 0.087 h^{-1}, respectively) and the non-degradable fraction (0.330 and 0.075 $g \cdot g^{-1}$, respectively) after rinsing at 30 and 40 spm did not differ with that upon rumen incubation. This new approach was applied to estimate the *in situ* fractional degradation rate of insoluble organic matter (OM) and insoluble nitrogen (N) in three different wheat yeast concentrates (WYC). These WYC were characterised by a high fraction of small particles and estimating their fractional degradation rate was not possible using the traditional washing machine rinsing method. The new rinsing method increased the mean non washout fraction of OM and N in these products from 0.113 and 0.084 $g \cdot g^{-1}$ (washing machine method) to 0.670 and 0.782 $g \cdot g^{-1}$, respectively. The mean effective degradation (ED) without correction for particulate matter loss of OM and of N was 0.714 and 0.601 $g \cdot g^{-1}$, respectively, and significant differences were observed between the WYC products. Applying the correction for particulate matter loss reduced the mean ED of OM to 0.676 (30 spm) and 0.477 $g \cdot g^{-1}$ (40 spm), and reduced the mean ED of N to 0.475 (30 spm) and 0.328 $g \cdot g^{-1}$ (40 spm). These marked reductions in fractional degradation rate upon correction for small particulate matter loss emphasised the pronounced effect of correction for non-degraded particulate matter loss on the fractional disappearance rates of OM and N in WYC products.

Keywords: Particulate matter loss; *in situ* protocol; rumen degradability; silica; wheat yeast concentrates.

Implication

The new approach of using mild rinsing conditions of rumen incubated nylon bags, combined with a correction for non-degraded particulate matter loss during incubation based on *in vitro* simulation of shaking conditions, can potentially be used to determine the fractional degradation rate of nutrients in feedstuffs with a high proportion of small particles. This approach offers a possibility to evaluate the assumptions about the degradation of nutrients in small particles made in feed evaluation systems.

Introduction

Feed evaluation systems, such as Norfor (Volden, 2011), DVE/OEB (van Duinkerken *et al.*, 2011), FiM (Thomas, 2004), and NRC (NRC, 2001) use the *in situ* method to predict the nutritional value of feed ingredients and forages. The basic assumption of this method is that disappearance of substrate from porous nylon (or Dacron) bags incubated in the rumen represents actual ruminal substrate degradation by rumen micro-organisms (Ørskov and McDonald, 1979; López, 2005).

A fundamental problem of the *in situ m*ethod is the disappearance of non-degraded small particles or particulate matter loss from the nylon bag, either during incubation itself or during rinsing after rumen incubation (Michalet-Doreau and Ould-Bah, 1992; Vanzant *et al.*, 1998; López, 2005). This problem occurs when there are particles in the feed material with a size smaller than the pore size of the nylon bag and can be observed as the loss of insoluble nutrients during rinsing of non-incubated bags (de Jonge *et al.*, 2013). Consequently, fractional degradation rate of nutrients in these small particles cannot be measured and the previously mentioned feed evaluation systems use assumptions for the degradation rate of these particles. Ruminal degradation is largely by surface erosion by bacteria, and smaller particles in a measured unit of substrate offer larger surface area and likely a faster rate of degradation (France *et al*., 1993). These assumptions are a potential source of uncertainty of the calculated feeding values as shown by Dhanoa *et al.* (1999). This problem is especially relevant for the determination of rumen degradation of feed ingredients that contain mainly small particles, such as wheat yeast concentrates (WYC), a

by-product from the bioethanol production. It would therefore be desirable to develop a rinsing method that would reduce the loss of particulate matter; however when applying such a method the issue of loss of particulate matter during *in situ* incubation in the rumen remains.

The hypothesis of this study is that a modification of the *in situ* protocol may reduce particulate matter loss during rinsing, which after correction for small particle loss during the actual incubation enables the estimation of the rumen effective degradation (ED) of nutrients in feedstuffs with a large proportion of small particles. The modification is based on two principles: firstly, a reduction of the particulate matter loss during rinsing; and secondly, a correction for the particulate matter loss during incubation in the rumen. Reduction of losses during rinsing is realised by applying a recently described rinsing method (de Jonge *et al.*, 2013). Correction for the particulate matter loss during incubation is based on simulation of the *in situ* particulate matter loss during incubation under laboratory conditions using silica as a marker.

This study comprises three experiments. In the first experiment, the particulate matter loss during incubation was investigated using silica with different particle size distributions. In the second experiment, the particulate matter loss during incubation in the rumen was simulated *in vitro* at different rinsing conditions using silica. In the final experiment, the modified *in situ* method was used to estimate the ED of organic matter (OM) and nitrogen (N) in three WYC with and without correction for particulate matter loss.

Materials and methods

Materials

The silica used include Silica gel 40 (< 400 mesh; Silica 1) and Davisil^{TM} grade 633 (200 – 425 mesh), both from Sigma-Aldrich (Steinheim, Germany). Davisil was divided in two fractions by sieving using a Retsch AS200 (Haan, Germany), *viz*. a fraction smaller than 53 µm (Silica 2) and a fraction between 53 and 71 µm (Silica 3). For the second and third experiment another batch of silica gel 40 was used (Silica 4). For the third experiment three WYC, labelled A, B, and C came from different producers and were obtained from Duynie B.V. (Alphen a/d Rijn, the Netherlands) and stored at approximately 4ºC during the experiment.

Methods

In the first experiment, the silica 1, 2, and 3 were incubated in nylon bags in the rumen of three rumen-cannulated dairy cows for 3, 6, 24, 48, and 96 h. Incubated and non-incubated (*i.e.* t = 0 h) bags were rinsed at 40 strokes per min (spm) according to the method of de Jonge *et al.* (2013) and the residues were analysed for ash.

In the second experiment, silica 4 was incubated in three rumen-cannulated dairy cows for 3, 6, 24, and 48 h. After incubation, bags (including non-incubated bags) were rinsed at 40 spm (method de Jonge *et al.*, 2013) and residues analysed for ash. To determine and simulate rumen particulate matter loss conditions, non-incubated bags containing silica 4 were rinsed in a beaker with 500 ml of buffer solution pH 6.2 (de Jonge *et al.*, 2013) in a shaking water bath at 20, 30, 40, and 50 spm for 3, 6, 24 and 48 h and subsequently dried and weighed. The comparison of the rate of disappearance of silica during rumen incubation with the disappearance of silica *in vitro* at 4 shaking speeds, gives information on the severity of "shaking" conditions in the rumen expressed in water bath shaking conditions.

In the third experiment, three different WYC's were incubated in nylon bags in three dairy cows for 2, 4, 8, 12, 24, and 48 h. Each bag contained approximately 5 g DM of WYC and 0.5 g silica 4 as internal marker for particulate matter loss. Afterwards incubated bags as well as non-incubated bags (*i.e.* t = 0 h) were rinsed at 40 spm (method de Jonge *et al.*, 2013). In addition, separate non-incubated bags were rinsed with the washing machine method. All residues were analysed for dry matter (DM), ash and N. The amount of silica in the bags was analysed by the determination of ash insoluble in HCl. The loss of particulate OM and N in WYC during rumen incubation was simulated with non-incubated nylon bags rinsed in a beaker with 500 ml of buffer solution pH 6.2 at 30 and 40 spm for 2, 4, 8, 24, and 48 h, as for shaking conditions 30 and 40 spm silica loss was similar between rumen incubation and *in vitro* simulation (results of experiment 2).

Rumen incubations. Rumen incubations were carried out with lactating Holstein-Friesian dairy cows and were approved by the Experimental Animal Committee of Wageningen University, The Netherlands. The cows were housed indoors and fed *ad libitum* a mixed ration of 50% grass silage (N, 16.6 $g \cdot kg^{-1}$ DM; NDF, 516 $g \cdot kg^{-1}$ DM) and 50% maize silage (N, 11.5 $g \cdot kg^{-1}$ DM; NDF, 397 $g \cdot kg^{-1}$ DM; starch, 374 $g \cdot kg^{-1}$ DM) at 7.00 am. Cows received each day an additional 2 kg of protein-rich concentrate feed (N, 53.0 $g \cdot kg^{-1}$), and commercial

concentrate feed (N, 29.8 g·kg^{-1}) according to milk production level up to a maximum of 7 kg. Cows were 216 ± 5 d in milk and produced 20.3 ± 2.9 kg milk·d^{-1}. All incubation times were conducted separately on different days, starting at 9.00 am (approximately 2 hours after feeding) according to the all in all out principle. Nylon bags were prepared according to the Dutch *in situ* protocol as described by Tas *et al.* (2006). Briefly, nylon bags with an inner size of 10 x 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30, Nybolt, Zurich, Switzerland) were filled with approximately 5 g of silica in the first and second experiment, and approximately 5 g DM WYC and 0.5 g of silica in the third experiment. Leakages of WYC during the weighing was very limited (*i.e.* < 0.1 g) and was considered to be part of the washout fraction. The number of bags for each WYC and incubation time combination was 8 per animal.

Modified rinsing methods. The modified rinsing method described by de Jonge *et al.* (2013) was used. Briefly, four nylon bags were placed in a glass vessel (Ø 19 cm, 7 cm height) containing 500 ml buffer solution (12.2 g·l^{-1} NaH_2PO_4 • H_2O and 8.9 g·l^{-1} $Na_2B_4O_7$ • $10H_2O$ and adjusted to pH 6.2 with hydrochloric acid). The vessels were placed in a mechanical shaker (Julabo SW-20c; Julabo GmhB, Seelbach, Germany) and were shaken during 60 min at 40 spm at room temperature. For WYC samples that were not incubated, the buffer solution in the vessel containing the soluble (S) and insoluble washout (W-S) fractions was centrifuged for 15 min at 20,000 x *g* at 25°C and the supernatant containing the S fraction as well as the pellet containing the W-S fraction were quantitatively collected and weighed.

Washing machine method. The washing machine method was performed as described by Tas *et al.* (2006), using a programmable washing machine (AEG Turnamat, Nuremberg, Germany) with tap water at ~18°C and a gentle wool wash program without centrifuging (40 min in ~ 80 l tap water with three swing turns).

Sample preparation and analyses. The nylon bags after rinsing, and the pellets of the WYC after centrifugation of the rinsing fluid were air-dried for at least 48 h at 70°C and weighed. For the first and the second experiment individual bags were analysed. For the third experiment, the content of the four bags from each rinsing vessel were combined and ground to pass a 1 mm sieve, leading to two samples for each combination of WYC, cow and incubation time point. Dry matter content was determined by drying to a constant weight at 103°C (ISO 6496, 1999). Ash content was determined by incineration to a constant weight at 550°C (ISO 5984, 2002). Ash insoluble acid was determined by incineration at 550°C during 3

h followed by boiling with 3 M HCl during 15 min and incineration of the residue at 550°C during 2 h (ISO 5985, 2002). N was measured by the Kjeldahl method (ISO 5983-2, 2005). Non protein N (NPN) in the S fraction was determined after addition of 1 ml 4% (w/v) trichloric acetate to 10 ml of the S fraction and centrifugation at 3000 x *g* during 10 min followed by N analysis of the supernatant by the Kjeldahl method.

Particle size distribution of the silica and WYC products was determined after suspending approximately 0.5 g sample into 10 ml water followed by laser diffraction using a Coulter LS 230 particle size analyser (Beckman Coulter Inc., Hialeah, Fl, USA). The particle size distribution of the W-S fraction was measured in the solution obtained after rinsing of the nylon bags by using the modified method. The particle size analyser was capable of measuring particle sizes from 0.04 to 2000 µm. The particle size distribution was expressed as a fraction of the total volume.

Statistical analyses and calculations. The fractional disappearance rate (k_d; h^{-1}) and the non-degradable fraction (U; $g \cdot g^{-1}$) of silica, insoluble OM and insoluble N were estimated with the PROC NLIN procedure of SAS (2002) using a first-order model:

$$Y(t) = (1 - U) \exp(-k_d \times t) + U \quad (1)$$

where Y(t) is the fractional residue of silica, insoluble OM or insoluble N after incubation during t hours expressed relative to residue after rinsing at t = 0 h. For insoluble OM and N, disappearance from the bag is assumed to occur due to degradation as well as due to particulate matter loss. For silica, disappearance is assumed to occur due to particulate matter loss only. The particulate matter loss of non-degraded insoluble OM and insoluble N of WYC during *in situ* rumen incubation was based on simulation of this process in a water bath at 30 and 40 spm shaking speeds, based on results of experiment 2 (see Results section). The fractional particulate matter loss rate (k_{pl}; h^{-1}), the fraction of WYC insensitive to particulate matter loss (F_{IS}; $g \cdot g^{-1}$) and the fraction sensitive to particulate matter loss (F_S; $g \cdot g^{-1}$; calculated as $1 - F_{IS}$) were estimated with the PROC NLIN procedure of SAS (2002) based on the simulation data using a first-order model:

$$Y(t) = F_S \times \exp(- k_{pl} \times t) + F_{IS} \quad (2)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of insoluble OM and insoluble N after rinsing during t hours. The fractional disappearance rate (k_d-corr; h^{-1}) and non-degradable fraction (U-corr; $g \cdot g^{-1}$) corrected for particulate matter loss of insoluble OM and insoluble N was estimated with the PROC NLIN procedure of SAS (2002) using a reduced second-order model, with F_S and k_{pl} taken from the *in vitro* simulation:

$$Y(t) = F_S \times \exp(- (k_d\text{-corr} + k_{pl}) \times t) + (1 - F_S - \text{U-corr}) \times \exp(- k_d\text{-corr} \times t) + \text{U-corr} \quad (3)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of insoluble OM and N after incubation during t hours. The effective degradation of OM and N was calculated as:

$$ED = S + (1 - S) \times [(1 - U) \times k_d / (k_d + k_p)] \quad (4)$$

where S is the soluble fraction, U is the non-degradable fraction of the insoluble fraction, and k_p is the fractional passage rate.

Analysis of variance was conducted using the GLM procedure of SAS (2002). In experiment 1, the model effects were type of silica (silica 1, silica 2, silica 3). In experiment 2 ,the model effect was the method (incubation *in situ*, 20 spm, 30 spm, 40 spm, 50 spm). In experiment 3, the model effects were WYC product (A, B, C). When treatment effects were detected (*i.e.* $P < 0.05$), in experiment 1 and 3 Tukey's test was used to test multiple pairwise comparisons. In experiment 2, the Dunnett test was used for pairwise comparison using the *in situ* method as reference.

Results

Nearly all the particles in silica 1 (*viz.*, 99.2%) were smaller than 40 µm (Table 1). The fraction of particles larger than 40 µm in silica 2 and silica 3 was 0.676 and 0.882, respectively. Silica 4 (used in experiments 2 and 3) had a particle size distribution that was comparable to that of silica 1 (experiment 1).

Table 1 Particle size distribution (average ± standard deviation) of the four silicas used in the three experiments (n = 4)

	First experiment			Second and third experiment
Particle size class (µm)	Silica 1	Silica 2	Silica 3	Silica 4
0 – 10	0.141 ± 0.009	0.038 ± 0.006	0.008 ± 0.001	0.115 ± 0.003
10 – 20	0.352 ± 0.004	0.054 ± 0.002	0.019 ± 0.001	0.345 ± 0.009
20 – 30	0.331 ± 0.010	0.005 ± 0.009	0.025 ± 0.001	0.394 ± 0.011
30 – 40	0.167 ± 0.025	0.227 ± 0.002	0.066 ± 0.001	0.146 ± 0.002
40 – 50	0.008 ± 0.005	0.205 ± 0.004	0.020 ± 0.001	
50 – 60		0.236 ± 0.004	0.217 ± 0.001	
60 – 70		0.155 ± 0.003	0.275 ± 0.002	
> 70		0.081 ± 0.003	0.370 ± 0.002	

First experiment

Substantial loss of silica during rinsing without incubation (t = 0 h) was only observed for silica 1 (Table 2). This fractional loss (*i.e.* 0.106 $g \cdot g^{-1}$) was smaller ($P < 0.001$) than obtained by using the washing machine (*i.e.* 0.627 $g \cdot g^{-1}$; result not shown). Only for silica 1 a marked increase in disappearance of material with incubation time was observed, which occurred mainly within the first 48 h. The mean fractional disappearance rate for silica 1 was 0.035 h^{-1} with a range from 0.028 to 0.050 h^{-1} for individual animals while no substantial non-degradable fraction was observed. For silica 2 and 3 the disappearance during the incubation was small and mainly between 0 and 3 h of incubation, leading to a non-degradable fraction of 0.909 and 0.924 $g \cdot g^{-1}$, respectively. The fractional disappearance rate for the remaining fraction in silica 2 and 3 was relatively large (0.771 and 0.993 h^{-1}).

Table 2 Experiment 1: Residues after *in situ* rumen incubation ($g \cdot g^{-1}$) of the three silicas at different incubation times, as well as the fractional disappearance rates (k_d; h^{-1}) and non-degradable fraction (U; $g \cdot g^{-1}$). Rinsing was done after incubation using the modified rinsing method at 40 spm

	Silica				
	1	2	3	SEM	*P*-value
Time (h)					
0	0.894[a]	0.994[b]	0.999[b]	0.006	< 0.001
3	0.865	0.915	0.921	0.015	0.113
6	0.729	0.888	0.910	0.043	0.079
24	0.475[a]	0.920[b]	0.939[b]	0.044	0.003
48	0.066[a]	0.919[b]	0.937[b]	0.014	< 0.001
96	0.046[a]	0.891[b]	0.902[b]	0.009	< 0.001
k_d (h^{-1})	0.035[a]	0.771[b]	0.993[b]	0.060	0.001
U ($g \cdot g^{-1}$)	0.001[a]	0.909[b]	0.924[c]	0.001	< 0.001

[a,b,c] Means in the same row with different letters differ ($P < 0.05$)

Second experiment

The mean *in situ* fractional disappearance rate and the mean non-degradable fraction of silica 4 was 0.073 h^{-1} and 0.050 $g \cdot g^{-1}$, respectively (Table 3). The non-degradable fraction of silica at 20 spm (0.461 $g \cdot g^{-1}$) was higher ($P < 0.05$) than that obtained *in situ*. At 50 spm, the fractional disappearance rate (0.215 h^{-1}) was higher ($P < 0.05$) than the *in situ* fractional disappearance rate. The fractional disappearance rate and non-degradable fraction obtained with 30 and 40 spm did not differ ($P > 0.05$) with the *in situ* fractional disappearance rate of silica, although the results found at 40 spm were numerically more comparable to those found *in situ*. Both shaking speeds were selected to estimate the particulate matter loss rate of insoluble OM and insoluble N in the third experiment.

Table 3 Experiment 2: Residues ($g \cdot g^{-1}$) of silica 4 after *in situ* incubation or after rinsing in a shaking water bath at 20, 30, 40 and 50 spm (strokes per min) during different times, as well as the fractional disappearance rate (k_d; h^{-1}) and the non-degradable fraction (U; $g \cdot g^{-1}$)

		Rinsing speed					
	In situ	20	30	40	50	SEM	*P*-value
Time (h)							
3	0.801	0.783	0.817	0.785	0.775	0.088	0.972
6	0.641[a]	0.860[a]	0.555[a]	0.687[a]	0.208[b]	0.064	0.003
24	0.361[a]	0.545[a]	0.462[a]	0.098[b]	0.190[a]	0.046	0.002
48	0.050[a]	0.483[b]	0.247[a]	0.148[a]	0.144[a]	0.080	0.045
k_d (h^{-1})	0.073[a]	0.086[a]	0.140[a]	0.087[a]	0.215[b]	0.021	0.014
U ($g \cdot g^{-1}$)	0.050[a]	0.461[b]	0.330[a]	0.075[a]	0.144[a]	0.074	0.024

[a,b] Means in the same row with different letters differ from the *in situ* ($P < 0.05$)

Third experiment

The DM and N content of the WYC ranged from 250 to 318 $g \cdot kg^{-1}$ and from 46.2 to 48.5 $g \cdot kg^{-1}$ DM, respectively (Table 4). Using the modified rinsing procedure, the insoluble washout fraction (W-S fraction) of OM varied between 0.060 and 0.067 $g \cdot g^{-1}$, whereas the non washout fraction (D+U fraction) ranged from 0.641 to 0.696 $g \cdot g^{-1}$ (Table 4). The mean calculated value for the soluble fraction (S fraction) of OM was 0.264 $g \cdot g^{-1}$. The D+U fraction of OM obtained with the washing machine (mean 0.113 $g \cdot g^{-1}$) was much smaller than with the modified rinsing method and varied between 0.091 and 0.150 $g \cdot g^{-1}$ for individual bags (results not shown). The S fraction of N varied between 0.104 and 0.165 $g \cdot g^{-1}$ and contained mainly NPN (0.88 to 0.98 $g \cdot g^{-1}$ of the total soluble N; data not shown). For N, the W-S fraction with the modified rinsing method ranged from 0.072 to 0.086 $g \cdot g^{-1}$ and the D+U fraction from 0.754 to 0.810 $g \cdot g^{-1}$. The D+U fraction of N obtained with the washing machine was much smaller (mean 0.084 $g \cdot g^{-1}$) and varied between 0.065 and 0.094 $g \cdot g^{-1}$ for individual bags (results not shown). Particle size analyses showed that the W-S fraction of these products mainly contained particles smaller than 40 µm with relative high fractions of particles smaller than 10 µm and between 20 and 30 µm (Fig. 1). Differences in the particle size distribution between the products were observed but not further analysed.

Table 4 Experiment 3: Dry matter (DM; $g \cdot kg^{-1}$), ash ($g \cdot kg^{-1}$ DM), and nitrogen (N; $g \cdot g^{-1}$ DM) of three Wheat Yeast Concentrates (WYC-A, WYC-B, and WYC-C) and the soluble (S; $g \cdot g^{-1}$), insoluble washout (W-S; $g \cdot g^{-1}$), and non-washout (D+U; $g \cdot g^{-1}$) fractions of organic matter (OM) and nitrogen (N) obtained by the modified rinsing method at 40 spm

	WYC-A	WYC-B	WYC-C
DM	250	268	318
Ash	46.7	91.1	102.8
N	46.7	48.5	46.2
Fractions OM			
S	0.244[1]	0.258[1]	0.292[1]
W-S	0.060	0.067	0.067
D+U	0.696	0.675	0.641
Fractions N			
S	0.104	0.144	0.165
W-S	0.086	0.072	0.081
D+U	0.810	0.784	0.754

[1] Calculated value (1 – (W-S) – (D+U))

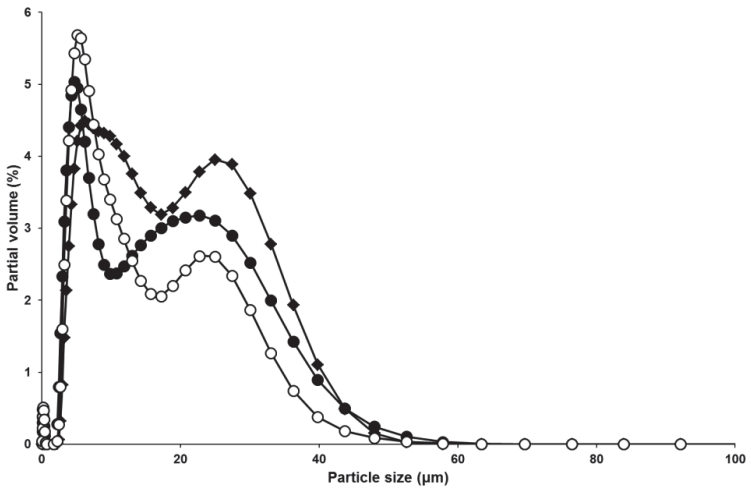

Figure 1 Particle size distribution of the W-S fraction of the three wheat yeast concentrates (WYC A, ♦; WYC B,●; WYC C, ○)

The *in situ* fractional disappearance rate and non-degradable fraction of the silica incubated together with the WYC were not significantly different for the three WYC products, ranging from 0.061 to 0.092 h^{-1} and from 0.015 to 0.080 $g \cdot g^{-1}$, respectively (Table 5). The average fractional residue of silica incubated together with WYC after rinsing only (t = 0 h) was 0.523 $g \cdot g^{-1}$, which was considerably less than found in the second experiment (*i.e.* 0.953 $g \cdot g^{-1}$, results not shown) when the bags contained only silica. However, the fractional disappearance rate and the non-degradable fraction for silica were numerically comparable to the values found for simulation at 40 spm and *in situ* in the second experiment.

Table 5 Experiment 3: Residues ($g \cdot g^{-1}$) after *in situ* rumen incubation of silica 4 incubated together with three Wheat Yeast Concentrates (WYC-A, WYC-B, and WYC-C) at different incubation times, the fractional disappearance rates (k_d; h^{-1}) and non-degradable fraction (U; $g \cdot g^{-1}$)

	Products				
	WYC-A	WYC-B	WYC-C	SEM	*P*
Time (h)					
0	0.567	0.499	0.504	0.050	0.293
2	0.555[a]	0.530[a]	0.456[b]	0.024	0.012
4	0.456	0.482	0.421	0.042	0.606
8	0.411[a]	0.303[ab]	0.237[b]	0.034	0.115
12	0.201	0.218	0.128	0.032	0.146
24	0.067	0.129	0.129	0.030	0.307
48	0.073	0.053	0.064	0.011	0.498
k_d (h^{-1})	0.074	0.061	0.092	0.006	0.065
U ($g \cdot g^{-1}$)	0.035	0.015	0.080	0.025	0.289

[a,b] Means in the same row with different letters differ ($P < 0.05$)

For insoluble OM and insoluble N significant differences between WYC samples were observed for residues in the nylon bags at all incubation times except 24 hours and a tendency only at 12 hours (Table 6 and 7). For insoluble OM, the fractional degradation rate ranged from 0.202 to 0.350 h^{-1}, and the non-degradable fraction varied between 0.220 and

0.235 g·g^{-1}. The calculated ED ranged from 0.678 to 0.754 g·g^{-1} and was higher ($P < 0.05$) for WYC C than for both other products. For insoluble N, the fractional degradation rate ranged from 0.094 to 0.188 h^{-1}, and the non-degradable fraction varied between 0.149 and 0.196 g·g^{-1}. The calculated ED ranged from 0.549 to 0.669 g·g^{-1} and (similar to the fractional degradation rate) was higher ($P < 0.05$) for WYC-C than for both other products.

Table 6 Experiment 3: Residues (g·g^{-1}) after *in situ* rumen incubation and rinsing at 40 spm (g·g^{-1}) of insoluble organic matter of the three wheat yeast concentrates at different incubation times and the fractional disappearance rates (k_d; h^{-1}), non-degradable fraction (U; g·g^{-1}), and effective degradation (ED; g·g^{-1}) without correction for particulate matter loss during rumen incubation

	Products				
	WYC-A	WYC-B	WYC-C	SEM	*P*-value
Time (h)					
0	1.000[1]	1.000	1.000	-	-
2	0.629[a]	0.615[a]	0.534[b]	0.009	< 0.001
4	0.572[a]	0.502[b]	0.473[b]	0.012	< 0.001
8	0.501[a]	0.478[a]	0.379[b]	0.013	< 0.001
12	0.353	0.350	0.270	0.024	0.085
24	0.250	0.212	0.213	0.013	0.121
48	0.208[a]	0.137[b]	0.155[b]	0.010	< 0.001
k_d(h^{-1})	0.202	0.221	0.350	0.030	0.048
U(g·g^{-1})	0.235	0.220	0.226	0.013	0.281
ED(g·g^{-1})[2]	0.678[a]	0.709[a]	0.754[b]	0.006	0.002

[a,b] Means in the same row with different letters differ ($P < 0.05$)

[1] Set value (total insoluble fraction)

[2] Calculated as $ED = S + (1 - S) \times [(1 - U) \times \{k_d / (k_d + k_p)\}]$; for S (soluble fraction) see Table 4; k_p (fractional passage rate) is 0.06 h^{-1}

Based on the results of the second experiment, the particulate matter loss during incubation for insoluble OM and insoluble N for the WYC was estimated by rinsing at 30 and

40 spm. At 30 spm, the mean fraction of WYC sensitive to particulate matter loss (*i.e.* F_S) was 0.48 $g \cdot g^{-1}$ for OM and 0.38 $g \cdot g^{-1}$ for N, respectively (Table 8), which increased to 0.62 (OM) and to 0.60 $g \cdot g^{-1}$ (N) at 40 spm. The mean fractional particulate matter loss rate of F_S for OM was 0.12 and 0.37 h^{-1} at 30 and 40 spm, respectively, and for N was 0.20 and 0.35 h^{-1} at 30 and 40 spm, respectively. For insoluble OM, the mean fractional degradation rate decreased from 0.258 (Table 6) to 0.177 h^{-1} using 30 spm as correction, and to 0.082 h^{-1} using 40 spm (Table 8) while the mean non-degradable fraction decreased from 0.233 to 0.221 $g \cdot g^{-1}$ using 30 spm, and to 0.072 $g \cdot g^{-1}$ using 40 spm. The mean ED of OM decreased from 0.714 to 0.676 $g \cdot g^{-1}$ using 30 spm and 0.477 $g \cdot g^{-1}$ using 40 spm. For insoluble N, the mean fractional degradation rate decreased from 0.126 (Table 7) to 0.056 h^{-1} using 30 spm, and to 0.030 h^{-1} using 40 spm while the mean non-degradable fraction decreased from 0.176 to 0.085 $g \cdot g^{-1}$ using 30 spm and 0.054 $g \cdot g^{-1}$ using 40 spm. The mean ED of N decreased from 0.601 to 0.475 $g \cdot g^{-1}$ using 30 spm and to 0.328 $g \cdot g^{-1}$ using 40 spm. For both OM and N, applying this correction had a larger numerical effect on the ED of WYC-A and B than on WYC-C.

Discussion

The aim of this study was to estimate the *in situ* ED of nutrients in small particles that in the conventional *in situ* nylon bag method are removed by rinsing in a washing machine. To that end a previously described modified protocol for rinsing the bags (De Jonge *et al.*, 2013) was applied to reduce the particulate matter loss during rinsing. If particulate matter losses during rinsing have been reduced, the subsequent issue concerns the loss of non-degraded particulate matter during incubation in the cow itself. Therefore, in the present paper we studied an approach to measure and account for the non-degraded particulate matter loss during *in situ* incubation itself. In comparison with the washing machine method, a marked reduction of the losses during rinsing for fine silica particles and for OM and N in WYC was realized by applying the modified rinsing method, which was in line with previous results for N and starch in dry feed ingredients (de Jonge *et al.*, 2013). The loss that still does occur for the three WYC products was mainly limited to particles smaller than approximately 40 μm which corresponded to observations from other studies with other feedstuffs using different rinsing methods (Michalet-Doreau and Ould-Bah,1992; de Jonge *et al.*, 2013). The results from experiment 1 and 2 showed that the particulate matter loss during incubation was mainly relevant for silica 1 and 4 which mainly contained particles

smaller than 40 µm. The results obtained for silica 2 and 3 also indicated that this loss was limited to particles smaller than 40 µm. The mean fractional disappearance rates of silica 1 and 4 found in this study were 0.035 and 0.073 h^{-1}. Differences between the shaking conditions in the rumen of individual cows and the particle size distribution of both silica gels could be the cause of this variation leading to different particulate matter loss rates, as was demonstrated by the simulation of this process in a waterbath at different shaking speeds (Table 3).

Table 7 Experiment 3: Residues ($g \cdot g^{-1}$) after *in situ* rumen incubation and rinsing at 40 spm of insoluble nitrogen of the three wheat yeast concentrates at different rumen incubation times and the fractional disappearance rates (k_d: h^{-1}), non-degradable fraction (U; $g \cdot g^{-1}$), and effective degradation (ED; $g \cdot g^{-1}$) without correction for particulate matter loss during rumen incubation

	Products				
	WYC-A	WYC-B	WYC-C	SEM	*P*-value
Time (h)					
0	1.000[1]	1.000	1.000	-	-
2	0.799[a]	0.778[a]	0.630[b]	0.011	< 0.001
4	0.733[a]	0.635[b]	0.585[b]	0.017	< 0.001
8	0.652[a]	0.637[a]	0.466[b]	0.023	< 0.001
12	0.413	0.452	0.311	0.041	0.083
24	0.256	0.247	0.232	0.018	0.652
48	0.207[a]	0.139[b]	0.154[ab]	0.015	0.020
k_d (h^{-1})	0.094[a]	0.095[a]	0.188[b]	0.014	0.016
U($g \cdot g^{-1}$)	0.184	0.149	0.196	0.012	0.123
ED($g \cdot g^{-1}$)[2]	0.549[a]	0.585[a]	0.669[b]	0.014	0.010

[a,b] Means in the same row with different letters differ ($P < 0.05$)

[1] Set value (total insoluble fraction)

[2] Calculated as $ED = S + (1 - S) \times [(1 - U) \times \{k_d / (k_d + k_p)\}]$; for S (soluble fraction) see Table 4; k_p (fractional passage rate) is 0.06 h^{-1}

For the silica added to the WYC in the bags, lower residues at 0 h incubation were found compared to the first two experiments where silica was present in bags without any WYC, which is an indication of an interaction with the WYC matrix. This difference was also observed for the individual incubation times, although, the fractional disappearance rates of the remaining silica when co-incubated with WYC were comparable to the results obtained from the first two experiments. This indicates that the process of particulate matter loss seems to be matrix depending and can vary between experiments.

The marked reduction of the loss of material during rinsing with the modified method might enable the estimation of the *in situ* degradation of nutrients in small particles in products such as WYC. However, this would require that non-degraded particulate matter loss from the bags during rumen incubation itself is minimal or can be accurately corrected for. Total disappearance of substrate during the incubation in the rumen is the result of degradation and particulate matter loss which in this study were assumed to be two independent processes. The silica experiments showed that small particles can leave the bag during *in situ* rumen incubation. Thus when using the modified rinsing method after *in situ* incubation, neglecting non-degraded particulate matter loss leads to an overestimation of the fractional degradation rate of WYC. *In vitro* simulation of the particulate matter loss during *in situ* incubation for WYC by rinsing at 30 and 40 spm revealed that the WYC products were very sensitive to shaking conditions. Correction for this loss reduced the average ED for OM to 0.94 (30 spm) and to 0.66 $g \cdot g^{-1}$ (40 spm) of the value obtained without correction. For N, this correction led to values that were 0.79 (30 spm) and 0.54 $g \cdot g^{-1}$ (40 spm) of the value obtained without correction. Such reductions, especially for N, have a considerable impact on the calculated feeding value in protein evaluation systems. Although shaking speeds of 30 and 40 spm did not result in significant differences with rumen (*in situ*) shaking conditions, the ED corrected for particulate matter loss differed substantially between both shaking speeds. Numerically, the silica disappearance rates obtained with 40 spm were closer to the *in situ* disappearance rates than those obtained with 30 spm (Table 2).

Table 8 Experiment 3: Fraction of the three wheat yeast concentrates sensitive to particulate matter loss (F_S; $g \cdot g^{-1}$), fraction of WYC insensitive to particulate matter loss (F_{IS}; $g \cdot g^{-1}$) and the fractional particulate matter loss rate (k_{pl}; h^{-1}) of F_S obtained with simulation at 30 and 40 spm and the fractional degradation rate (k_d-corr; h^{-1}), non-degradable fraction (U-corr; $g \cdot g^{-1}$), and the effective degradation (ED; $g \cdot g^{-1}$) for organic matter (OM) and nitrogen (N) of after correction of *in situ* measured data for simulated particulate matter loss during incubation

		OM			N		
		WYC-A	WYC-B	WYC-C	WYC-A	WYC-B	WYC-C
Simulation							
30 spm	F_S[1]	0.48	0.52	0.44	0.39	0.38	0.37
	F_{IS}[2]	0.52	0.48	0.56	0.61	0.62	0.63
	k_{pl}	0.14	0.10	0.13	0.21	0.16	0.23
	k_d-corr[3]	0.110	0.150	0.270	0.032	0.036	0.100
	U-corr[3]	0.235	0.207	0.220	0.044	0.049	0.163
	ED[4]	0.621	0.671	0.736	0.388	0.439	0.598
40 spm	F_S	0.61	0.67	0.59	0.61	0.61	0.58
	F_{IS}	0.39	0.33	0.41	0.39	0.39	0.42
	k_{pl}	0.43	0.51	0.16	0.40	0.49	0.16
	k_d-corr	0.010	0.010	0.226	0.005	0.008	0.077
	U-corr	< 0.001	< 0.001	0.215	< 0.001	< 0.001	0.163
	ED	0.352	0.363	0.717	0.183	0.251	0.549

[1] Expressed as fraction of the total insoluble fraction

[2] Expressed as fraction of the total insoluble fraction

[3] Calculated as $Y(t) = F_S \times \exp[-(k_{pl} + k_d\text{-corr}) \times t] + (1 - F_S - \text{U-corr}) \times \exp(-k_d\text{-corr} \times t)$.

[4] Calculated as $ED = S + (1 - S) \times [(1 - \text{U-corr}) \times k_d\text{-corr} / (k_d\text{-corr} + k_p)]$; for S (soluble fraction) see Table 4; k_p (fractional passage rate) is 0.06 h^{-1}

The method described, based on reduction of particulate matter loss during rinsing and applying a correction for particulate matter loss during incubation, seems to be a potential new approach to determine the ED of nutrients in small particles in feed ingredients. A methodological challenge of this approach remains the accurate estimation of

the particulate matter loss during the incubation. Although the method presented seems to be a good approach to simulate this process, additional measurements with different batches of silica and feed ingredients and comparison between the incubation in animals are needed to improve the accuracy of the simulation and to estimate the effects of variation in conditions on the results found. The marked effect of applying this correction on the calculated ED values for the WYC products emphasises the importance of this issue. Another issue for further research is the effect of the modified rinsing method on the ED values of feed ingredients. The use of this more gentle rinsing method could affect the degradation characteristics and consequently the calculated ED-values for feed ingredients compared to the standard procedure that involves washing machine rinsing. A comparison between both rinsing methods using other feed ingredients is needed to fully evaluate such differences. Both issues should be the subject for further investigation before this new approach can be applied to supply data that can be used in feed evaluation systems.

The WYC products containing a large fraction of small particles showed relatively low ED values for N with a significant difference between product A and B and product C. The relatively low degradation rate for N in the WYC products could be related to the larger contribution of yeast protein to total WYC protein and the location of the proteins in yeast cells. These cells have a rigid cell wall containing mainly β1-3 and β1-6 glucans, glycoproteins, and chitin (Lipke and Ovalle, 1998) that form a barrier for the degradation of the proteins in these cells. Various glucanases and proteases are required to break down the cell wall structure and release the protein inside the cell. The size of yeast cells, between 5 and 10 µm, made their disappearance from the nylon bag very sensitive to variations in the shaking conditions during the incubation. Differences in the degree of aggregation (*i.e.* flocculation) between the yeast cells could be a reason for variation in disappearance during rinsing of the WYC. Differences between the WYC products with respect to their *in situ* fractional degradation rate for N could be related to the variation of native proteins from wheat in these products. With product A and B and according to the producer, the protein fraction (*i.e.* gluten) of wheat was almost completely removed during the process, whereas for product C this fraction was not removed. Consequently, product C contained more native proteins compared to the other products which could be an explanation for its significantly higher kd and ED value and differences in particulate matter loss especially at 40 spm (Table

8). Other factors, such as differences between wheat, yeast types, and production conditions could also contribute to the variation between the products.

Conclusions

An alternative approach for the *in situ* method based on reduction of the loss of particulate matter during rinsing was applied and a correction for the loss of particulate matter during *in situ* incubation in the rumen was developed. The *in situ* particulate matter loss was mainly limited to particles smaller than 40 µm and could be simulated by *in vitro* rinsing in a waterbath at 30 and 40 spm, whereas 20 and 50 spm led to a significant lower and higher disappearance rate, respectively, compared with the *in situ* particulate matter loss. Application of this new approach for WYC products increases the residues of OM and N in nylon bags after rinsing. Correction for the loss of non-degraded particulate matter during incubation markedly reduced the calculated ED for OM and for N in all WYC products. More research work is needed to fully evaluate this alternative approach and its application in current protein evaluation systems.

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Chapter 5

Estimation of the *in situ* degradation of the washout fraction of starch by using a modified *in situ* protocol and *in vitro* measurements

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Abstract

The *in situ* degradation of the washout fraction of starch in six feed ingredients (*i.e.* barley, faba beans, maize, oats, peas, and wheat) was studied by using a modified *in situ* protocol and *in vitro* measurements. In comparison with the washing machine method, the modified protocol comprises a milder rinsing method to reduce particulate loss during rinsing. The modified method markedly reduced the average washout fraction of starch in these products from 0.333 to 0.042 $g \cdot g^{-1}$. Applying the modified rinsing method, the fractional degradation rate (k_d) of starch in barley, oats and wheat decreased from on average 0.327 to 0.144 h^{-1} whereas for faba beans, peas, and maize no differences in k_d were observed compared to the traditional washing machine rinsing. For barley, maize, and wheat, the difference in non-fermented starch in the residue between both rinsing methods during the first 4 hours of incubation increased, which indicates secondary particle loss. The average effective degradation (ED) of starch decreased from 0.761 to 0.572 $g \cdot g^{-1}$ when using the new rinsing method and to 0.494 $g \cdot g^{-1}$ when applying a correction for particulate matter loss during incubation. The *in vitro* k_d of starch in the non-washout fraction did not differ from that in the total product. The calculated ratio between the k_d of starch in the washout and non-washout fraction was on average 1.59 and varied between 0.96 for oats and 2.39 for maize. The fractional rate of gas production was significantly different between the total product and the non-washout fraction. For all products, except oats, this rate of gas production was larger for the total product compared to the non-washout fraction whereas for oats the opposite was observed. The rate of increase in gas production was, especially for grains, strongly correlated with the *in vitro* k_d of starch. The results of the present study do not support the assumption used in several feed evaluation systems that the degradation of the washout fraction of starch in the rumen is much faster than that of the non-washout fraction.

Keywords: Particulate matter loss; *in situ* protocol; *in vitro*; rumen degradability; starch

Implication

This study showed that the fractional degradation rate (k_d) of starch of the washout fraction was lower than generally assumed. Also, the rinsing method affected the k_d caused by particulate matter loss. The effect of both observations on the effective degradation of

starch varied between feed ingredients, and can therefore change the ranking in nutritive value.

Introduction

To meet nutrient requirements for high milk yields, diets for dairy cows are formulated for high intake of absorbable nutrients. In practical terms, this has been accomplished mainly through the addition of starch rich feed ingredients. Perceived benefits of feeding starch include increased metabolizable energy and metabolizable protein supply per unit of feed intake and thus greater milk and milk protein yield compared to feeding fibre, although there is no clear evidence as to the advantages of postruminal digestion of starch to enhance milk yield or to change milk composition compared with ruminal digestion of starch (Nocek and Tamminga, 1991). Starch digestion site is important for calculating the total fermentable carbohydrate supply to the rumen, which is relevant for managing rumen fermentation level, or potentially acidosis. Moreover, the site of starch digestion determines the type of nutrient (VFA *vs.* glucose) that is available for the animal, which is relevant for mechanistic nutritional models (Mills *et al.*, 1999). Most starch is subject to extensive anaerobic degradation in the rumen. The standard procedure to measure ruminal degradation is the *in situ* method which is based on the disappearance of substrate from porous nylon (or dacron) bags in the rumen of rumen fistulated animals as described by Ørskov and McDonald (1979) which is also used for starch (Cerneau and Michalet-Doreau, 1991). The starch degradation rates obtained by the *in situ* method are used in numerous feed evaluation systems, such as DVE/OEB (van Duinkerken *et al*., 2011), Norfor (Volden, 2011), and PDI (Sauvant *et al.*, 2004) and mechanistic rumen models (Mills *et al.*, 1999) to predict the nutritional value of feed ingredients.

One of the methodological problems of the *in situ* procedure is the loss of non-degraded particulate matter from the bags by rinsing both incubated and non-incubated nylon bags. This loss by rinsing is influenced by the interaction of the particle size of the feed material in relation to the pore size of the bag (Michalet-Doreau and Ould-Bah, 1992; Vanzant *et al*., 1998; López, 2005) and the severity of rinsing. The degradation rate of this washout fraction cannot be measured *in situ* and therefore in various feed evaluation systems assumptions on this degradation rate are made to be able to estimate the fermentability of the washout fraction (Offner *et al*., 2003; van Duinkerken *et al*., 2011;

Volden, 2011). The washout fraction of starch is relatively large and highly variable between feeds (Offner *et al*., 2003; de Jonge *et al.*, 2013) and is generally considered to be rapidly degradable based on theoretical assumptions about the degradation of small particles (France *et al.*, 1993) and comparison between *in situ* and *in vivo* data (van Duinkerken *et al.*, 2011). However, the assumption that material washed out of nylon bags is rapidly and completely degraded in the rumen is not supported by *in vitro* gas production results (Yang *et al.*, 2005; Cone *et al.*, 2006; Stevnebø *et al.*, *2009*). It should be noted that the rate of gas production reflects fermentation of the organic matter present, not solely the starch fraction. The size of the washout fraction in combination with the assumptions on its degradation rate strongly influence the effective *in situ* rumen degradation (ED) of starch in feed ingredients (Offner *et al*., 2003; Huhtanen and Sveinbjörnsson, 2006) and the evaluation of the effects of expansion, extrusion, and toasting on *in situ* starch degradation (Goelema *et al*., 1998; Offner *et al*., 2003). Significant relationships with *in vivo* ruminal starch degradability have been found for both *in situ* measurements and *in vitro* approaches based on incubation with rumen fluid followed by determination of starch in the residue, with a stronger correlation for *in situ* than for *in vitro* estimates (r = 0.84 and r = 0.76; Weisbjerg *et al.*, 2011). Tahir *et al.* (2013) reported a somewhat higher correlation (R^2 = 0.81) between the predicted ruminal neutral detergent soluble digestibility based on *in vitro* gas production with ruminal starch digestibility for several products.

The hypothesis of this study is that the degradation of the washout fraction of starch can be estimated by using a modified *in situ* method combined with *in vitro* measurements. The modified *in situ* method uses a gentle rinsing method that strongly reduces the washout fraction of starch in feed ingredients compared to the conventional washing machine procedure (de Jonge *et al.*, 2013). The differences found between the fractional degradation rate (k_d) of starch as determined by either rinsing method will depend on whether the k_d of the washout and the non-washout fraction of starch are actually different. To gain insight into potential differences in k_d between washout and non-washout fraction, the *in vitro* measurements are based on the comparison of starch degradation between the total feed ingredient and their non-washout fraction.

Materials and methods

Rumen incubations were carried out with four lactating Holstein-Friesian dairy cows and were approved by the Experimental Animal Committee of Wageningen University, The Netherlands.

Materials

The feed ingredients barley, faba beans, maize, peas, oats, and wheat were obtained from local commercial suppliers and were ground to pass a 3 mm sieve (Retsch ZM100, Haan, Germany). For the *in situ* and *in vitro* experiment, different batches of these feed ingredients are used.

Methods

Design This study consisted of an *in situ* and an *in vitro* experiment which were conducted with the six feed ingredients. The *in situ* experiment involved the comparison of rinsing nylon bags after rumen incubation, using a moderate rinsing method as described by de Jonge *et al.* (2013) *vs.* rinsing by a wool wash program of a commercial washing machine (details in the rinsing methods section). The k_d of starch obtained with both rinsing methods was estimated. The particulate matter loss from the bag that occurs during incubation in the rumen was estimated by *in vitro* simulation and was used to correct the k_d obtained with the moderate rinsing method. The *in vitro* experiment involved the incubation of the feed ingredients and their non-washout fractions obtained by the washing machine, in buffered rumen fluid during 48 h. The disappearance of starch in the residue and the gas production was measured during this incubation.

Rumen incubations. The cows were housed indoors and fed *ad libitum* a mixed ration of 50% grass silage (N, 16.6 $g \cdot kg^{-1}$ dry matter (DM); NDF, 516 $g \cdot kg^{-1}$ DM) and 50% maize silage (N, 11.5 $g \cdot kg^{-1}$ DM; NDF, 397 $g \cdot kg^{-1}$ DM; starch, 374 $g \cdot kg^{-1}$ DM) at 7.00 am. Cows received each day an additional 2 kg of protein-rich concentrate feed (N, 53.0 $g \cdot kg^{-1}$), and commercial concentrate feed (N, 29.8 $g \cdot kg^{-1}$) according to milk production level up to a maximum of 7 kg (on average 3 ± 1 kg). Cows were 290 ± 53 d in milk and produced 23.9 ± 4.0 kg $milk \cdot d^{-1}$. All incubation times were conducted separately on different days, starting at 9.00 am, according to the all in all out principle. Samples were incubated for 2, 4, 8, 12, 24 and 48 h. Nylon bags were prepared according to the Dutch *in situ* protocol as described by Tas *et al.* (2006).

Briefly, nylon bags with an inner size of 10 x 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30, Nybolt, Zurich, Switzerland) were filled with approximately 5 g of feed ingredient. The number of bags for each feed ingredient, rinsing method and incubation time combination was 4 per animal.

Rinsing methods. The modified rinsing method described by de Jonge *et al.* (2013) was used. Briefly, four nylon bags were placed in a glass vessel (Ø 19 cm, 7 cm height) containing 500 ml buffer solution (12.2 $g \cdot l^{-1}$ $NaH_2PO_4 \bullet H_2O$ and 8.9 $g \cdot l^{-1}$ $Na_2B_4O_7 \bullet 10H_2O$, adjusted to pH 6.2 with hydrochloric acid). The vessels were placed in a mechanical shaker (Julabo SW-20c; Julabo GmhB, Seelbach, Germany) and were shaken during 60 min at 40 spm at room temperature. For feed ingredient samples that were not rumen incubated, the buffer solution after rinsing was centrifuged for 15 min at 20,000 x *g* at 25°C and the pellet containing the washout fraction (*i.e.* W-S fraction) of starch was quantitatively collected and weighed. The washing machine method was performed as described by Tas *et al.* (2006), using a programmable washing machine (AEG Turnamat, Nuremberg, Germany) with tap water at ~18°C and a gentle wool wash program without centrifuging (40 min in ~ 80 l tap water with three swing turns). For both rinsing methods, the residue of starch in the nylon bags of non-rumen incubated feeds was defined as the non-washout fraction (*i.e.* D-fraction).

Nylon bags after rinsing and the isolated pellets, obtained from non-incubated nylon bags using the modified rinsing method, were air-dried for at least 48 h at 70°C and weighed. The content of the four bags was combined by feed ingredient, animal and incubation time and ground to pass a 1 mm sieve. The samples were analysed for DM and starch.

In vitro *simulation of particulate matter loss.* An *in vitro* simulation, as described by de Jonge *et al*. (2015), was conducted to mimic the process of particulate loss of starch during the incubation. For this purpose, nylon bags containing the feed ingredients were continuously rinsed at a shaking speed of 40 spm that previously showed the best correspondence to the rumen conditions (de Jonge *et al*., 2015). Data obtained for the decrease of starch in the residue from the nylon bag as a function of the incubation time were used to estimate the fractional particulate matter loss rate (k_{pl}; h^{-1}), the fraction of starch insensitive (F_{IS}; $g \cdot g^{-1}$) and sensitive (F_S; $g \cdot g^{-1}$, calculated as $1 - F_{IS}$) to particulate matter loss, with the PROC NLIN procedure of SAS (2002) using a first-order model:

$$Y(t) = F_S \times \exp(- k_{pl} \times t) + F_{IS} \quad (1)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of starch after rinsing during t hours.

In vitro *method.* The *in vitro* fermentation was performed using a fully automated gas production technique as described by Cone *et al.* (1996). Rumen fluid was collected 2 h after morning feeding from three lactating rumen-cannulated cows (220 ± 38 days in milk; production 27.9 ± 3.3 kg milk$\cdot d^{-1}$), fed as described in the previous section. Samples of 0.5 g of the total feed ingredient and their isolated non-washout fraction obtained by washing machine rinsing were incubated in 60 ml buffered rumen fluid in 250 ml bottles in a shaking water bath at 39°C, and gas production was recorded for 48 h as described by Cone *et al.* (1996). Individual incubations were stopped at 4, 8, 24 and 48 h by addition of hydrochloric acid and contents of the bottles were freeze dried. The amount of starch was quantitatively determined in the lyophilized residue. All incubations were repeated in two independent runs containing one replicate within a run.

Chemical analyses. Dry matter content was determined by drying to a constant weight at 103°C (ISO 6496, 1999). Starch was determined by an enzymatic method (ISO 15914, 2004).

Statistical analyses and calculations. The k_d (h^{-1}) of starch in both the *in situ* and the *in vitro* incubations was estimated with the PROC NLIN procedure of SAS (2002) using a first-order model:

$$Y(t) = \exp(- k_d \times t) \quad (2)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of starch after incubation during t hours either expressed relative to residue after rinsing at t = 0 h (*in situ*) or as relative to total starch incubated (*in vitro*). This model was fitted without a non-degradable fraction as starch was assumed to be totally degradable in both the *in situ* and *in vitro* method (Offner *et al.*, 2003).

The effect of microbial contamination on the amount of starch in the nylon bag was regarded to be very small and was therefore ignored (Volden, 2011).

The k_d of starch corrected for particulate matter loss during incubation (k_d-corr; h^{-1}) was estimated as described by de Jonge *et al*. (2015) with the PROC NLIN procedure of SAS (2002) using a first-order model, with F_S, F_{IS} and k_{pl} taken from the *in vitro* simulation of particulate matter loss:

$$Y(t) = F_S \times \exp(- (k_d\text{-corr} + k_{pl}) \times t) + F_{IS} \times \exp(- k_d\text{-corr} \times t) \quad (3)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of starch after incubation during t hours. The effective degradation (ED; $g \cdot g^{-1}$) of starch was calculated as:

$$ED = 1 - D + D \times [k_d / (k_d + k_p)] \quad (4)$$

where D is the non-washout fraction of starch ($g \cdot g^{-1}$) and k_p is the fractional passage rate (h^{-1}). In this study, a fixed value of 0.06 was used as k_p.

In the *in vitro* method, the residues of the W-S fraction for all incubation times were estimated by:

$$\text{Residue-washout } (t) = \exp(- k_d(\text{total}) \times t) - D \times \exp (- k_d(D) \times t) \quad (5)$$

where k_d(total) is the fractional disappearance rate of starch in the total feed ingredient, and k_d(D) is the fractional disappearance rate of starch in the D fraction. The *in vitro* k_d of the W-S fraction was estimated with the PROC NLIN procedure of SAS (2002) by using the estimated residue W-S as function of the incubation time as input variable.

The gas production results were fitted to a mono-phasic Gompertz-curve (equation 6) using the PROC NLIN procedure of SAS (2002):

$$gas(t) = A \times \exp (- B \times \exp (- C \times t)) \quad (6)$$

Where gas(t) is the total volume of produced gas (ml) per g organic matter (OM) at time t, A is the maximal gas production ($ml \cdot g^{-1}$ OM incubated), and B and C are parameters

that are related to the start time of gas production and the fractional rate of gas production, respectively. Parameter C is related to the k_d obtained with the *in vitro* incubation, which were analysed with a first order model.

Analysis of variance was conducted using the GLM procedure of SAS (2002). For the *in situ* measurements, the model consisted of the effect of rinsing (modified method and washing machine) for each feed ingredient. For the analysis of the difference of starch in the residue obtained with both rinsing methods, the model consisted of the effect of incubation time within feed ingredient. For the *in vitro* measurements, the model consisted of the effects of whole material or non-washout fraction and the feed ingredient. When treatment effects were detected (*i.e.* $P < 0.05$), Tukey's test was used to test multiple pairwise comparisons.

Results

In situ experiment

The new rinsing method increased ($P < 0.05$) the non-washout fraction of starch for all feed ingredients compared to washing machine rinsing (Table 1). This increase ranged from 0.100 $g \cdot g^{-1}$ for barley to 0.496 $g \cdot g^{-1}$ for oats, leading to a non-washout fraction for starch between 0.890 $g \cdot g^{-1}$ for oats and 0.993 $g \cdot g^{-1}$ for barley using the modified rinsing method. The k_d of starch for faba beans, maize, and peas were comparable between both rinsing methods and varied between 0.040 and 0.055 h^{-1}. For barley, oats, and wheat, the k_d of starch was lower with the new rinsing method compared with the washing machine method. For these products the average k_d for starch decreased from 0.327 h^{-1} using the washing machine to 0.144 h^{-1} using the new rinsing method. The average ED decreased from 0.761 $g \cdot g^{-1}$ using the washing machine to 0.572 $g \cdot g^{-1}$ using the new rinsing method. The differences in ED of starch between rinsing methods varied between 0.141 $g \cdot g^{-1}$ for maize and 0.269 $g \cdot g^{-1}$ for faba beans.

Table 1 Non-washout fraction (D; $g \cdot g^{-1}$, n = 2), fractional degradation rate (k_d; h^{-1}, n = 4), and the calculated effective degradation (ED; $g \cdot g^{-1}$) of starch in six feed ingredients using the modified rinsing method (40 spm) or the washing machine method

Ingredients	Parameter	40 spm	Washing machine	SEM	*P*
Barley	D	0.993[a]	0.893[b]	0.005	0.008
	k_d	0.092[a]	0.220[b]	0.021	0.005
	ED[1]	0.608	0.809	-	-
Faba beans	D	0.972[a]	0.505[b]	0.002	< 0.001
	k_d	0.044	0.044	0.008	0.841
	ED	0.440	0.709	-	-
Maize	D	0.971[a]	0.846[b]	0.005	0.008
	k_d	0.040	0.055	0.005	0.087
	ED	0.417	0.559	-	-
Oats	D	0.890[a]	0.394[b]	0.006	< 0.001
	k_d	0.234[a]	0.520[b]	0.080	0.049
	ED	0.816	0.959	-	-
Peas	D	0.938[a]	0.552[b]	0.009	0.001
	k_d	0.052	0.049	0.005	0.737
	ED	0.498	0.696	-	-
Wheat	D	0.980[a]	0.813[b]	0.001	< 0.001
	k_d	0.110[a]	0.240[b]	0.010	0.001
	ED	0.654	0.837	-	-

[a,b] Means in the same row with different letters differ ($P < 0.05$)

[1] Calculated as $ED = (1 - D) + D \times (k_d / (k_p + k_d))$, where k_p (fractional passage rate) is 0.06 h^{-1}

Starch content ($g \cdot kg^{-1}$ DM): barley 574; faba beans 441; maize 681; oats 427; peas 476; wheat 674

The difference in the amount of starch in the residue between 40 spm. rinsing and the washing machine rinsing, expressed as fraction of the original amount of starch, as a function of the incubation time showed two patterns (Table 2). For faba beans, oats, and peas, the difference between starch in the residue obtained with both rinsing methods decreased with longer incubation time. For these 3 feeds with high washout fraction the average difference between both rinsing methods decreased from 0.449 $g \cdot g^{-1}$ at 0 h to 0.021

g·g^{-1} at 48 h. For barley, maize, and wheat, the difference between the relative amount of starch in the residue obtained with both rinsing methods increased during the first 4 hours and then decreased with incubation time. For these ingredients, the average difference between both rinsing methods increased from 0.129 g·g^{-1} at 0 h to 0.286 g·g^{-1} at 4 h and subsequently decreased to 0.034 g·g^{-1} at 48 h.

Table 2 Difference between the amount of starch in the residue after rinsing at 40 spm and after the washing machine, expressed as fraction (g·g^{-1}) of the original amount, for different incubation times for six feed ingredients (n = 4)

	Feed ingredients					
Incubation time (h)	Barley	Faba beans	Maize	Oats	Peas	Wheat
0	0.099^{ab}	0.466^{a}	0.124^{ab}	0.496^{a}	0.386^{a}	0.165^{ab}
2	0.207^{ab}	0.366^{a}	0.130^{ab}	0.279^{b}	0.379^{a}	0.320^{a}
4	0.313^{a}	0.313^{ab}	0.208^{a}	0.299^{b}	0.260^{b}	0.339^{a}
8	0.270^{ab}	0.220^{abc}	0.161^{ab}	0.108^{c}	0.220^{bc}	0.228^{a}
12	0.285^{ab}	0.072^{c}	0.162^{ab}	0.016^{c}	0.105^{cd}	0.183^{ab}
24	0.200^{ab}	0.112^{bc}	0.109^{ab}	0.005^{c}	0.110^{cd}	0.024^{b}
48	0.040^{b}	0.022^{c}	0.028^{b}	0.006^{c}	0.037^{d}	0.036^{b}
SEM	0.056	0.056	0.042	0.035	0.023	0.042
P	0.038	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

^{a,b,c,d} Means in the same column with different letters differ ($P < 0.05$)

Particulate matter loss of starch during simulation of the rumen incubation by rinsing at 40 spm (Table 3) was different for the feed ingredients. For barley and maize only a small fraction of starch (*i.e.* < 0.1 g·g^{-1}) was sensitive (*i.e.* F_s) to particulate matter loss and correction for this loss led to a decrease for the k_d of starch of barley and maize of 0.012 and 0.003 h^{-1} respectively. For faba beans, peas, and wheat, F_s varied between 0.27 and 0.40 g·g^{-1}, with a lower fractional disappearance rate (*i.e.* k_{pl}) for wheat (0.071 h^{-1}) than for peas and faba beans (0.287 and 0.266 h^{-1}, respectively). Correction for particulate matter loss for these feed ingredients led to an average decrease in k_d of 0.022 h^{-1}. For oats, F_s was large

(*i.e.* 0.82 $g \cdot g^{-1}$) and correction led to a decrease of 0.104 h^{-1} in k_d. Upon correction for particulate matter loss, the average ED decreased from 0.572 to 0.494 $g \cdot g^{-1}$. The decrease in ED ranged from 0.018 $g \cdot g^{-1}$ for maize to 0.159 $g \cdot g^{-1}$ for faba beans.

Table 3 Fraction of starch in six feed ingredients sensitive to particulate matter loss (F_S; $g \cdot g^{-1}$), fraction of starch insensitive to particulate matter loss (F_{IS}; $g \cdot g^{-1}$), and fractional particulate matter loss rate (k_{pl}; h^{-1}) of F_S obtained with simulation by rinsing at 40 spm, as well as fractional degradation rate (k_d-corr; h^{-1}) and effective degradation (ED; $g \cdot g^{-1}$) for starch after correction of *in situ* k_d for simulated particulate matter loss during incubation

Ingredient	F_s	F_{IS}	k_{pl}	k_d-corr[1]	ED[2]
Barley	0.06	0.94	0.446	0.080	0.574
Faba beans	0.40	0.60	0.266	0.021	0.281
Maize	0.09	0.91	0.069	0.037	0.399
Oats	0.82	0.18	0.151	0.130	0.719
Peas	0.30	0.70	0.287	0.032	0.388
Wheat	0.27	0.73	0.071	0.088	0.603

[1] Calculated as residue(t) = $F_S \times \exp(-(k_{pl} + k_d\text{-corr}) \times t) + F_{IS} \times \exp(-k_d\text{-corr} \times t)$

[2] Calculated as ED = $(1 - D) + D \times (k_d\text{-corr} / (k_p + k_d\text{-corr}))$, where k_p is 0.06 h^{-1} and D in Table 1

In vitro *experiment*

The non-washout fraction of starch of this batch of the feed ingredients ranged from 0.135 $g \cdot g^{-1}$ for oats to 0.930 $g \cdot g^{-1}$ for maize (Table 4) and differed from the results from the previous batch (Table 1). These differences ranged from 0.001 $g \cdot g^{-1}$ for faba beans to 0.259 $g \cdot g^{-1}$ for oats. The *in vitro* k_d of starch in the total product significantly differed between the feed ingredients and ranged from 0.140 h^{-1} for maize to 0.212 h^{-1} for wheat (Table 4). The k_d of the non-washout fraction was on average 0.93 of that of the total fraction and the difference was not significant. The calculated k_d of the washout fraction (*i.e.* W) varied between 0.184 h^{-1} for faba beans and 0.374 h^{-1} for barley. The ratio between the k_d of the washout and non-washout fraction ranged from 0.96 for oats to 2.39 for maize.

Table 4 Starch non-washout fraction (D; $g \cdot g^{-1}$) obtained with the washing machine, *in vitro* fractional degradation rate (k_d; h^{-1}) of starch in the total feed ingredient, and starch in the D fraction, and based on these the calculated k_d in the washout fraction (W) of six feed ingredients (n = 2)

		In vitro fractional degradation rate (k_d ; h^{-1})			
Ingredient	D	Total	D	W[1]	Ratio[2]
Barley	0.852	0.184[abc]	0.161[abc]	0.374	2.32
Faba beans	0.506	0.172[abc]	0.161[abc]	0.184	1.14
Maize	0.930	0.140[a]	0.131[a]	0.313	2.39
Oats	0.135	0.193[bc]	0.201[c]	0.192	0.96
Peas	0.587	0.170[ab]	0.147[ab]	0.218	1.48
Wheat	0.719	0.212[c]	0.199[c]	0.250	1.26

[a,b,c] Means in the same column with different letters differ ($P < 0.05$).

For k_d of total and D-fraction: Feed ingredient: $P = 0.001$ with SEM 0.009; Fraction: $P = 0.126$ with SEM 0.005; no interaction between feed ingredient and fraction was observed ($P = 0.83$)

[1] Calculated based on the difference between the degradation of the total and D fraction

[2] Ratio is k_d(W) / k_d(D)

For all ingredients, all gas production characteristics (*i.e.* A, B, and C in equation 5) were significantly affected by the feed and the interaction between feed and fraction while B and C were also significantly affected by the fraction (Table 5). The maximal gas production (A) ranged from 136 $ml \cdot g^{-1}$ OM for the non-washout fraction of oats to 428 $ml \cdot g^{-1}$ OM for the non-washout fraction of peas. For maize and peas, the maximal gas production of the total product was lower than that of the non-washout fraction, but for oats it was the reverse. The correlation between the maximal gas production and the content of starch in the samples was high ($R^2 = 0.69$) and further increased when the data set was limited to grains only ($R^2 = 0.94$). The fractional rate of gas production (C) for the total product differed significantly from the non-washout fraction. For all ingredients, except for oats, this rate was numerically larger for the total product than for the non-washout fraction, whereas for oats it was the reverse. The correlation between the rate of increase of the gas production characteristic C and the *in vitro* k_d of starch (see Table 4) was high ($R^2 = 0.76$) and further increased when the data set was limited to grains ($R^2 = 0.91$).

Table 5 Gas production characteristics[1] (A, maximal amount of gas produced; B, scaling parameter related to the start of gas production; C, parameter related to fractional rate of gas production) of the total feedstuff and the non-washout starch (D) fraction of 5 feedstuffs

				P		
Ingredient	Total	D	SEM	Feed	Fraction	Feed × Fraction
A (ml·g^{-1} OM)						
Barley	343^{bc}	332^{bc}	7.1	< 0.001	0.81	< 0.001
Faba beans	329^{b}	346^{bc}				
Maize	378^{c,x}	428^{y,d}				
Oats	245^{a,x}	136^{a,y}				
Peas	318^{b,x}	369^{c,y}				
Wheat	324^{b}	318^{b}				
B						
Barley	2.88^{ab}	3.06^{bcd}	0.060	< 0.001	0.019	0.001
Faba beans	3.19^{bc}	2.97^{bc}				
Maize	2.49^{a}	2.77^{b}				
Oats	2.72^{a,x}	2.20^{a,y}				
Peas	3.32^{c}	3.22^{cd}				
Wheat	3.78^{d}	3.45^{d}				
C (h^{-1})						
Barley	0.138^{ab}	0.127^{b}	0.014	< 0.001	0.044	0.021
Faba beans	0.182^{b}	0.123^{ab}				
Maize	0.061^{a}	0.048^{a}				
Oats	0.184^{b}	0.239^{c}				
Peas	0.186^{b}	0.142^{b}				
Wheat	0.215^{b}	0.179^{bc}				

^{a,b,c} Means in the same column with different letters differ for each parameter ($P < 0.05$)

^{x,y} Means in the same row with different letters differ ($P < 0.05$)

[1] Calculated as Gas(t) = A × exp (-B × exp (-C × t)) (Gompertz-curve)

Starch content (g·kg^{-1}): barley 520 (total) and 565 (D); faba beans 350 (total) and 385 (D); maize 620 (total) and 715 (D); oats 390 (total) and 106 (D); peas 400 (total) and 460 (D); wheat 550 (total) and 595 (D)

Discussion

The aim of this study was to estimate the *in situ* rumen k_d of starch of feed materials in both the washout and non-washout fraction by using a modified *in situ* protocol and additional *in vitro* measurements. The modified *in situ* measurement was based on reduction of the washout fraction and comparison of the k_d to that obtained by the traditional washing machine procedure. Although with the modified method the reduction in washout fraction was successfully realized, which was in line with previous observations (de Jonge *et al*., 2013), this did not lead to a significant increase of k_d of starch, as would be expected based on the assumptions on k_d of the washout fraction used in the Dutch DVE/OEB system (*i.e.* 2 × k_d(D-fraction) + 0.375; van Duinkerken *et al*., 2011) or the Scandinavian Norfor system (*i.e.* 1.5 h^{-1} ; Volden, 2011). For faba beans, maize and peas, the k_d remained the same despite the marked reduction of the washout fraction when using the new rinsing method, whereas for barley, oats, and wheat the k_d decreased compared to the washing machine rinsing method. These observations would suggest that the washout fraction of starch has a lower degradation rate than the non-washout fraction, which seems to be unrealistic based on the physical state of this fraction (France *et al.*, 1993). An alternative explanation for this decrease in degradation rate is the process of particle size reduction during the incubation in combination with particulate matter loss, which was different for the two rinsing methods. This process is described as secondary particulate matter loss by Huhtanen and Sveinbjörnsson (2006) which is the breakdown of particles during the incubation to a size smaller than the pore size of the nylon bag, which makes them potentially sensitive to losses during rinsing. Results obtained for the washout fraction clearly demonstrated that the washing machine rinsing was much more effective in removing these small particles from the nylon bag compared to the modified method. Consequently, the *in situ* method was more affected by the process of particle size reduction when applying washing machine rinsing. For barley, wheat and to a lesser extent maize, this effect could be observed by an increase of the difference in the residual starch between both rinsing methods during the first 4 h of incubation, which implicates an increase of small particles in the polyester bags during the first stage of the incubation (Table 2). The effect of secondary particulate matter loss was also found in the study of Tothi *et al*. (2003). They showed a decrease of the k_d of starch in barley and maize by reducing the pore size of the nylon bag from 36 to 15 µm. The consequence of secondary particulate matter loss is that

the difference found for the k_d of starch between both rinsing methods was not exclusively caused by the degradation rate of the washout fraction and could not be used to estimate the k_d of this fraction.

The *in vitro* starch degradation results were not affected by the problem of secondary particulate matter loss and did not indicate any significant difference in k_d between both methods. The ratio between the calculated k_d of the washout fraction and the k_d of the non-washout fraction averaged 1.59, but varied between 0.96 for oats and 2.39 for maize. The highest ratio was still lower than that which may be calculated using feed evaluation systems (van Duinkerken *et al.*, 2011; Volden, 2011). The results from the gas production, especially for the fractional rate of gas production, were clearly correlated to the *in vitro* results and therefore also did not support the general assumption about the very fast degradation of the washout fraction of starch in feed ingredients. The negative effect of the presence of the washout fraction in oats on both the *in vitro* k_d and the rate of increase of the gas production was quite remarkable. The results for the gas production were in line with the study of Stevnebø *et al.* (2009) that showed no great differences between the small and large particles in several barley cultivars.

The *in situ* method is the standard method to predict the ruminal ED of nutrients and is used to rank feed ingredients according to nutritional value. The methodological weaknesses of applying washing machine rinsing were the use of assumptions for the washout fraction and the effect of secondary particle loss on the k_d which led for starch to an overestimation of the *in situ* degradation. This overestimation, however, seems to partly compensate for the unfavourable fermentation conditions caused by limited access of microbes and accumulation of end products in the nylon bag compared to *in vivo* rumen conditions (Offner *et al.*, 2003), and for the high fractional passage rate assumed that ignores selective retention of feed particles in the rumen (Allen and Mertens, 1988). Hindle *et al.* (2005) showed that the efficiency of this compensation for unfavourable fermentation conditions varied strongly between feed ingredients. In that study a good match between the *in vivo* and *in situ* degradation of starch in wheat was obtained whereas for starch in maize a very large difference was found. The modified method reduced the methodological weakness of the washout fraction, but also reduced this compensation and therefore emphasised the difference between the calculated ED and the *in vivo* results for these ingredients as reported by Larsen *et al.* (2009). This difference even further increased when a full

correction was made for possible particulate loss during the incubation leading to very low k_d for starch (see Table 3). A regression between *in vivo* and *in situ* data, as given by Offner and Sauvant (2004), seems to be a good approach to convert *in situ* results into *in vivo* data.

The *in vitro* method could be an attractive alternative for the *in situ* method because of the absence of the problem of particulate matter loss which makes the need for assumptions on the k_d of the washout fraction redundant. The gas production method, however, contains the disadvantages that it is not specific for starch and that the relation between starch degradation and gas production differs between feed ingredients (Chai *et al.*, 2004). In this study, this problem was observed by the decrease of the correlation between *in vitro* degradation and the rate of increase in gas production when the results from legume seed, containing a high amount of soluble proteins (de Jonge *et al.*, 2013), were added to those from grains. An *in vitro* method based on measuring the starch degradation could be an attractive alternative, although the results of Weisbjerg *et al.* (2011) showed that this approach needs further improvement and validation. This approach, however, also required a conversion of *in vitro* results to the *in vivo* situation. To evaluate the benefit of this method above the *in situ* procedure additional research involving a larger set of feed ingredients with *in vivo*, *in vitro* and *in situ* techniques is required.

Conclusions

The *in vitro* results showed for all ingredients that the k_d of starch in the non-washout fraction did not differ from that in the total product. The differences between k_d of the washout and the non-washout fraction were much less than typically assumed in feed evaluation systems

The *in situ* k_d of starch in barley, oats, and wheat was affected by the particle size reduction of these products during the incubation, especially when using washing machine rinsing. The use of the modified rinsing method reduced the problem of particulate matter loss, but also led to a lower effective degradability when compared to *in vivo* results.

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Chapter 6

Effect of rinsing method on the *in situ* degradation of nitrogen in grains and legume seeds

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Abstract

This study compared a modified rinsing method, based on reduction of small particulate matter loss during rinsing, with the conventional washing machine method, for the effect on *in situ* degradation of nitrogen (N) in barley, faba beans, maize, oats, peas, and wheat. In the modified method, nylon bags after rumen incubation were rinsed with a buffer in a shaking water bath under well controlled conditions instead of rinsing with tap water in a washing machine. The modified method reduced the loss of N in small particles during rinsing, compared to the use of the washing machine, and also reduced the proportion of soluble N especially for legume seeds. The average non-washout fraction of N in the feed ingredients increased from 0.644 (washing machine) to 0.863 $g \cdot g^{-1}$ (modified method). Therefore the calculated effective degradation (ED) was less dependent on assumptions about the fractional degradation rate (k_d) of the washout fraction. In comparison to the washing machine method, the k_d for N obtained with the modified method was higher for legume seeds (except faba beans where kd did not differ significantly between both methods), but lower for grains. The average non-degradable fraction (U) increased from 0.048 (washing machine method), to 0.144 $g \cdot g^{-1}$ (modified method). The average ED for the feed ingredients decreased from 0.621 (washing machine method) to 0.458 $g \cdot g^{-1}$ (modified method). The modified method decreased the ED of N in the grains stronger than in legume seeds. The difference in ED between both rinsing methods was mainly caused by the reduction of the soluble fraction for the legume seeds and by the reduction of the k_d for the grains. For barley, faba beans, maize, and peas a higher ratio diaminopimelic acid (DAPA) / N was found for the modified method compared to the washing machine method. The DAPA based estimated microbial contamination was larger than 100% of the total protein present at the short incubation times (*i.e.* < 8 h) when using the modified method, and this suggests that lysis of bacterial cells during the modified rinsing method occurred. Correction for estimated degree of lysis (by a modified microbial N – DAPA ratio) strongly influenced the calculated ED of N.

Keywords: Particulate matter loss; *in situ* protocol; microbial contamination; rumen degradability; protein

Implication

This study showed that a modified rinsing method influences the results obtained from the *in situ* method compared with the washing machine method, by increasing the non-washout fraction, decreasing the soluble fraction, and increasing (legumes) or decreasing (grains) the fractional degradation rate of the non-washout fraction. These changes affect the calculated effective degradation of N and may influence the nutritional ranking between feed ingredients.

Introduction

The basic structure of all protein evaluation models of cattle is similar, with rumen degradable and non-degradable protein being distinguished, and with ruminally synthesised microbial protein from dietary and endogenous nitrogen (N) sources as well as non-degraded protein contributing to total absorbable protein from the small intestine (Bach *et al.*, 2005). The rate and extent of protein degradation in the rumen is not only a major determinant of protein outflow from the rumen, but will also significantly affect the efficiency of N use and the level and form of N excreted in urine (Dijkstra *et al.*, 2013). A widely used procedure to measure ruminal degradation of nutrients is the *in situ* method which is based on the disappearance of substrate from porous nylon (or dacron) bags incubated in the rumen of rumen fistulated animals (Ørskov and McDonald, 1979; López, 2005). Results of the *in situ* method are used in numerous feed evaluation systems, such as Norfor (Volden, 2011), DVE/OEB (van Duinkerken *et al*., 2011), FiM (Thomas, 2004), and NRC (NRC, 2001). A methodological problem of the *in situ* procedure is the inevitable loss of non-degraded matter from the bags (washout fraction, W), during both incubation and subsequent rinsing (De Jonge *et al.*, 2015a), which is influenced by the particle size of the feed material in relation to the pore size of the bag (Michalet-Doreau and Ould-Bah, 1992; Vanzant *et al*., 1998; López, 2005) and the severity of rinsing. This fraction of material lost from the bags comprises two subfractions of presumably different kinetic behaviour, *i.e.* a truly soluble fraction (S) and a particulate matter loss fraction (W-S) (Dhanoa *et al*., 1999). The fractional degradation rates (k_d) of these fractions cannot be measured *in situ* and therefore in various feed evaluation systems assumptions on the degradation rates of these fractions are made to be able to estimate its fermentability (Offner *et al*., 2003; van Duinkerken *et al.,* 2011; Volden, 2011).

To resolve this problem, a modified *in situ* method is developed that contains a gentle rinsing method that markedly reduces the washout fraction of nutrients in feed ingredients, especially starch, compared to the conventional washing machine procedure (de Jonge *et al.*, 2013). This modified method also enabled the determination of the *in situ* degradation of organic matter (OM) and nitrogen (N) in wheat yeast concentrates characterised by a high fraction of small particles (de Jonge *et al.*, 2015a). A recent study (de Jonge *et al*., 2015b), however, showed that for grains not only the size of the washout fraction decreased compared with the traditional washout method, but also the measured k_d of the non-washout fraction of starch, leading to a lower effective degradation (ED) compared to the standard washing machine method. It is possible that application of the modified method affects the ED of other nutrients, especially proteins. Compared to starch, the modified method can affect the estimated degradation rate of protein (*i.e.* N) in two additional ways. Firstly, the use of a different solvent, compared to rumen fluid, during rinsing can affect the solubility of N in feed ingredients (de Jonge *et al.*, 2009). Secondly, a lower severity of rinsing can lead to a higher degree of microbial contamination of the residues.

The objective of this study is to investigate the effects of a modified method with gentle rinsing of nylon bags, on the measured *in situ* degradation rate of N in six feed ingredients, as compared to the standard method. The contribution of the different processes to the total effect on the ED and the effect of microbial contamination were investigated.

Materials and methods

Materials

The feed ingredients, barley, faba beans, maize, peas, oats, and wheat were obtained from local commercial suppliers and were ground to pass a 3 mm sieve (Retsch ZM100, Haan, Germany), and are equal to those used in a previous study (de Jonge *et al*., 2015b).

Methods

Design. The *in situ* experiment involved the use of a moderate rinsing method (termed modified method) and a vigorous rinsing method (termed washing machine method) after rumen incubation, which results in low and high particulate matter loss, respectively. The k_d

and the non-degradable (U) fraction obtained with both rinsing methods were estimated. The particulate matter loss during incubation was estimated by *in vitro* simulation and used to correct the degradation characteristics obtained with the moderate rinsing method. Correction for microbial contamination was conducted by the analysis of diaminopimelic acid (DAPA) and the use of various ratios between DAPA and microbial N.

Rumen incubations. Rumen incubations were carried out with four lactating Holstein-Friesian dairy cows (290 ± 53 d in milk and producing 23.9 ± 4.0 kg milk·d^{-1} and were approved by the Experimental Animal Committee of Wageningen University, The Netherlands. The cows were housed indoors and fed *ad libitum* a mixed ration of 50% grass silage and 50% maize silage at 7.00 am each day, and cows received each day an additional 2 kg of protein-rich concentrate feed and commercial concentrate feed according to milk production level up to a maximum of 7 kg (for chemical composition, see De Jonge *et al.*, 2015b). All incubation times were conducted separately on different days, starting at 9.00 am according to the all in all out principle. Samples were incubated for 2, 4, 8, 12, 24, 48, 96 and 336 h. Nylon bags were prepared according to the Dutch *in situ* protocol as described by Tas *et al.* (2006). Briefly, nylon bags with an inner size of 10 × 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30, Nybolt, Switzerland) were filled with approximately 5 g of feed ingredient. The number of bags for each feed ingredient, rinsing method and incubation time combination was 4 per animal.

Rinsing methods. The modified rinsing method described by de Jonge *et al.* (2013) was used. Briefly, four nylon bags were placed in a glass vessel (Ø 19 cm, 7 cm height) containing 500 ml buffer solution (12.2 g·l^{-1} $NaH_2PO_4 \bullet H_2O$ and 8.9 g·l^{-1} $Na_2B_4O_7 \bullet 10H_2O$, adjusted to pH 6.2 with hydrochloric acid). The vessels were placed in a mechanical shaker (Julabo SW-20c; Julabo GmhB, Seelbach, Germany) and were shaken during 60 min at 40 spm at room temperature. For feed ingredient samples that were not rumen incubated, the buffer solution after rinsing was centrifuged for 15 min at 20,000 × *g* at 25°C and the supernatant containing the S fraction of N and the pellet containing the W-S fraction of N were quantitatively collected and weighed.

The washing machine method was performed as described by Tas *et al.* (2006), using a programmable washing machine (AEG Turnamat, Nuremberg, Germany) with tap water at ~

18°C and a gentle wool wash program without centrifuging (40 min in ~ 80 l tap water with three swing turns). The S fraction of N was determined by extraction of 3 g of feedstuff with 75 ml tap water during 30 min under mechanical stirring at room temperature. The solution was centrifuged for 15 min at 3,000 × *g* and an aliquot of the supernatant was analysed for N. The W-S fraction of N was calculated by difference.

Nylon bags after rinsing and the isolated pellets, obtained from non-incubated nylon bags using the modified rinsing method, were air-dried for at least 48 h at 70°C and weighed. The contents of the four bags were combined by feed ingredient, animal and incubation time and ground to pass a 1 mm sieve. The samples were analysed for DM and N and additionally for two cows for diaminopimelic acid (DAPA) as marker for microbial contamination. For some feed ingredients, in particular wheat and peas, insufficient residual matter was present at later incubation times to allow DAPA determination.

Chemical analyses. Dry matter content was determined by drying to a constant weight at 103°C (ISO 6496, 1999). Nitrogen was determined by the Kjeldahl method (ISO 5983-2, 2005). DAPA was determined by the amino acid method including previous oxidation (ISO 13903, 2005).

In vitro *simulation of particulate matter loss.* Disappearance from the nylon bags during incubation is due to degradation and due to loss of small particles during incubation. For insoluble N, the particulate matter loss during incubation was simulated by *in vitro* rinsing of non-incubated nylon bags in buffer solution of pH 6.2 at a shaking speed of 40 spm for 2, 4, 8, 24 and 48 h, as it has been shown previously that at this shaking speed the loss of small particles was similar between rumen incubation and *in vitro* simulation (de Jonge *et al*., 2015a). Data obtained for the decrease of N in the residue as a function of the incubation time were used to estimate the fractional particulate matter loss rate (k_{pl}; h^{-1}), the fraction of N insensitive to particulate matter loss (F_{IS}; $g \cdot g^{-1}$) and the fraction of N sensitive to particulate matter loss (F_S; $g \cdot g^{-1}$, calculated as $1 - F_{IS}$), with the PROC NLIN procedure of SAS (2002) using a first-order model:

$$Y(t) = F_S \times \exp(- k_{pl} \times t) + F_{IS} \quad (1)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of insoluble N after rinsing during t hours.

Correction for microbial contamination. The ratio between DAPA and N in the residue was used to determine the degree of microbial N contamination. The ratio between microbial N and DAPA was based on an assumed degree of lysis of the bacterial cells after washing. The ratio without lysis was assumed to be 34.3 based on the study of Nocek and Grant (1987), whereas the ratio after lysis of cells, assuming all bacterial cell contents are released, was 1.4 since only 0.04 of the total microbial N is located in the cell wall (based on data as summarized by Dijkstra *et al*., 1992). This ratio was calculated as $34.3 \times (1 - \alpha) + 1.4 \times \alpha$, where α is the level of lysis. In this study, the effect of four different levels of lysis, *i.e.* 0, 0.25, 0.50, 0.75, and 1.00 were investigated. Correction for microbial contamination for the individual residues was conducted by subtraction of the calculated microbial N from the N analysed.

Statistical analyses and calculations. The k_d (h^{-1}) and the non-degradable fraction (U; $g \cdot g^{-1}$) of the non-washout N (*i.e.* D+U; $g \cdot g^{-1}$), with various levels of correction of microbial contamination, were estimated with the PROC NLIN procedure of SAS (2002) using a first-order model:

$$Y(t) = (1 - U) \exp(-k_d \times t) + U \quad (2)$$

where Y(t) is the fractional residue ($g \cdot g^{-1}$) of N after incubation during t hours expressed relative to residue after rinsing at t = 0 h (*in situ*). The effective degradation (ED; $g \cdot g^{-1}$) of N was calculated as:

$$ED = S + (1 - S - U) \times [k_d / (k_d + k_p)] \quad (3)$$

where S ($g \cdot g^{-1}$) is the soluble fraction and assumed to be fully degraded, U ($g \cdot g^{-1}$) is the non-degradable fraction, and k_p is the fractional passage rate assumed to be 0.06 h^{-1} for compound feed materials. The k_d of the W-S fraction is assumed to be equal to that of the D fraction (van Duinkerken *et. al.*, 2011).

The difference found in ED (ΔED) using the modified method compared to the standard method was divided into three processes that contribute to this difference (see Figure 1):

1. Change in S fraction: $\Delta ED(S\text{-fraction}) = [S(40\ spm) - S(washing\ machine)] \times \{1 - [k_d(40\ spm) / (k_d(40\ spm) + k_p)]\}$
2. Change in U fraction: $\Delta ED(U\text{-fraction}) = - [U(40\ spm) - U(washing\ machine)] \times [k_d(washing\ machine) / (k_d(washing\ machine) + k_p)]$,
3. Change in k_d of W-S and D fraction: $\Delta ED(k_d\text{-fraction}) = [1 - S(washing\ machine) - U(40\ spm)] \times \{[k_d(40\ spm)/ (k_d(40\ spm) + k_p)] - [k_d(washing\ machine) / (k_d(washing\ machine) + k_p)]\}$

The fractional disappearance rate (k_d-corr; h^{-1}) and non-degradable fraction (U-corr; $g \cdot g^{-1}$) of N corrected for particulate matter loss of insoluble N was estimated as described by de Jonge *et al*. (2015a) with the PROC NLIN procedure of SAS (2002) using a reduced second-order model, with F_S and k_{pl} taken from the *in vitro* simulation of particulate matter loss:

$$Y(t) = F_S \times \exp(- (k_d\text{-corr} + k_{pl}) \times t) + (1 - F_S - U\text{-corr}) \times \exp(- k_d\text{-corr} \times t) + U\text{-corr} \quad (4)$$

where Y(t) is the fractional residue of insoluble N after incubation during t hours and with F_S and k_{pl} obtained as described previously (equation 1). The effective degradation corrected for particulate matter loss (ED_c; $g \cdot g^{-1}$) of N was calculated as:

$$ED_c = S + (1 - S) \times [(1 - U\text{-corr}) \times k_d\text{-corr} / (k_d\text{-corr} + k_p)] \quad (5)$$

Analysis of variance was conducted using the GLM procedure of SAS (2002). For the *in situ* measurements, the model consisted of the effect of rinsing (modified method and

washing machine) for each feed ingredient. For the microbial contamination, the model consisted of the effect of rinsing, time, and their interaction for each feed ingredient. When treatment effects were detected (*i.e.* $P < 0.05$), Tukey's test was used to test multiple pairwise comparisons.

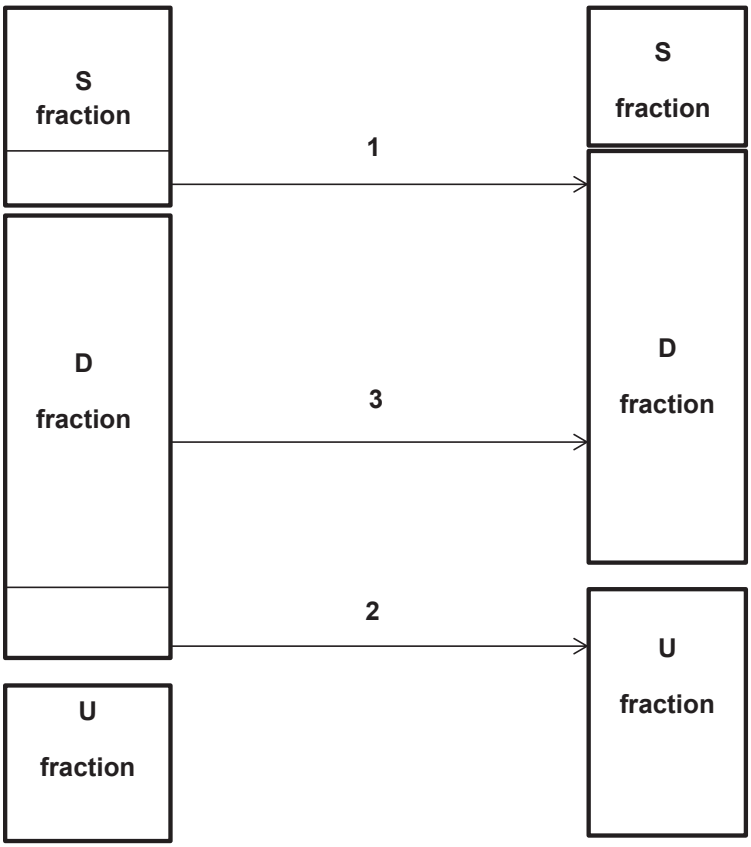

Figure 1 Different effects of changing the rinsing method that influenced the calculated effective degradation (ED): decrease of S fraction (line 1), increase of U fraction (line 2), and change of k_d (fractional degradation rate) of W-S and D fraction (line 3)

Results

The modified rinsing method decreased the S fraction of N for all feed ingredients compared to washing machine rinsing, except for maize (Table 1). For the grains, this decrease ranged from 0.044 $g \cdot g^{-1}$ for maize to 0.065 $g \cdot g^{-1}$ for oats, whereas for the legume seeds the decrease was more pronounced, *viz*. 0.267 and 0.279 $g \cdot g^{-1}$ for peas and faba beans, respectively. The modified rinsing method also decreased the W-S fraction of N for all feed ingredients, with the decrease ranging from 0.035 $g \cdot g^{-1}$ for wheat to 0.200 $g \cdot g^{-1}$ for oats. Consequently, the modified rinsing method increased the non-washout fraction (D+U) of N for all feed ingredients, the increase ranging from 0.083 $g \cdot g^{-1}$ for wheat, to 0.435 $g \cdot g^{-1}$ for faba beans. For the grains, the k_d of N was lower with the new rinsing method compared with the washing machine method. For these products the average k_d for N decreased from 0.122 h^{-1} using the washing machine to 0.062 h^{-1} using the new rinsing method. The k_d of N for peas increased using the modified rinsing method whereas for faba beans no significant difference between both rinsing methods was found. The modified rinsing method increased the average non-degradable fraction (U) of all feed ingredients from 0.048 $g \cdot g^{-1}$ when using the washing machine, to 0.144 $g \cdot g^{-1}$ when using the modified method. These increases varied between 0.073 $g \cdot g^{-1}$ for faba beans and 0.131 $g \cdot g^{-1}$ for maize.

Table 1 Soluble (S; $g \cdot g^{-1}$), insoluble washout (W-S; $g \cdot g^{-1}$) and non-washout (D+U; $g \cdot g^{-1}$) fractions, fractional degradation rate (k_d; h^{-1}), non-degradable fraction (U; $g \cdot g^{-1}$), and effective degradation (ED; $g \cdot g^{-1}$) of nitrogen in six feedstuff obtained with the modified rinsing method (40 spm) and washing machine rinsing method (n = 4)

		Rinsing methods			
Feedstuff	Parameter	40 spm	Washing machine	SEM	*P*
Barley	S	0.049	0.106	0.005	0.014
	W-S	0.003	0.070[1]	-	-
	D+U	0.947	0.822	0.005	0.003
	k_d	0.039	0.081	0.009	0.017
	U	0.153	0.068	0.014	0.005
	ED[2]	0.363	0.580	-	-
Faba beans	S	0.187	0.466	0.004	< 0.001
	W-S	0.006	0.160[1]	-	-
	D+U	0.805	0.370	0.018	0.003
	k_d	0.050	0.028	0.009	0.130
	U	0.077	0.004	0.006	< 0.001
	ED[2]	0.521	0.634	-	-
Maize	S	0.042	0.086	0.014	0.154
	W-S	0.011	0.073[1]	-	-
	D+U	0.946	0.840	0.016	0.041
	k_d	0.018	0.031	0.002	0.011
	U	0.226	0.095	0.015	< 0.001
	ED[2]	0.211	0.365	-	-
Oats	S	0.071	0.136	0.001	0.003
	W-S	0.058	0.258[1]	-	-
	D+U	0.870	0.605	0.004	< 0.001
	k_d	0.140	0.290	0.035	0.020
	U	0.174	0.060	0.009	< 0.001
	ED[2]	0.599	0.805	-	-
Peas	S	0.249	0.516	0.008	0.002
	W-S	0.041	0.079[1]	-	-
	D+U	0.709	0.404	0.010	0.002
	k_d	0.070	0.047	0.003	0.003
	U	0.090	0.003	0.014	0.005
	ED[2]	0.605	0.727	-	-
Wheat	S	0.092	0.142	0.002	0.004
	W-S	0.001	0.036[1]	-	-
	D+U	0.906	0.823	0.001	< 0.001
	k_d	0.052	0.087	0.004	0.002
	U	0.144	0.058	0.008	< 0.001
	ED[2]	0.446	0.615	-	-

[1] Calculated as W-S = 1 – S – (D+U)

[2] Calculated as ED = S + (1 – S – U) × (k_d / (k_d + k_p)) where k_p = 0.06 h^{-1}

Nitrogen content ($g \cdot kg^{-1}$ DM): barley 18.9, faba beans 38.1, maize 15.4, oats 20.4, peas 38.0, and wheat 21.3

The average ED decreased from 0.621 $g \cdot g^{-1}$, using the washing machine, to 0.457 $g \cdot g^{-1}$, using the modified method. The total effect of the rinsing method on the ED was larger for the grains than for the legume seeds. The contribution to this decrease by the shifts in S and U fractions and k_d was different for grains *vs.* legume seeds (Table 2). For the grains, all three shifts reduced ED with the reduction due to change in k_d being the largest. For the legume seeds the decrease of the S fraction had the largest contribution to the total effect which was partially compensated by an increase of the k_d for these products.

Table 2 Effect of the change of the soluble fraction (S fraction; $g \cdot g^{-1}$), of the non-degradable fraction (U fraction; $g \cdot g^{-1}$), and of the fractional degradation rate (k_d; h^{-1}) on the change in effective degradation (ΔED; $g \cdot g^{-1}$) between both rinsing methods (modified method at 40 spm *vs.* washing machine method) for six feed ingredients (n = 4)

	ΔED			
Feedstuff	S fraction[1]	U fraction[2]	k_d[3]	Total
Barley	- 0.034	- 0.049	- 0.134	- 0.217
Faba beans	- 0.152	- 0.023	0.062	- 0.113
Maize	- 0.033	- 0.045	- 0.076	- 0.154
Oats	- 0.020	- 0.095	- 0.091	- 0.206
Peas	- 0.123	- 0.038	0.039	- 0.122
Wheat	- 0.027	- 0.051	- 0.091	- 0.169

[1] Calculated as ΔED(S-fraction) = [S(40 spm) – S(washing machine)] × {1 – [k_d(40 spm) / (k_d(40 spm) + k_p)]}, where k_p (fractional passage rate) is 0.06 h^{-1}

[2] Calculated as ΔED(U-fraction) = – [U(40 spm) – U(washing machine)] × [k_d(washing machine) / (k_d(washing machine) + k_p)], where k_p (fractional passage rate) is 0.06 h^{-1}

[3] Calculated as ΔED(k_d-fraction) = [1 – S(washing machine) – U(40 spm)] × {[k_d(40 spm) / (k_d(40 spm) + k_p)] – [k_d(washing machine) / (k_d(washing machine) + k_p)]}, where k_p (fractional passage rate) is 0.06 h^{-1}

Particulate matter loss of N during simulation of the rumen incubation by rinsing at 40 spm (Table 3) was different for the feed ingredients. The fraction sensitive to particulate matter loss (*i.e.* F_s) ranged from 0.15 $g \cdot g^{-1}$ for maize to 0.60 $g \cdot g^{-1}$ for oats while the fractional particulate matter loss rate (*i.e.* k_{pl}) varied between 0.13 h^{-1} for oats and 0.55 h^{-1} for faba

beans. The k_d corrected for particulate matter loss (k_d-corr) was on average 0.033 h^{-1} which was lower than the uncorrected k_d (0.062 h^{-1}). This difference varied between 0.006 h^{-1} for maize and 0.072 h^{-1} for oats. The non-degradable fraction corrected for particulate matter loss (U-corr) was on average 0.129 $g \cdot g^{-1}$ which was lower than the uncorrected U (0.144 $g \cdot g^{-1}$). The greatest absolute difference was 0.030 $g \cdot g^{-1}$ for faba beans. Upon correction for particulate matter loss, the average ED decreased from 0.457 to 0.360 $g \cdot g^{-1}$ with the individual decrease ranging from 0.043 $g \cdot g^{-1}$ for maize to 0.169 $g \cdot g^{-1}$ for faba beans.

Table 3 Fraction sensitive to particulate matter loss (F_S; $g \cdot g^{-1}$), fraction insensitive to particulate matter loss (F_{IS}; $g \cdot g^{-1}$) and the fractional particulate matter loss rate (k_{pl}; h^{-1}) of F_S obtained with simulation at 40 spm and the fractional degradation rate (k_d-corr; $g \cdot g^{-1}$), non-degradable fraction (U-corr; $g \cdot g^{-1}$) and the effective degradation (ED_c; $g \cdot g^{-1}$) for nitrogen in the feed ingredients after correction of *in situ* measured data for particulate matter loss during incubation

Feedstuff	F_S[1]	F_{IS}[1]	k_{pl}	k_d-corr[2]	U-corr[2]	ED_c[3]
Barley	0.17	0.83	0.22	0.030	0.151	0.315
Faba beans	0.46	0.54	0.55	0.017	0.047	0.352
Maize	0.15	0.85	0.18	0.012	0.199	0.168
Oats	0.60	0.40	0.13	0.068	0.181	0.467
Peas	0.30	0.70	0.18	0.042	0.083	0.522
Wheat	0.31	0.69	0.14	0.027	0.117	0.339

[1] Expressed as fraction of the total insoluble fraction

[2] Calculated as $Y(t) = F_S \times \exp[-(k_{pl} + k_d\text{-corr}) \times t] + (1 - F_S - \text{U-corr}) \times \exp(-k_d\text{-corr} \times t)$

[3] Calculated as $ED_c = S + (1 - S - \text{U-corr}) \times [k_d\text{-corr} / (k_d\text{-corr} + k_p)]$; for S (soluble fraction, 40 spm) see Table 1; k_p (fractional passage rate) is 0.06 h^{-1}

The ratio DAPA / N of the residues using the modified method was larger ($P < 0.022$) than using the washing machine for barley, faba beans, maize, and peas whereas for wheat a trend ($P = 0.093$) was observed (Table 4). The average DAPA / N ratio using the modified method was 0.022 and varied between 0.008 and 0.055, whereas using the washing machine the average DAPA / N ratio was 0.011 and varied between 0.003 and 0.038. For barley, faba beans, and maize, the average relative microbial contamination using the modified method

decreased from 0.031 at 2 h to 0.013 at 96 h. For barley and maize an increase of the microbial contamination was observed between 96 and 336 h of incubation. No significant time effects were found for the oats, peas, and wheat. Only for faba beans, a significant interaction between rinsing and incubation time was observed. For this product, the difference in DAPA / N ratio between rinsing methods was high at 2 and 4 h of incubation (0.020), and from 8 h onwards this difference was 0.006 on average.

Table 4 The average ratio of DAPA and N in incubation residues obtained with the modified rinsing method (40 spm) and washing machine rinsing method for six feed ingredients at different rumen incubation times

Feed	Time (h)	DAPA / N ratio		SE	*P*		
		40 spm	Washing machine		Rinsing	Time	Rinsing × time
Barley	2	0.033	0.012	0.004	0.001	0.049	0.098
	4	0.030	0.007				
	8	0.024	0.010				
	12	0.008	0.006				
	24	0.014	0.011				
	48	0.012	0.006				
	96	0.008	0.011				
	336	0.018	0.012				
Faba beans	2	0.032	0.007	0.002	< 0.001	0.003	0.008
	4	0.020	0.005				
	8	0.014	0.011				
	12	0.011	0.005				
	24	0.012	0.004				
	48	0.011	0.004				
	96	0.015	0.008				
Maize	2	0.027	0.007	0.003	< 0.001	0.002	0.290
	4	0.016	0.007				
	8	0.021	0.008				
	12	0.010	0.004				
	24	0.015	0.009				
	48	0.012	0.005				
	96	0.016	0.006				
	336	0.030	0.015				
Oats	2	0.012	0.009	0.014	0.172	0.651	0.663
	4	0.045	0.020				
	8	0.055	0.018				
	12	0.033	0.014				
	24	0.020	0.018				
	48	0.023	0.031				
	96	0.036	0.024				
	336	0.027	0.038				
Peas	2	0.033	0.005	0.006	0.022	0.481	0.481
	4	0.017	0.010				
	8	0.024	0.011				
	12	0.027	0.008				
	24	0.014	0.003				
Wheat	2	0.025	0.011	0.009	0.093	0.450	0.999
	4	0.038	0.026				
	8	0.035	0.022				
	12	0.027	0.016				

When using the washing machine method, the average microbial contamination (*i.e.* N-microbial / N-total) assuming no lysis of microbial cells was 0.40 and ranged from 0.10 to 1.30. In case of the modified method, the average contamination was 0.77 and ranged from 0.27 to 1.88. Especially at short incubation times (*i.e.* < 8 h) theoretical impossible values (*i.e.* contamination > 1.0) were found for most feed ingredients when using the modified method. For the modified method, the assumption of the degree of lysis of the bacterial cells markedly influenced the degradation characteristics and therefore the calculated ED (Table 5; no ED for peas and wheat could be calculated due to absence of results at later incubation times). Using a moderate degree of lysis (*i.e.* 0.25 and 0.50), the correction for the microbial contamination at short incubation times seemed still too high leading to a lower amount of N corrected for microbial contamination in the residue compared to longer incubation times. At higher degree of lysis (*i.e.* $\geq$ 0.50), the degradation curve showed a good first-order fit and leading to a modest increase of the average ED of 0.086 and 0.021 at a degree of lysis of 0.75 and 1.00, respectively. In case of the washing machine, the calculated ED was less sensitive for microbial contamination and the degree of lysis used for the correction, except for maize. The decrease of the average ED was 0.108 and 0.012 at a degree of lysis of 0.00 and 1.00, respectively.

Table 5 Effective degradation ($g \cdot g^{-1}$) of nitrogen in barley, faba beans, maize and oats with and without correction for microbial contamination based on different degrees of lysis of bacterial cells obtained with the modified method (40 spm) and the washing machine method for cow 1 and 2 (n = 2)

Feed	Method	Without correction	Degree of lysis				
			0.00	0.25	0.50	0.75	1.00
Barley	40 spm	0.325	0.489	0.449	0.423	0.418	0.338
	Washing machine	0.577	0.684	0.657	0.631	0.613	0.584
Faba beans	40 spm	0.482	0.723	0.682	0.629	0.589	0.518
	Washing machine	0.591	0.612	0.605	0.603	0.596	0.591
Maize	40 spm	0.229	0.443	0.371	0.396	0.308	0.242
	Washing machine	0.372	0.539	0.498	0.457	0.416	0.376
Oats	40 spm	0.545	0.817	0.750	0.679	0.609	0.568
	Washing machine	0.744	0.881	0.856	0.831	0.806	0.779

Discussion

The aim of this study was to evaluate the effect of a modified method for rinsing nylon bags after incubation on the measured values for *in situ* N degradation in six feed ingredients. The calculated ED of N in all feed ingredients decreased significantly when applying the modified rinsing method compared to the standard washing machine rinsing method. This effect increased when the correction for particulate matter loss during the incubation was applied within the modified method. For legume seeds, the greatest contribution to the decrease of the total ED was the reduction of the S fraction. The lower S fraction in the modified method was mainly caused by the use of a different solvent with a lower pH that affected the solubility of the proteins in these products, compared with use of tap water to estimate the S-fraction in the washing machine method. De Jonge *et al.* (2009) showed that the difference in pH especially affected the solubility of the 7S and 11S globulin proteins in these products which were regarded to be relatively fast fermentable (Spencer *et al*., 1988). A greater amount of these proteins in the non-washout fraction (*i.e.* D) led to an

increase of the k_d of this fraction as was observed in this study. Based on the comparison of the results found with both rinsing methods and assuming that the degradation of other proteins in the D fraction was not influenced by the rinsing method used, the calculated k_d of these globulins was approximately 0.10 h^{-1} for both products. This k_d is much smaller than the fixed values used in feed evaluation systems (*e.g.* 1.5 h^{-1} in Norfor (Volden, 2011) and 2.0 h^{-1} in DVE/OEB (van Duinkerken *et al.*, 2011)) but more in line with the electrophoresis results obtained from *in situ* residues that indicated a lower degradation rate for globulins than assumed (Aufrere *et al*., 2001; Spencer *et al*., 1988), and with the *in vitro* results that showed differences between the degradation of buffer (pH 6.8) soluble proteins (Hedqvist and Udén, 2006). Messman *et al*. (1994) also reported that some high-soluble protein compounds in various ensiled forages had small rumen degradability. Based on gas production profiles Yang *et al.* (2005) showed that the soluble fraction of barley and maize was fermented faster than the insoluble fraction, but differences were much less than typically assumed. *In vivo* studies using the omasal sampling technique in cattle have indicated a substantial escape of dietary soluble amino acids from ruminal degradation (Choi *et al.*, 2002; Reynal *et al.*, 2007). Overall, the modified method was better capable to reveal these differences compared to the washing machine method.

For grains, especially barley, the greatest contribution to the decrease in ED was the reduction of the k_d of the D fraction which may be related to the occurrence of secondary particulate matter loss (Huhtanen and Sveinbjörnsson, 2006). Secondary particulate matter loss concerns removal during rinsing of particles broken down during the actual incubation. The modified method is less rigorous and therefore less effective in removing these reduced size particles compared to the washing machine. Therefore the level of degradation of N estimated with the modified method is lower, in line with previous observations for starch in these products (de Jonge *et al*., 2015b).

For both grains and legume seeds, the U fraction increased leading to a lower ED when using the modified method compared to the washing machine. This larger U fraction was partially related to a higher degree of microbial contamination found with the modified method. This effect was largest for grains, which was mainly caused by their low N content making them more sensitive for the effect of microbial contamination (Rodríguez and González, 2006).

In this study, DAPA was used to evaluate the effect of rinsing methods on microbial contamination of the residues. Microbial colonization of feed inside the nylon bag is required for degradation to proceed, but its presence in the residue may lead to substantial underestimation of ED (López, 2005). For all feed ingredients, a relative high degree of contamination was found during the first 8 hours of incubation which was in line with observations of Koike *et al*. (2003) who showed a fast attachment of ruminal fibrolytic bacteria during the first 6 h of incubation. No clear accumulation of microbial contamination by prolonged incubation time was observed, which seemed to be in contrast with the results found by Nocek and Grant (1987). In that study, however, forages with a high cellulose content, which is positively related with microbial contamination (Rodríguez and González, 2006), were investigated and a much shorter rinsing procedure (*i.e.* 2 min) was applied which both could cause a higher degree of microbial contamination at later incubation times. The difference between both rinsing methods could be related to the preference of bacteria to attach to small particles (Yang *et al*., 2001) and the more effective removal of small particles from the nylon bag by the washing machine method compared with the modified method. The impact of the correction for microbial contamination on the ED of N using DAPA as a marker depends on several assumptions including assumptions on the ratio of N and DAPA in microbes and assumptions on the level of lysis of cells after rinsing. The ratio of N and DAPA in microbes depends on various factors and different ratios have been reported in the literature. Olubobokum *et al*. (1990) found a ratio of approximately 40 whereas Martin *et al*. (1984) even reported a ratio between 76 and 100 for particle associated bacteria. The ratio calculated from the values found by Storm and Ørskov (1983) was approximately 20. Applying a high N to DAPA ratio will lead to a marked correction for microbial contamination. The assumed level of lysis of bacterial cells has a strong effect on the ratio between microbial N and DAPA in the residue. Although in some studies (Broderick and Merchen, 1992; Cecava *et al.*, 1990) this aspect has been included, mostly it was neglected within *in situ* studies. The calculated ratio between microbial N and total N greater than 1 found at short incubation times with the modified method suggested that the feedstuff itself contained DAPA, or that either the assumed ratio of N to DAPA was too high or a high degree of lysis of bacterial cells, with only bacterial cell wall N assumed to remain attached to substrate within the bag, occurred during rinsing, which markedly reduced the effect of microbial contamination on the calculated ED.

The modified methods showed some methodological advantages and disadvantages compared to the traditional washing machine method. The most pronounced methodological advantage was the increase of the non-washout fraction of N which made the calculation of the ED less depended on assumptions used for the soluble and insoluble washout fraction. For the modified method, the calculated ED was based on the measured *in situ* degradation of an average fraction of 0.863 $g \cdot g^{-1}$ whereas for the washing machine this average fraction was 0.644 $g \cdot g^{-1}$. An additional advantage of the modified method was that the *in situ* k_d of substrate reflected better the ruminal fermentation of nutrients and was less affected by the mechanical removing of particles during rinsing compared to the washing machine method. A methodological disadvantage was the higher degree of contamination of the residues which made the modified method more sensitive with respect to assumptions on the ratio of N and DAPA in bacteria and the level of lysis of the bacterial cells. The modified method led to a higher decrease of the ED of N in grains compared to legume seeds, and therefore may change the relative ranking of the ruminal fermentation of N in these products.

Conclusions

A modified method to reduce the loss of non-degraded substrate from nylon bags resulted in an increase of the non-washout fraction of N in the feed ingredients investigated and a decrease of the calculated ED compared with the washing machine method. This decrease in ED was more pronounced for grains than for legume seeds. For legume seeds the decrease of the ED was mainly caused by a lower solubility, whereas for grains a reduction of the k_d of the potentially degradable fraction was the main reason of decreased ED. The modified method was more sensitive to microbial contamination compared to the washing machine method. The ratios between DAPA and N in the residues indicated the occurrence of lysis of bacterial cells during rinsing with the modified method.

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Chapter 7

General Discussion

Introduction

In this thesis, the development and evaluation of a modified *in situ* method for the ruminal degradation of protein (N) and starch in grains, legume seeds and wheat yeast concentrates is described, as well as a comparison of the modified method with the conventional method, based on washing machine rinsing. The modifications within the *in situ* method focussed on the development and implementation of a rinsing method with a buffer instead of water that reduced particulate matter loss and had a smaller impact on the solubility of the N, and an approach to estimate the particulate loss during the incubation. The differences found between both methods find their origin in the processes that influence the disappearance of substrate from the nylon bag during the *in situ* method. To conceptually describe these processes, the feedstuffs are divided into a group of large particles (*i.e.* larger than the pore size of the nylon bag) and small particles (*i.e.* smaller than the pore size of the nylon bag). For starch, these processes (Figure 1) are less complex than for N because of the absence of a soluble and a non-degradable fraction (Offner *et al.*, 2003), and the negligible effect of microbial contamination (Volden, 2011). The disappearance rate of the large particles from the bag is the sum of the degradation rate ($F_{D,LP}(t)$) and the breakdown rate ($F_B(t)$) leading to the formation of small particles. The disappearance rate of the small particles from the bag is the sum of the degradation rate ($F_{D,SP}(t)$) and the particulate loss during the incubation ($F_{PL}(t)$). The difference between the modified and the conventional method concerns their effectiveness to remove the fraction of small particles during the rinsing step. The conventional method nearly completely removes the small particles, whereas with the modified method only a relatively small part of small particles is removed. The process of secondary particulate loss, as described by Huhtanen and Sveinbjörnsson (2006), depends mainly on the breakdown of large particles to small particles ($F_B(t)$) and the subsequent effectivity of removing these small particles during rinsing. In comparison with the modified method, the results obtained with the conventional method are therefore more affected by the process of secondary particulate loss.

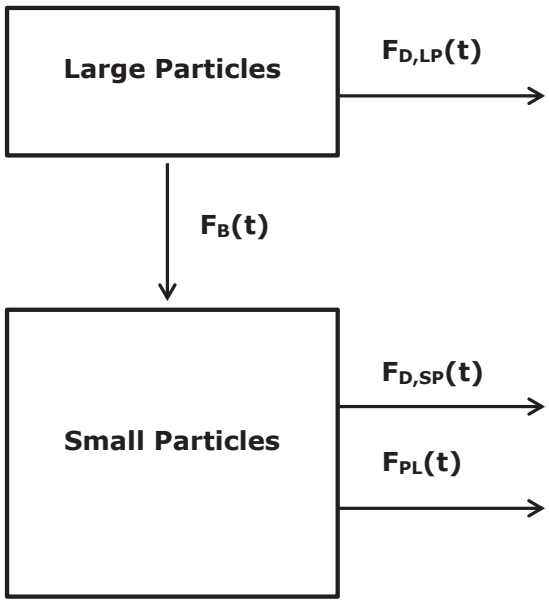

Figure 1 Disappearance processes of starch during the *in situ* incubation where $F_{D,LP}(t)$ and $F_{D,SP}(t)$ are the degradation rate of the large and small particles, respectively, $F_B(t)$ is the breakdown rate of large particles, and $F_{PL}(t)$ is the particulate loss rate of small particles.

For protein or N, the disappearance processes are more complex than for starch (Figure 2). A part of N located in both types of particles is soluble and is assumed to be directly removed from the nylon bag. This disappearance is therefore a fixed value and not a function of the incubation time ($F_{S,LP}$ and $F_{S,SP}$). In contrast to starch, N contains a non-degradable fraction located in both types of particles which is not affected by degradation in the rumen. For N, microbial contamination by the influx of microbes ($F_{MC,LP}(t)$ and $F_{MC,SP}(t)$) usually cannot be neglected and has a negative effect on the net disappearance rate. The difference in disappearance of N between the modified and the conventional method is primarily caused by the difference in effectivity to remove small particles during rinsing (as for starch) but additionally also by the difference in solubility of N ($F_{S,LP}$ and $F_{S,SP}$) and the difference in effectivity to remove microbial material contaminating the incubated feed. The

potential difference in solubility of N is caused by the difference between the solvents used during rinsing in both methods, which had an effect on the solubility of N especially for legume seeds (Chapter 6, Table 1). The conventional method contains a more vigorous rinsing method which was more effective to remove microbial contamination during this step, as indicated by the smaller amount of microbial marker (*i.e.* DAPA) in the residue compared to the modified method (Chapter 6, Table 4).

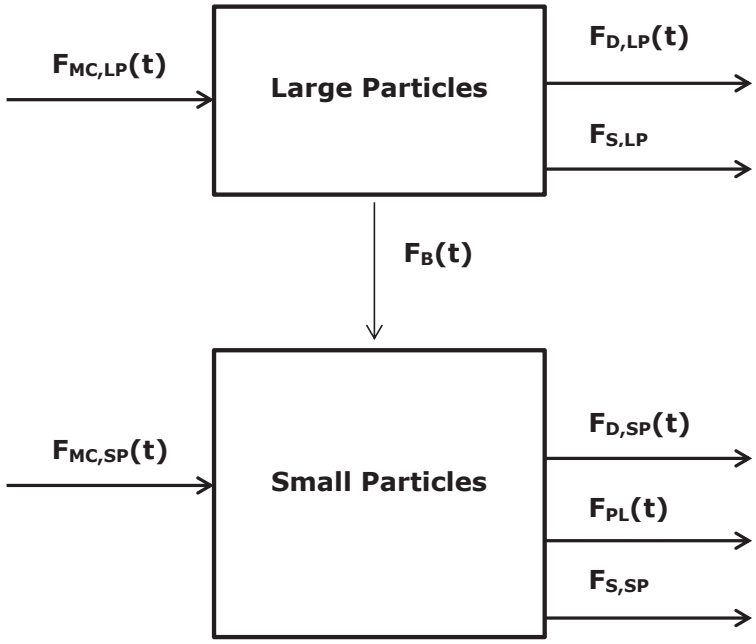

Figure 2 Disappearance processes of N during the *in situ* incubation where $F_{D,LP}(t)$ and $F_{D,SP}(t)$ are the degradation rate of the large and small particles, respectively, $F_B(t)$ is the breakdown rate of large particles, $F_{PL}(t)$ is the particulate loss rate of small particles, $F_{S,LP}$ and $F_{S,SP}$ are the instantaneous removal of the soluble material in the large particles and in the small particles, respectively, and $F_{MC,LP}(t)$ and $F_{MC,SP}(t)$ are the influx rate of microbial N into the large and the small particles, respectively.

The aim of the *in situ* method is to estimate the degradation rate of each relevant nutrient in the total feedstuff ($F_{D,LP}(t)$ and $F_{D,SP}(t)$) based on the measured disappearance rate from the nylon bag. The conventional method removes nearly all small particles and therefore can only estimate the degradation rate of the large particles, and requires assumptions regarding the degradation rate of small particles. The net disappearance rate of the large particles is affected by both the degradation rate as well as by the breakdown rate of particles ($F_B(t)$), and by influx of microbial material and effectivity of removing microbial contamination. The modified method removes only a small part of the small particles and therefore enables the estimation of degradation rate of the large particles and of a large fraction of the small particles. The disappearance rate of the small particles is affected by the degradation rate as well as by the particulate loss rate during incubation ($F_{PL}(t)$), which is indirectly influenced by the breakdown rate of large particles ($F_B(t)$) by increasing the amount of small particles. The influx of microbial material and the effectivity of removing microbial contamination from both fractions will also affect the net disappearance rate.

The aim of this thesis was to reduce the bias caused by the *in situ* method itself by modifying the procedure. The difference between the *in situ* and *in vivo* method was outside the scope of this thesis. Firstly, the effects of both modifications found in this thesis on the bias of the estimated effective degradation based on *in situ* (ED) caused by the potential impact of the use of assumptions, which depends on the size of the soluble and small particle fraction, breakdown of particles, and microbial contamination, will be discussed and evaluated based on the results found in this study. Secondly, the effect of the modified method on protein evaluation based on the Dutch DVE/OEB 1991 system and partly the 2007 system (Tamminga *et al.,* 1994; van Duinkerken *et al*., 2011) will be evaluated and compared to the conventional method. The discussion ends with some remarks and suggestions for further research followed by conclusions.

Effect modifications on bias

The new rinsing method significantly decreased the S fraction for N and the W-S fraction for N and starch (Table 1), which reduced the impact of the assumption used for the fractional degradation rate (k_d) of both fractions on the estimated ED. The average S fraction of N decreased from 0.242 $g \cdot g^{-1}$ using the conventional method to 0.115 $g \cdot g^{-1}$ using the modified method. The greatest reduction was found for the legume seeds, which has a large

impact on the estimated ED for these products (Chapter 6, Table 2). The W-S fraction of N and starch was also decreased by the modified method compared to the conventional method. For N, the average W-S fraction decreased from 0.112 to 0.020 $g \cdot g^{-1}$ with the largest effect for oats (*i.e.* a reduction of 0.200 $g \cdot g^{-1}$). For starch, a more pronounced decrease in average W-S fraction was obtained with the modified method compared with the conventional method, and the average W-S fraction of starch decreased from 0.333 to 0.042 $g \cdot g^{-1}$. A large reduction of the W-S fraction of starch was found for faba beans (*i.e.* 0.467 $g \cdot g^{-1}$), oats (*i.e.* 0.496 $g \cdot g^{-1}$), and peas (*i.e.* 0.386 $g \cdot g^{-1}$). The modified method strongly reduced the sum of the S and W-S fraction of N in wheat yeast concentrations (WYC), that did not contain a detectable amount of starch, from 0.915 to 0.217 $g \cdot g^{-1}$ compared to the conventional method and enabled the estimation of the *in situ* degradation of these products (Chapter 4). The decrease of the S and W-S fraction by the modified method reduced the potential bias caused by the assumption about the degradation of these fractions on the estimated ED.

The modified method also reduced the variation in S and W-S fraction between the feedstuffs compared to the conventional method. For N, the range between the feedstuffs reduced from 0.430 to 0.207 $g \cdot g^{-1}$ and from 0.222 to 0.057 $g \cdot g^{-1}$ for the S and W-S fraction, respectively. For starch, the range of the W-S fraction was reduced from 0.499 to 0.103 $g \cdot g^{-1}$. This decrease of the differences in S and W-S fraction between the feedstuffs also reduced the variation of the potential bias on the estimated ED caused by the assumption about the degradation of these fractions. The results found for starch by *in situ* and *in vitro* method is study (Chapter 5), additionally, indicated that the assumption used for the W-S fraction seems to overestimate the fractional degradation rate of this fraction which was in line with the observation of Stevnebø *et al.* (2009).

The effect of the large particle breakdown rate (F_B) on the disappearance rate was indicated by the differences found for the fractional degradation rate of starch in grains using both methods (Chapter 5). The fractional degradation found with the conventional method is potentially biased by the breakdown rate of large particles, and was higher than that found with the modified method. The impact of the breakdown rate strongly varies between the feedstuffs and for grains ranged from 0.015 h^{-1} for maize to 0.286 h^{-1} for oats (Table 2). For legume seeds, this effect was not observed which could be caused by the presence of a large W-S fraction for starch in these products which might also affect the k_d

found with both methods. The modified method reduced the average k_d of starch from 0.188 to 0.095 h^{-1} and decreased the range between the feedstuffs from 0.476 to 0.194 h^{-1} compared to the conventional method. The modified method reduced the level of and variation in the bias of the estimated ED between feedstuffs caused by the breakdown rate of large particles.

Table 1 The soluble fraction (S; $g \cdot g^{-1}$) of N and the non-soluble washout fraction (W-S; $g \cdot g^{-1}$) of N and starch in six feedstuffs obtained with the modified method (MM) and the conventional method (CM)

		N		Starch	
Parameter	Feedstuff	MM	CM	MM	CM
S	Barley	0.049	0.106		
	Faba beans	0.187	0.466		
	Maize	0.042	0.086		
	Oats	0.071	0.136		
	Peas	0.249	0.516		
	Wheat	0.092	0.142		
W – S	Barley	0.003	0.070	0.007	0.107
	Faba beans	0.006	0.160	0.028	0.495
	Maize	0.011	0.073	0.029	0.154
	Oats	0.058	0.258	0.110	0.606
	Peas	0.041	0.079	0.062	0.448
	Wheat	0.001	0.036	0.020	0.187

The modified method is sensitive to particulate loss of small particles during incubation which is a potential source of bias for the estimation of the ED. In this thesis, an *in vitro* method was developed to simulate this process based on the results found for the disappearance of an inert marker (*i.e.* silica gel, Chapter 4). The results found for the particulate loss of N and starch with this method, were used to correct the disappearance rate of both nutrients (Chapter 5 and 6). This correction decreased the average k_d of starch from 0.095 to 0.064 h^{-1} and the range between the feedstuffs from 0.194 to 0.109 h^{-1}.

Table 2 Fractional degradation rate (k_d; h^{-1}) of starch in six feed ingredients using the modified method with and without correction for particulate matter loss during *in situ* incubation and the conventional method

	Modified method		Conventional method
Feedstuff	With correction	Without correction	
Barley	0.080	0.092	0.220
Faba beans	0.021	0.044	0.044
Maize	0.037	0.040	0.055
Oats	0.130	0.234	0.520
Peas	0.032	0.052	0.049
Wheat	0.088	0.110	0.240

The modified method potentially increased bias caused by microbial contamination as indicated by the ratio between DAPA and N in the residue compared to the conventional method (Chapter 6). The effect of microbial contamination was observed by an increase of the non-degradable (U) fraction of N using the modified method compared to the conventional method (Table 3). The average U fraction and the range between the feedstuffs increased from 0.048 to 0.144 $g \cdot g^{-1}$ and from 0.092 to 0.149 $g \cdot g^{-1}$, respectively. The modified method increased the level and variation in the bias of the estimated ED between feedstuffs caused by the microbial contamination.

Table 3 The non-degradable fraction (U; $g \cdot g^{-1}$) of nitrogen in six feedstuffs obtained with the modified method and the conventional method

Feedstuff	Modified method	Conventional method
Barley	0.153	0.068
Faba beans	0.077	0.004
Maize	0.226	0.095
Oats	0.174	0.060
Peas	0.090	0.003
Wheat	0.144	0.058

The result above showed that the modified method was successful to reduce the potential bias on the estimated ED caused by the assumptions and breakdown of large particles but increased the potential bias caused by microbial contamination.

Consequences for protein evaluation

The fractions, fractional degradation rate and non-degradable fraction of N and starch were in general different between the modified and the conventional method. The effect of the differences between both methods on protein evaluation was evaluated by comparing the fraction of ruminal bypass dietary protein (%BRE; $g \cdot g^{-1}$) and starch (%BZET; $g \cdot g^{-1}$) calculated according to the DVE/OEB 1991 (Tamminga *et al*., 1994) and DVE/OEB 2007 (van Duinkerken *et al*., 2011) systems. The results obtained with the DVE/OEB 1991 method were used to estimate the amount of intestinal digestible protein (DVE) and the balance between nitrogen and energy available for microbial synthesis (OEB) according to the equations of the DVE/OEB 1991 system. The DVE/OEB 2007 was not used because not all the required input parameters were available.

The equations in the DVE/OEB 1991 system to calculate the %BRE and %BZET are:

$$\%BRE = D \times [0.06 / (k_d + 0.06)] + U \quad (1)$$

$$\%BZET = (W\text{-}S) \times 0.10 + D \times [0.06 / (k_d + 0.06)] \quad (2)$$

where 0.06 h^{-1} is the fractional passage rate of the D fraction. The S and W-S fraction of N are assumed to be complete degraded in rumen, whereas for starch 0.1 of the W-S fraction is regarded to be rumen bypass starch.

The modified method increased the average %BZET from 0.272 to 0.510 $g \cdot g^{-1}$ compared to the conventional method. This increase varied between 0.147 for maize to 0.382 $g \cdot g^{-1}$ for faba beans (Table 4). For %BZET, the ranking of feedstuffs differed between the conventional and modified method. The conventional method showed a higher %BZET for maize compared to the legume seeds whereas the opposite was found with the modified method. The average %BRE increased from 0.333 $g \cdot g^{-1}$ using the conventional method to 0.626 $g \cdot g^{-1}$ using the modified method. This increase ranged from 0.225 for peas to 0.383 $g \cdot g^{-1}$

for faba beans. The ranking of feedstuffs for %BRE was slightly affected by the method used. The conventional method showed a higher %BRE for peas than oats, whereas the opposite was found with the modified method.

Table 4 Ruminal escape fraction of starch (%BZET; $g \cdot g^{-1}$) and N (%BRE; $g \cdot g^{-1}$) estimated with the conventional method (CM) and the modified method (MM) according to the DVE/OEB 1991 system

	%BZET		%BRE	
Feedstuff	CM	MM	CM	MM
Barley	0.202	0.426	0.390	0.682
Faba beans	0.341	0.723	0.256	0.639
Maize	0.457	0.604	0.587	0.822
Oats	0.101	0.292	0.154	0.504
Peas	0.349	0.618	0.227	0.452
Wheat	0.181	0.399	0.387	0.662

The %BRE of the three WYC found by the modified method using equation 1 (DVE/OEB 1991) varied between 0.422 and 0.748 $g \cdot g^{-1}$, and was in the range of the other products.

The equations in the DVE/OEB 2007 system to calculate the %BRE and %BZET are:

$$\%BRE = S \times [0.11 / 2.11] + (W\text{-}S) \times [0.08 / (k_d + 0.08)] + D \times [0.06 / (k_d + 0.06)] + U \quad (3)$$

$$\%BZET = (W\text{-}S) \times \{0.08 / [(2 \times k_d + 0.375) + 0.08]\} + D \times [0.06 / (k_d + 0.06)] \quad (4)$$

where 2.11 h^{-1} is derived from the fractional passage rate (0.11) and the fractional degradation rate (2.00) of the S fraction, respectively, and 0.08 h^{-1}, and 0.06 h^{-1} are the fractional passage rates of the W-S and D fraction, respectively. The fractional degradation rate of the W-S fraction of N is equal to that of the D fraction (k_d) whereas for starch the fractional degradation of the W-S fraction is assumed to be 2 × k_d(D fraction) + 0.375 (h^{-1}).

The modified method reduced the W-S fraction or small particle loss during rinsing which automatically would lead to a reduction of the passage rate of the fraction of small particles that is not removed during rinsing. To avoid a difference caused by the passage rate, the D fraction obtained with the modified method is divided into a fraction of small particles (D1) with a passage rate of 0.08 h^{-1} and a remaining fraction of large particles (D2) with a passage rate of 0.06 h^{-1}. Fraction D1 and D2 are calculated as:

$$D1 = (W\text{-}S)(\text{conventional method}) - (W\text{-}S)(\text{modified method}) \quad (5)$$

$$D2 = D(\text{modified method}) - D1 \quad (6)$$

The equations used to calculated %BRE and %BZET with the modified method are :

$$\%BRE = S \times (0.11 / 2.11) + (W\text{-}S) \times [0.08 / (k_d + 0.08)] + D1 \times [0.08 / (k_d + 0.08)] + D2 \times [0.06 / (k_d + 0.06)] + U \quad (7)$$

$$\%BZET = (W\text{-}S) \times \{0.08 / [(2 \times k_d + 0.375) + 0.08]\} + D1 \times [0.08 / (k_d + 0.08)] + D2 \times [0.06 / (k_d + 0.06)] \quad (8)$$

The modified method increased the average %BZET from 0.274 to 0.529 $g \cdot g^{-1}$ compared to the conventional method. This increase varied between 0.150 for maize to 0.384 $g \cdot g^{-1}$ for faba beans (Table 5). The ranking between the %BZET of the legume seeds and maize was affected by the method used. The conventional method showed a higher %BZET for maize compared to the legume seed whereas the opposite was found with the modified method. The effect found for %BZET were comparable to those found by using the equations from the DVE/OEB 1991 system. The average %BRE increased from 0.400 $g \cdot g^{-1}$ using the conventional method to 0.650 $g \cdot g^{-1}$ using the modified method. This increase ranged from 0.190 $g \cdot g^{-1}$ for peas to 0.338 $g \cdot g^{-1}$ for faba beans. The ranking between the %BRE of oats and peas was affected by the method used. The conventional method showed a higher %BRE for peas whereas the opposite was found with the modified method. The effect found for %BRE were comparable to those found by using the equations from the DVE/OEB 1991 system.

Table 5 Ruminal escape fraction of starch (%BZET; $g \cdot g^{-1}$) and N (%BRE; $g \cdot g^{-1}$) estimated with the conventional method (CM) and the modified method (MM) according to the DVE/OEB 2007 system

	%BZET		%BRE	
Feedstuff	CM	MM	CM	MM
Barley	0.201	0.434	0.430	0.691
Faba beans	0.364	0.748	0.399	0.661
Maize	0.463	0.613	0.644	0.836
Oats	0.073	0.326	0.216	0.554
Peas	0.369	0.645	0.304	0.494
Wheat	0.179	0.412	0.412	0.669

The results for %BRE and %BZET of the different products calculated with the DVE/OEB 1991 and with the DVE/OEB 2007 were very comparable (Table 4 and 5).

The average DVE based on the DVE/OEB system 1991 increased from 85 to 112 $g \cdot kg^{-1}$ DM using the modified method compared to the conventional method (Table 6). This increase was caused by a strong increase of the fraction ruminal bypass protein (%BRE) which was only partly compensated by a reduction of the synthesis of microbial protein. The increase of the DVE for the feedstuffs ranged from 10 for maize to 58 $g \cdot kg^{-1}$ DM for faba beans. The modified method led in general to a larger difference for the DVE between legume seeds and grains. The average OEB decreased from 11 $g \cdot kg^{-1}$ DM using the conventional method to -12 $g \cdot kg^{-1}$ DM using the modified method (Table 6). This decrease in OEB indicates that the decrease in rumen fermentable N (because of the increase of fraction ruminal bypass protein (%BRE)) could not be compensated for by the reduction in N required for microbial protein due to the reduction of ruminal starch fermentation (because of the increase in ruminal bypass starch (%BZET)). The decrease of the OEB for the feedstuffs ranged from 6 for maize to 54 $g \cdot kg^{-1}$ DM for faba beans.

Table 6 Estimated intestinal digestible protein (DVE; g·kg^{-1} DM) and ruminal N balance (OEB; g·kg^{-1} DM) based on the results from the conventional method (CM) and the modified method (MM) using the DVE/OEB system 1991

	DVE		OEB	
Feedstuff	CM	MM	CM	MM
Barley	85	102	– 27	– 39
Faba beans	99	157	64	10
Maize	82	92	– 31	– 38
Oats	55	84	11	– 17
Peas	97	131	71	43
Wheat	91	106	– 22	– 33

Remarks and suggestions for further research

To further improve the accuracy of the modified method, reduction of the potential bias caused by microbial contamination is necessary. Measurement of this microbial contamination seems to be the most direct approach to solve this problem. In this thesis, the approach of using diaminopimelic acid (DAPA) as a marker and a fixed ratio between microbial N and DAPA to estimate the microbial contamination as used by Nocek and Grant (1987), failed, which was probably caused by lysis of bacteria cells during rinsing (Chapter 6). A more accurate method could be the use of ^{15}N labelling type of microbes or feed to estimate the degree of contamination as done in studies by Rodríguez and González (2006), or the use of the general formula to estimate microbial contamination as presented in that study. An alternative approach could be to combine the results obtained with the modified and the conventional method in a model which corrects for the breakdown and secondary loss of particles and reduced the effect of microbial contamination. A full mathematical description of this model, however, should first be further developed and evaluated.

To evaluate the full impact of the modified method on protein evaluation, a larger set of feedstuffs including forage should be investigated. In this thesis, the number of feedstuffs was limited, and additionally focussed only on N and starch. For the feedstuffs investigated, the effect of the modified method on the results found by the protein evaluation system was

substantial. For other feedstuffs, with a smaller S and W-S fraction, and less breakdown of large particles, the impact of the modified method could be much smaller and therefore potential affects the ranking between products with high S and W-S fractions and products with low S and W-S fractions. This study should also include the effect of the modified method on the fractional degradation rate of NDF. Preliminary results from maize silage (data not shown) indicate that the effect of the modified method on the *in situ* degradation of NDF is less than for N and starch.

The modified method can also have a positive effect on the precision of the *in situ* method between and within laboratories. The conditions in the new rinsing method can be fully controlled, which offers the opportunity for standardisation between laboratories. The lack of a standardized rinsing method is regarded as a major source for variation between laboratories (Vanzant *et al*., 1998) and therefore the implementation of this new rinsing method could improve the precision between laboratories. Additionally, the use of silica gel as inert marker could help to identify differences in particulate matter loss during the incubation between different laboratories. The developed *in vitro* simulation method offers the opportunity to correct for these differences and therefore increases the comparability of the results found by different laboratories. These potential advantages should be further explored by collaborative trials between laboratories using the modified method.

The difference between the *in situ* and *in vivo* degradation is the last topic that should be addressed. This thesis focussed on reducing the bias within the *in situ* method itself without taking the bias between the *in situ* and *in vivo* into account. In general, the modified method enlarged the difference between the *in situ* and in vivo degradation which might be related to the less favourable conditions for degradation compared to the *in vivo* situation (Nozière and Michalet-Doreau, 2000; López, 2005; Offner and Sauvant, 2004). To translate the *in situ* results into *in vivo* data the development of regression equations, as presented for starch by Offner and Sauvant (2004), is needed for the modified method.

Conclusions

The developed modified method has some advantages compared to the conventional method used to estimate the *in situ* degradation:

- The reduction of the S fraction of N and the W-S fraction of N and starch in several feedstuffs enlarged the fraction of these nutrients from which the *in situ* fractional degradation rate is measured.
- The reduction of the particle loss during rinsing enables estimation of the *in situ* fractional degradation rate of nutrients in WYC.
- The potential bias on the estimated ED caused by the assumptions with respect to the degradation rate of the S and W-S fraction and by the breakdown of large particles, is strongly reduced when using the modified method.
- The modified method offers the opportunity to simulate the particulate matter loss during the incubation, and to correct for this loss.

The disadvantages of the modified method compared to the conventional method are:

- The potential bias caused by microbial contamination is larger than for the conventional method.
- The difference between the *in situ* and *in vivo* degradation rates is larger with the modified method than with the conventional method.

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Summary

The most widely used method to estimate the rumen degradation of dietary components in feedstuffs is the *in situ* or *in sacco* method which is based on rumen incubation of substrate (feed) in porous (nylon or dacron) bags. This method allows influx of microbes leading to degradation of the feed components. Despite its large scale use and acceptance, the *in situ* method has a number of methodological weaknesses, such as the low precision and lack of standardization, that negatively affects the quality and the comparability of the results obtained by various laboratories. Another problem is the inaccuracy in the *in situ* method which is divided in a bias in the estimation of the degradation rate within the nylon bag and the difference between the *in situ* and *in vivo* degradation rate. This thesis focuses on the bias of the method which is caused by the use of several assumptions regarding the degradation rate of the washable fraction, breakdown of particles, and microbial contamination. The aim of this thesis is to reduce this bias through the use of a modified *in situ* method that involves a new rinsing method and an *in vitro* method that simulates particulate matter loss during incubation.

In Chapter 2, the solubility of N in various feedstuffs as function of different rumen conditions, such as pH, osmolality and temperature of solvents, was examined in two experiments. In the first experiment, the effect of the solvent, *i.e.* tap water, borate-phosphate buffer of pH 6.8 and sodium acetate buffer of pH 5.0, and temperature, *i.e.* 18 and 38°C was investigated for 17 feedstuffs including legumes, oilseeds, grains, ryegrass and silages. In the second experiment, N solubility of soybean, soybean meal, peas, faba beans and lupins was evaluated in buffers at 4 pH values (5.0, 5.6, 6.2 and 6.8) and 2 osmolality values (300 and 400 $mOsm \cdot l^{-1}$) at 38°C.The N solubility in feedstuffs was mainly affected by the pH and to a lesser extent to osmolality of the solvent used. For many feedstuffs, the N solubility decreased upon reduction in pH from 6.8 to 5.0 with the largest effect for untreated legumes. The reduction in pH increased the fraction non-protein N (NPN) as a fraction of soluble N but decreased the solubility of globulins, especially 11S, in these legume seeds as was observed by SDS-Page electrophoresis. Increasing osmolality from 300 to 400 $mOsm \cdot l^{-1}$ generally increased N solubility but this effect was less pronounced than that of pH. Results show that pH of the solvent, and to a lesser extent osmolality, affect N solubility,

and suggests that evaluation of CP degradation in the rumen requires consideration of rumen fluid pH and osmolality.

Chapter 3 describes the development and evaluation of the new rinsing approach to separate, isolate and characterise the soluble (S), the insoluble washout (W-S) and the non-washout fractions (D+U) within one procedure which was used within the modified method. This new approach was based on rinsing of nylon bags in a closed vessel containing a buffer solution (pH 6.2) during 1 h, where shaking speeds of 40, 100, and 160 strokes per min (spm) were evaluated, and tested for 6 dry feed ingredients (faba beans, maize, oats, peas, soybean, and wheat) and 4 forages (2 ryegrass silages and 2 maize silages). The average recoveries as the sum of all fractions were 0.972 $\pm$ 0.041 for N and 0.990 $\pm$ 0.050 for starch (mean $\pm$ SD). The mean W-S fraction increased with increasing shaking speed and varied between 0.017 (N) and 0.083 $g \cdot g^{-1}$ (starch) at 40 spm and 0.078 (N) and 0.303 $g \cdot g^{-1}$ (starch) at 160 spm, respectively. For ryegrass silages, the W-S fraction was absent at all shaking speeds, but was present in the conventional method, based on washing machine rinsing. The new rinsing method, in particular at 40 and 100 spm, reduced the loss of small particles during rinsing, resulting in lower W-S and higher D+U fractions for N and starch compared with the conventional method. For soybean and ryegrass silage, the modified method reduced the S fraction of N compared with the conventional method. The results obtained at 160 spm showed the best comparison to the results of the conventional method based on washing machine rinsing and separate estimation of the N solubility in tap water. The W-S fraction of the feedstuffs obtained at 160 spm contained mainly particles smaller than 40 µm (0.908 $\pm$ 0.086). In most feedstuffs, starch was the most abundant chemical component in the W-S fraction and its content (726 $\pm$ 75 $g \cdot kg^{-1}$ DM) was higher than in the D+U fraction (405 $\pm$ 177 $g \cdot kg^{-1}$ DM). Alkaline soluble proteins were the dominant N containing components in the W-S fraction of dry feed ingredients obtained at 160 spm and its relative content (0.79 $\pm$ 0.18 $g \cdot g^{-1}$ of total N in W-S) was higher than that in the D+U fraction (0.59 $\pm$ 0.07 $g \cdot g^{-1}$ of total N in D+U) for all feedstuffs except maize. The molecular weight distribution of the alkaline soluble proteins differed between the W-S and the D+U fractions of all dry feed ingredients, except soybean and wheat.

In Chapter 4, the modified method is presented which includes the new rinsing approach at 40 spm, as described in Chapter 3, as well as quantification the particulate matter loss during incubation in the rumen. The aim of these modifications was to estimate

the fractional degradation rate of nutrients in small particles. To quantify particulate matter loss during incubation, loss of small particles during the *in situ* incubation was studied using non-degradable silica with different particle sizes. Particulate matter loss during incubation was limited to particles smaller than approximately 40 µm with a mean fractional particulate matter loss rate of 0.035 h^{-1} (first experiment) and 0.073 h^{-1} (second experiment) and a non-degradable fraction of 0.001 and 0.050 $g \cdot g^{-1}$, respectively. This fractional particulate matter loss could be simulated by rinsing of nylon bags containing this silica at 30 and 40 spm. The modified method was applied to estimate the *in situ* fractional degradation rate of insoluble organic matter (OM) and insoluble nitrogen (N) in three different wheat yeast concentrates (WYC). These WYC were characterised by a high fraction of small particles and estimating their fractional degradation rate was not possible using the conventional method. The new rinsing approach increased the mean non D+U fraction of OM and N in these products from 0.113 and 0.084 $g \cdot g^{-1}$ (conventional method) to 0.670 and 0.782 $g \cdot g^{-1}$, respectively. The mean effective degradation (ED) without correction for particulate matter loss of OM and of N was 0.714 and 0.601 $g \cdot g^{-1}$, respectively, and significant differences were observed between the WYC products. Applying the correction for particulate matter loss reduced the mean ED of OM to 0.676 (30 spm) and 0.477 $g \cdot g^{-1}$ (40 spm), and reduced the mean ED of N to 0.475 (30 spm) and 0.328 $g \cdot g^{-1}$ (40 spm). These marked reductions in ED upon correction for small particulate matter loss emphasised the pronounced effect of correction for non-degraded particulate matter loss on the ED of OM and N in WYC products.

In Chapter 5, the *in situ* degradation of the W-S fraction of starch in six feed ingredients (*i.e.* barley, faba beans, maize, oats, peas, and wheat) was studied by using the modified method, as described in Chapter 4, and additional *in vitro* measurements. The modified method markedly reduced the average washout fraction of starch in these products from 0.333 to 0.042 $g \cdot g^{-1}$ compared to the conventional method. Applying the modified method, the fractional degradation rate (k_d) of starch in barley, oats and wheat decreased from on average 0.327 to 0.144 h^{-1} whereas for faba beans, peas, and maize no differences in k_d were observed compared to the conventional method. For barley, maize, and wheat, the difference in non-fermented starch in the residue between both rinsing methods during the first 4 hours of incubation increased, which indicates secondary particle loss. The average ED of starch decreased from 0.761 to 0.572 $g \cdot g^{-1}$ when using the modified method and to 0.494 $g \cdot g^{-1}$ when applying a correction for particulate matter loss during

incubation. The *in vitro* k_d of starch in the non-washout fraction did not differ from that in the total product. The calculated ratio between the k_d of starch in the washout and non-washout fraction was on average 1.59 and varied between 0.96 for oats and 2.39 for maize. The fractional rate of gas production was significantly different between the total product and the non-washout fraction. For all products, except oats, this rate of gas production was larger for the total product compared to the D fraction whereas for oats the opposite was observed. The rate of increase in gas production was, especially for grains, strongly correlated with the *in vitro* k_d of starch. The results of the present study do not support the assumption used in several feed evaluation systems that the degradation of the W-S fraction of starch in the rumen is much faster than that of the D fraction.

In Chapter 6, the *in situ* degradation of nitrogen (N) in barley, faba beans, maize, oats, peas, and wheat found with the modified method was compared to that with the conventional method. The modified method reduced the S fraction especially for legume seeds, as well as the W-S fraction, compared to the conventional method. The average D+U fraction of N in the feed ingredients increased from 0.644 (conventional method) to 0.863 $g \cdot g^{-1}$ (modified method). The k_d for N obtained with the modified method was higher for legume seeds (except faba beans where k_d did not differ significantly between both methods), but lower for grains compared to the conventional method. The average non-degradable fraction (U) increased from 0.048 (conventional method) to 0.144 $g \cdot g^{-1}$ (modified method). The average ED for the feed ingredients decreased from 0.621 (conventional method) to 0.458 $g \cdot g^{-1}$ (modified method). The modified method decreased the ED of N in the grains stronger than in legume seeds. The difference in ED between both methods was mainly caused by the reduction of the soluble fraction for the legume seeds and by the reduction of the k_d for the grains. For barley, faba beans, maize, and peas a higher ratio diaminopimelic acid (DAPA) / N and, therefore, a higher level of microbial contamination was found for the modified method compared to the conventional method. The DAPA based estimated microbial contamination was larger than 100% of the total protein present at the short incubation times (*i.e.* < 8 h) when using the modified method, and this suggests that lysis of bacterial cells during the modified rinsing method occurred. Correction for estimated degree of lysis (by a modified microbial N – DAPA ratio) strongly influenced the calculated ED of N.

In Chapter 7, several aspect of the modified method as compared with the conventional method were discussed. The developed modified method was successful in reducing the S fraction of N and the W-S fraction of N and starch and, therefore, enlarged the fraction of these nutrients from which the *in situ* fractional degradation rate can be measured. The reduction of the particle loss during rinsing enables estimation of the *in situ* fractional degradation rate of nutrients in WYC and reduced the potential bias on the estimated ED caused by the assumptions regarding to the degradation rate of the S and W-S fraction. The modified method also offers the opportunity to correct for particulate matter loss during the incubation and reduced the bias on the ED caused by the breakdown of particles that strongly affects the k_d of starch in grains when using the conventional method. The disadvantages of the modified method were a greater potential bias caused by microbial contamination and a larger difference between the *in situ* and *in vivo* degradation rates compared to the conventional method.

Samenvatting

De *in situ* of *in sacco* methode is de meest gebruikte techniek om de afbraak van voercomponenten in de pens van herkauwers te bepalen. Deze methode is gebaseerd op de incubatie in de pens van voeders in poreuze nylon zakjes waardoor een instroom van microben mogelijk is die leidt tot de afbraak van het aanwezige substraat. Ondanks het gebruik op grote schaal kent de *in situ* methode enkele methodologische zwakheden zoals de matige herhaalbaarheid en het gebrek aan standaardisatie die de kwaliteit en vergelijkbaarheid van resultaten gevonden door verschillende instituten negatief beïnvloedt. Een bijkomend probleem betreft de nauwkeurigheid van de *in situ* methode die kan worden verdeeld in een systematische fout in de bepaling van afbreekbaarheid in het nylon zakje zelf en het verschil tussen de *in situ* en *in vivo* (werkelijke) afbreekbaarheid. Deze studie richt zich op de systematische fout binnen de *in situ* methode die wordt veroorzaakt door het gebruik van aannames ten aanzien van de afbreekbaarheid van uitwasbare componenten, de afbraak (verkleining) van deeltjes en microbiële verontreiniging. Het doel van deze thesis is om mogelijkheden te onderzoeken om de systematische fout binnen de *in* situ methode te beperken door het gebruik van een nieuwe methode voor het wassen van nylon zakjes na incubatie en een *in vitro* simulatie voor het verlies aan deeltjes tijdens de incubatie.

Hoofdstuk 2 beschrijft het verband tussen de oplosbaarheid van N en de variatie in penscondities zoals pH, osmolaliteit en temperatuur verkregen door het gebruik van diverse oplossingen. In het eerste experiment werden een aantal oplossingen (namelijk kraan water, boraat-fosfaat buffer pH 6.8 en natriumacetaat buffer van pH 5.0) bij twee temperaturen (18 en 38°C) onderzocht voor 17 grondstoffen waaronder vlinderbloemigen, oliehoudende zaden, granen, gras en gras- en snijmaissilage. In het tweede experiment is voor bonen, erwten, lupine, sojabonen en sojaschroot de oplosbaarheid van N in buffers met 4 verschillende pH's (5.0, 5.6, 6.2 en 6.8) en 2 verschillende osmolaliteit waarden (300 en 400 $mOsm \cdot l^{-1}$) bij 38°C bepaald. De N oplosbaarheid in deze diervoedergrondstoffen werd voornamelijk door de pH en in mindere mate door de osmolaliteit beïnvloed. Voor de meeste grondstoffen nam de N oplosbaarheid af bij een verlaging van de pH van 6.8 naar 5.0 waarbij het sterkste effect bij onbehandelde vlinderbloemigen werd gevonden. De verlaging van de pH verhoogde ook het aandeel niet-eiwit N (NPN) binnen de fractie oplosbare N en

reduceerde de oplosbaarheid van globulines, met name 11S, in vlinderbloemigen zoals werd aangetoond door middel van SDS-Page electroforese. Het verhogen van de osmolaliteit van 300 naar 400 mOsm·l^{-1} leidde in het algemeen tot een toename van de oplosbaarheid van N maar dit effect was minder duidelijk dan voor de pH. De resultaten toonden duidelijk het effect van de pH en in mindere mate de osmolaliteit van het oplosmiddel op de N oplosbaarheid hetgeen impliceert dat bij de evaluatie van de eiwit afbraak in de pens rekening moet worden gehouden met de pH en de osmolaliteit van de pens-vloeistof.

Hoofdstuk 3 beschrijft de ontwikkeling en evaluatie van een nieuwe was methodiek om de oplosbare (S), de niet-oplosbare uitwasbare (W-S) en de niet-uitwasbare fractie (D+U) met één methode te scheiden, te isoleren en te karakteriseren. Deze nieuwe methodiek was gebaseerd op het schudden van de nylon zakjes gedurende 1 uur in een glazen bak gevuld met een buffer oplossing (pH 6.2). De onderzochte schudsnelheden bedroegen 40, 100 en 160 slagen per minuut (spm) en werden toegepast op 6 droge diervoedergrondstoffen (bonen, erwten, haver, mais, sojabonen en tarwe) en 4 silages (2 grassilages en 2 snijmaissilages). De gemiddelde recovery van de som van alle fracties bedroeg 0.972 $\pm$ 0.041 voor N en 0.990 $\pm$ 0.050 voor zetmeel (gemiddelde $\pm$ SD). De gemiddelde W-S fractie nam toe bij een hogere schudsnelheid en varieerde van 0.017 (N) en 0.083 g·g^{-1} (zetmeel) bij 40 spm tot 0.078 (N) en 0.303 g·g^{-1} (zetmeel) bij 160 spm. In grassilage werd bij geen van de schudsnelheden een W-S fractie aangetoond in tegenstelling tot de conventionele methode die gebaseerd is op het gebruik van een wasmachine. De nieuwe methode, met name bij 40 en 100 spm, reduceerde het verlies aan kleine deeltjes gedurende het wassen wat resulteerde in een lagere W-S en een hogere D+U fractie voor N en zetmeel in vergelijking met de conventionele methode. De nieuwe methode verkleinde ook de S fractie van N voor sojabonen en grassilage ten opzichte van de conventionele methode. De resultaten van de nieuwe methode verkregen bij 160 spm kwamen het beste overeen met de resultaten verkregen met de conventionele methode gebaseerd op het gebruik van de wasmachine en een aanvullende bepaling van de oplosbare N fractie. De W-S fractie verkregen bij 160 spm bestond voornamelijk uit deeltjes kleiner dan 40 µm (0.908 $\pm$ 0.086) en voor de meeste grondstoffen was zetmeel de meest voorkomende chemische component. Het gehalte aan zetmeel in W-S (726 $\pm$ 75 g·kg^{-1} DM) was hoger dan in de D+U fractie (405 $\pm$ 177 g·kg^{-1} DM). Alkalisch oplosbare eiwitten waren de meest voorkomende N verbindingen in de W-S fractie van de onderzochte droge grondstoffen verkregen bij 160 spm en deze fractie (0.79 $\pm$ 0.18

$g \cdot g^{-1}$ van totaal N in W-S) was hoger dan in de D+U fractie (0.59 $\pm$ 0.07 $g \cdot g^{-1}$ van totaal N in D+U) voor alle grondstoffen behalve mais. De moleculair gewichtsverdeling van deze alkalisch oplosbare eiwitten in de W-S fractie verschilde van de verdeling in de D+U fractie voor alle droge grondstoffen behalve sojabonen en tarwe.

Hoofdstuk 4 beschrijft de nieuwe aangepaste *in situ* methode die bestaat uit de nieuwe was methode bij 40 spm, zoals beschreven in hoofdstuk 3, en de kwantificering van de kleine deeltjes verdwijning tijdens de incubatie. Het doel van deze aanpassingen is om de fractionele afbraak van nutriënten in kleine deeltjes te bepalen. Voor het kwantificeren van deze verdwijning werden *in situ* incubaties met onafbreekbaar silica gel met verschillende deeltjes verdelingen uitgevoerd. Het verlies tijdens de incubatie was beperkt tot deeltjes kleiner dan ongeveer 40 µm met een fractionele verdwijningssnelheid van 0.035 h^{-1} (eerste experiment) en 0.073 h^{-1} (tweede experiment) en een rest fractie van respectievelijk 0.001 en 0.050 $g \cdot g^{-1}$. Deze verdwijningscurve kon worden gesimuleerd door het schudden van nylon zakjes met deze silica volgens de nieuwe methode bij 30 en 40 spm. De aangepaste methode werd gebruikt voor het bepalen van de *in situ* fractionele afbraaksnelheid (k_d) van onoplosbare organische stof (OM) en onoplosbare N in drie verschillende tarwegist-concentraten (TGC). Deze TGC's bezitten een zeer hoge fractie aan kleine deeltjes waardoor het onmogelijk is om de *in situ* afbraak via de conventionele methode betrouwbaar te meten. De nieuwe was methode verhoogde de gemiddelde D+U fractie voor OM en N in deze producten van 0.113 en 0.084 $g \cdot g^{-1}$ (conventionele methode) tot respectievelijk 0.670 en 0.782 $g \cdot g^{-1}$. De gemiddelde effectieve degradatie (ED) zonder correctie voor deeltjes verlies was voor OM en N respectievelijk 0.714 en 0.601 $g \cdot g^{-1}$ waarbij significante verschillen tussen de TGC's werden waargenomen. Toepassing van een correctie voor het deeltjes verlies tijdens de incubatie reduceerde de gemiddelde ED voor OM tot 0.676 (30 spm) en 0.477 $g \cdot g^{-1}$ (40 spm) en voor N tot 0.475 (30 spm) en 0.328 $g \cdot g^{-1}$ (40 spm). Deze opvallende afname in ED ten gevolge van de correctie voor deeltjes verlies bevestigde het effect van deze correctie op de ED van OM en N in TGC's.

Hoofdstuk 5 behandelt de *in situ* afbraak van de W-S fractie van zetmeel in zes grondstoffen (bonen, erwten, gerst, haver, mais en tarwe) via de nieuwe methodiek, zoals beschreven in hoofdstuk 4, en aanvullende *in vitro* metingen. De nieuwe methode reduceerde de gemiddelde uitwasbare fractie van zetmeel in deze producten van 0.333 tot 0.042 $g \cdot g^{-1}$ ten opzichte van de conventionele methode. Het gebruik van de nieuwe methode

verminderde de gemiddelde k_d van zetmeel in gerst, haver en tarwe van 0.327 tot 0.144 u^{-1} terwijl voor bonen, erwten en mais geen verschil in k_d ten opzichte van de conventionele methode werd waargenomen. Voor gerst, mais en tarwe nam het verschil tussen niet-afgebroken zetmeel in het residu tussen beide methoden gedurende de eerste vier uur van de incubatie duidelijk toe hetgeen een indicatie is voor het plaatsvinden van secundair deeltjesverlies. De gemiddelde ED van zetmeel daalde van 0.761 tot 0.572 $g \cdot g^{-1}$ bij gebruik van de nieuwe method en tot 0.494 $g \cdot g^{-1}$ na correctie voor deeltjes verlies tijdens de incubatie. De in vitro k_d van zetmeel in de niet-uitwasbare fractie verschilde niet van de waarde voor het gehele product. De berekende verhouding tussen de k_d voor zetmeel in de uitwasbare en niet-uitwasbare fractie was gemiddeld 1.59 en varieerde tussen 0.96 voor haver en 2.39 voor mais. De fractionele snelheid van gas productie verschilde significant tussen het gehele product en de niet-uitwasbare fractie. Voor alle producten, behalve haver, was deze snelheid groter voor het gehele product dan voor de niet-uitwasbare fractie terwijl voor haver het tegengestelde werd waargenomen. De snelheid in toename van de gasproductie was met name voor de granen sterk gecorreleerd met de *in vitro* k_d voor zetmeel. De resultaten van deze studie bevestigen niet de aanname gebruikt in diverse eiwit evaluatie systemen dat de afbraak van de W-S fractie van zetmeel veel sneller is dan de afbraak vanuit de D fractie.

Het onderwerp van hoofdstuk 6 is de vergelijking van de *in situ* afbraak van N in bonen, erwten, gerst, haver, mais en tarwe gevonden met de nieuwe en de conventionele methode. De nieuwe methode verminderde de S fractie met name voor de vlinderbloemigen, en de W-S fractie ten opzichte van de conventionele methode. De gemiddelde D+U fractie van N van deze producten nam toe van 0.644 (conventionele methode) tot 0.863 $g \cdot g^{-1}$ (nieuwe methode). De k_d van N verkregen met de nieuwe methode was hoger voor de vlinderbloemigen (behalve voor bonen waarvoor geen verschil in k_d verkregen met beide methoden werd waargenomen), maar lager voor de granen ten opzichte van de conventionele methode. De gemiddelde niet-afbreekbare fractie (U) nam toe van 0.048 (conventionele methode) tot 0.144 $g \cdot g^{-1}$ (nieuwe methode). De gemiddelde ED van de grondstoffen verminderde van 0.621 (conventionele methode) tot 0.458 $g \cdot g^{-1}$ (nieuwe methode). De nieuwe methode verminderde de ED van N voor granen sterker dan voor vlinderbloemigen. De verschillen in ED bij het gebruik van beide methoden werden voornamelijk veroorzaakt door de afname van de oplosbare fractie voor de

vlinderbloemigen en de afname van de k_d voor de granen. Voor bonen, erwten, gerst en mais werden hogere verhoudingen tussen diaminopimeline zuur (DAPA) en N gevonden met de nieuwe methode ten opzichte van de conventionele methode hetgeen wees op een grotere mate van microbiële besmetting. De op basis van DAPA berekende microbiële eiwit besmetting was hoger dan 100% van het totaal aanwezige eiwit voor de korte incubatie tijden (kleiner dan 8 uur) bij gebruik van de nieuwe methode. Deze hoge mate van besmetting duidt op het openbreken van bacterie cellen gedurende het wassen van de nylon zakjes. Correctie voor de mate van het openbreken van bacterie cellen, door het aanpassen van de verhouding microbiële N – DAPA, had een sterk effect op de berekende ED van N.

Hoofdstuk 7 vergelijkt de nieuwe methode ten opzichte van de conventionele methode. De ontwikkelde nieuwe methode reduceerde de S fractie van N en de W-S fractie van N en zetmeel sterk en vergrootte daardoor de fractie van de nutriënten waarvan de *in situ* fractionele afbraaksnelheid kan worden bepaald. De vermindering van het deeltjes verlies tijdens het wassen stelde de nieuwe methode in staat om de *in situ* fractionele afbraaksnelheid van OM en N in TGC's te bepalen en reduceert de potentiele systematische fout in de berekening van de ED veroorzaakt door de gebruikte aannames ten aanzien de afbraaksnelheid van de S en W-S fractie. De nieuwe methode bood ook de mogelijkheid om te corrigeren voor het verlies aan deeltjes tijdens de incubatie waardoor de systematische fout in de berekening van de ED veroorzaakt door de verkleining van deeltjes kan worden beperkt. Deze verkleining heeft met name een groot effect op de k_d van zetmeel in granen bij gebruik van de conventionele methode. De nadelen van de nieuwe methode waren een toename van de systematische fout in de berekening van ED ten gevolge van een hogere mate van microbiële besmetting en een groter verschil tussen de *in situ* en *in vivo* afbraak ten opzichte van de conventionele methode.

Dankwoord

Het verschijnen en verdedigen van een proefschrift betekent voor elke PhD kandidaat de formele afronding van een wetenschappelijk avontuur waarvan het verloop vooraf moeilijk is in te schatten. Dit geldt zeker voor mijn avontuur toen ik in 2004 na een gesprek met Jan Dijkstra het plan opvatte om te gaan promoveren op het terrein van de voeding van de herkauwers. De eerste fase (tot 2009) was voornamelijk gericht op het inwerken, het vaststellen van het onderwerp en het formuleren van concrete onderzoeksvragen. Gedurende deze fase werd duidelijk dat mijn PhD onderzoek zich zou richten op het verbeteren van de *in situ* methodiek hetgeen voortkwam uit mijn belangstelling voor methodologische problemen en de praktische relevantie van het onderwerp. In de tweede fase (na 2009) vond het experimenteel *in situ* werk plaats en kreeg het onderzoek zijn huidige vorm. De ontwikkelde, aangepaste wasmethode en de correctiemethode voor deeltjesverlies tijdens de pensincubatie hebben mijns inziens tot meer inzicht in de toepasbaarheid van de *in situ* methode geleid. Mijn werk met de nylon zakjes heeft mij binnen de leerstoelgroep de titel “zakkenwasser” opgeleverd, echter deze zal ik als een Geuzennaam in de toekomst blijven gebruiken.

Dit avontuur heb ik alleen met de hulp van velen tot een goed einde kunnen brengen en hen wil ik hiervoor van harte bedanken. Zonder anderen tekort te doen, wil ik in dit Dankwoord enkele mensen noemen die op een speciale wijze aan de voltooiing van dit proefschrift hebben bijgedragen. Ten eerste gaat mijn dank uit aan Wouter Hendriks die als hoogleraar van leerstoelgroep Diervoeding mij in staat heeft gesteld om naast het gewone werk in rust aan deze promotie te mogen werken en te voltooien. Zijn inhoudelijk commentaar en het opspeuren van layout - technische onjuistheden hebben de kwaliteit van mijn proefschrift duidelijk verbeterd.

Mijn dagelijkse begeleiders, Jan en Harmen, wil ik bedanken voor hun ondersteuning tijdens dit proces. Harmen, de discussie met jou over de *in situ* methode en de toepassing hiervan in de praktijk waren altijd zeer nuttig en levendig. Jouw strikte wetenschappelijke benadering was altijd een goede buffer als mijn gedachten weer eens in een vlaag van wild enthousiasme op hol sloegen en leidde tot allerlei speculatieve beschouwingen. Samen zorgden we voor een mooie balans en ik hoop dat onze samenwerking binnen het *in situ*

onderzoek ook na deze promotie wordt gecontinueerd. Jan, ondanks jouw zeer drukke agenda wist je toch tijd vrij te maken om mij te begeleiden en de diverse papers aan een kritische beoordeling te onderwerpen. Deze beoordeling liet vaak wel even op zich wachten echter was dan ook van een uitzonderlijk hoog en gedetailleerd niveau. Jouw hulp met betrekking tot het gebruik van de Engelse taal heb ik altijd zeer gewaardeerd en hiervoor nogmaals mijn dank.

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De studenten die bij de uitvoering van dit onderzoek betrokken zijn geweest, wil ik van harte bedanken voor hun inzet. Het uitvoeren van experimentele *in situ* werk is zeer tijdrovend en elke ondersteuning was derhalve welkom. Een speciaal woord van dank gaat uit naar Johan Heeren die eerst via een BSc-project en later als student assistent veel praktisch werk binnen dit project heeft uitgevoerd. Zijn toewijding en betrokkenheid bij de uitvoering van de experimenten hebben een significante bijdrage aan de kwaliteit van dit proefschrift geleverd waarvoor ik hem van harte wil bedanken.

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resultaten van dit onderzoek een bijdrage leveren aan de verbetering van het in de praktijk gebruikte eiwitwaarderingssysteem voor herkauwers.

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Een promotie op gevorderde leeftijd vergroot de kans dat dierbaren de voltooiing hiervan niet meer kunnen bijwonen. Dit geldt met name voor mijn ouders en schoonouders die allen gedurende het promotieproces zijn overleden. Speciaal denk ik hierbij aan mijn vader die alleen het begin van het traject heeft meegemaakt. Zijn oprechte betrokkenheid heeft mij altijd gestimuleerd en zijn Groningse agrarische achtergrond is allicht een reden voor mijn belangstelling voor diervoeding. Uit dankbaarheid draag ik mijn proefschrift aan hem op.

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krijgen voor een volgende wetenschappelijk avontuur dat echter betrekking heeft op gebeurtenissen die 350 jaar geleden zijn gebeurd.

Curriculum Vitae

Leon de Jonge werd geboren op 13 september 1962 te Kerkrade. In 1980 behaalde hij zijn Atheneum diploma aan het Sint Antonius Doktor College te Kerkrade en begon hij met de studie Scheikunde aan de Rijksuniversiteit Utrecht. In 1987 studeerde hij af met als hoofdvak analytische chemie en als bijvak bodemkunde. Vanaf 1983 studeerde hij aanvullend geschiedenis aan de Rijksuniversiteit Utrecht die hij in 1989 afronden met als specialisatie Nieuwe geschiedenis. Vanaf 1990 was hij werkzaam als hoofd chemische en endocrinologisch laboratorium bij het Instituut voor Veevoedingsonderzoek (IVVO) en later het ID-Lelystad. Sinds 2003 was hij gedeeltelijk en vanaf 2008 volledig werkzaam als senior onderzoeker // hoofd laboratorium bij de leerstoelgroep Diervoeding van de Universiteit van Wageningen. Hiernaast is hij verantwoordelijk voor het onderwijs op het gebied van de diervoedingsanalyses binnen het cursorisch onderwijs van de leerstoelgroep. Leon treedt op auditor voor ISO 17025 in dienst van de Raad van accreditatie en participeert in diverse FAO werkgroepen met betrekking tot het ontwikkelen van kwaliteitssystemen binnen chemische laboratoria. In zijn vrije tijd is hij actief binnen diverse historische verenigingen.

Leon de Jonge was born on 13 September 1962 in Kerkrade. In 1980 he graduated from secondary grammar school Sint Antonius Doktor College in Kerkrade. In the same year he began his graduate studies in Chemistry at the State University of Utrecht. In 1987, he obtained his Master's degree in Chemistry with analytical chemistry as specialisation. During the period 1983 – 89, he additionally studied History for which he obtained his Master's degree with early modern history as specialisation. In 1990 he started working as head of the chemical laboratory at the Research Institute for Livestock Feeding and Nutrition (IVVO) and later at the Institute for Animal Health and Welfare (ID-Lelystad) in Lelystad. Since 2003 he initially worked part-time but from 2008 onwards full-time as senior researcher // head of laboratory at the Animal Nutrition Group (ANU) of the Wageningen University. He is also responsible for the education regarding feedstuff analysis within the BSc and MSc courses of ANU. He is auditor for ISO 17025 in service of the Dutch Council for Accreditation and member of several FAO working groups regarding the development of quality systems within

chemical laboratories. Besides his work, Leon is an active member of several historical groups.

Training and Supervision Plan[1]

Description	Year
The basic Package (3 ECTS[2])	
WIAS Introduction Course	Exemption
Course on philosophy of science and/or ethics	2010
International Conferences (4 ECTS)	
ISRP, Clermont Ferrand, France	2009
ISNH8, Wales, UK	2011
3rd International conference on food digestion (Cost), Wageningen	2014
EAAP, Copenhagen, Denmark	2014
Seminar and workshops (2 ECTS)	
Seminar Dietary lysine, Wageningen, The Netherlands	2010
Meetonzekerheid invoering NEN 7777, Delft, The Netherlands	2010
Forum ANR, Lelystad, The Netherlands	2010
Forum ANR, Leuven, Belgium	2011
Seminar Development in Ruminant Nutrition, Wageningen, The Netherlands	2013
Presentations (6 ECTS)	
Poster, ISRP, Clermont Ferrand, France	2009
Oral, ANR, Lelystad, The Netherlands	2010
Oral, ANR, Leuven, Belgium	2011
Oral, ISNH8, Wales, UK	2011
Oral, EAAP, Copenhagen, Denmark	2014
Poster, EAAP, Copenhagen, Denmark	2014

[1] Completed in fulfilment of the requirements for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Science)

[2]One ECTS (European Credit Transfer System) equals a study load of 28 hours

In-depth studies (6 ECTS)	
WBS course Rundveevoeding, Wageningen, The Netherlands	2010
WIAS Advanced statistical course: design of Animal Experiments, Wageningen, The Netherlands	2010
Feed evaluation Course, Wageningen, The Netherlands	2011/13
Statistics for Life Sciences, Wageningen, The Netherlands	2011
Summer Course Glycoscience VLAG, Wageningen, The Netherlands	2014
Quality of protein in animal diets, Wageningen, The Netherlands	2015
Statuary courses (6 ECTS)	
Stralingsdeskundigheid niveau 3, NRG Petten, The Netherlands	2001
Professional skills support courses (4 ECTS)	
Scientific writing, Lelystad, The Netherlands	2001
Personal developments training, Lelystad, The Netherlands	2002-4
Project work, Lelystad, The Netherlands	2002
GLP training, Lelystad, The Netherlands	2005
Didactic skills training (18 ECTS)	
Lecturing Principles of Animal Nutrition (ANU 20306)	2009-15
Lecturing Nutrition and Physiology (ANU 30806)	2009-15
Supervision practical ANU 20306	2009-15
Supervision practical ANU 30806	2009-15
Supervision 5 MSc and 3 BSc students	2008-15
Preparing course Advances in Feed Evaluation Sciences	2009-13
Management skills training (6 ECTS)	
Organizing Animal Feed analysis for PTC+ (Barneveld)	2008-12
Member Dutch Normalisation Committee (NEN, section Feed analysis)	2000-13
Member Quality committee for laboratories within the Animal Feed sector (KDLL)	2000-15
Education and training total	**55 ETCS**

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