An alternative method to assess 24-h ruminal in vitro neutral detergent fiber digestibility¹

J. P. Goeser and D. K. Combs²

Department of Dairy Science, University of Wisconsin-Madison, Madison 53706

ABSTRACT

Interassay error caused by the inconsistent nature of rumen fluid inoculum confounds comparisons of forage in vitro neutral detergent fiber (NDF) digestibility (NDFD) analyzed in different repetitions or laboratories. Our objective was to determine if priming rumen fluid and allowing it to produce a standard amount of gas before inoculating samples improved assay repeatability. In 2 experiments, we compared interassay error of NDFD estimates between several in vitro assays. In both experiments, dried, ground (1 mm) alfalfa samples (0.5 g) sealed in bags were placed in 125-mL Erlenmeyer flasks and incubated with in vitro media and 10 mL of rumen fluid. In experiment A, rumen fluid was collected from a cannulated cow fed a high forage diet and prepared one of 2 ways; rumen fluid was either used immediately after it was collected and strained through cheese cloth (GVA), or strained fluid was combined with buffer, reducing solution, and 12.5 mg of cellulose/mL of rumen fluid and allowed to produce a consistent amount of gas before inoculation (RPA). The assay was repeated 5 times, with 13 samples per method. In experiment B, inoculum was prepared one of 3 ways; RPA, GVA except rumen fluid was collected and pooled from 2 cows (GVB), or RPA with fluid pooled from 2 cows. The in vitro assays were repeated 5 times with 8 samples per method. Neutral detergent fiber was analyzed using a forage fiber analyzer and 24-h NDFD was determined as: $\text{NDFD (\% of NDF)} = 100 \times [(\text{NDF}_{0\text{h}} - \text{NDF}_{\text{residue}})/(\text{NDF}_{0\text{h}})]$. Data for each experiment were analyzed using a mixed model procedure and repetition sum of squares for each technique was determined and compared with an F-test to assess technique interassay error. In both experiments, rumen fluid priming significantly reduced repetition sums of squares, 51.2 versus 503 and 23.3 versus 164, compared with the respective GVA or GVB. However, priming

significantly decreased NDFD values, 22.5 versus 24.8 and 23.9 versus 26.6%, compared with GVA and GVB, respectively. Priming rumen fluid with cellulose improved in vitro NDFD assay precision, but depressed in vitro NDFD.

Key words: in vitro, neutral detergent fiber digestion, rumen fluid standardization

INTRODUCTION

Forage digestibility is greatly affected by the high proportion of forage DM as NDF and the variability of fiber digestibility. Neutral detergent fiber digestibility (NDFD) is one of the most variable forage parameters, ranging from less than 40% of NDFD for highly lignified mature legumes to greater than 90% for unlignified immature grass. Ruminant nutritionists and forage plant breeders use in vitro laboratory measures of NDFD to assess forage quality, predict diet digestibility, and select plant genotypes for advanced breeding. Many commercial forage testing laboratories offer in vitro digestion assays based on the in vitro technique developed by Tilley and Terry (1963) and modified by Goering and Van Soest (1970). Briefly, the technique involves collection of rumen fluid and immediate inoculation of dried, ground forage samples contained in a flask with a buffering and nutritive in vitro medium. Sample digestion is measured following 48 h of anaerobic degradation by rumen fluid bacteria. The technique has been modified by others, and alternative techniques have been shown to affect estimates of in vitro NDF digestibility (ivNDFD; Craig et al., 1984, Grant and Mertens, 1992a,b). The variable activity of rumen fluid inoculum is a major factor introducing interassay error into ivNDFD assays. Rymer et al. (2005) attributed the largest source of run-to-run variation with an in vitro rumen technique to inoculum. Interassay error with ruminal in vitro digestion techniques has been acknowledged in replicated experiments by Hall et al. (1998) and Schofield and Pell (1995). Schofield and Pell (1995) accounted for interassay error by including a repetition term in multiple linear regression models and Hall et al. (1998) used paired *t*-tests to remove the influence of inoculum collected on different days. Studies

Received February 28, 2008.

Accepted April 19, 2009.

¹This research was funded by USDA Hatch multistate project NE-1024.

²Corresponding author: dkcombs@wisc.edu

by Grant and Mertens (1992a) and Hall and Mertens (2008) examining technique effects on *ivNDFD* have minimized the relevance of repetition variance by including a repetition term within multiple linear regression models and not discussing the significance of the effect or practical implications for commercial forage testing laboratories repeating the techniques. Commercial forage testing laboratories that do not replicate forage samples in multiple runs are not able to account for run-to-run variance introduced by rumen fluid inoculum using statistical techniques. Forage *ivNDFD* estimates from a single run may be presented without inference to how variable assay results are from one run to the next. An internal standard feed should be included with each run; however, the standard only indicates variance between runs. The inability of commercial laboratories to control repetition variance makes interpretation of *ivNDFD* estimates difficult.

Rumen fluid collection procedures have been modified in many ways in an attempt to reduce variability of inoculum activity. Collection methods that rapidly transfer inoculum from donor animal to digestion flasks, control inoculum temperature, and minimize inoculum exposure to oxygen are thought to be ways to minimize death losses of rumen microbes. Strategies such as feeding donor animals standardized diets, collecting inoculum at a fixed time each day, and pooling rumen fluid from multiple donor animals have also been proposed to improve the fermentative consistency of rumen fluid (Rymer et al., 2005). However, little published data exists that documents by how much and how consistently these modifications reduce run-to-run variability of *in vitro* digestibility methods. Our objective was to determine if a rumen fluid priming technique would improve *ivNDFD* precision relative to a modified Goering and Van Soest (1970) technique by reducing repetition variance.

MATERIALS AND METHODS

All experimental protocols were approved by the Research Animal and Resource Center of the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

In experiment A, a rumen fluid inoculum priming technique was compared with a modified Goering and Van Soest (1970) technique and both techniques used rumen fluid inoculum from a single lactating cow. In experiment B, the rumen fluid priming technique used in experiment A was compared with a rumen fluid priming technique and a modified Goering and Van Soest (1970) technique that both used inoculum collected and pooled from 2 lactating dairy cows.

The 2 experiments were completed by comparing 24-h *in vitro* NDF digestibility obtained using modified Goering and Van Soest (1970) techniques and rumen fluid priming techniques in a randomized complete block design.

An alfalfa silage sample was dried at 60°C for 48 h, ground to pass a 1-mm Wiley mill screen (Arthur H. Thomas, Philadelphia, PA), and used as a forage substrate in experiments A and B. Alfalfa silage samples were analyzed by Dairyland Laboratories Inc. (Arcadia, WI) by AOAC (2006) methods for DM (method 930.15), CP (method 954.01), and ash (method 942.15). Lignin and ADF were determined using methods described by Goering and Van Soest (1970).

Experiment A

Approximately 0.5 g of dried, ground alfalfa silage sample was weighed into tared, labeled filter bags (F57, Ankom Technology, Macedon, NY), which then were heat-sealed. Each repetition included 13 samples, 2 blank filter bags, and 2 zero-hour samples, and 5 *ivNDFD* repetitions were completed for each of the 2 techniques being compared; a rumen fluid inoculum priming *ivNDFD* technique (**RPA**) and a modified Goering and Van Soest (1970) *ivNDFD* technique (**GVA**). Both techniques were completed using inoculum collected from a single region of the rumen of one cannulated lactating dairy cow with a stainless steel hand-operated vacuum pump into a glass-lined, insulated container that had been prewarmed with approximately 70°C tap water.

In Vitro Solution and Sample Preparation

Two solutions, A and B, were formulated. Solution A contained 18.0 L of distilled H₂O, 102.6 g of Na₂HPO₄, 111.6 g of KH₂PO₄, and 10.5 g of MgSO₄·7H₂O. Solution B contained 13.2 g of CaCl₂·2H₂O, 10.0 g of MnCl₂·4H₂O, 1.0 g of CoCl₂·6H₂O, and 8.0 g of FeCl₃·6H₂O, and was brought to 100 mL with distilled H₂O.

For each repetition, the day before inoculation at approximately 1500 h, filter bags containing a forage sample were placed in 125-mL Erlenmeyer flasks, one bag per flask, secured in a shaking, heated (39°C) water bath. A mineral solution was then prepared for each repetition that contained 800 mL of distilled H₂O, 400 mL of solution A, 4.0 g of trypticase peptone, 2.0 mL of solution B, and 2.0 mL of resazurin indicator (0.1% wt/vol).

A 30-mL aliquot of mineral solution was added to each flask designated for the RPA treatment. Six hundred milliliters of the remaining mineral solution was then combined with 200 mL of a buffer solution that

Figure 1. Interassay variation in rumen fluid inoculum gas pressure readings, an indication of rumen fluid activity, for a primed (12.5 mg of cellulose/mL of rumen fluid inoculum) and unprimed inoculum for various mean gas production levels across 5 assay repetitions; results from a pilot study (J. P. Goesser and D. K. Combs, unpublished data).

contained 18.0 L of distilled H_2O , 630 g of $NaHCO_3$, and 72.0 g of NH_3HCO_3 .

A 40-mL aliquot of mineral-buffer solution was added to each flask designated for the GVA treatment. All flasks were sealed with a 2-hole rubber stopper. Each rubber stopper was fitted with 2 glass tubing pieces, one sealed with a rubber policeman with a 4-mm vertical cut and the other attached to a gas manifold for continuous CO_2 gassing. The flasks containing mineral or mineral-buffer solution, and filter bags with sample were purged with CO_2 for 15 min and warmed overnight. In addition, the day before inoculation, reducing solution was prepared that contained 0.505 g of cysteine HCl, 0.505 g of $Na_2S \cdot 9H_2O$, 76.8 mL of distilled H_2O , and 3.2 mL of 1 N NaOH.

Rumen Fluid Collection and GVA Flask Inoculation

At approximately 0630 h on the day of inoculation, all flasks were purged continuously with CO_2 , and 2 mL of reducing solution was added to each flask designated for the GVA treatment. At 0645 h, approximately 1 L of rumen fluid was collected from one cannulated, lactating cow into a prewarmed, glass-lined thermos. The donor cow was fed a 60% forage (alfalfa and corn silage) and 40% concentrate diet ad libitum once daily at 0700 h. The rumen fluid inoculum was transported to the laboratory and, in a warm ($39^\circ C$) room, strained through 4 layers of cheesecloth while being gassed continuously with CO_2 . Approximately 800 mL of strained rumen fluid was used to immediately inoculate the GVA flasks with 10 mL of rumen fluid inoculum per flask. The period from rumen fluid collection to GVA flask inoculation was approximately 15 min.

Rumen Fluid Priming Technique and RPA Flask Inoculation

A pilot study was completed before experiments A and B comparing rumen fluid activity, as indicated by gas pressure measurements. Variation in gas pressure from a primed inoculum (12.5 mg of cellulose/mL of rumen fluid inoculum) and an unprimed inoculum were compared over 5 repetitions. Figure 1 summarizes the average variation in gas pressure measurements relative to the median value of gas pressure measurements of rumen fluid, for primed and unprimed (control) rumen fluid. For the control rumen fluid, interassay variance increased as more gas per milliliter of rumen fluid inoculum was produced. For the primed rumen fluid, however, interassay variance of gas pressure measurements became more consistent after rumen fluid inoculum produced approximately 0.1 mL of gas/mL of rumen fluid with the primed inoculum. Results of the pilot study suggested that rumen fluid reached a more consistent activity when primed and allowed to produce at least 0.1 mL of gas production/mL of rumen fluid, so 12.5 mg of cellulose/mL of rumen fluid inoculum and a gas pressure reading that corresponded to more than 0.1 mL of gas production/mL of rumen fluid were chosen for further evaluation.

At 0630 h on the day of inoculation, 2.5 g of crystalline cellulose ground to pass a 1-mm Wiley screen (Whatman 42 Ashless Filter Paper Circles, Whatman Int. Ltd., Maidstone, UK) was combined with 200 mL of buffer solution and 40 mL of reducing solution in a 1,000-mL side-arm Erlenmeyer flask. The 1,000-mL Erlenmeyer flask was gassed with CO_2 for 15 min while rumen fluid inoculum was collected. At 0645 h, 200 mL of filtered rumen fluid was added to the flask. The flask was then sealed with a rubber stopper, set in an incubating ($39^\circ C$) shaker and allowed to reach 46.7 mmHg, which corresponded to 60 mL of gas produced or 0.3 mL of gas production/mL of rumen fluid inoculum. The amount of gas production to reach 46.7 mmHg was determined through manual calibration by forcing known amounts of gas into a sealed 1,000-mL side-arm flask and reading corresponding pressures with the pressure meter. After reaching 46.7 mmHg, the contents of the flask were used to inoculate, using 22 mL of rumen fluid inoculum per flask, the RPA samples.

Sample Analysis

At approximately 1500 h for each repetition, filter bags for both methods were deflated by tamping each filter bag down with a plastic rod while purging the flask with CO_2 . Samples were removed at 0 and 24 h after inoculation for each technique. The forage fiber

bags were rinsed with ambient temperature (approximately 20°C) distilled water until effluent was clear to terminate the fermentations, similar to the method described by Eun et al. (2007).

Sample NDF content was determined using a neutral detergent solution containing α -amylase and sodium sulfite using the procedure described by Goering and Van Soest (1970) adapted for an Ankom²⁰⁰ Fiber Analyzer (Ankom Technology). Neutral detergent fiber percentages of all samples were determined using the following equation:

$$\text{NDF (\% of DM)} = \frac{[(\text{bag wt.} + \text{residue}) - (\text{bag wt.} \times \text{bag correction factor})]}{[(\text{bag wt.} + \text{sample}) - (\text{bag wt.})] \times 100}.$$

The bag correction factor is the weight of an empty, sealed bag divided by the weight of the same bag after going through the in vitro procedure. In vitro NDF digestibility was determined using the following equation:

$$\text{ivNDFD (\% of NDF)} = 100 \times \frac{[(\text{NDF}_{0 \text{ h}} - \text{NDF}_{\text{residue}})/(\text{NDF}_{0 \text{ h}})]}{1}.$$

Statistical Analysis

The complete data set was analyzed as a randomized complete block design with subsampling using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The model used was

$$Y_{ijkl} = \mu + R_i + M_j + RM_{ij} + e_{ijkl},$$

where Y_{ijkl} = NDFD, the dependent variable, μ = population mean, R_i = fixed effect of repetition i , M_j = fixed effect of method, RM_{ij} = interaction between repetition and method, and e_{ijkl} = random residual error, assumed to be normally distributed. The data sets were also separated by technique and analyzed using PROC GLM of SAS (SAS Institute Inc.) to obtain sums of squares. The model used was

$$Y_{ijkl} = \mu + R_i + e_{ijkl},$$

where Y_{ijkl} = NDFD, the dependent variable, μ = population mean, R_i = fixed effect of repetition i , and e_{ijkl} = random residual error, assumed to be normally distributed. Repetition variance for each technique was compared using an F -test. Significance was declared at $P < 0.05$. Variance within runs was evaluated using

Levene's test (Levene, 1960) where ANOVA was performed on the absolute deviance of each observation from the median of its group.

Experiment B

The alfalfa silage sample described in experiment A was weighed (0.5 g) into Ankom F57 filter bags as described previously. The bags were sealed and 5 ivNDFD repetitions were completed with 36 samples analyzed per repetition. Each repetition included 8 samples, 2 blank filter bags, and 2 zero-hour samples for each ivNDFD technique. The 3 ivNDFD techniques used in experiment B were the RPA described in experiment A, a rumen fluid priming ivNDFD technique using rumen fluid inoculum pooled from 2 cows (**RPB**), and a modified Goering and Van Soest (1970) ivNDFD technique using rumen fluid inoculum pooled from 2 cows (**GVB**). Experiment B used the same cow as described in experiment A and an additional cannulated lactating Holstein cow.

In Vitro Solution and Sample Preparation

The A, B, buffer, mineral, and reducing solutions were formulated as described in experiment A. For each repetition, at approximately 1500 h on the day before inoculation, filter bags containing sample were placed in 125-mL Erlenmeyer flasks secured in a shaking water bath set at 39°C. A 30-mL aliquot of mineral solution was added to each flask designated for the RSA and RSB treatments. A 400-mL aliquot of mineral solution was combined with 133 mL of buffer solution, and 40 mL of the mineral-buffer solution was added to each flask designated for the GVB treatment. All flasks were then sealed as described in experiment A, purged with CO_2 for 15 min, and warmed overnight.

Rumen Fluid Collection and GVB Flask Inoculation

At approximately 0645 h on the day of inoculation, approximately 1 L of rumen fluid was collected from each of 2 cannulated cows as described in experiment A. The donor cows were fed a 60% forage and 40% concentrate diet ad libitum, once daily at 0700 h. The rumen fluid inoculum was strained through 4 layers of cheesecloth while being gassed continuously with CO_2 , and 700 mL of strained fluid from each cow was pooled in a 2-L Erlenmeyer flask. Approximately 800 mL of strained, pooled rumen fluid was used to immediately inoculate the GVB flasks with 10 mL of rumen fluid inoculum per flask. The period from rumen fluid collection to GVB flask inoculation was approximately 15 min.

Rumen Fluid Priming Techniques and RPA and RPB Flask Inoculation

The RPA and RPB inoculums were prepared in 1,000-mL side-arm Erlenmeyer flasks. Crystalline cellulose, buffer solution, and reducing solution were added to each flask as described in experiment A. Strained rumen fluid (200 mL) from a single cow was added to one flask for the RPA, and 200 mL of strained, pooled rumen fluid was added to the second flask for the RPB. The two 1,000-mL flasks were then sealed, set in an incubating (39°C) shaker, and allowed to reach 46.7 mmHg gas pressure as described in experiment A. Twenty-two milliliters of primed RSA or RSB inoculum was then added to the 125-mL Erlenmeyer in vitro flasks.

Sample Analysis

All samples were analyzed as described in experiment A.

Statistical Analysis

The complete data set was analyzed as a randomized complete block design with subsampling using PROC MIXED of SAS (SAS Institute Inc.). The model used was as follows:

$$Y_{ijkl} = \mu + R_i + M_j + RM_{ij} + e_{ijkl},$$

where Y_{ijkl} = NDFD, the dependent variable, μ = population mean, R_i = fixed effect of repetition i , M_j = fixed effect of method, RM_{ij} = interaction between repetition and method, and e_{ijkl} = random residual error, assumed to be normally distributed. The data sets were also separated by technique and analyzed using PROC GLM (SAS Institute Inc.) to obtain sums of squares. The model used was as follows:

$$Y_{ijkl} = \mu + R_i + e_{ijkl},$$

where Y_{ijkl} = NDFD, the dependent variable, μ = population mean, R_i = fixed effect of repetition i , and e_{ijkl} = random residual error, assumed to be normally distributed.

Repetition variance for each technique was compared using an F -test. Significance was declared at $P < 0.05$. Variance within runs was evaluated using Levene's test (Levene, 1960) where ANOVA was performed on the absolute deviance of each observation from the median of its group.

RESULTS AND DISCUSSION

The in vitro methods described in experiments A and B use Goering and Van Soest (1970) buffer, macro-,

and micro-mineral solutions. The in vitro techniques described here differ from that of Goering and Van Soest (1970) through the following modifications: the use of a 24-h incubation time, the use of Ankom F57 forage fiber bags in 125-mL Erlenmeyer flasks for digestion and NDF assays, no blending of inoculum was done based upon the observations of Rymer et al. (1999), and an ambient temperature distilled water rinse was used to terminate fermentations. In addition, for the priming method, the rumen fluid was combined with buffer and reducing solution before inoculation and cellulose was added to inoculum solution.

A 24-h incubation time may be a harsher test of differences in in vitro NDFD methodology, because at early stages of digestion the corresponding residue disappearances are small, making digestion more difficult to study (Pell and Schofield, 1993). The use of Ankom F57 forage fiber bags may limit forage sample surface area available to rumen microbes. We maximized fluid and sample contact by deflating the forage fiber bags manually. Adesogan (2005) used Ankom forage fiber bags in a bulk in vitro digestion system and observed that the Ankom bags gave a more precise prediction ($R^2 = 0.77$) of the Tilley and Terry method than alternative types of forage digestion bags. Eun et al. (2007) also used individual bags within 125-mL Erlenmeyer flasks for in vitro digestions and used cold distilled water to terminate the digestions. Using forage fiber bags reduces the losses of residue when transferring between in vitro vessel and NDF beaker, an issue that has been acknowledged recently by Hall and Mertens (2008). Without the use of forage fiber bags for sample handling, fiber particles may be retained on the sidewall of the digestion vessel during the digestion procedure and when transferring fluids to a beaker for residue NDF analysis. The use of forage fiber bags also improves the efficiency of NDF analysis of degraded residue. Up to 24 in vitro samples can be simultaneously refluxed and rinsed using the Goering and Van Soest (1970) NDF procedure modified for the Ankom²⁰⁰ forage fiber analyzer (Ankom Technologies) in approximately a 2-h period and using one Ankom²⁰⁰ analyzer. Efficiency can be improved beyond this level by employing multiple forage fiber analyzers, with the potential to reflux and rinse 48 or 72 samples in a similar period.

The remaining differences between our technique and Goering and Van Soest's (1970) in vitro rumen digestion, specifically combining rumen fluid inoculum with 12.5 mg of cellulose/mL of rumen fluid inoculum, buffer, and reducing solution before inoculation and allowing the mixture to produce 0.3 mL of gas/mL of rumen fluid, are unique and are reported here for the first time. The Tilley and Terry (1963) technique developed in the 1960s and modified by Goering and Van Soest (1970)

and others over the following 30 yr (Craig et al., 1984; Grant and Mertens, 1992a; Pell and Schofield, 1993) is based upon collecting rumen fluid inoculum from one or several ruminants and promptly inoculating samples to be digested. The effects of time from rumen fluid inoculum collection to sample inoculation before in vitro gas production studies have been summarized in a review by Mould et al. (2005). They concluded that there was no significant correlation between the time interval between collection and inoculation and in vitro gas production. However, there was not an attempt to stimulate microbial activity before inoculation by adding fermentable carbohydrate in any of the studies summarized in this review.

To our knowledge, this experiment is the first attempt to standardize microbial activity of rumen fluid by mixing inoculum with a substrate and allowing the inoculum to produce a predetermined amount of gas before sample inoculation. We used cumulative gas pressure of the inoculum as an indicator of microbial activity. Our hypothesis was that inoculum microbial activity might be more consistent if the stresses associated with collection (e.g., oxygen exposure, temperature changes) or the inconsistencies in activity caused by normal eating and drinking behavior were accounted for by monitoring gas production and using gas pressure within the collection flask as a crude measure of microbial activity compared with inoculum that was collected and used as quickly as feasible.

Nutrient composition for the alfalfa silage used in both experiments A and B is presented in Table 1. In experiment A, both techniques used inoculum from a

Table 1. Alfalfa silage nutrient composition in experiments A and B

Item	% of DM
NDF	43.83
ADF	39.73
CP	21.12
Lignin	8.71
Fat	2.42
Ash	9.41

single donor cow. The amount of time for the flasks to reach 46.7 mmHg ranged from 2 to 5.5 h. The rumen fluid priming technique decreased ($P < 0.024$) repetition sums of squares for 24-h ivNDFD compared with a modified Goering and Van Soest (1970) technique (Table 2). This observation suggests that primed rumen fluid from a single donor animal reduced some of the variable nature of rumen fluid inoculum. Priming provided a measure of standardization beyond collecting rumen fluid inoculum from the same animal, on a prescribed diet, at the same time each day (before feeding). Both methods displayed comparable intraassay error. Levene's test was insignificant for each repetition and method (Table 2). The standard deviations of means for each method and repetition also did not exhibit any patterns.

Although priming improved repeatability, the RPA also significantly decreased 24-h ivNDFD relative to the GVA. The decreased ivNDFD most likely resulted from an increase in substrate to inoculum ratio by priming inoculum with cellulose. The RPA flasks contained 12.5 mg more substrate per flask than did the GVA

Figure 2. Relationship between repetition mean in vitro NDF digestibility (ivNDFD) and the time required for inoculum to reach standard pressure (priming technique) in experiment A. The unprimed inoculum was plotted against the time to pressure of the corresponding priming technique. No significance was observed. RSA = rumen fluid inoculum standardization using inoculum from a single cow; GVA = modified Goering and Van Soest (1970) using inoculum from a single cow.

Figure 3. Relationship between repetition mean in vitro NDF digestibility (ivNDFD) and the time required for inoculum to reach standard pressure (priming technique) in experiment B. The unprimed inoculum was plotted against the time to pressure of the corresponding priming technique. No significance was observed. GVB = modified Goering and Van Soest (1970) using inoculum pooled from 2 dairy cows; RSA and RSB = rumen fluid inoculum standardization using inoculum from 1 and 2 cows, respectively.

Table 2. In vitro NDF digestion (ivNDFD) parameters for 2 in vitro techniques in experiment A

Item	ivNDFD technique ¹		SEM ²	<i>F</i> -value ³	<i>P</i> -value <
	GVA	RSA			
Repetition sum of squares ⁴	503.31	51.17		9.84	0.02
Repetition ivNDFD, % of NDF ⁵					
1	24.70 ± 3.40	21.84 ± 3.29			
2	26.71 ± 3.34	23.41 ± 3.93			
3	21.74 ± 3.55	21.34 ± 4.87			
4	28.92 ± 3.75	23.65 ± 4.02			
5	21.84 ± 4.14	22.34 ± 5.03			
Repetition mean absolute deviation from median of each group, % of NDF (Levene's test) ⁶					
1	2.80	2.70		0.47	NS
2	2.75	2.72			
3	3.06	4.34			
4	2.90	3.05			
5	3.00	3.68			
Mean 24-h ivNDFD, % of NDF	24.78 ^a	22.52 ^b	0.49		0.01

^{a,b}Means within a row with differing superscripts differ ($P < 0.05$).

¹In vitro NDF digestion techniques: GVA = modified Goering and Van Soest (1970); RSA = rumen fluid inoculum standardization; both techniques used inoculum from a single cow.

²SEM = standard error of the treatment means.

³*F*-value = larger sum of squares value/smaller sum of squares value on 4, 4 degrees of freedom.

⁴Repetition Type III sum of squares = sum of squares attributed to repetition following ANOVA.

⁵Repetition ivNDFD, % of NDF = mean ivNDFD as a % of NDF for each technique and repetition, each value is the mean of 13 observations ± SD.

⁶Repetition mean absolute deviation from median of each group, % of NDF = variable for Levene's test, ANOVA completed on absolute deviation from median of each group for each observation.

flasks. The sample to inoculum ratios were 50 and 62.5 mg of substrate/mL of rumen fluid inoculum for GVA and RPA, respectively, and were within the range of substrate per milliliter of rumen fluid calculated from reviews by Getachew et al. (1998) and Rymer et al. (2005), however the unprimed vessels contained 25% less substrate. The time to reach 46.7 mmHg was not an index of inoculum activity. The time to reach 46.7 mmHg for each repetition was not related to average NDFD from that repetition in either experiment A or B (Figures 2 and 3).

In experiment B, we tested the priming technique precision and the effect of pooled rumen fluid inoculum, which has been suggested to reduce repetition variance Williams (2000). As observed with experiment A, intraassay error in experiment B was similar for the 3 methods. The standard deviation of the means for each repetition and method did not exhibit any clear trends and Levene's test was again insignificant (Table 3). The amount of time for each flask to reach 46.7 mmHg varied from 2 to 6.5 h, and the RPA consistently took longer to reach 46.7 mmHg in each repetition. Our observations were in accordance with the suggestions of Williams (2000), in that the modified Goering and Van Soest (1970) technique using pooled rumen fluid inocu-

lum produced repetition variance comparable to that of the RPA (Table 3). This observation suggests that priming rumen fluid inoculum from one donor animal yields similar assay precision to using pooled rumen fluid inoculum from 2 donor cows, and both techniques may offer improved precision over using unprimed inoculum from one animal. However, the rumen fluid priming technique using pooled rumen fluid further reduced interassay error compared with the GVB and tended to be lower than RPA, indicating that a further improvement in precision was achieved by priming pooled rumen fluid inoculum in this experiment. The ivNDFD estimates from the primed techniques were significantly lower than the modified Goering and Van Soest (1970) method (Table 3). However, primed rumen fluid inoculum pooled from 2 donor cows had lower interassay error than any other technique tested.

The collective results of experiments A and B suggest that the rumen fluid priming techniques described here decreased ivNDFD interassay error relative to unprimed rumen fluid inoculum, using either a single cow inoculum or inoculum pooled from 2 donor cows. The methods did not differ in intraassay precision, but ivNDFD precision was improved from repetition to repetition using priming techniques. The priming methods

Table 3. In vitro NDF digestion (ivNDFD) parameters for 3 techniques in experiment B

Item	ivNDFD technique ¹			SEM	<i>F</i> -value ²	<i>P</i> -value <
	GVB	RSA	RSB			
Repetition type III sum of squares ³	163.70		23.34		7.01	0.04
		133.12	23.34		5.70	0.06
Repetition ivNDFD, % of NDF ⁴						
1	28.31 ± 3.81	20.84 ± 1.43	24.26 ± 2.73			
2	26.87 ± 1.99	18.40 ± 2.80	23.14 ± 4.33			
3	23.12 ± 3.62	22.51 ± 3.28	22.67 ± 2.48			
4	25.36 ± 2.08	24.08 ± 2.72	24.73 ± 2.87			
5	28.99 ± 3.21	21.06 ± 1.69	24.20 ± 2.65			
Repetition mean absolute deviation from median of each group, % of NDF ⁵						
1	2.80	1.17	1.85			NS
2	1.10	1.89	3.23			
3	2.32	2.12	1.83			
4	1.73	2.15	2.36			
5	2.14	1.26	1.61			
Mean 24-h ivNDFD, % of NDF	26.55 ^a	21.43 ^c	23.83 ^b	0.47		0.01

^{a-c}Means within a row with differing superscripts differ ($P < 0.05$).

¹In vitro NDF digestion techniques: GVB = modified Goering and Van Soest (1970) using inoculum pooled from 2 dairy cows; RSA = rumen fluid inoculum standardization using inoculum from a single cow; RSB = rumen fluid inoculum standardization using inoculum from 2 cows.

²*F*-value = largest sum of square value/smallest sum of squares value on 4, 4 degrees of freedom.

³Repetition type III sum of squares = sum of squares attributed to repetition following ANOVA.

⁴Repetition ivNDFD, % of NDF = mean ivNDFD as a percentage of NDF for each technique and repetition, each value is the mean of 8 observations (or 7 in the case of a missing value) ± SD.

⁵Repetition mean absolute deviation from median of each group, % of NDF = variable for Levene's test, ANOVA completed on absolute deviation from median of each group for each observation.

also produced significantly lower ivNDFD estimates, which most likely occurred because of an increase in substrate to inoculum ratio.

We hypothesize that providing rumen inoculum microflora with a cellulose substrate and allowing the inoculum to produce 0.3 mL of gas/mL of rumen fluid permitted the ruminal microbial populations to propagate to a more consistent activity level, designated by a gas pressure reading, allowing more repeatable 24-h ivNDFD measurements to be made. Although rumen fluid is not a reagent, the primed inoculum appears to have more consistent fermentative activity than unprimed fluid.

CONCLUSIONS

The in vitro NDF digestion technique using cellulose primed rumen fluid reduced ivNDFD interassay error compared with a traditional laboratory technique using inoculum from a single or 2 donor animals but yielded lower estimates of 24-h ivNDFD.

REFERENCES

Adesogan, A. T. 2005. Effect of bag type on the apparent digestibility of feeds in ANKOM Daisy(II) incubators. *Anim. Feed Sci. Technol.* 119:333–344.

AOAC. 2006. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists International, Washington, DC.

Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In vitro inoculum enriched with particle-associated microorganisms for determining rates of fiber digestion and protein degradation. *J. Dairy Sci.* 67:2902–2909.

Eun, J. S., K. A. Beauchemin, and H. Schulze. 2007. Use of exogenous fibrolytic enzymes to enhance in vitro fermentation of alfalfa hay and corn silage. *J. Dairy Sci.* 90:1440–1451.

Getachew, G., M. Blummel, H. P. S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: A review. *Anim. Feed Sci. Technol.* 72:261–281.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). *Agric. Handbook No. 379*. ARS-USDA, Washington, DC.

Grant, R. J., and D. R. Mertens. 1992a. Impact of in vitro fermentation techniques upon kinetics of fiber digestion. *J. Dairy Sci.* 75:1263–1272.

Grant, R. J., and D. R. Mertens. 1992b. Influence of buffer pH and raw corn starch addition on in vitro fiber digestion kinetics. *J. Dairy Sci.* 75:2762–2768.

Hall, M. B., and D. R. Mertens. 2008. In vitro fermentation vessel type and method alter fiber digestibility estimates. *J. Dairy Sci.* 91:301–307.

Hall, M. B., A. N. Pell, and L. E. Chase. 1998. Characteristics of neutral detergent-soluble fiber fermentation by mixed ruminal microbes. *Anim. Feed Sci. Technol.* 70:23–39.

Levene, H. 1960. Robust test for equality of variances. Pages 278–292 in *Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling*. I. Olkin, S. G. Ghurye, W. Hoeffding, W. G. Madow, and H. B. Mann, ed. Stanford University Press, Stanford, CA.

- Mould, F. L., K. E. Kliem, R. Morgan, and R. M. Mauricio. 2005. In vitro microbial inoculum: A review of its function and properties. *Anim. Feed Sci. Technol.* 123:31–50.
- Pell, A. N., and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion in vitro. *J. Dairy Sci.* 76:1063–1073.
- Rymer, C., J. A. Huntington, and D. I. Givens. 1999. Effects of inoculum preparation method and concentration, method of inoculation and pre-soaking the substrate on the gas production profile of high temperature dried grass. *Anim. Feed Sci. Technol.* 78:199–213.
- Rymer, C., J. A. Huntington, B. A. Williams, and D. I. Givens. 2005. In vitro cumulative gas production techniques: History, methodological considerations and challenges. *Anim. Feed Sci. Technol.* 123:9–30.
- Schofield, P., and A. N. Pell. 1995. Validity of using accumulated gas pressure readings to measure forage digestion in vitro—A comparison involving 3 forages. *J. Dairy Sci.* 78:2230–2238.
- Tilley, J., and R. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. *J. Br. Grassl. Soc.* 18:104–111.
- Williams, B. A. 2000. Cumulative gas production techniques for forage evaluation. Pages 189–213 in *Forage Evaluation in Ruminant Nutrition*. D. I. Givens, E. Owen, R. F. E. Axford, and H. M. Omed, ed. CABI Publishing, Wallingford, UK.