Variability in feed and total mixed ration neutral detergent fiber and crude protein analyses among commercial laboratories¹

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ABSTRACT

The objective of this study was to assess the variability in amylase-treated neutral detergent fiber (aNDF) and crude protein (CP) analyses of feed and total mixed ration (TMR) samples among feed analysis laboratories. Two TMR were prepared that varied in the dry matter proportion of forage and concentrates: 45% forage (LF-TMR) versus 60% forage (HF-TMR). Replicated TMR and individual feed samples were dried, ground through a 4-mm screen, and sent to 10 commercial and 4 research or development laboratories for aNDF and CP analyses. Laboratories were asked to complete a detailed questionnaire regarding the aNDF procedure used. Variability in aNDF and CP analyses was assessed using univariate statistics and mixed modeling procedures. Significant variability in the aNDF analysis of individual feeds was found among the participating laboratories. The variability was particularly large for low-aNDF feeds such as distillers and barley grains. The variability among laboratories in the aNDF analysis of low-fiber TMR was greater than of high-fiber TMR, with the most likely reason being their greater proportions of grains and protein concentrates and the effect of variation in the aNDF protocols, particularly α -amylase use, on the analysis of these types of feeds. Variability due to the technique used for aNDF analysis was not statistically significant when outlier labs using the filter bag technique were removed; however, laboratories using the filter bag technique tended to produce more variable results than did laboratories using variation of the crucible technique (SE = 2.542 vs. 0.930, respectively). Calculated aNDF values for TMR, based

on proportions and aNDF analysis of individual feeds, were slightly greater than analyzed aNDF values for TMR. Results from this ringtest emphasize the need for feed analysis laboratories to follow the official aNDF method exactly. Variation within and among laboratories can be reduced by replicating analysis and including reference materials in each analytical run. Results of CP analysis were more consistent among laboratories, and variability in CP analysis of individual feeds or TMR was marginally acceptable.

Key words: neutral detergent fiber, crude protein, total mixed ration, ringtest

INTRODUCTION

Dietary fiber, specifically NDF, is one of the most important constituents of dairy cow diets, controlling both intake and digestibility of dietary DM (Mertens, 1994). Accurate quantification of dietary fiber in dairy cow rations is critical for cost-efficient milk production and reduction of the environmental impact of the dairy industry. Inaccurate feed and TMR fiber analysis can lead to inaccurate prediction of the cow's energy requirements and loss of production or inefficient nutrient utilization. Although the original method for analyzing NDF has been in place for several decades (Goering and Van Soest, 1970), it has been modified to more accurately measure insoluble fiber in nonforage feeds (Van Soest et al., 1991; Mertens, 2002). These modifications, primarily in the use of heat-stable α -amylase and sodium sulfite, generate fiber measurements that are highly correlated but that can differ significantly in magnitude. These subtle, but important, modifications of the NDF method were intended to improve the measurement of insoluble fiber in nonforage feeds; however, they produce results that vary among modifications, and measurement of NDF in nonforage feeds can be difficult to accomplish without attention to methodological details (Mertens, 2003). Poor reproducibility among feed analysis laboratories was often attributed to the specific modifications of the method used by each laboratory (Mertens, 2003; Gustavsson and Mar-

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tinsson, 2004; Lund et al., 2004). Currently, the AOAC official method for amylase-treated neutral detergent fiber (**aNDF**), which uses both amylase and sulfite, is accepted as the standard method for fiber measurement by feed analysis laboratories. However, an automated filter bag technique introduced by Ankom Technology (Macedon, NY; Komarek, 1993), which was not evaluated in the collaborative study for aNDF (Mertens, 2002), is also used to measure aNDF. Because fiber methods are empirical; that is, the fiber measured is determined by the specifics of the method, slight variation in protocols and technique can result in aNDF values that vary considerably among laboratories. This variation in aNDF results could have a significant effect on the accurate formulation of dairy diets, leading to inefficient utilization of feed resources and impaired utilization of the nonfiber components of the ration.

The objective of this study was to assess the variability in the aNDF of feed and TMR samples among primarily commercial feed analysis laboratories. Variability in CP, which is a more standardized and reproducible procedure, was also monitored for comparison with the variation in aNDF analysis.

MATERIALS AND METHODS

Two TMR were prepared (Table 1) that varied in the DM proportion of forage by replacing alfalfa hay and corn silage with corn and barley grains: 45% forage (low forage, **LF-TMR**) versus 60% forage (high forage, **HF-TMR**). The TMR were based on feed ingredients commonly used in dairy rations in the Pacific Northwest [corn silage, alfalfa hay, corn, barley, whole cottonseed, dry distiller grains with solubles (**DDGS**), and solvent-extracted soybean meal (**SSBM**)]. All feeds were from the University of Idaho Dairy Teaching and Research Center (Moscow) feed inventory and were purchased from commercial feed suppliers. Barley grain was locally grown and dry-rolled at the dairy center feed mill. The alfalfa hay was chopped through a hammer mill (model 200, New Holland, PA) to approximately 5-cm particle length. Feeds for each TMR were mixed in a Data Ranger (American Calan Inc., Northwood, NH) in one batch of 150 kg (as-is basis, or 82.5 kg of DM). The DM content of the feed ingredients was determined before mixing the TMR by drying at 65°C for 72 h in a forced-air oven. To mimic field conditions when dry hay is used in TMR, water was added to bring the moisture content of both TMR to approximately 55% ($54.6 \pm 0.46\%$). Following an initial 5-min mixing cycle of the feed ingredients, water was added and mixing continued for another 10 min. Samples for analyses were collected directly from the Data Ranger as it was being unloaded. Samples of approximately 10 kg each

(as-is basis) were collected from each TMR at 3 different times of the unloading process. The samples were oven-dried (as for the feed ingredients) and ground through a 4-mm sieve (Wiley mill, Thomas Scientific, Swedesboro, NJ). After grinding, each sample was split into 2 subsamples. Therefore, 6 samples (splits) from each TMR were prepared for analyses. In addition, representative samples of all feed ingredients (except the mineral mix) were also prepared for analyses. Samples of the chopped hay and the corn silage (10 kg each, as-is basis) and 3-kg samples of the concentrate feeds and the whole cottonseed were dried (at 65°C for 72 h in a forced-air oven) and ground through a 4-mm sieve (except the SSBM and DDGS). All samples (150 g each) were stored in airtight plastic bags before being shipped to the participating laboratories. Nineteen samples (7 individual feeds and 12 TMR samples) were submitted to each laboratory for aNDF and CP analyses. The aNDF content of the TMR were also calculated based on aNDF content of the individual feeds reported by each laboratory and feed mixing proportions.

Ten National Forage Testing Association (**NFTA**)-certified commercial laboratories were contacted and agreed to participate in this ringtest. In addition, samples were analyzed at 4 research or development laboratories. Two of the laboratories participated in the AOAC collaborative study for the aNDF method (Mertens, 2002). All samples were submitted after drying at 65°C for 72 h, and laboratory results were processed on an as-received basis. After the goals of the project were explained, participants were asked to complete a questionnaire related to the type of aNDF procedure used (Table 2). Questions included NDF procedure used and modifications applied; processing of sample before analysis (drying, grinding); procedure of weighing of crucibles (if a crucible procedure was used); amount of sample analyzed; use of amylase and sodium sulfate (including type and amount); fat extraction of high-fat samples; calibration of the Ankom fiber analyzer (when used); and others. A similar survey was not submitted regarding the CP analysis. The 2 official AOAC techniques for CP analysis (official methods 990.03 and 2001.11; AOAC, 1990) are based on nitrogen analysis but use very different techniques (dry combustion and Kjeldahl digestion, respectively). Both techniques produced similar results with a variety of samples including ruminant feeds (Bicsak, 1993; Etheridge et al., 1998). A recent study with distiller grains found excellent precision of the 2 techniques for nitrogen determination with a very low relative standard deviation (Thiex, 2009).

Based on the returned questionnaires, classification variables such as aNDF procedure used, additional grinding of the sample and size of the screen, drying tem-

Table 1. Ingredient composition of the TMR used for the amylase-treated neutral detergent fiber (aNDF) ringtest

Ingredient	TMR ¹ (% DM basis)	
	LF-TMR	HF-TMR
Alfalfa hay	20	28
Corn silage ²	25	32
Cottonseed, whole with lint (whole cottonseed)	7	7
Corn grain, steam-flaked (flaked corn)	20	12
Soybean meal, solvent-extracted 48% CP (SSBM)	8	7
Dry distillers grain with solubles (distillers grains)	6	5
Barley grain, dry-rolled (rolled barley)	12	7
Mineral/vitamin mix ³	2	2

¹LF-TMR = low-forage TMR; HF-TMR = high-forage TMR.

²Corn silage was 30% DM.

³Mineral vitamin premix (Land O'Lakes, St. Paul, MN) composition (as-is basis): Ca, 15.8%; P, 4.7%; Mg, 5.2%; Cl, 2.8%; K, 0.3%; Na, 6.5%; S, 0.3%; Co, 4.36 mg/kg; Cu, 928 mg/kg; I, 42 mg/kg; Fe, 189 mg/kg; Mn, 4.7 mg/kg; Se, 15.7 mg/kg; Zn, 4,649 mg/kg; vitamin A, 258,000 IU/kg; vitamin D, 45,900 IU/kg; and vitamin E, 1,839 IU/kg.

perature before analysis, crucible weighing, instrument calibration, use of desiccant pouch, use of α -amylase (source, amount), use of sodium sulfite (amount), and size of sample analyzed were considered as sources of variation for the statistical analyses. All laboratories except one re-ground the samples. One laboratory analyzed 0.25 g, one 0.4 g, and all other laboratories analyzed 0.5 g of sample. In all cases, aNDF results were expressed inclusive of residual ash. Although one laboratory indicated they were using the Goering and Van Soest (1970) method for NDF, they also indicated that they were using amylase, so it was assumed they were actually measuring aNDF with sulfite and amylase. The only variation in methodology that was used consistently enough for statistical analysis was the cru-

cible (**CR**) technique for aNDF (Mertens, 1992; available at http://www.foragetesting.org/lab_procedure/sectionC/5/part5.1.htm; or Mertens, 2002) and the filter bag (**FB**) technique based on the use of Ankom fiber analyzer (Ankom Technology; the aNDF procedure is available at http://www.ankom.com/media/documents/NDF_081606_A200.pdf).

Of the 14 laboratories evaluated, 6 used FB and 8 used CR techniques to measure aNDF. Before estimating the within-laboratory repeatability and among-laboratory reproducibility of measuring aNDF, it was necessary to determine if the FB and CR techniques were different and whether each should have separate estimates of repeatability and reproducibility. The results obtained from participating laboratories required

Table 2. Analysis questionnaire submitted to the laboratories participating in the amylase-treated neutral detergent fiber (aNDF) ringtest

Question
1. What procedure are you using for NDF analysis?
a. Are samples ground before analysis and through what size sieve?
b. Are crucibles weighed hot or after cooling in a desiccator?
c. What is the temperature and time the samples are dried before and after the analysis?
d. Are heating units calibrated for temperature?
e. What amount of test sample is analyzed?
f. How many replicates were analyzed (e.g., 1, 2)?
g. What criteria are used to identify unacceptable results?
2. Is the method published (provide reference)?
a. Are you using any modifications to the original procedure? If so, what are these modifications?
3. Do you use amylase? How much? Name of supplier?
4. Do you use sodium sulfite? How much?
5. If using the Ankom Filter Bag apparatus, did you modify the method (method attached)? If so, what were the modifications?
a. Is a desiccant pouch used to weigh the filter bags?
b. What is the temperature and time the samples are dried before and after the analysis?
c. Are the instruments calibrated for temperature and agitation?
d. Is a pre-fat extraction performed on the soybean samples and high fat samples?
e. What amount of test sample is analyzed?
f. What criterion is used to determine acceptable results?
6. Is the data reported on a DM or as-received basis?
a. If reported on a DM basis, what method was used to determine DM (give equipment, sample size, time, and temperature of drying)?

2 statistical approaches to make inferences. The TMR results were replicated with 6 samples (splits) for each HF-TMR and LF-TMR. The RANDOM statement of the PROC GLM procedure was used to determine the expected mean squares for effects and interactions that were used to determine AOAC performance statistics (Mertens, 2002). Type 3 ANOVA was used to determine the significance of random and fixed effects, whereas least squares means and pairwise comparisons were used to detect differences among model effects (PROC MIXED). All computations were carried out using SAS software (ver. 9.1.3; SAS Institute Inc., Cary, NC).

To evaluate differences between CR and FB techniques for split, replicated TMR results, the statistical model was

$$Y_{ijlm} = R_l + S_j(R_l) + T_m + L_i(T_m) + \varepsilon_{ijlm}, \quad [1]$$

where Y_{ijlm} = the aNDF or CP result for each TMR and split, R_l = LF-TMR or HF-TMR, $S_j(R_l)$ = the split nested within R_l , T_m = CR or FB technique, $L_i(T_m)$ = laboratory nested within T_m , and ε_{ijlm} = residual error assumed to be independent and distributed normally with mean = 0 and constant variance. The $S_j(R_l)$ term allows the testing of splits within TMR, and the technique effect (T_m) was tested using $L_i(T_m)$ as the error term. The main effect (T_m) was fixed and all other main effects and interactions were assumed random. The technique effect was tested for significant differences using all 14 laboratories, the 12 laboratories without the extreme outliers, and the 10 commercial laboratories. If T_m was not significant, it was replaced with laboratory (L_i) to evaluate splits within TMR.

Although replicated results for feed ingredients were not obtained, the results can be analyzed as Youden pairs (Youden and Steiner, 1975). Six feeds with single results were divided into 1 of 3 Youden pairs based on similarity of ingredients (forages = alfalfa hay and corn silage; grains = rolled barley and steam flaked corn; and protein supplements = SSBM and DDGS). In addition, using individual feed results and DM proportions in the ration, TMR results were calculated to create a fourth Youden pair (CalR, MR = HF-TMR and LF-TMR). The ANOVA model needed to evaluate CR and FB techniques for Youden pairs was

$$Y_{ikmn} = P_n + F_k(P_n) + T_m + L_i(T_m) + \varepsilon_{ikmn}, \quad [2]$$

where Y_{ikmn} = the aNDF or CP result for each Youden pair and feed, P_n = Youden pair (forage, grain, CalR, MR, or protein supplement), $F_k(P_n)$ = the feeds nested within pair P_n , T_m = the CR or FB technique, $L_i(T_m)$ = laboratory nested within technique T_m , and ε_{ikmn} =

residual error with similar distributional assumptions as model (1). The technique effect (T_m) was a fixed effect and all other main effects and interactions were assumed random.

Different ANOVA models were needed to calculate the AOAC performance statistics for within-laboratory repeatability and the within- and among-laboratory reproducibility of repeated and single results. It was assumed that the repeated samples for the 2 TMR (LF-TMR and HF-TMR; each $n = 6$) were unknown or blind to the analyst because they were simply labeled TMR1 to TMR12. Assuming each split was an equally representative replicate of each TMR, the ANOVA model used to calculate the repeatability and reproducibility of each set of blind replicated TMR was

$$Y_i = L_i + \varepsilon_i, \quad [3]$$

where Y_i = the aNDF or CP result for each replicate, L_i = the random effect of laboratory, and ε_i = the residual error assumed to be distributed normally with a mean of 0 and constant variance σ^2 . Because the number of TMR replicates (splits) was greater than 2, it was possible to test the variation among the splits within each TMR and remove any variation associated with differences among splits (S_j) using the model

$$Y_{ij} = L_i + S_j + \varepsilon_{ij}. \quad [4]$$

The residual mean squared error (**MSE**) in this model is an estimate of the variance of the within-laboratory repeatability of a result averaged across all laboratories, and the square root of the mean squared residual error (**RMSE**) is an estimate of within-laboratory repeatability. The expected mean squares of this model indicate that the MSE of L is a function of residual MSE and the "pure" among-laboratory (systematic biases among laboratory means) variance (σ^2_{among}):

$$\sigma^2_L = \sigma^2_r + n \times \sigma^2_{\text{among}},$$

which can be solved for σ^2_{among} ,

$$\sigma^2_{\text{among}} = (\sigma^2_L - \sigma^2_r) \div n, \quad [5]$$

where σ^2_L = MSE of L , σ^2_r = RMSE, and n = average number of replicates per TMR. Within-laboratory repeatability is the square root of σ^2_r , and resulting reproducibility is the square root of $(\sigma^2_r + \sigma^2_{\text{among}})$.

The ANOVA model used to calculate the repeatability and reproducibility of each Youden pair was as Eq. [4] with the effect of feed (random) replacing the effect of split. The MSE in this model is also an estimate

of the variance of the within-laboratory repeatability of a result averaged across all laboratories, and the square root of the mean squared residual error (RMSE) is an estimate of within-laboratory repeatability. The expected mean squares of this model indicate that the MSE of L is a function of residual MSE and the “pure” among-laboratory (systematic biases among laboratory means) variance (σ^2_{among} ; see Eq. [5]).

One laboratory returned extremely high aNDF values (33 and 23% higher than the average of all other laboratories for LF-TMR and HF-TMR, respectively) and was excluded from the statistical analysis. This particular laboratory reground the samples and used the FB technique with α -amylase and sodium sulfite based on the returned questionnaire. There was no obvious reason for the high aNDF results, except that the fiber analyzer was calibrated for temperature but not agitation.

Ranking of laboratory results is used by AOAC to identify laboratories with pronounced systematic errors that are not indicative of acceptable method compliance. For each feed, the results for laboratory were ranked from highest to lowest and the rankings were summed for each laboratory (Youden, 1963). When the sum was greater than the upper or less than the lower limits (Biometrika Trust, 1963), the laboratory was deemed an outlier at the $P < 0.05$ level of significance. Individual results were declared outliers when their studentized residual indicated that the difference between predicted and observed values was greater than 4.5 times the SE of the least squares predicted value using Eq. [4] with the effect of feed replacing the effect of split. To assess the effect of outlying observations, separate analyses using all 14 laboratories, the 12 laboratories without outliers, and the 10 commercial laboratories were carried out.

Although laboratories were not requested to and did not provide results for individual assays, they did provide result means and the corresponding number of replicated assays. For comparison with AOAC method performance parameters, the reported standard errors of mean of replicated results were subsequently adjusted to the sample standard deviation.

RESULTS AND DISCUSSION

All laboratories used α -amylase and sodium sulfite in the aNDF analysis, but the amounts and source of α -amylase varied greatly. Most laboratories used 0.4 or 0.5 mL/g; however, some reported “2 to 3 drops” (1 laboratory), 0.2, 0.25, 2, or 4 mL/g. The enzyme was purchased from 4 suppliers, with 9 out of 14 laboratories using α -amylase from Ankom Technology. The amount of sodium sulfite varied from 0.3 to 0.83 g/

sample. The size of the sample analyzed was relatively consistent among laboratories, with 12 out of 14 using 0.5 g. Most laboratories re-ground the samples through a 1-mm sieve, although the type of grinder (when specified) differed among laboratories. All but 4 laboratories dried the samples before analysis. Two laboratories used microwave drying and the remaining laboratories used initial drying regimens varying from 55°C for 10 h to 65°C for 75 h. Some laboratories did not calibrate their instrument (for temperature and agitation, in the case of Ankom fiber analyzer). Of the laboratories using CR, some (5) weighed the crucibles hot, whereas others weighed them cool (3), after desiccation. A few laboratories using FB did not use desiccant pouches and did not pre-extract high-fat feeds. This variability in procedural details further emphasizes the need for commercial feed analysis laboratories to follow the aNDF procedure exactly. Not surprisingly, an NDF ringtest among several European countries concluded that a standard technique needed to be followed (Lund et al., 2004).

Laboratory 4 ranked consistently low and laboratory 12 ranked consistently high; these laboratories were identified as outliers using the method of Youden (1963) for the analysis of individual feeds (Table 3). They also generated the lowest and highest averages, respectively, of the TMR analyses (Table 4), but the ranking test could not be applied to TMR results because samples were replicated within TMR and therefore were not independent. Both of these laboratories used the FB technique, as did the laboratory that was eliminated because of extremely high aNDF results. For both replicated TMR and Youden pairs, laboratory 4 was lower and laboratory 12 was higher than all other laboratories based on pairwise differences ($P < 0.05$), which confirms the conclusions of the ranking test that these laboratories were outliers for the measurement of aNDF. Laboratories 4 and 12 were noncommercial laboratories. Subsequent analyses were, therefore, done with and without laboratory outliers 4 and 12 to assess bias in data selection.

The result for rolled barley by laboratory 11 was an individual outlier for this feed. The result was almost 10 percentage units higher than the next highest result from nonoutlier laboratories. Laboratory 11 used only 2 to 3 drops of amylase, which was substantially less than that used by other laboratories. It is also interesting that this laboratory obtained higher than average results for corn silage and TMR, which also contain starch. Mertens (2002) provided a method for standardizing the amount of heat-stable α -amylase needed to remove starch from unprocessed corn under the conditions for aNDF analysis. The results of laboratory 11, and the large variation in usage of amylase from the

Table 3. Results for individual feeds from collaborating laboratories for amylase-treated neutral detergent fiber (aNDF; %, as-received basis)

Item	Pair ¹	Collaborating laboratory													
		1 ²	2 ²	3	4 ³	5 ²	6 ²	7 ^{2,4}	8 ⁴	9 ²	10 ²	11 ²	12 ³	13 ²	14 ²
Technique ⁵		CR	FB	FB	FB	CR	CR	CR	CR	FB	CR	CR	FB	FB	CR
Replicates ⁶		1	1	3	3	2	2	2	2	3	2	3	2	1	1
Alfalfa hay	For	36.9	40.8	39.5	38.1	34.2	38.1	38.1	36.7	35.7	41.3	38.1	41.6	38.8	38.5
Corn silage	For	52.0	48.0	47.6	41.8	46.8	45.9	49.2	48.6	48.9	48.2	50.1	49.9	48.5	47.3
Flaked corn	Gr	8.9	8.0	8.9	6.1	8.2	8.4	5.6	7.3	7.3	9.2	8.9	9.0	7.4	10.4
Rolled barley	Gr	19.1	22.7	21.0	14.0	20.6	18.0	15.9	18.6	19.0	21.4	31.0 ⁷	26.4	20.4	17.0
Solvent-extracted soybean meal (SSBM)	Pro	10.7	9.8	8.7	9.9	9.8	9.4	7.9	9.3	9.1	11.0	9.9	16.2	10.4	9.1
Distillers grains	Pro	36.1	33.1	35.2	27.1	31.6	30.3	31.3	26.9	33.2	32.8	35.2	38.5	32.0	30.5
Whole cottonseed		52.7 ⁷	47.3	46.7	46.3	45.0	48.9	35.9 ⁷	41.3	46.4	35.5 ⁷	44.9	56.2 ⁷	49.2	42.6
Measured HF-TMR ⁸	MR	33.9	35.2	34.6	30.9	32.7	33.1	33.4	32.6	33.1	35.1	35.8	37.0	32.3	33.0
Measured LF-TMR ⁹	MR	29.5	30.5	30.6	27.6	28.5	27.8	28.6	28.5	29.8	30.4	32.3	33.1	28.2	28.0
Calculated HF-TMR ¹⁰	CalR	35.6	35.0	34.5	31.0	32.4	33.2	32.8	32.9	33.4	34.5	35.5	37.6	34.5	33.5
Calculated LF-TMR ¹¹	CalR	31.2	30.6	30.2	26.6	28.5	28.9	27.9	28.4	29.1	30.1	31.7	33.3	30.0	29.2
Least squares means ¹²		31.0	31.0	30.7	27.2	28.9	29.3	28.7	28.3	29.6	31.0	31.2	33.9	30.2	29.0

¹Youden pairing used for estimating AOAC method performance parameters for repeatability and reproducibility. For = forage; Gr = grain; Pro = protein supplement; MR = measured results; CalR = calculated results.

²Commercial feed analysis laboratory.

³Laboratory outlier detected by ranking of results using the method of Youden (1963).

⁴Laboratories participating in the AOAC collaborative study for the aNDF official method (Mertens, 2002).

⁵CR = aNDF using the crucible technique; FB = aNDF using the filter bag technique.

⁶Number of replicated analyses per result.

⁷Outlier detected with studentized residuals greater than 4.5 times the standard error of the predicted value.

⁸Average analytical results of replicated samples of the high-fiber TMR.

⁹Average analytical results of replicated samples of the low-fiber TMR.

¹⁰Calculated results based on feed ingredient proportions and analyses for the high-fiber TMR.

¹¹Calculated results based on feed ingredient proportions and analyses for the low-fiber TMR.

¹²Least squares means calculated without data in bold font.

Table 4. Results of replicated TMR from collaborating laboratories for amylase-treated neutral detergent fiber (aNDF; %, as-received basis)

Item	Collaborating laboratory														TMR mean
	1 ¹	2 ¹	3	4	5 ¹	6 ¹	7 ^{1,2}	8 ²	9 ¹	10 ¹	11 ¹	12	13 ¹	14 ¹	
Technique ³	CR	FB	FB	FB	CR	CR	CR	CR	FB	CR	CR	FB	FB	CR	
Replicates ⁴	1	1	3	3	2	2	2	2	3	2	3	2	1	1	
High-fiber TMR 1	33.7	33.8	35.8	31.0	32.0	32.6	33.5	32.7	32.8	34.5	35.2	35.4	29.2	31.6	33.1
High-fiber TMR 2	34.4	37.0	35.3	32.5	31.2	33.0	32.9	32.8	33.2	35.2	35.4	36.8	31.9	33.8	33.9
High-fiber TMR 3	33.5	34.8	34.5	30.8	34.8	32.1	32.7	31.3	33.3	34.5	36.6	36.9	32.5	34.1	33.7
High-fiber TMR 4	34.3	34.8	33.5	31.2	32.2	33.6	33.3	32.6	33.3	36.5	35.4	37.1	33.1	33.8	33.9
High-fiber TMR 5	34.4	36.4	35.6	30.5	32.0	34.7	34.5	33.4	34.3	35.8	36.2	38.2	34.8	33.4	34.6
High-fiber TMR 6	33.0	34.3	33.0	29.4	34.0	32.4	33.2	32.6	31.9	33.9	36.4	37.6	32.5	31.5	33.2
High-fiber TMR Mean	33.9	35.2	34.6	30.9	32.7	33.1	33.4	32.6	33.1	35.1	35.9	37.0	32.3	33.0	
Low-fiber TMR 7	28.9	29.1	28.0	27.0	26.0	26.3	28.6	27.8	29.5	28.9	32.0	33.1	27.2	25.9	28.4
Low-fiber TMR 8	29.6	29.0	32.4	26.7	29.8	28.7	27.6	29.2	29.5	29.5	33.3	33.7	28.4	28.5	29.7
Low-fiber TMR 9	31.0	32.8	32.5	29.4	24.8	26.8	29.1	30.2	29.4	30.9	32.1	30.6	28.4	27.6	29.7
Low-fiber TMR 10	29.5	31.1	30.8	28.9	27.8	28.5	28.5	27.8	29.9	29.8	31.3	32.3	27.8	27.4	29.4
Low-fiber TMR 11	28.3	30.6	29.9	26.7	32.6	27.4	27.9	27.2	31.7	30.8	33.4	35.7	28.0	30.0	30.0
Low-fiber TMR 12	29.8	30.3	29.7	26.8	30.2	29.1	29.6	29.1	28.9	32.5	31.6	33.4	29.5	28.7	29.9
Low-fiber TMR Mean	29.5	30.5	30.6	27.6	28.5	27.8	28.6	28.6	29.8	30.4	32.3	33.1	28.2	28.0	

¹Commercial feed analysis laboratory.

²Laboratories participating in the AOAC collaborative study for the aNDF method (Mertens, 2002).

³CR = aNDF using the crucible technique; FB = aNDF using the filter bag technique.

⁴Number of replicated analyses per result.

same source, suggest that amylase standardization is one aspect of the aNDF method that was not followed among the participating laboratories.

Four individual results were outliers (laboratories 1, 7, 10, and 12) for whole cottonseed, and outlier results were both high and low. Based on our (D. Mertens) experience, these deviations in results for whole cottonseed may be due to the heterogeneous nature of ground cottonseed. We typically observe that grinding segregates cottonseed into a meal and balls of lint that make it difficult to obtain a representative sample for analysis. We often separate these heterogeneous samples into meal and lint fractions, determine the proportions of each, and create a composite sample for analysis. We conclude that the numerous outliers for whole cottonseed are not the result of aNDF methodology differences, but are related to feed preparation and sampling for analysis. The higher variability in CP of whole cottonseed presented later also suggests problems in sampling for analysis. Therefore, we did not use the outlier whole cottonseed results in methodology evaluation.

Although we concluded that preparation and sampling could explain the high and low outliers for whole cottonseed, the high results could be related to its high fat content. It is recommended that feeds containing >10% lipid should be pre-extracted to minimize extraction difficulties and reduce variability in aNDF results (Mertens, 2002). Pre-extraction of high-fat feeds, however, was inconsistently used by the participating laboratories. Although the FB procedure (Ankom Technology) recommends pre-extraction of samples containing >5% fat, some laboratories were pre-extracting samples only in cases where their clients required fat analysis on the samples.

One possible explanation for the higher variability in aNDF results for barley grain could be its relatively high concentration of β -glucans (Bergh et al., 1999), which are highly viscous substances and may cause filtration problems. These polysaccharides are not recovered in the NDF residues (Van Soest et al., 1991), and their solubility depends on the destruction of the endosperm cell walls (McAllister and Cheng, 1996), which may be affected by the use, or not, of sodium sulfite. A Scandinavian ringtest among 5 research laboratories reported high NDF (and indigestible NDF) variability for barley grain and particularly rapeseed meal (CV of 9.4 and 19.0%, respectively; Lund et al., 2004). In addition, starch can cause filtration problems if α -amylase is not used in the NDF analysis. This has been shown previously to be a problem with the FB technique (Ferreira and Mertens, 2007). Variability in the amount of α -amylase used by the participating laboratories could account for the high variability of aNDF concentra-

tion in corn and barley grain samples observed in this study. D'heer et al. (2000) reported higher variability for starchy feeds with the FB technique than with the CR technique, particularly when samples greater than 0.5 g were used in the former procedure. Generally, the 2 techniques gave similar NDF results for a large spectrum of feeds (Komarek et al., 1994; D'heer et al., 2000), although in some cases significant discrepancies were reported (Gustavsson and Martinsson, 2004) when the FB technique was compared with a variation of the CR technique (i.e., Chai and Udén, 1998).

Figure 1a shows the relationship between measured aNDF in TMR and aNDF calculated from feed ingredient composition and proportions. In general, a good relationship was observed between measured and calculated aNDF results with most values within 1 percentage unit of one another. The calculated values for laboratory 1 were greater than the measured values, perhaps because of the high outlier aNDF for whole cottonseed. The high calculated values compared with measured values for laboratory 13 is difficult to explain, although their aNDF value for whole cottonseed was above the average for this feed. The greater number of deviations >1 percentage unit between calculated and measured results for LF-TMR compared with HF-TMR may be related to the greater proportions of concentrates in the former TMR and the difficulties in aNDF analyses of concentrates.

Before estimating the repeatability and reproducibility of the results reported by laboratories in this study and comparing them with those of the AOAC official method for aNDF (Mertens, 2002), it is relevant to determine the effect of technique on aNDF results. Based on replicated results for TMR, techniques CR and FB were not different with $P = 0.62$, 0.67 and 0.95 for all 14 laboratories, 12 laboratories without ranking outliers, and 10 commercial laboratories, respectively. This result is in agreement with other similar analyses (Komarek et al., 1994; D'heer et al., 2000). However, in all instances differences among laboratories within technique were significant ($P < 0.001$). Thus, for subsequent analyses, CR or FB technique differences were removed from statistical models and differences among laboratories were evaluated. Splits within TMR also differed for all combinations of laboratories tested (14, 12, or 10). Interestingly, in both HF-TMR and LF-TMR, the aNDF of the first split (TMR1 or TMR7) was lower ($P = 0.008$ and $P < 0.001$, respectively) than the fifth split (TMR5 or TMR11). The SD among splits for HF-TMR and LF-TMR were ± 0.55 and ± 0.62 , respectively, which is one-third or more of the SD among commercial laboratories for HF-TMR and LF-TMR (± 1.33 and ± 1.85 , respectively). The differences among carefully collected replicates document the difficulty of

Figure 1. Comparison of amylase-treated neutral detergent fiber (aNDF) and CP concentration of low-fiber (LF-TMR) or high-fiber (HF-TMR) TMR when measured or calculated from feed ingredient composition and proportions (numbers indicate identification of laboratories with largest differences; line indicates ideal, one-to-one relationship).

obtaining equally representative samples from TMR in their native form.

As with replicated TMR, analysis of Youden pairs also indicated that techniques CR and FB were not different, with $P = 0.55$, 0.45 , and 0.81 , for all 14 laboratories, 12 laboratories without ranking outliers, and 10 commercial laboratories, respectively. However, in all instances, differences among laboratories within technique were significant ($P < 0.001$). Our analysis of replicated TMR or single result Youden pairs indicates no difference between CR and FB technique; however, it should be remembered that all 3 outlier laboratories used the FB technique. This suggests that although acceptable methodologies using the FB technique for aNDF analysis exist, FB methodologies may result in extremely high or low results. Mertens (1998) performed a ruggedness test on the FB technique and observed several procedural steps that improved agreement between FB and CR techniques for all or some individual feeds. These procedures included (1) mixing sodium sulfite and amylase in neutral detergent (ND) solution before adding it to the extraction chamber, (2) mixing amylase with hot wash water before adding to the chamber, (3) soaking with water for 5 min each time (first 2 washes with amylase followed by 2 without), (4) using procedure (2) with 100 mL of hot water (80 – 90°C) and heating the chamber with the lid sealed during each wash cycle, and (5) after ND extraction/washing, soaking bags in 240 mL of acetone for 5 min with swirling at 0, 2, and 4 min. From questionnaire responses, it was not possible to determine if any or all of these procedures were followed for the FB technique used by participating laboratories.

Performance parameters for aNDF results of replicated TMR were estimated according to AOAC and adjusted to a single analysis basis because most commercial results are reported for a single analysis of the feed (Table 5). The performance parameters were also estimated using the same ANOVA model as AOAC, assuming the TMR were random representatives of all TMR and the laboratories were random representatives of all commercial laboratories. As expected, the mixed model estimates are slightly higher. To compare the reproducibility (adjusted SD_R) observed in this study with those of collaborative study for the aNDF method (Mertens, 2002), the AOAC results were weighted by the proportions of forage and concentrates in the feed. The AOAC reproducibility estimated for a high-fiber and low-fiber TMR was ± 1.25 and ± 1.30 , respectively. The observed SD_R , determined by fixed effects and mixed models, were 1.24 and 1.36 times those of AOAC, respectively, for high-fiber TMR, and 1.55 and 1.65 times those of AOAC, respectively, for low-fiber TMR (Table 5). The higher SD_R observed for commercial laboratories suggests that the analytical methods used by some participating laboratories were different from the AOAC official method for aNDF.

To confirm that results for commercial laboratories were more variable than expected based on AOAC performance estimates for aNDF, the 95% CI was calculated for the AOAC SD_R and adjusted for the number of replicate analyses for each laboratory and the mean of each TMR replicate. For the high-fiber TMR, 8 of the 60 results were outside the 95% CI, and 3 of the 8 were from one laboratory (laboratory 11). For the low-fiber TMR, 11 of the 60 results exceeded the 95% CI, and 5

Table 5. Statistical analysis of amylase-treated neutral detergent fiber (aNDF) variation for TMR among laboratory groups using replicated analyses

Feed type, laboratory or feed	No. of feeds ¹	No. of labs ²	No. of reps ³	Mean (%)	SD _r ⁴	Adj. SD _r ⁵	SD _R ⁶	Adj. SD _R ⁷	Adj. SD _r Mixed ⁸	Adj. SD _R Mixed ⁹
High-fiber TMR ¹⁰										
All ¹¹	1	14	2.00	33.8	0.95	1.34	1.68	1.93	1.34	2.06
Without outliers ¹²	1	12	1.92	33.7	0.96	1.33	1.30	1.59	1.33	1.72
Commercial ¹³	1	10	1.80	33.8	0.95	1.27	1.30	1.55	1.27	1.70
Low-fiber TMR ¹⁰										
All	1	14	2.00	29.5	1.33	1.88	1.96	2.36	1.87	2.46
Without outliers	1	12	1.92	29.4	1.28	1.78	1.62	2.03	1.78	2.17
Commercial	1	10	1.80	29.4	1.26	1.69	1.68	2.02	1.69	2.14
Both TMR ¹⁰										
All	2	14	2.00	31.6	1.15	1.63	1.97	2.28	1.61	2.26
Without outliers	2	12	1.92	31.6	1.13	1.57	1.64	1.97	1.55	1.95
Commercial	2	10	1.80	31.6	1.12	1.50	1.66	1.94	1.49	1.93
AOAC ¹⁴										
Forage	5	11	2	52.2	0.59	0.84	0.93	1.10		
Concentrates <10% fat	3	11	2	33.5	0.83	1.18	1.15	1.42		
Concentrates >10% fat	3	11	2	8.7	0.71	1.01	1.44	1.61		
All feeds	11	11	2	38.7	0.74	1.05	1.10	1.33		

¹Number of feeds used to obtain statistical data.²Number of laboratories submitting results.³Average number of replicated analyses per laboratory mean.⁴Standard deviation of repeatability within laboratory for a reported replicated result.⁵Standard deviation of repeatability within laboratory adjusted to a single analytical result.⁶Standard deviation of reproducibility within and among laboratories for a reported replicated result.⁷Standard deviation of reproducibility within and among laboratories adjusted to a single analytical result.⁸Standard deviation of repeatability within laboratory adjusted to a single analytical result using mixed model variance estimates.⁹Standard deviation of reproducibility within and among laboratories adjusted to a single analytical result using mixed model variance estimates.¹⁰Experimental results for replicated analyses of high- and low-fiber TMR.¹¹All participating laboratories.¹²Participating laboratories without laboratory ranking outliers.¹³Participating commercial laboratories.¹⁴Results adapted from AOAC collaborative study (Mertens, 2002) and published here for comparison.

of the 11 were from one laboratory (laboratory 11). The greater number of results outside of the AOAC 95% CI for low-fiber compared with high-fiber TMR suggests that aNDF analysis of concentrate feeds, which are a greater proportion of the low-fiber TMR, may be responsible for much of the increased variation among commercial laboratories in this study compared with those laboratories in the AOAC collaborative study for aNDF (Mertens, 2002).

To determine if aNDF results among laboratories differed among feeds, AOAC analysis of Youden pairs (Youden and Steiner, 1975) was used. To confirm that Youden pairs resulted in similar estimates of repeatability (SD_r) and SD_R compared with blind replicates, the results from the collaborative study for the aNDF method (Mertens, 2002), which used blind duplicates, was also analyzed as Youden pairs. Using Youden pairs with AOAC collaborative data (Table 6), the SD_R of forage pairs (alfalfa silage and corn silage or grass hay and corn stover; $SD_R = \pm 0.86$ and ± 1.29 , respectively; average = ± 1.08) was lower than that of grains (corn

with cobs and brewers grains; $SD_R = \pm 1.58$), which was less than that of protein sources (mixed dairy supplement and roasted soybeans; $SD_R = \pm 1.84$). These SD_R are similar to those obtained using blind replicates, indicating that Youden pair analysis generates comparable estimates of SD_R . In general, all laboratories and laboratories without ranking outliers generated larger SD_R than commercial laboratories or those in the AOAC collaborative study (Mertens, 2002). This raises concerns that research or development laboratories may generate unacceptable results, which may explain some atypical results that have been published. This inaccuracy can have an important effect in the field because such data are routinely used in meta-analyses and feeding model development. Research or development laboratories should participate in laboratory proficiency programs, such as the NFTA proficiency certification or the American Association of Feed Control Officials (AAFCO) check sample programs to assure their results are comparable to those observed in the field.

The greater variability in the analysis of LF-TMR compared with HF-TMR suggested that commercial laboratories might have difficulty in measuring aNDF in concentrates. However, commercial laboratories had similar SD_R for protein supplements when compared with laboratories in the AOAC collaborative study. This suggests that protein contamination of aNDF is not a factor affecting results for TMR analysis. Hintz

et al. (1996) demonstrated that sodium sulfite is crucial for removing protein from fiber residues. The results of the questionnaire and results for protein supplements indicate that commercial laboratories are using adequate amounts of sodium sulfite in aNDF assays. The source of the variation in aNDF analysis by commercial laboratories appears to be analysis of grains and starch removal. For grains, the SD_R of commercial laboratories

Table 6. Statistical analysis of amylase-treated neutral detergent fiber (aNDF) variation among laboratory groups using Youden pair analysis for feed ingredients used to make high- and low-fiber TMR

Feed type, laboratory, or feed	n ¹	Reps ²	Mean (%)	SD_r ³	Adj SD_r ⁴	SD_{Lab} ⁵	SD_R ⁶	Adj SD_R ⁷	Adj SD_r Mixed ⁸	Adj SD_R Mixed ⁹
Forage										
AOAC ¹⁰ AS-CS ¹¹	12	2.00	38.9	0.37	0.52	0.68	0.77	0.86		
AOAC GH-CSt ¹¹	12	2.00	65.5	0.72	1.02	0.79	1.07	1.29		
All ¹²	14	2.00	43.2	2.15	3.04	0.52	2.21	3.09	3.05	3.09
Without outliers ¹³	12	1.92	43.2	1.87	2.59	0 ¹⁴	1.80	2.54	2.56	2.56
Commercial ¹⁵	10	1.80	43.3	1.96	2.63	0	1.92	2.60	2.68	2.68
Grain										
AOAC Youden ¹⁶	12	2.00	35.3	1.08	1.53	0.40	1.16	1.58		
All	13	1.92	13.8	1.96	2.72	1.39	2.41	3.06	2.78	3.11
Without outliers	11	1.82	13.8	1.48	2.00	0.79	1.68	2.15	2.07	2.21
Commercial	9	1.67	13.7	1.65	2.12	0.74	1.80	2.25	2.23	2.34
ProtSupp										
AOAC Youden ¹⁷	11	2.00	12.9	1.27	1.80	0.37	1.33	1.84		
All	14	2.00	21.2	1.90	2.68	1.89	2.68	3.28	2.68	3.27
Without outliers	12	1.92	21.0	1.72	2.38	0.81	1.90	2.52	2.38	2.51
Commercial	10	1.80	21.2	1.16	1.55	0.93	1.48	1.81	1.56	1.81
MeasTMR ¹⁸										
AOAC Youden	12	2.00	34.4	0.73	1.04	0.67	0.98	1.23		
All	14	12.00	31.6	0.42	1.47	1.59	1.65	2.17	1.47	2.17
Without outliers	12	11.50	31.6	0.40	1.37	1.19	1.26	1.82	1.37	1.82
Commercial	10	10.80	31.6	0.43	1.42	1.23	1.31	1.88	1.42	1.88
CalcTMR ¹⁸										
All	14	2.00	31.9	0.17	0.24	1.65	1.66	1.66	0.25	1.66
Without outliers	12	1.92	31.8	0.19	0.26	1.10	1.12	1.13	0.26	1.13
Commercial	10	1.80	31.9	0.20	0.27	1.14	1.16	1.17	0.27	1.17

¹Number of laboratories submitting results.

²Average number of replicated analyses per laboratory mean.

³Standard deviation of repeatability within laboratory for a reported replicated result.

⁴Standard deviation of repeatability within laboratory adjusted to a single analytical result.

⁵Standard deviation of reproducibility among laboratories for a reported replicated result.

⁶Standard deviation of reproducibility within and among laboratories for a reported replicated result.

⁷Standard deviation of reproducibility within and among laboratories adjusted to a single analytical result.

⁸Standard deviation of repeatability within laboratory adjusted to a single analytical result using mixed model variance estimates.

⁹Standard deviation of reproducibility within and among laboratories adjusted to a single analytical result using mixed model variance estimates.

¹⁰All AOAC results adapted from AOAC collaborative study (Mertens, 2002) and published here for comparison.

¹¹Forage Youden pair of alfalfa silage (AS) and corn silage (CS) and grass hay (GH) and corn stalks (CSt) calculated from the AOAC collaborative study for amylase-treated NDF (Mertens, 2002).

¹²All participating laboratories.

¹³Participating laboratories without laboratory ranking outliers.

¹⁴Set to 0 by the estimation process.

¹⁵Participating commercial laboratories.

¹⁶Grain Youden pair of brewers grains and corn and cob meal calculated from the AOAC collaborative study for amylase-treated NDF (Mertens, 2002).

¹⁷Protein supplements (ProtSupp) Youden pair of soybean meal and dairy mixed protein supplement calculated from the AOAC collaborative study for amylase-treated NDF (Mertens, 2002).

¹⁸MeasTMR = average analytical results of replicated samples; CalcTMR = calculated results based on feed ingredient proportions and analyses.

is 1.4 times that of AOAC laboratories, and the among-laboratory SD_R (SD_{Lab}) is 1.8 times that of the AOAC collaborative study.

Surprisingly, the greatest source of variability between commercial and AOAC laboratories was forages. The SD_R for commercial laboratories was 2.4 times that of AOAC results and within-laboratory SD_r was 3.4 times that of AOAC results (Table 6). The SD_r within laboratories was so large that the GLM estimate of SD_{Lab} among laboratories was negative (PROC MIXED estimated the SD_{Lab} to be 0). The large SD_r of commercial laboratories for the forage Youden pair (alfalfa hay and corn silage) provides another clue that starch removal may be a culprit in variable aNDF analysis among and within laboratories. Youden pair analysis is based on the assumption that no or negligible interaction exists between the laboratory effect and the feed

within-pair effect. It essentially assumes that each feed in the Youden pair is analyzed with the same accuracy so the difference between the feed in a pair is an unbiased estimate of within-laboratory precision. When the estimate of SD_r is large for Youden pairs, especially when the estimate of among-variation is small, it is an indication that the repeatability within laboratory is not the same for both feeds of the pair (a significant laboratory by feed within-pair interaction exists). This is confirmed by identifying laboratories within alfalfa hay and corn silage that fall outside the 95% CI based on AOAC results. For alfalfa hay, commercial laboratories 5 and 9 were below and laboratories 2 and 10 were above the confidence interval. For corn silage, commercial laboratory 6 was below and laboratories 1 and 10 were above the confidence interval. The high results for corn silage could be attributed to starch contamination,

Table 7. Statistical parameters for amylase-treated neutral detergent fiber (aNDF) analyses of individual feeds (%; as-received basis) used to prepare the low- and high-fiber TMR

Laboratory group and ingredient ¹	n	No. of reps	Mean	Median	SD	SD Adj. ²	Minimum	Maximum
All laboratories								
Alfalfa hay	14	2	38.3	38.1	2.08	2.94	34.2	41.6
Corn silage	14	2	48.1	48.4	2.35	3.32	41.8	52.0
Flaked corn	14	2	8.1	8.3	1.28	1.81	5.6	10.4
Rolled barley	13	2	19.5	19.1	3.14	4.45	14.0	26.4
Solvent-extracted soybean meal (SSBM)	14	2	10.1	9.8	1.93	2.74	7.9	16.2
Distillers grains	14	2	32.4	32.4	3.24	4.58	26.9	38.5
Whole cottonseed	10	2	45.9	46.4	2.51	3.55	41.3	49.2
Measured HF-TMR	14	12	33.8	33.3	1.61	5.58	30.9	37.0
Measured LF-TMR	14	12	29.5	29.0	1.69	5.84	27.6	33.1
Calculated HF-TMR	14	2	34.0	34.0	1.62	2.29	31.1	37.6
Calculated LF-TMR	14	2	29.7	29.6	1.69	2.39	26.6	33.3
Without outliers								
Alfalfa hay	12	1.92	38.1	38.1	2.01	2.78	34.2	41.3
Corn silage	12	1.92	48.4	48.4	1.59	2.19	45.9	52.0
Flaked corn	12	1.92	8.2	8.3	1.22	1.69	5.6	10.4
Rolled barley	11	1.92	19.4	19.1	2.02	2.79	15.9	22.7
SSBM	12	1.92	9.6	9.6	0.87	1.20	7.9	11.0
Distillers grains	12	1.92	32.4	32.4	2.54	3.52	26.9	36.1
Whole cottonseed	9	1.92	45.8	46.4	2.66	3.68	41.3	49.2
Measured HF-TMR	12	11.50	33.7	33.3	1.17	3.96	32.3	35.8
Measured LF-TMR	12	11.50	29.4	29.0	1.35	4.58	27.8	32.3
Calculated HF-TMR	12	1.92	34.0	34.0	1.08	1.50	32.4	35.6
Calculated LF-TMR	12	1.92	29.6	29.6	1.15	1.59	27.9	31.7
Commercial								
Alfalfa hay	10	1.80	38.1	38.1	2.12	2.85	34.2	41.3
Corn silage	10	1.80	48.5	48.4	1.73	2.32	45.9	52.0
Flaked corn	10	1.80	8.2	8.3	1.30	1.74	5.6	10.4
Rolled barley	9	1.80	19.3	19.1	2.17	2.91	15.9	22.7
SSBM	10	1.80	9.7	9.8	0.90	1.21	7.9	11.0
Distillers grains	10	1.80	32.6	32.4	1.90	2.55	30.3	36.1
Whole cottonseed	7	1.80	46.3	46.4	2.36	3.17	42.6	49.2
Measured HF-TMR	10	10.80	33.8	33.3	1.19	3.92	32.3	35.8
Measured LF-TMR	10	10.80	29.4	29.0	1.41	4.64	27.8	32.3
Calculated HF-TMR	10	1.80	34.0	34.0	1.13	1.51	32.4	35.6
Calculated LF-TMR	10	1.80	29.7	29.6	1.19	1.60	27.9	31.7

¹Measured HF-TMR and LF-TMR = average analytical results of replicated samples of the high- and low-fiber TMR, respectively; Calculated HF-TMR and LF-TMR = calculated results based on feed ingredient proportions and analyses for the high- and low-fiber TMR, respectively.

²Standard deviation adjusted to a single analytical result.

but the variation in alfalfa hay results is unexplained. Summary analysis (Table 7) confirms that both of the TMR, rolled barley (one high outlier result removed), DDGS, and corn silage had large SD when adjusted to a result of a single analysis on a single sample. Flaked corn SD was low, which may be related to the gelatinization of the starch in this feed during flaking that would allow starch to be more easily removed by hot neutral detergent extraction.

The high SD for aNDF analysis of alfalfa hay and corn silage by commercial laboratories (Table 7) is surprising given that all laboratories participated in the NFTA proficiency certification program; however, the proficiency grades were not obtained. Proficiency certification by NFTA is based on DM, CP, aNDF, and ADF analysis of a finely ground sample of 4 alfalfa hays, a corn silage, and a grassy hay each year. It is

possible that sample preparation, which is not a factor in NFTA certification, may have contributed to some of the variation observed in this study. However, a range of 34.2 to 41.3% aNDF for alfalfa hay or 45.9 to 52.0% aNDF for corn silage is not acceptable for feed evaluation or ration formulation.

Two laboratories in this study also participated in the original AOAC collaborative study. Their results were compared with the 10 laboratories that were not outliers. Using either mean separation or least-squared differences across all Youden pairs or replicated TMR, it was observed that they did not differ from 6 of the 10 nonoutlier laboratories. For all feeds (except whole cottonseed) and TMR, the average aNDF of the 2 AOAC laboratories was less than that of the 4 laboratories from which they differed. This difference was -2.0 , -2.0 , and -1.6 percentage units for feed ingredients,

Table 8. Statistical analysis of percentage CP variation among laboratory groups using Youden pair analysis for feed ingredients and using replicated analyses for TMR

Feed type, laboratory, and feed	n ¹	Mean (%)	SD _r ²	SD _{Lab} ³	SD _R ⁴	SD _r Mixed ⁵	SD _R Mixed ⁶
High-fiber TMR ⁷							
All ⁸	13	15.5	0.39	0.59	0.70	0.39	0.73
Commercial ⁹	11	15.5	0.41	0.62	0.74	0.41	0.76
Low-fiber TMR ⁷							
All	13	15.4	0.43	0.49	0.65	0.43	0.70
Commercial	11	15.4	0.46	0.54	0.71	0.46	0.74
Both TMR ⁷							
All	13	15.5	0.41	0.58	0.71	0.41	0.71
Commercial	11	15.5	0.44	0.60	0.74	0.44	0.74
Forage							
Horwitz SD _R ¹⁰					0.35		
All	12	12.8	0.72	0.48	0.86	0.72	0.86
Commercial	10	12.8	0.58	0.53	0.79	0.58	0.79
Grain							
Horwitz SD _R					0.30		
All	12	10.6	0.52	0.13	0.53	0.52	0.53
Commercial	10	10.6	0.55	0.13	0.57	0.55	0.57
ProtSupp							
Horwitz SD _R					0.91		
All	12	39.4	0.54	1.12	1.24	0.54	1.24
Commercial	10	39.6	0.53	0.97	1.11	0.53	1.11
MeasTMR							
Horwitz SD _R					0.41		
All	12	15.5	0.48	0.61	0.77	0.48	0.77
Commercial	10	15.5	0.53	0.63	0.82	0.53	0.82
CalcTMR							
Horwitz SD _R					0.42		
All	12	15.8	0.07	0.45	0.51	0.07	0.48

¹Number of laboratories submitting results.

²Standard deviation of repeatability within laboratory for a reported replicated result.

³Standard deviation of reproducibility among laboratories for a reported replicated result.

⁴Standard deviation of reproducibility within and among laboratories for a reported replicated result.

⁵Standard deviation of repeatability within laboratory for a reported replicated result using mixed model variance estimates.

⁶Standard deviation of reproducibility within and among laboratories for a reported replicated result using mixed model variance estimates.

⁷Experimental results for replicated analyses of high- and low-fiber TMR.

⁸All participating laboratories.

⁹Participating commercial laboratories.

¹⁰Horwitz standard deviation of reproducibility (Horwitz, 1982).

Table 9. Statistical parameters for CP analyses of individual feeds (% as received basis) used to prepare the low- and high-fiber TMR

Laboratory group or ingredient ¹	n	Mean	Median	SD	Minimum	Maximum	CV (%)
All laboratories							
Alfalfa hay	12	19.69	19.69	1.115	18.13	21.25	5.66
Corn silage	12	5.89	5.63	0.496	5.00	6.88	8.42
Flaked corn	12	8.44	8.75	0.421	7.50	8.75	4.99
Rolled barley	12	12.76	12.50	0.623	11.88	13.75	4.88
Solvent-extracted soybean meal (SSBM)	12	48.28	48.44	1.309	45.63	50.00	2.71
Distillers grains	12	30.57	30.63	1.176	28.13	32.50	3.85
Whole cottonseed	12	21.88	21.25	1.903	19.38	26.88	8.70
Measured HF-TMR	13	15.51	15.19	1.575	14.88	16.88	10.15
Measured LF-TMR	13	15.42	15.31	1.409	14.44	16.25	9.14
Calculated HF-TMR	12	15.74	15.78	0.508	15.06	16.50	3.23
Calculated LF-TMR	12	15.84	15.88	0.457	15.19	16.50	2.89
Commercial							
Alfalfa hay	10	19.69	19.69	0.988	18.13	21.25	5.02
Corn silage	10	5.81	5.63	0.515	5.00	6.88	8.86
Flaked corn	10	8.38	8.44	0.437	7.50	8.75	5.21
Rolled barley	10	12.75	12.50	0.672	11.88	13.75	5.27
SSBM	10	48.38	48.44	1.222	45.63	50.00	2.53
Distillers grains	10	30.81	30.94	0.979	28.75	32.50	3.18
Whole cottonseed	10	21.88	21.25	2.083	19.38	26.88	9.52
Measured HF-TMR	11	15.54	15.19	1.628	14.94	16.88	10.48
Measured LF-TMR	11	15.43	15.31	1.467	14.44	16.25	9.51
Calculated HF-TMR	10	15.73	15.78	0.458	15.06	16.38	2.91
Calculated LF-TMR	10	15.83	15.88	0.419	15.19	16.44	2.65

¹Measured HF-TMR and LF-TMR = average analytical results of replicated samples of the high- and low-fiber TMR, respectively; Calculated HF-TMR and LF-TMR = calculated results based on feed ingredient proportions and analyses for the high- and low-fiber TMR, respectively.

LF-TMR, and HF-TMR, respectively. Higher aNDF results could be related to incomplete extraction, which is often associated with low boiling temperatures that do not agitate particles vigorously during extraction, or to

inadequate soaking of fiber residues with boiling water to remove detergent and extracted solubles.

The SD_R of within- and among-laboratory variation of commercial laboratories for CP (Table 8) was about

Table 10. Variance estimates for amylase-treated neutral detergent fiber (aNDF) and CP analyses among participating laboratories

Item	n	Estimated variance	SE	Pr > Z ¹
aNDF analysis: type of TMR ²				
LF-TMR	168	2.35	1.096	0.032
HF-TMR	168	1.81	0.872	0.038
aNDF analysis: aNDF technique ³				
FB technique	72	3.06	2.542	0.23
CR technique	96	1.17	0.930	0.21
aNDF analysis: TMR × aNDF technique interaction				
FB technique ³				
LF-TMR	36	1.50	0.978	0.13
HF-TMR	36	1.08	0.748	0.15
CR technique ³				
LF-TMR	48	4.52	3.428	0.19
HF-TMR	48	3.55	2.748	0.20
CP analysis: type of TMR				
LF-TMR	156	0.0077	0.00348	0.027
HF-TMR	156	0.0097	0.00430	0.024

¹Test for significance of the variance.

²LF-TMR = low-forage TMR; HF-TMR = high-forage TMR.

³CR = aNDF using the crucible technique; FB = aNDF using the filter bag technique.

half of that for aNDF of TMR, but the CP concentration was also about half of that for aNDF. A UK collaborative study reported low CV (2.7 to 5.4%) for CP analysis of plant materials among 10 commercial laboratories (Crosland et al., 2001) and a more recent collaborative study with DDGS reached a similar conclusion (Thiex, 2009). For the Youden feed pairs, the SD_R of CP (Table 8) for commercial laboratories was usually less than half of that for aNDF (Table 6). However, the SD_R of CP was generally twice that expected for AOAC official methods (Horwitz, 1982). Horwitz (1982) observed a nonlinear relationship between the reproducibility and the value of the mean result across all types of official methods. The SD_R estimated by the equation of Horwitz (1982) can be used to determine if the variation of any analytical result is comparable to those observed for all AOAC official methods. The univariate statistical parameters in Table 9 suggest that the variation in CP analysis by commercial laboratories is probably less than acceptable for feed evaluation or ration formulation, assuming that the expected CV of most feed analyses (with means of 10 to 60% of DM) would be 2 to 3% (Mertens, 2003).

Table 10 depicts the estimated variability among laboratories within TMR and analytical technique for aNDF and CP analyses. Overall, the variability in aNDF and CP analyses among laboratories was significant (trends at $P = 0.06$ and 0.019 , respectively). Technique of analysis had no effect ($P = 0.52$) on the aNDF values, but variability among laboratories was significant ($P = 0.032$ and 0.038 for LF-TMR and HF-TMR, respectively). The variance estimates for the 2 techniques of aNDF analysis were not statistically significant ($P = 0.23$ and 0.21 for CR and FB, respectively). The technique of aNDF analysis and TMR interactions were also not significant ($P = 0.13$ to 0.20). Variability among participating laboratories was significant ($P = 0.027$ and 0.024 for LF-TMR and HF-TMR, respectively) for the CP analysis.

CONCLUSIONS

This ringtest showed that commercial laboratories were not a significant source of variation in the analysis of TMR aNDF even though the SD of reproducibility was greater than that reported by laboratories participating in the AOAC evaluation of the aNDF method. Although the FB and CR techniques did not differ among commercial laboratories, several research and development laboratories using the FB technique generated results that were outliers. Compared with AOAC performance statistics for the aNDF official method, the SD of reproducibility among and within commercial laboratories was higher for forages and

grains primarily because the variability of analysis of alfalfa hay and rolled barley were particularly large. For all feeds and TMR, the variation among laboratories was primarily related to higher values for aNDF than those obtained by laboratories participating in the AOAC collaborative study evaluating the aNDF method. This suggests that inconsistencies exist among laboratories in following the official aNDF method that may be related to extraction temperatures and soaking of fiber residues. Crude protein analysis results were more consistent among laboratories, and variability in CP analysis of individual feeds or TMR was marginally acceptable. Variability in aNDF analysis was not statistically significant although laboratories using the filter bag technique tended to produce more variable results than laboratories using variation of the crucible technique. Calculated aNDF values for TMR, based on aNDF analysis of individual feeds, were slightly greater than measured aNDF values. Results from this ringtest emphasize the need for feed analysis laboratories to follow the official aNDF method exactly. Within-laboratory repeatability was a major source of variation within and among commercial laboratories. This variation could be reduced by replicating analysis and including reference materials in each analytical run.

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