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ORIGINAL ARTICLE

Comparison of fractionation methods for nitrogen and starch in maize and grass silages

M. Ali[1,2,3], L. H. de Jonge[1], J. W. Cone[1], G. van Duinkerken[2], M. C. Blok[4], M. H. Bruinenberg[5] and W. H. Hendriks[1,6]

1 Animal Nutrition Group, Wageningen University, Wageningen, The Netherlands
2 Wageningen UR Livestock Research, Lelystad, The Netherlands
3 Institute of Animal Sciences, University of Agriculture, Faisalabad, Pakistan
4 Product Board Animal Feed, Zoetermeer, The Netherlands
5 BLGG AgroXpertus, Wageningen, The Netherlands, and
6 Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Summary

In *in situ* nylon bag technique, many feed evaluation systems use a washing machine method (WMM) to determine the washout (W) fraction and to wash the rumen incubated nylon bags. As this method has some disadvantages, an alternate modified method (MM) was recently introduced. The aim of this study was to determine and compare the W and non-washout (D+U) fractions of nitrogen (N) and/or starch of maize and grass silages, using the WMM and the MM. Ninety-nine maize silage and 99 grass silage samples were selected with a broad range in chemical composition. The results showed a large range in the W, soluble (S) and D+U fractions of N of maize and grass silages and the W, insoluble washout (W-S) and D+U fractions of starch of maize silages, determined by both methods, due to variation in their chemical composition. The values for N fractions of maize and grass silages obtained with both methods were found different ($p < 0.001$). Large differences ($p < 0.001$) were found in the D+U fraction of starch of maize silages which might be due to different methodological approaches, such as different rinsing procedures (washing vs. shaking), duration of rinsing (40 min vs. 60 min) and different solvents (water vs. buffer solution). The large differences ($p < 0.001$) in the W-S and D+U fractions of starch determined with both methods can led to different predicted values for the effective rumen starch degradability. In conclusion, the MM with one recommended shaking procedure, performed under identical and controlled experimental conditions, can give more reliable results compared to the WMM, using different washing programs and procedures.

Keywords nitrogen, starch, washing machine method, modified method, silages

Correspondence M. Ali, Institute of Animal Sciences, University of Agriculture, Faisalabad, 38040, Pakistan.
Tel: +92-34-68590098; Fax: +92-41-9200193; E-mail: drmubarak434@gmail.com

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Introduction

Different ruminant feed evaluation systems, such as the DVE/OEB_{2010} system (Van Duinkerken et al., 2011) in the Netherlands, the Feed into Milk (FiM) system (Thomas, 2004) in the UK, the North American NRC system (NRC, 2001), the PDI system (Vérité et al., 1979) in France and the Nordic NorFor system (Volden, 2011) in Scandinavian countries use data of *in situ* nylon bag experiments to estimate the rumen degradation characteristics of dietary nutrients in different feedstuffs. The *in situ* nylon bag technique (Ørskov and McDonald, 1979) divides feed or feed ingredients into a washout (W) fraction, a potentially rumen degradable (D) fraction and a rumen undegradable (U) fraction. Within the *in situ* nylon bag technique, the washing machine method (WMM) has been extensively used to determine the W fraction and to wash the rumen incubated nylon bags to remove contaminations (Gierus et al., 2006). The washing of non-incubated nylon bags with this method separates the feed into a W and a non-washout (D+U) fraction. Different washing machines, rinsing procedures, washing programmes and washing times have been used in different *in situ* studies, which cause variation in the *in situ* results (Lindberg, 1985;

Madsen and Hvelplund, 1994; Vanzant et al., 1998). Different researchers tried to standardize a procedure without washing machines (Licitra et al., 1996; Shannak et al., 2000). The W fraction of feed or feed ingredients can be divided into a soluble (S) fraction and an insoluble washout (W-S) fraction of small particles (Gierus et al., 2005; de Jonge et al., 2013). Using the WMM, the S fraction has to be determined separately by an additional analysis and the W-S fraction can be calculated by subtraction of the S fraction from the W fraction (Van Duinkerken et al., 2011). Recently, de Jonge et al. (2013) introduced a modified method (MM) that in contrast to WMM determines all fractions, for example S, W-S and D+U for nitrogen (N) and starch in feed ingredients. The MM is based on washing of nylon bags with a buffer solution in a closed system that enables to isolate and determine all fractions. With the MM, the S, W-S and D+U fraction of chemical components of feed or feed ingredients are determined directly unlike with the WMM. As all the fractions are separated with one rinsing procedure, the accuracy of their sum can be verified, based on total recovery. Although the MM has some methodological advantages, it also affects the values found for the different fractions in feed ingredients (de Jonge et al., 2013), and consequently, the predicted rumen degradation by the different feed evaluation systems. In the previous mentioned study, however, the comparison between both methods was limited to a small number of feed ingredients and forages, which made it impossible to evaluate the full impact of this difference.

It is hypothesized that the different methodological approaches used in MM and WMM results in different N and starch fractionation values. Therefore, the aim of this study was to evaluate the effect of the new method on the values for the different fractions conducting a comparison between the WMM and the new MM for a large set of maize and grass silages.

Materials and methods

Sample selection and processing

Ninety-nine maize silage and 99 grass (mainly *Lolium perenne*) silage samples were obtained from various Dutch commercial farms, located in different regions in the Netherlands. After collection, individual silages were stored at −20 °C. Each frozen silage (maize or grass) was cut using a bread slicer (JAC Duro BEL 450; ABO, Leek, The Netherlands) having a distance of 11 mm between the discs and then thoroughly mixed by hand.

Standard washing machine method

Approximately 5 g DM of each maize and grass silage sample was weighed into 10 cm × 19 cm nylon bags (porosity 24%, pore size 37 μm; Nybolt, Zurich, Switzerland), in duplicate. The bags were washed in a washing machine (AEG-Electrolux Öko Turnamat 2800, Stockholm, Sweden) for 40 min, using tap water at 25 °C, according to the method described by Rodrigues et al. (2009), to determine D+U fraction and the W fraction was calculated as 1-(D+U). The washed bags were stored at −20 °C and subsequently freeze-dried. For each maize and grass silage, the washed residues were pooled and the contents were ground over a 1-mm sieve, using a hammer mill (Pepping, 200 AN-797002, Deventer, The Netherlands). The washed maize silage residues were analysed for DM, N and starch, while grass silage residues were analysed for DM and N.

Modified method

Approximately 5 g DM of maize or grass silage was weighed into 10 cm × 19 cm nylon bags (porosity 24%, pore size 37 μm; Nybolt, Zurich, Switzerland). Two bags of each maize or grass silage 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/l $NaH_2PO_4.H_2O$ and 8.9 g/l $Na_2B_4O_7.10H_2O$ (Merck, Darmstadt Germany), and the pH was adjusted to 6.2 with HCl (de Jonge et al., 2009). The glass vessel, containing the buffer solution with the bags, was placed in a mechanical water shaker (160 rpm) for 1 h at room temperature. Separation of the different fractions was performed according to the procedure described by de Jonge et al. (2013). After 1 h, the bags were removed and dried for 48 h at 70 °C. This sample corresponded to the D+U fraction. The buffer solution in the vessel was quantitatively centrifuged for 15 min at 20 000 *g* at 25 °C, and the supernatant was quantitatively collected and weighed (S fraction). The pellet (W-S fraction) was quantitatively collected and dried for 48 h at 70 °C.

Chemical analysis

The DM content was determined by oven drying at 103 °C for 4 h (ISO 6496), and ash content was determined by incineration at 550 °C for 4 h (ISO 5984). The N content was determined using the Kjeldahl method (ISO 5983-2), and CP was calculated as N × 6.25. Neutral detergent fibre was determined

according to the modified method of Van Soest et al. (1991), using amylase and expressing values exclusive of residual ash (ISO 16472). Acid detergent fibre was determined by boiling with acid detergent reagent and expressed exclusive of residual ash (ISO 13906: 2008). Acid detergent lignin was determined after boiling with acid detergent reagent and treatment with sulphuric acid (ISO 13906:2008). Starch was determined using the amyloglucosidase method (ISO 15914) after dissolving in dimethyl sulfoxide. Crude fat and sugar contents were determined according to ISO 6492 and the Luff–Schoorl method (NEN 3571: 1947 nl), respectively.

Statistical analysis

The data on the N and starch fractions of the maize silages and N fractions of the grass silages determined using the WMM and the MM were summarized by Descriptive Statistics using SAS 9.2 (2009). The values for N and starch fractions determined by both methods were compared using a pairwise *t*-test.

Results

The chemical composition and silage quality parameters of the 99 maize and 99 grass silages showed a large variation (Table 1). The DM content of maize silages ranged from 272 to 440 g/kg fresh matter. The range in the CP, starch and NDF contents of maize silages was 53–81, 76–427 and 278–503 g/kg DM, respectively. The DM content of grass silages was ranged from 201 to 685 g/kg fresh matter. The range in the CP and NDF contents of grass silages was 102–222 and 326–611 g/kg DM, respectively.

The results of the N fractionation of maize and grass silages into W and D+U fractions, determined with the WMM and into S and D+U fractions, determined with the MM, are presented in Table 2. The D+U fraction of N of the maize silages, determined with the WMM, ranged from 0.266 to 0.796, whereas the D+U fraction of N, determined with the MM, ranged from 0.335 to 0.754. The N fractions of maize silages determined with both methods were found different ($p < 0.001$). Figure 1(a) shows a positive linear relationship between the D+U values of N of the maize silages determined with the both methods (WMM and MM). The range in the D+U fraction of starch determined with the WMM was 0.270–0.938, whereas the range in the D+U fraction determined with the MM was 0.502–0.948. The values for starch fractions of maize silages obtained with both methods were also differed ($p < 0.001$). Figure 1(b) shows a positive relationship ($R^2 = 0.46$) between the D+U fractions of starch of the maize silages, determined by both methods.

For all the grass silages, no W-S fraction was observed using the MM, and therefore, the W fraction of N calculated with the WMM was compared with the S fraction with the MM. The W fraction for N of grass silages determined with the WMM ranged from 0.116 to 0.638 whereas the S fraction determined with the MM ranged from 0.237 to 0.639. The N fractions of grass silages determined with both methods were different ($p < 0.001$). The positive linear relationship between the two methods for the D+U fraction of N of the grass silages is shown in Figure 1(c).

Table 1 Chemical composition of maize and grass silages

	Maize silages (n = 99)				Grass silages (n = 99)			
Variable	Mean	SD*	Minimum	Maximum	Mean	SD	Minimum	Maximum
Chemical composition (g/kg DM)								
Dry matter (g/kg)	350.9	35.9	272.2	440.4	445.0	116.2	201.0	685.0
Ash	37.3	9.6	21.0	79.0	103.4	19.4	70.0	192.0
Crude protein	65.9	6.2	52.6	81.0	165.8	31.1	102.0	222.0
Crude fat	35.9	4.4	27.0	47.0	42.0	7.6	27.0	65.0
Starch	332.2	48.7	176.0	427.0	–	–	–	–
Sugar	11.5	4.7	3.0	43.0	87.8	46.2	11.0	246.0
Neutral detergent fibre	387.1	44.1	278.0	503.0	498.8	54	326.0	611.0
Acid detergent fibre	216.0	26.2	152.0	289.0	272.6	30.6	157.0	347.0
Acid detergent lignin	17.5	3.4	11.0	27.0	19.6	6.6	10.0	40.0

*Standard deviation.

Table 2 Nitrogen and starch fractions of maize and grass silages, determined using the washing machine and a modified method

Variable	Maize silages (n = 99)				Grass silages (n = 99)			
	Mean	SD*	Minimum	Maximum	Mean	SD	Minimum	Maximum
Nitrogen								
Washing machine method								
W fraction[†]	0.531	0.091	0.204	0.734	0.435	0.099	0.116	0.638
D+U fraction[‡]	0.469	0.091	0.266	0.796	0.565	0.099	0.362	0.884
Modified method								
S fraction[§]	0.509	0.085	0.246	0.665	0.467	0.085	0.237	0.639
D+U fraction	0.491	0.085	0.335	0.754	0.533	0.085	0.361	0.763
Starch								
Washing machine method								
W fraction	0.440	0.142	0.062	0.730	–	–	–	–
D+U fraction	0.560	0.142	0.270	0.938	–	–	–	–
Modified method								
W-S fraction[¶]	0.232	0.089	0.052	0.498	–	–	–	–
D+U fraction	0.768	0.089	0.502	0.948	–	–	–	–

*Standard deviation.
[†]Washout fraction; the fraction disappeared from non-incubated nylon bags after washing in the washing machine (calculated value).
[‡]Non-washout fraction.
[§]Soluble fraction.
[¶]Insoluble washout fraction, the insoluble small particles which escape from the nylon bag during incubation in the buffer solution.

Discussion

In the present study, the results of the N and starch fractions in maize and grass silages determined with the WMM and the MM were compared to determine the effect of different methodological approaches used in these methods on the fractionation values. Different values were obtained for the D+U fraction of N and/or starch of maize silages, using the WMM and MM, as well as for the N fraction of grass silages. A large range in the N and starch fractions of maize and grass silages might be due to the broad range in their chemical composition. The values for N fractions (W fraction determined with the WMM vs. S fraction determined with the MM and D+U fraction determined with the WMM vs. D+U fraction determined with the MM) of maize and grass silages were different ($p < 0.001$) between the methods. The different values for N fractions obtained with the WMM and the MM might be due to different rinsing procedures under different experimental conditions used in these methods. The positive slopes in Fig. 1 indicate that the D+U fraction of N of maize and grass silages determined by both methods shows a similar increasing or decreasing trend between different samples. The data points away from the regression lines indicate that the relationships were not aligned and differed between the individual samples. Residual analysis shows random distribution for D+U fraction of N of maize and grass silages. For few maize and grass silages, the D+U fraction of N was higher for the MM, and for other samples, the D+U fraction was higher for the WMM.

There is no S fraction of starch and the W fraction determined by the WMM contains only the W-S fraction (Chai et al., 2004; Cone et al., 2006). Therefore, in the present study, the W-S fraction of starch determined with the MM was compared with the W fraction determined with the WMM. The results found for the fractions of starch were significantly ($p < 0.001$) affected by the method used. In the MM, there was less loss of starch particles from the nylon bags leading to higher D+U fraction and lower W-S fractions. The obtained results for starch fractions of maize silages are in line with the previous observations made by de Jonge et al., 2013. The large differences in the values of starch fractions determined with the WMM and the MM might be due to the different rinsing procedures (washing vs. shaking), duration of rinsing (40 min vs. 60 min) and different solvents (water vs. buffer solution) used in these methods. The W or W-S fraction affects the calculated value of effective rumen degradability of nutrients. The lower W-S values of starch determined with the MM can lead to different predicted effective rumen starch degradability.

As the different methodological approaches used in both methods resulted in different values of the different fractions, the question arises which method pro-

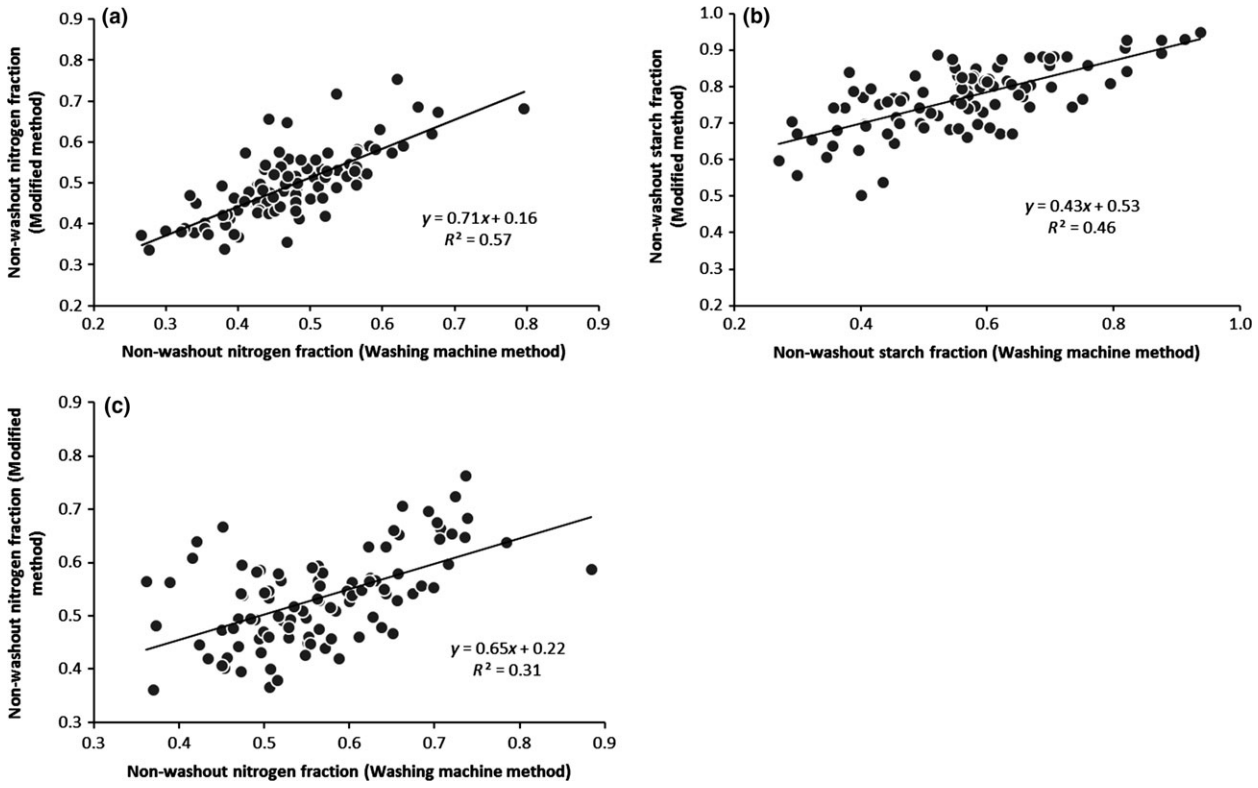

Fig. 1 Relationship between (a) non-washout nitrogen fractions of maize silages, (b) non-washout starch fractions of maize silages, (c) non-washout nitrogen fractions of grass silages, determined using the washing machine method and a modified method.

vides the most feasible results. In the MM, one recommended shaking procedure, performed under identical and controlled experimental conditions, can give more reliable results compared to the WMM, using different washing programs and times. In addition, the use of a buffer solution in the MM makes it physiological more close to the situation in the rumen. Therefore, the controlled experimental conditions, one shaking procedure and use of buffer solution in the MM make this method reliable for fractionation.

In the present study, the average W fraction for N of maize silages, determined by the WMM, was 0.531 ranging from 0.204 to 0.734. Average values of 0.632, 0.618 and 0.637 for the W fraction of crude protein (CP) of maize silages were reported by Von Keyserlingk et al. (1996), De Boever et al. (2005) and González et al. (2010), respectively. The higher values for the W fraction of CP, obtained in these studies, might be due to the high CP content present in those maize silages and different numbers of samples used, compared with the mean CP content of 66 g/kg DM of the 99 maize silages used in the present study. The CP content of the maize silages used by Von Keyserlingk et al. (1996), De Boever et al. (2005) and González et al. (2010) were 80 (n = 12), 73 (n = 26) and 69 g/kg DM (n = 30), respectively. In the present study, the average value for the W fraction of starch of maize silages was 0.440. A high value of 0.529 was reported by De Boever et al. (2005) for W fraction of maize silages. The higher value may be explained by 50-min washing program with no spin cycle used by De Boever et al. (2005), whereas a 40-min wool washing program was used in the present study. The average W fraction of N of the grass silages, using the WMM in the present study, was 0.428. Gierus et al. (2005) reported W fractions of 0.258 for low DM grass silages (251 g/kg fresh matter) and 0.480 for high DM grass silages (529 g/kg fresh matter). The large difference in the W fraction might be due to different DM contents of the grass silages, although the CP contents (295 and 288 g/kg DM) were comparable (Gierus et al., 2005).

In conclusions, different values of N and starch fractions were obtained for maize and grass silages due to the different methodological approaches of both fractionation methods (WMM and MM) used. The values obtained for N and/or starch fractions of maize and grass silages with both methods are different ($p < 0.001$).

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