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Technical note: Precision and accuracy of in vitro digestion of neutral detergent fiber and predicted net energy of lactation content of fibrous feeds

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ABSTRACT

The objective of this study was to test the precision and agreement with in situ data (accuracy) of neutral detergent fiber degradability (NDFD) obtained with the rotating jar in vitro system (Daisy^{II} incubator, Ankom Technology, Fairport, NY). Moreover, the precision of the chemical assays requested by the National Research Council (2001) for feed energy calculations and the estimated net energy of lactation contents were evaluated. Precision was measured as standard deviation (SD) of reproducibility (S_R) and repeatability (S_r) (between- and within-laboratory variability, respectively), which were expressed as coefficients of variation (SD/mean × 100, S_R and S_r, respectively). Ten fibrous feed samples (alfalfa dehydrated, alfalfa hay, corn cob, corn silage, distillers grains, meadow hay, ryegrass hay, soy hulls, wheat bran, and wheat straw) were analyzed by 5 laboratories. Analyses of dry matter (DM), ash, crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) had satisfactory S_r, from 0.4 to 2.9%, and S_R, from 0.7 to 6.2%, with the exception of ether extract (EE) and CP bound to NDF or ADF. Extending the fermentation time from 30 to 48 h increased the NDFD values (from 42 to 54% on average across all tested feeds) and improved the NDFD precision, in terms of both S_r (12 and 7% for 30 and 48 h, respectively) and S_R (17 and 10% for 30 and 48 h, respectively). The net energy for lactation (NE_L) predicted from 48-h incubation NDFD data approximated well the tabulated National Research Council (2001) values for several feeds, and the improvement in NDFD precision given by longer incubations (48 vs. 30 h) also improved precision of the NE_L estimates from 11 to 8%. Data obtained from the rotating jar in vitro technique compared well with in situ data. In conclusion, the adoption of a 48-h period of incubation improves repeatability and reproducibility of NDFD and accuracy and reproducibility of the associated calculated NE_L. Because the in vitro rotating jar technique is a simple apparatus, further improvement would probably be obtained by reducing the laboratory differences in rumen collection procedures and type of animal donors, which, however, reflect practical conditions.
Key words: in vitro rumen fermentation, neutral detergent fiber, net energy of lactation

The NRC (2001) model provides a summative equation to predict the energy content of feeds for dairy cattle, based on some analytical determinations [ash, ether extracts (**EE**), CP, NDF, CP bound to NDF (**NDIN**), and NDF degradability (**NDFD**)]. The rotating jar in vitro system (Daisy^{II} incubator, Ankom Technology, Fairport, NY) is a fast, simple, and inexpensive in vitro rumen fermentation technique to measure NDFD. Despite widespread utilization of this technique, there is limited research aimed at verifying its accuracy and precision (Spanghero et al., 2007). The objective of this study was to test the precision and accordance with in situ data (accuracy) of the NDFD obtained with the rotating jar in vitro system. Moreover, the precision of the chemical assays, requested by NRC (2001) for feed energy calculations, and the estimated NE_L contents have been evaluated.

Five Italian university laboratories executed the same analytical assays [DM, ash, EE, CP, NDF, ADF, NDIN, CP bound to ADF (**ADIN**)], and NDFD on 10 fibrous feed samples (alfalfa dehydrated, alfalfa hay, corn cob, corn silage, distillers grains, meadow hay, ryegrass hay, soy hulls, wheat bran, and wheat straw). Dried and milled samples (1-mm sieve, Wiley mill, Arthur H. Thomas, Philadelphia, PA) were analyzed in duplicate by each laboratory. Moisture was determined by a forced-air oven method, ash as gravimetric residue after incineration at 550°C, EE by ether extraction, and

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CP (nitrogen × 6.25) by the Kjeldahl method (AOAC, 1995). The Ankom^{200} Fiber Analyzer (Ankom Technology) was used to determine NDF and ADF following the procedure of Mertens (2002) for NDF and Van Soest et al. (1991) for ADF (AOAC, 1995). Neutral detergent fiber analyses (**aNDF**) utilized a neutral detergent solution containing sodium sulfite and a heat-stable bacterial α-amylase (activity = 17,400 Liquefon units/mL, Ankom Technology). The aNDF and ADF contents were corrected for residual ash content (**aNDFom** and **ADFom**, respectively). The NDIN and ADIN were determined as residual nitrogen in Ankom fiber bags after extraction with neutral detergent (containing sodium sulfite) or acid detergent solution. The NFC content was calculated as follows: 100 − [CP + ash + EE + (aNDFom – NDIN)].

Samples were also analyzed in each laboratory for NDFD by the Ankom Daisy^{II} incubator, according to Robinson et al. (1999). Two digestion jars were filled with prewarmed (39°C) buffer solutions (266 mL of solution A: KH_2PO_4 10 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L, urea 0.5 g/L; 1,330 mL of solution B: Na_2CO_3 15.0 g/L, $Na_2S \cdot 9H_2O$ 1.0 g/L) and placed into the Daisy^{II} incubator. For each sample, 4 bags (Ankom F57) were filled with ground material (1-mm sieve, 250 mg) and the bags were sealed. Jars were divided vertically by using a perforated plastic separator, and 2 bags for each sample were inserted on either side of the separator, giving a total of 20 bags per jar with 10 bags on each side. Rumen fluid was collected from rumen fistulated cows (2 laboratories), from rumen contents obtained at a slaughterhouse (2 laboratories), and via esophageal tube (1 laboratory). Rumen fluid was then filtered through 2 layers of cheesecloth. Four hundred milliliters of the filtered rumen fluid was introduced into each jar with the filter bags in place. After 30 h of incubation, the jars were opened under a CO_2 flow, the bags on one side of the jar were quickly collected, and the jar was closed again. The remaining bags were removed after 48 h of incubation. The bags removed from the jars were rinsed thoroughly with cold tap water and immediately analyzed for NDF content using the Ankom^{200} Fiber Analyzer and incinerated to correct the residual NDF for the residual ash. The residual NDF was also used to calculate NDF degradability as percentage of DM (**dNDF**). The experiment was repeated in a second fermentation run.

Two laboratories also measured the in situ NDFD after incubation of the bags in the rumens of cannulated dry cows (2 cows from each laboratory) fed a fibrous diet (90% DM as forage) at maintenance level (NRC, 2001) divided into 2 meals per day (at 0800 and 1800 h). The bag preparation and feed samples were identical to that used for the in vitro assay, and bags (4 bags per feed for each cow) were placed in string net bags (10 × 15 cm; 15-mm pore size), which were then inserted in the rumens of cows. The bags were removed after 30 and 48 h of incubation and were treated as those of the in vitro assay.

The CP, ADIN, NFC, and NDF contents (in % DM) and NDFD (measured in vitro at 30 and 48 h) were used to calculate the truly digestible amounts for each feed as described in equations 2–4a,b,d,e of NRC (2001). The digestible energy content at maintenance level (**DE_{1X}**) was calculated with equation 2–8a (NRC, 2001; fatty acids (**FA**) content as (EE − 1)]. For each sample, 2 DE_{1X} values were calculated using the NDFD obtained from in vitro measurements at both 30 and 48 h of fermentation. The DE_{1X} values were adjusted for a level of intake of 3× maintenance and for a dietary total digestible nutrient content (**TDN**) of 0.74 (**DE_p**, Eq. 2–9; NRC, 2001). For each sample, the 2 DE_p values were the basis for the calculation of the 2 NE_L values, using the equations 2–10, 2–11, and 2–12 of the NRC (2001). The NE_L values were then converted to kilocalories.

Duplicate measured values (k = 1,2) from chemical assays and in vitro NDFD (obtained from 2 fermentation runs) and single calculated values of NFC and NE_L contents obtained in different laboratories (α; i = 1,5) and samples (β; j = 1,10) were analyzed with GLM procedures of SAS (SAS Institute, 1999) using the following linear model (μ = overall mean; ε = residual error):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk.}$$

For chemical assays and in vitro NDFD, the variance components of laboratory effect, its interaction with the feed effect and the error variance (σ_L^2, σ_i^2, and σ_e^2, respectively) were used to calculate the standard deviation (**SD**) of repeatability ($\mathbf{S_r}, \sqrt{\sigma_e^2}$) and reproducibility ($\mathbf{S_R}$, $\sqrt{\left\{\sigma_e^2 + \left[\left(\sigma_i^2 - \sigma_e^2\right)/k\right] + \left[\left(\sigma_L^2 - \sigma_i^2\right)/jk\right]\right\}}$; Youden and Steiner, 1975). The SD reproducibility of NFC and NE_L (1 determination for each sample and laboratory) was calculated as $\sqrt{\left\{\sigma_i^2 + \left[\left(\sigma_L^2 - \sigma_i^2\right)/j\right]\right\}}$. Repeatability and reproducibility were then expressed as coefficients of variation (SD/mean × 100, S_r and S_R respectively), which were used as precision terms in this work.

The collaborative trial presented in this paper involved 5 laboratories and 10 samples (giving a total of 50 observations) and therefore it satisfies the absolute minimum design (5 labs and 3 pairs of materials) for a collaborative study indicated by Youden and Steiner (1975) and the minimum number needed to obtain an

Table 1. Chemical composition of feeds and estimates of repeatability and reproducibility for chemical analysis

Item	Analytical DM	Ash	CP	Ether extract	NDF	ADF	NFC	NDIN	ADIN
Feeds	(%)	(% DM)							
Alfalfa dehydrated	90.4	7.6	15.8	1.2	53.9	44.2	24.5	0.50	0.25
Alfalfa hay	88.2	8.4	15.4	0.9	58.5	50.6	21.0	0.65	0.27
Corn cob	94.9	1.9	2.6	0.5	78.8	43.7	17.7	0.23	0.14
Corn silage	89.7	3.6	8.1	2.9	37.2	22.5	49.7	0.24	0.16
Distillers grains	88.3	5.5	28.9	12.2	35.8	18.6	29.4	1.89	0.96
Meadow hay	89.7	10.3	8.6	1.7	63.8	40.7	19.7	0.66	0.25
Ryegrass hay	91.0	9.7	5.3	0.9	67.7	46.2	18.8	0.36	0.21
Soyhulls	88.8	5.2	12.1	2.0	64.8	50.0	21.3	0.87	0.24
Wheat bran	87.1	6.0	16.3	3.1	44.9	14.9	35.3	0.92	0.19
Wheat straw	89.8	9.1	6.8	0.8	78.0	53.9	7.7	0.38	0.25
Variances[1]	($\%^2$)	($\%^2$ DM)							
Residual error[2]	0.10	0.01	0.12	0.05	0.73	0.33	3.80	0.004	0.003
Laboratory	3.97	0.68	6.40	0.46	10.40	24.60	32.20	1.360	0.160
Laboratory × feed	0.42	0.06	0.38	0.16	5.00	3.60	—	0.230	0.014
Precision parameters	(%)								
Repeatability[3]	0.35	1.70	2.90	8.87	1.46	1.49	—	9.44	18.88
Reproducibility[3]	0.74	3.85	6.18	13.28	3.04	4.51	10.52	62.17	43.34

[1]Variances obtained from the statistical analysis, which considered the effect of feed, laboratory, and their interaction (see text).
[2]50 df (36 df for NFC).
[3]Coefficient of variation = SD/mean × 100.

acceptable estimate of standard errors (Wernimont and Spendley, 1985).

Feeds used for the ring test were forages, crop residues, and by-products having medium or high fiber contents (Table 1). The aNDFom content of feeds ranged from 36 to 54% of DM for 4 feeds (alfalfa dehydrated, corn silage, distillers grains, and wheat bran), from 59 to 68% of DM for other 4 feeds (alfalfa hay, meadow hay, ryegrass hay, and soyhulls) and was equal to 78 and 79% of DM for wheat straw and corn cobs, respectively. Feeds generally had medium to low contents of CP and EE, with the exception of distillers grains, which were high in both CP and EE contents (28.9 and 12.2% DM, respectively).

The analytical DM, ash, CP, aNDFom, and ADFom had satisfactory values of repeatability (S_r range 0.4–2.9%), with the exception of EE, which showed higher values (8.9%). Very poor repeatability was found for NDIN and ADIN (S_r of 10 and 19%, respectively). As expected, reproducibility values, which are inclusive of the interlaboratory variations, were approximately 3 times higher than repeatability values (Mertens, 2003). The S_R ranged from 0.7 to 6.2% for the analytical DM, ash, CP, aNDFom, and ADFom and achieved a value of 13.3% for the EE content. Because the NFC fraction is calculated from the others assays (ash, CP, NDF, EE, NDIN), its value of reproducibility is the result of the sum of reproducibility of its components. Therefore, the S_R of the NFC (10.5%) was poor. The worst reproducibility values were obtained again for NDIN and ADIN (around 60 and 40%, respectively).

In the present work, the fiber analyses (e.g., NDF, ADF, NDIN, and ADIN) were executed by the filter bag procedure and not by using crucible filters. Measurements of NDF and ADF gave contrasting results in terms of repeatability and reproducibility: the coefficients of variation obtained for ADF in the present study were higher than those obtained by Van Soest (1973) in an AOAC collaborative study to test AOAC official method 973.18 (S_r and S_R of 1.0 and 2.9%, respectively). The S_r and S_R of aNDFom, however, were better than those published by Mertens (2002; S_r and S_R of 2.7 and 3.4%).

Feeds analyzed had a wide range of variation for the NDFD in vitro values: 48-h incubation values (Table 2) were low for alfalfa forages, ryegrass hay, and straw (36 to 40%), intermediate for corn silage, hay, wheat bran, and corn cob (47 to 60%), and reached values of 84 and 90% for distillers grains and soyhulls, respectively. As expected, an increase in the length of fermentation increased average NDFD values from 42 to 54% across all tested feeds. This increase (+29%) is higher than that obtained by Hall and Mertens (2008), who studied 4 feeds (alfalfa hay, corn silage, ryegrass, and soyhulls) and measured an increment from 62 to 71% (+15%), using different in vitro vessels. Therefore, opening the jars to collect bags at 30 h did not appear to depress fermentation because NDF degradation increased substantially during the remaining 18 h.

The within-laboratory variability of in vitro NDFD was calculated from duplicate values obtained within laboratories from 2 consecutive runs and therefore it

Table 2. Neutral detergent fiber degradability (NDFD) after 30 and 48 h of incubation and calculated contents of NE_L of feeds and estimates of repeatability and reproducibility for NDF degradability and NE_L contents

	NDFD[1]		dNDF[2]		NE_L	
Item	30 h	48 h	30 h	48 h	30 h	48 h
Feeds	(%)		(% DM)		(kcal/kg DM)	
Alfalfa dehydrated	32.7	39.7	17.6	21.4	956	1,059
Alfalfa hay	26.3	36.3	15.6	21.7	764	924
Corn cob	41.6	54.1	32.7	42.7	693	964
Corn silage	31.5	47.4	11.7	17.7	1,325	1,488
Distillers grains	67.6	84.4	24.2	30.1	2,340	2,504
Meadow hay	46.4	59.5	30.3	37.9	932	1,159
Ryegrass hay	29.8	38.6	19.7	25.5	486	649
Soyhulls	73.7	90.1	47.7	58.4	1,618	1,909
Wheat bran	43.2	51.7	19.3	23.1	1,452	1,558
Wheat straw	24.1	36.5	18.8	28.5	185	448
Variances[3]	($\%^2$)		($\%^2$ DM)		($kcal^2/kg^2$ DM)	
Residual error[4]	24.90	13.53	8.36	3.91	9,550	6,557
Laboratory	347.07	264.77	85.46	49.57	62,893	39,979
Laboratory × feed	45.81	26.02	16.59	12.23	—	—
Precision parameters	(%)		(%)		(%)	
Repeatability[5]	11.97	6.83	12.18	6.43	—	—
Reproducibility[5]	17.03	10.46	16.78	10.30	11.30	7.70

[1]NDF degradability, as percentage of NDF.

[2]NDF degradability, as percentage of DM.

[3]Variances obtained from the statistical analysis, which considered the effect of feed, laboratory, and their interaction (see text).

[4]50 df (36 df for NE_L).

[5]Coefficient of variation = SD/mean × 100.

is mainly due to the interassay variations in handling and characteristics of rumen fluid. For both NDFD and dNDF determinations, the S_r was approximately 12% after 30 h of incubation, which improved to 6.4 to 6.8% when the incubation was prolonged to 48 h. The same effect was noticed for S_R, which was very poor at 30 h of fermentation (approximately 17% for both NDFD and dNDF), but it improved to 10% at 48 h. The poor reproducibility is probably because of differences in rumen inocula among laboratories, which adopted different procedures to collect the rumen fluid (e.g., immediately after slaughtering, via esophageal tube, or through ruminal cannula) and to the type of animal donors (fattening bulls, lactating or dry dairy cows). However, these conditions may reflect practical conditions, given the difficulty in standardizing the origin of rumen fluid from different laboratories in collaborative studies.

The low S_r and S_R at 30 h probably indicate insufficient time for complete degradation of the potentially degradable NDF fraction. It is well known that fiber degradation requires an adequate time to start because cellulolytic microbes have to adhere to the fibrous material. Likely, the initial degradation phase is the most sensitive to different conditions (e.g., different inoculum, inoculum handling) and is the most difficult to reproduce in different labs. The longer incubation time (e.g., 48 h) allowed a more complete NDF degradation, and thus improved the precision of the results. In an automated gas production system, the reproducibility of total gas production was also improved with progressively increased times of incubation (Van Laar et al., 2006).

Goeser et al. (2009) speculated that NDFD has a higher intrinsic assay error compared with dNDF, because the NDF assay error is included within NDFD calculation twice, whereas the dNDF calculation uses the NDF determination once. However, the present study was not able to determine any improvement in precision parameters by changing the expression of NDF degradation from NDFD to dNDF.

The regression between NDFD data measured in vitro (average of 5 laboratories) and in situ (average of 2 laboratories) at 30 and 48 h of incubation (Figure 1) had high coefficients of determination ($R^2 = 0.92$ and 0.98 for 30 and 48 h, respectively) and slopes very close to 1. Satisfactory in vitro versus in situ relationships were found in previous research on whole-crop forages (Robinson et al., 1999) and mountain hays (Spanghero et al., 2003).

The reduction of the incubation time from 48 to 30 h is thought to better describe the potential digestion of NDF in high-producing lactating dairy cows, which retain feed in the rumen for less than 48 h (Hoffman et

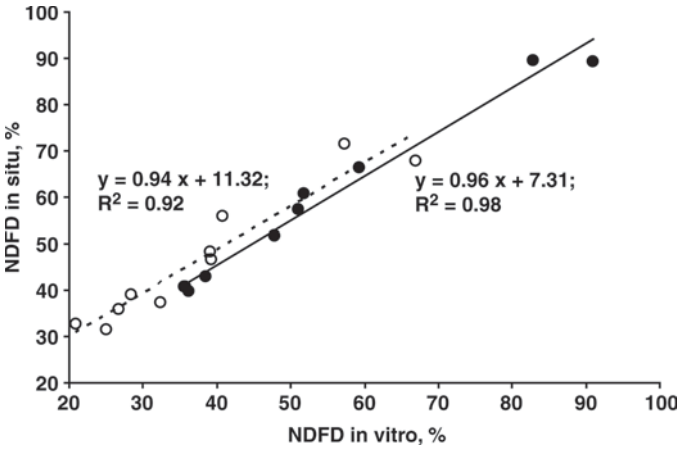

Figure 1. Regressions between NDF degradability (NDFD) measured in vitro (x, average of 5 laboratories) and in situ (y, average of 2 laboratories) after 30 (○, ----) and 48 h (●, —) of incubation.

al., 2003). Overall values of NE_L predicted from 48-h NDFD data (Table 2) approximated well the tabulated NRC (2001) values for hay, corn silage, wheat bran, and corn cobs (1,159, 1,488, 1,558, and 964 kcal/kg of DM, respectively). The remaining feeds differed from tabulated values according to the measured values of NDFD: for feeds having a low NDFD (between 36.3 and 39.7%, such as ryegrass hay, alfalfa, and wheat straw), the estimated NE_L content was lower than that reported by NRC, whereas the opposite was true for soy hulls and distillers dried grains (NDFD of 84.4 and 90.1%, respectively). Differences of NDFD due to incubation time (48 versus 30 h) translated into differences in the predicted NE_L contents of the feedstuffs that were approximately 18% higher when calculated from 48-h incubations. The improvement in NDFD reproducibility given by longer incubations (48 versus 30 h) also affected NE_L reproducibility (S_R), which was reduced from 11 to 8%, when the in vitro incubation was prolonged from 30 to 48 h.

Overall, the rotating jar in vitro technique has a high degree of accuracy compared with in situ data, but it is, in general, still marginally precise. Our results suggest that adoption of a 48-h period of incubation would improve repeatability and reproducibility of NDFD and accuracy and reproducibility of the associated calculated NE_L. Because the in vitro rotating jar technique is a simple apparatus, further improvement would probably be obtained by reducing the laboratory differences in rumen collection procedures and type of animal donors, which reflect practical conditions.

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