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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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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/g. 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 with 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 h of incubation increased, which indicates secondary particle loss. The average effective degradation of starch decreased from 0.761 to 0.572 g/g when using the new rinsing method and to 0.494 g/g 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 with 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. In addition, 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 with 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 (volatile fatty acid v. glucose) that is available for the animal, which is relevant

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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; Stevnebo *et al.*, 2009). It should be noted that the rate of gas production reflects fermentation of the organic matter (OM) 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 (effective 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 with 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.

Material 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 (ZM100; Retsch, 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 that 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) *v.* rinsing by a wool wash programme of a commercial washing machine (see 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/kg dry matter (DM); NDF, 516 g/kg DM) and 50% maize silage (N, 11.5 g/kg DM; NDF, 397 g/kg DM; starch, 374 g/kg DM) at 0700 h. Cows received each day an additional 2 kg of protein-rich concentrate feed (N, 53.0 g/kg) and commercial concentrate feed (N, 29.8 g/kg) according to milk production level up to a maximum of 7 kg (on average 3 ± 1 kg). Cows were 290 ± 53 days in milk and produced 23.9 ± 4.0 kg milk/day. All incubation times were conducted separately on

different days, starting at 0900 h, 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 × 8 cm, a pore size of 40 µm and porosity of 0.30 (PA 40/30; Nybolt, Zurich, Switzerland) were filled with ~5 g of feed ingredient. The number of bags for each feed ingredient, rinsing method and incubation time combination was four 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 NaH₂PO₄·H₂O and 8.9 g/l Na₂B₄O₇·10H₂O, adjusted to pH 6.2 with hydrochloric acid). The vessels were placed in a mechanical shaker (SW-20c; Julabo, Seelbach, Germany) and were shaken during 60 min at 40 s.p.m. 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 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 programme 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 s.p.m. 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_{pi} ; h⁻¹), the fraction of starch insensitive (F_{IS}) and sensitive (F_S ; calculated as 1 - F_{IS}) to particulate matter loss, with the PROC NLIN procedure of SAS (2002) using a first-order model:

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

where $Y(t)$ is the fractional residue 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/day), 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. DM 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 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_{dcorr} ; 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_{pi} taken from the *in vitro* simulation of particulate matter loss:

$$Y(t) = F_S \times \exp(-(k_{dcorr} + k_{pi}) \times t) + F_{IS} \times \exp(-k_{dcorr} \times t) \quad (3)$$

Where $Y(t)$ is the fractional residue of starch after incubation during t hours. The ED (g/g) 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/g) and k_p 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 $k_d(\text{total})$ is the fractional disappearance rate of starch in the total feed ingredient and $k_d(D)$ 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):

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

where $\text{gas}(t)$ is the total volume of produced gas (ml) per g OM at time t , A the maximal gas production (ml/g 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.

ANOVA 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 with washing machine rinsing (Table 1). This increase ranged from 0.100 g/g for barley to 0.496 g/g for oats, leading to a non-washout fraction for starch between 0.890 g/g for oats and 0.993 g/g 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/g using the washing machine to 0.572 g/g using the new rinsing method. The differences in ED of starch between rinsing methods varied between 0.141 g/g for maize and 0.269 g/g for faba beans.

The difference in the amount of starch in the residue between 40 s.p.m. 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 three feeds with high washout fraction the average difference between both rinsing methods decreased from 0.449 g/g at 0 h

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

Ingredients	Parameter	40 s.p.m.	Washing machine	s.e.m.	<i>P</i>
Barley	<i>D</i>	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	<i>D</i>	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	<i>D</i>	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	<i>D</i>	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	<i>D</i>	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	<i>D</i>	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		

Starch content (g/kg DM): barley 574; faba beans 441; maize 681; oats 427; peas 476; wheat 674.

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

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

to 0.021 g/g 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 h and then decreased with incubation time. For these ingredients, the average difference between both rinsing methods increased from 0.129 g/g at 0 h to 0.286 g/g at 4 h and subsequently decreased to 0.034 g/g at 48 h.

Particulate matter loss of starch during simulation of the rumen incubation by rinsing at 40 s.p.m. (Table 3) was different for the feed ingredients. For barley and maize only a small fraction of starch (i.e. <0.1) 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, with a lower fractional disappearance rate (i.e. k_{p1}) 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/g) 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/g. The decrease in ED ranged from 0.018 g/g for maize to 0.159 g/g for faba beans.

In vitro experiment

The non-washout fraction of starch of this batch of the feed ingredients ranged from 0.135 g/g for oats to 0.930 g/g for

Table 2 Difference between the amount of starch in the residue after rinsing at 40 s.p.m. and after the washing machine, expressed as fraction of the original amount, for different incubation times for six feed ingredients (n = 4)

Incubation time (h)	Feed ingredients					
	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
s.e.m.	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 ($P < 0.05$).

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

Ingredient	F_S (g/g)	F_{IS} (g/g)	k_{pi} (h^{-1})	k_d -corr (h^{-1}) 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

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

²Calculated 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.

maize (Table 4) and differed from the results from the previous batch (Table 1). These differences ranged from 0.001 g/g for faba beans to 0.259 g/g 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.

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/g OM for the non-washout fraction of oats to 428 ml/g 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

Table 4 Starch non-washout fraction (D ; g/g) 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)

Ingredient	<i>In vitro</i> fractional degradation rate (k_d ; h^{-1})				
	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

For k_d of total and D fraction: feed ingredient: $P = 0.001$ with s.e.m. 0.009; fraction: $P = 0.126$ with s.e.m. 0.005; no interaction between feed ingredient and fraction was observed ($P = 0.83$).

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

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

²Ratio is $k_d(W)/k_d(D)$.

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 ($R^2 = 0.91$).

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

Table 5 Gas production characteristics (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 five feedstuffs

Ingredient	Total	D	s.e.m.	Feed	Fraction	Feed × fraction
A (in ml/g 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 (in h)						
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}				

OM = organic matter.

Calculated as $\text{Gas}(t) = A \times \exp(-B \times \exp(-C \times t))$ (Gompertz curve).

Starch content (g/kg): 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).

^{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 ($P < 0.05$).

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. $2 \times k_d(\text{D fraction}) + 0.375$; 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 with 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 with 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 by 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 by Stevnebo *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 with *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 emphasized 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 Sauvart (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 that 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 with *in vivo* results.

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