Comparison of In Vitro Long Digestion Methods and Digestion Rates for Diverse Forages

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RESEARCH

Comparison of In Vitro Long Digestion Methods and Digestion Rates for Diverse Forages

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ABSTRACT

Unreliable measures of undigested neutral detergent fiber (uNDF) can influence animal nutrition and performance when balancing diets based on flawed forage quality estimates. Two common techniques for in vitro long digestions are the conventional flask method and the ANKOM filtration bag procedure. An ANKOM filter bag has been developed (F58) with an 8- to 10-μm pore size, decreasing the chance of losing particles during neutral detergent fiber (NDF) procedure, but it has not been evaluated for use with in vitro digestions. Our objective was to compare ANKOM F58 bags with F57 bags and the conventional flask method for in vitro long digestions. Analyses incorporated 24 forage samples representing a broad range of temperate and tropical grasses and legumes. A commercial laboratory analyzed the same samples using the conventional flask procedure. Separate analyses evaluated the effect of Na_2SO_3 for ANKOM F57 and F58 methods and the effect of ruminal fluid plus buffer refreshing at 2-d intervals with ANKOM F57. Undigested NDF at 240 h on an organic matter basis (uNDF240om) values between methods were different from one another, but rate calculations derived from uNDF240om values were not different. Method pore size was highly correlated (r = −0.993) with uNDF240om values. Results showed that refreshing ruminal inoculum plus buffer at 2-d intervals for ANKOM F57 and the addition of Na_2SO_3 during the analysis of ash-free NDF on an organic matter basis (aNDFom) after ANKOM F57 and F58 in vitro digestions both had significant effects on lowering uNDF240om values.

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Abbreviations: aNDF, ash-free neutral detergent fiber; aNDFom, ash-free neutral detergent fiber on an organic matter basis; BMR, brown-midrib; HSD, honestly significant difference; iNDF, indigestible neutral detergent fiber; kd, rate of digestion; NDF, neutral detergent fiber; pdNDF, potentially digestible neutral detergent fiber; uNDF, undigested neutral detergent fiber; uNDFom, undigested neutral detergent fiber on an organic matter basis; uNDF30om, undigested neutral detergent fiber at 30 h on an organic matter basis; uNDF120om, undigested neutral detergent fiber at 120 h on an organic matter basis; uNDF240om, undigested neutral detergent fiber at 240 h on an organic matter basis.

Neutral detergent fiber (NDF) is composed of a truly indigestible portion (iNDF) and a potentially digestible portion (pdNDF). The iNDF limits the overall extent of digestion for forages at higher NDF levels and influences feed quality, rumen digesta load, and voluntary intake (Lippke, 1986; Mertens, 2010). Accurate measurement of iNDF is important for predicting the pdNDF fraction and for predicting the fractional rate constant (Cherney and Mertens, 1998). The pdNDF is digested by microorganisms in the rumen or exits by passage, whereas iNDF can exit the rumen only by passage (Mertens, 1993). The iNDF is unaffected by the average retention time in the rumen, and time of fermentation required to attain 95 to 98% completion of pdNDF is a function of the rate of digestion (Buxton et al., 1996).

Mertens (2013) identified the term undigested NDF (uNDF) as the amount of NDF remaining at a specific fermentation time

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because, in theory, iNDF would require infinite time to achieve true indigestibility. Concerns regarding costs and labor requirements of feed digestibility determinations in vivo have furthered the utilization of alternative laboratory techniques. In vitro cultures with flasks (Tilley and Terry, 1963; Goering and Van Soest, 1970) are widely used and provide estimates of feedstuff digestibility at defined times of incubation. In vitro digestion, however, is considered only a guide to the potential, rather than realizable, value of a feed (Tilley and Terry, 1963). Although it is desirable to have consistent guidelines relating to host animals, sampling, inoculum preparation, and digestion system (Yáñez-Ruiz et al., 2016), it is unlikely that any particular system would be accepted as a standard procedure (Mould et al., 2005). Long-term ruminal in vitro methodologies, generally modifications of the Goering and Van Soest's (1970) procedure, have been commonly used to estimate feedstuff digestibility values such as rate of degradation and uNDF.

Laboratory methods for routine forage digestibility analysis of large numbers of samples must be relatively rapid, easy to perform, and have acceptable precision and accuracy (Weiss, 1994). The ANKOM filter bag method with ANKOM Daisy Incubators (ANKOM Technology) is a simpler procedure than traditional flask methods for in vitro digestibility because samples can run in batch, and researchers can avoid the individual sample filtering procedure after digestion (Adesogan, 2005). Multiple studies have demonstrated that ANKOM digestibility values were comparable with traditional procedures for a variety of ruminant feeds (Holden, 1999; Vogel et al., 1999; Mabjeesh et al., 2000; Brons and Plaizier, 2005) at shorter durations such as 48 h, although some studies did show differences for higher energy feeds such as grains and meals (Mabjeesh et al., 2000; Brons and Plaizier, 2005). All previous studies have used ANKOM F57 filter bags. Where there have been several studies comparing digestibility for in vitro digestion methods up to 48 h, research is required for comparisons of ANKOM methods with the conventional flask method for a long-term ruminal in vitro digestion (240 h).

The pore size of digestion bags is a compromise between allowing an influx of microorganisms and efflux of digested material and preventing loss of undigested material (Kitessa et al., 1999). ANKOM's first and most extensively used bag is the F57 (25-μm pore size). Critics of ANKOM's bag method indicate the potential loss of small indigestible particles through F57's 25-μm pores that could overestimate digestibility. ANKOM recently released a new F58 bag (8- to 10-μm pore size) that should decrease small particle losses without restricting access of protozoa and bacterial populations (Kitessa et al., 1999). ANKOM recommends F58 for crude fiber, NDF, and acid detergent fiber analyses, but F58 bags had not been tested or validated for use in the ANKOM Daisy Incubator for in vitro digestion studies. Pore sizes of <10 μm can restrict the number of protozoa and bacteria entering digestion bags (Meyer and Mackie, 1986), such that a bag pore size smaller than found in the F58 is not advisable.

Inclusion of Na_2SO_3 during NDF analysis is a debated matter in the literature. Van Soest (2015) omitted Na_2SO_3 from his simple NDF procedure, and Van Soest and Robertson (1980) advised how Na_2SO_3 attacks lignin and causes significant losses to recovery values (Robertson, 1978; Van Soest, 1994). Hintz and Mertens (1996) recommended the 0.5 g Na_2SO_3 sample^{-1} be added before ash-free NDF (aNDF) analysis because it decreased nitrogenous concentration in residues of animal products and heated feeds. Sodium sulfite did reduce lignin values for grasses more than legumes but also reduced within-sample variance for all fiber measurements (Hintz and Mertens, 1996).

Ruminal fermentation is often considered a continuous system because the rumen experiences constant apparent turnover of microorganisms (Van Soest, 1994). However, continuous in vitro systems are impractical for measurement of the extent of digestion. In vitro analyses aim to recreate and correlate with the rumen environment with repeatable and reliable procedures. An NDF analysis of the residue after digestion attempts to remove all rumen microbial residue to provide a true undigested residue. To better reflect the rumen scenario, it seems logical to reinoculate in vitro cultures with ruminal fluid plus buffer at regular intervals. Reinoculation of ruminal fluid at 120 h for long-digesting feeds has been recommended by multiple sources (Vogel et al., 1999; Raffrenato and Van Amburgh, 2010; Harper and McNeill, 2015).

Our objective was to determine the variability of digestibility values between the conventional flask system and the ANKOM system, with both ANKOM F57 and ANKOM F58 filter bags, for long-term ruminal in vitro digestions (240 h). A significant advantage of the ANKOM system is the ability to easily change ruminal inoculum plus buffer at regular intervals during long in vitro digestions. Therefore, a second objective was to determine whether digestibility of samples measured with the ANKOM F57 method was affected by changing ruminal fluid plus buffer every 2 d. Comparisons of ruminal fluid plus buffer refreshing pose an obstacle for comparing methods because ruminal fluid cannot easily be refreshed with the conventional flask method. Also, considering that many of the forages in this study did not have high levels of protein but did have relatively high levels of lignin, the effect of Na_2SO_3 during the aNDF procedure was quantified.

MATERIALS AND METHODS

We selected 24 samples to represent a comprehensive range of temperate and tropical grasses and legumes of differing

maturities that would potentially exhibit a variety of digestibility behaviors and uNDF values (Table 1).

Sample Preparation, Fermentation, and Chemical Analysis

Forage samples were oven dried to a constant weight at 60°C and ground in a Wiley mill to pass a 1-mm sieve. Ground forage samples were divided into separate containers using a sample splitter. For ANKOM laboratory procedures (ANKOM Technology, 2005), forages were weighed into either ANKOM F57 or ANKOM F58 filter bags for each digestion time period (0, 30, 120, and 240 h) in duplicate. A sample size of 0.25 g is permitted in the ANKOM system procedure and avoids the filtering challenges after neutral detergent extraction caused by larger sample weights. Reliable results for neutral detergent extractions have been obtained for samples >0.10 g (Cherney et al., 1985). The Kansas State buffer nutrient solution, as described by Marten and Barnes (1980), was used for ANKOM digestions. A 240-h ultimate extent of fermentation was used based on studies using long-term digestions in an in vitro anaerobic environment (Raffrenato and Van Amburgh, 2010; Raffrenato, 2011) and because commercial laboratories commonly use 240 h. ANKOM F57 bags have a 25-μm porosity, and ANKOM F58 bags have a pore size of 8 to 10 μm. Bags were heat sealed using a hand-operated, 2-mm impulse heat sealer (American International Electronics).

Approval was received for cow use from the Cornell University Institutional Animal Care and Use Committee. Ruminal fluid was collected from a fistulated nonlactating cow fed first-cut grass–legume hay ad libitum with 1.13 kg (2.5 lbs) of a 14% crude protein stock diet daily, and a salt lick. After ruminal fluid was collected, it was filtered through two layers of Grade no. 60 cheesecloth into a thermos. In the laboratory, ruminal fluid was again filtered through four layers of cheesecloth and purged with CO_2. After the addition of 1600 mL of Kansas State buffer and 400 mL of ruminal fluid to each digestion jar, jars were purged with CO_2 for 30 s. Complete sets of 24 sample bags per jar were put into 12 jars and randomly placed into ANKOM Daisy Incubators (ANKOM Technology) maintained at 39°C. The time period from collection to ruminal fluid addition to jars was <0.5 h. All 24 forages were present in each digestion jar, with two jars per time period for each bag type. Three blanks

Table 1. Composition of forages.

Forage	Scientific name	aNDFom†	ADF‡	ADL§	CP¶
		g kg^{-1} dry matter			
Legumes					
Alfalfa 1	*Medicago sativa* L.	277	226	47.4	296
Alfalfa 2	*Medicago sativa* L.	348	289	59.9	230
Alfalfa 3 (low lignin)	*Medicago sativa* L.	343	267	52.9	208
Birdsfoot trefoil	*Lotus corniculatus* L.	281	242	66.6	225
Red clover	*Trifolium pratense* L.	304	234	50.1	259
Grasses					
Big bluestem	*Andropogon gerardii* Vitman	743	486	81.5	57.9
Brachiaria (Mulato)	*Brachiaria ruziziensis* Germ. & Evrard × *B. decumbens* (Stapf) × *B. brizantha* (Hochst. ex A. Rich.) Stapf	627	333	31.9	133
Corn silage 1	*Zea mays* L.	427	225	24.0	74.6
Corn silage 2 (BMR#)	*Zea mays* L.	417	261	17.3	90.6
Coastal panic grass	*Panicum amarum* Elliott (Hitchc. & Chase) P.G. Palmer	696	411	68.7	59.1
Festulolium	*Festulolium braunii* (K. Richt.) Stace	484	303	22.1	191
Meadow fescue	*Schedonorus pratensis* (Huds.) P. Beauv.	531	312	18.8	206
Miscanthus	*Miscanthus* ×*giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize	764	533	97.4	44.1
Oat straw	*Avena sativa* L.	775	560	80.8	53.4
Orchardgrass 1	*Dactylis glomerata* L.	494	320	37.7	182
Orchardgrass 2	*Dactylis glomerata* L.	602	350	28.5	151
Quackgrass	*Elytrigia repens* (L.) Nevski	569	328	30.1	213
Reed canarygrass	*Phalaris arundinacea* L.	528	290	17.4	262
Rice straw	*Oryza sativa* L.	708	508	44.4	60.0
Smooth bromegrass	*Bromus inermis* Leyss.	555	378	27.4	170
Sorghum × sudangrass	*Sorghum bicolor* (L.) Moench	640	397	39.6	33.7
Switchgrass	*Panicum virgatum* L.	761	510	86.3	39.2
Timothy	*Phleum pratense* L.	569	338	29.2	144
Eastern gamagrass	*Tripsacum dactyloides* L.	688	393	45.4	142
Mean ± SD		547 ± 161	354 ± 102	46.1 ± 23.7	147 ± 81.2

† aNDFom, neutral detergent fiber with Na_2SO_3 included in neutral detergent solution and α-amylase added during rinsing, expressed on an organic matter basis (Mertens, 2002).

‡ ADF, acid detergent fiber (AOAC, 1990).

§ ADL, acid detergent lignin (AOAC, 1990).

¶ CP, crude protein (AOAC, 1995).

BMR, brown-midrib.

were included in each jar for a blank correction. Every 2 d of incubation, a second set of 12 jars containing buffer at 39°C was inoculated with ruminal fluid plus buffer, and bags were quickly transferred to the second set of jars. Any gas accumulated in bags was gently expelled by hand during ruminal fluid plus buffer changes, and the transfer required <1 min per jar. Blank bags did not accumulate any gas. Expelling of gas was not done in runs that did not have ruminal fluid plus buffer changes. Jars were removed from the ANKOM Daisy Incubators at their designated times, and bags with digested residues were subject to neutral detergent extraction using ANKOM fiber extractors to determine in vitro true digestibility. Each batch of 24 samples was suspended in an ANKOM NDF digestion vessel with 2000 mL of neutral detergent solution, 20 g of Na_2SO_3, and 4.0 mL of amylase and heated to 100°C for 75 min. After two rinses with 70 to 90°C water and amylase and one rinse with only 70 to 90°C water, bags were soaked in acetone for 5 min and dried at 105°C. Digested forage samples were analyzed both with and without Na_2SO_3 in the neutral detergent solution. α-Amylase (thermostable form from *Bacillus licheniformis*, ANKOM Technology) was added to two of the three hot water rinses after neutral detergent extraction and weighing, and dried bags with residues were ashed in tared beakers in a muffle furnace at 510°C for 4 h. Amylase-treated neutral detergent fiber residues were ash corrected and blank corrected and reported on an organic matter basis. All weighing of sample residues after drying was conducted after cooling residues to room temperature in desiccators. Neutral detergent extraction of forage samples, including amylase treatment of residues, are reported on an organic matter basis (aNDFom), as are neutral detergent extractions of residues after specific periods of digestion (uNDF at 120 h on an organic matter basis [uNDF120om] vs. uNDF at 240 h on an organic matter basis [uNDF240om]). All experiments were repeated in a second digestion run.

Cumberland Valley Analytical Services (Waynesboro, PA) analyzed the same 24 forages with the conventional flask in vitro procedure (Goering and Van Soest, 1970) in duplicate for each time point (0, 30, 120, and 240 h) in two replicated runs. Sodium sulfite and α-amylase were included during NDF processing, and a Fisher G8 filter mat with a porosity of 2.5 μm was used (Fisher Scientific). Ruminal inoculum for commercial laboratory analyses differed from ANKOM F57 and F58 analyses, and nonlactating cows at the commercial laboratory consumed a haylage and corn (*Zea mays* L.) silage total mixed ration. Ruminal fluid plus buffer was not refreshed at 2-d intervals with the conventional flask in vitro digestions.

A separate experiment with the same 24 forages was conducted with two in vitro digestion runs using ANKOM F57 bags with ruminal fluid plus buffer refreshed every 2 d, compared with ANKOM F57 bags without any refreshing during the in vitro digestion run. Gas was expelled gently by hand from sample bags during ruminal fluid plus buffer transfer but was not expelled from bags that were not refreshed with ruminal fluid plus buffer. Samples for all in vitro digestion runs were included in duplicate. Only ANKOM F57 bags were included in this experiment because there was only room for two methods (refreshing vs. not refreshed) with 12 jars (three digestion times, replicated in separate jars).

Statistical Analysis

All statistical analyses were done in R version 3.2.3 (R Core Team, 2015). Observed means, SDs, and CVs were calculated for the 30-, 120-, and 240-h time points and rates of digestion. Coefficients of variation were calculated as SD divided by observed means multiplied by 100 throughout statistical analyses. An ANOVA model with uNDF240om as the dependent variable with pore size and forage as main effects was used to estimate the R^2 and the slope of the three methods containing Na_2SO_3. The slope of methods with Na_2SO_3 was compared with the slope of methods without Na_2SO_3 with a mixed model that had uNDF240om as the dependent variable, pore size and Na_2SO_3 inclusion as fixed effects, the pore size × Na_2SO_3 inclusion interaction, and forage as a random effect. Estimates for the kinetics of digestion were determined with a dynamic system model with a nonlinear equation fitting multiple regressions to the curve (Ventana Simulation Environment, Ventana Systems) currently used by commercial laboratories and integrated into the Cornell Net and Carbohydrate Protein System (Cornell University, Ithaca, NY). The dynamic system requires uNDF time points at 0, 30, 120, and 240 h and generates an integrated rate of a two-pooled model. Statistical analysis of uNDF on an organic matter basis (uNDFom) at 30, 120, and 240 h used a two-way ANOVA with uNDFom as the dependent variable and method and forage as main effects to compare five methods. Least squares means were separated by Tukey's honestly significant difference (HSD) test. The method × forage interaction was excluded from these models because the interest lies in method comparisons across forages rather than for individual forages, and later analyses address differences among legume and grass forages. Statistical analysis of the rate of digestion used a two-way ANOVA with rate of digestion (kd) as the dependent variable and method and forage as main effects.

We used a two-way ANOVA model with uNDF240om SD between methods as the dependent variable with method comparison and forage as main effects to compare differences between each method that could then be contrasted against the variation we observed within method replicates. Interaction terms were excluded because the topic of interest lies at the method level rather than differences for individual forages. Spearman correlations compared how the order of method means over each of the two runs ranked uNDF240om and rates of digestion for the 24 forages.

Grass and legume forages were compared for uNDF240om and rates of digestion values with a linear regression containing uNDF240om or the kd as the dependent variable and method and grass or legume as fixed effects, the interaction between method and grass or legume, and forage was nested within grass or legume. Least squares means were separated by Tukey's HSD test.

Comparisons of in vitro ruminal inoculum plus buffer refreshing at 2-d intervals vs. no ruminal inoculum refreshing on ANKOM F57 bags were evaluated with a two-way ANOVA model with uNDF240om as the dependent variable and method and forage as main effects. The method × forage interaction was excluded because the topic of interest was the comparison at the method level rather than individual forages. Least squares means were separated by Tukey's HSD test.

RESULTS

Forage Composition

Table 1 shows the chemical composition of the forages. There was considerable variation among forages, which ranged from 277 to 775 g aNDFom kg^{-1}, 225 to 510 g acid detergent fiber kg^{-1} on a dry matter basis, 17.3 to 97.4 g acid detergent lignin kg^{-1} on an organic matter basis, and 33.7 to 262 g crude protein kg^{-1} on a dry matter basis. One of the three alfalfa (*Medicago sativa* L.) forages was a reduced-lignin cultivar, and one of the two corn silage samples contained the brown-midrib (BMR) reduced-lignin trait. Immature and mature cool-season and warm-season grass forages were represented.

Indigestibility

The numerically maximum method SD for uNDF at 30 h on an organic matter basis (uNDF30om) among forages was with the conventional flask method for big bluestem (*Andropogon gerardii* Vitman), the numerically maximum CV was observed with the conventional flask method for reed canarygrass (*Phalaris arundinacea* L.), and the conventional flask also had the numerically greatest average SD and CV (Table 2). The numerically minimum method SD and CV for uNDF30om among forages was the F57 with Na_2SO_3 method for switchgrass (*Panicum virgatum* L.), and the F58 with Na_2SO_3 method had the numerically smallest average SD and CV when considering all forages.

The numerically maximum method SD for uNDF120om among forages was the conventional flask method for oat straw (*Avena sativa* L.), the numerically maximum CV was the conventional flask method for reed canarygrass, and the conventional flask also had the numerically greatest average SD and CV (Table 3). The numerically minimum method SD and CV for uNDF120om among forages was the F57 with Na_2SO_3 method for alfalfa-3 (low lignin), and the F58 with Na_2SO_3 method had the numerically smallest average SD and CV for all forages.

The numerically maximum method SD and CV for uNDF240om among forages were with the conventional flask method for red clover (*Trifolium pratense* L.), and the conventional flask also had the numerically greatest average SD and CV (Table 4). The numerically minimum method SD and CV for uNDF240om among forages was the F57 without Na_2SO_3 method for corn silage 2 (BMR), and the F58 with Na_2SO_3 method had the numerically smallest average SD and CV for all forages.

Table 2. Observed means of undigested neutral detergent fiber at 30 h on an organic matter basis (uNDF30om), SDs, and CVs for 24 forages.

	Method														
	Flask SS†			F57 SS			F57 no SS			F58 SS			F58 no SS		
Forage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
	— g kg^{-1} DM‡ —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%
Alfalfa 1	163	0.89	0.54	116	4.03	3.49	117	0.71	0.60	123	1.98	1.61	128	5.94	4.64
Alfalfa 2	214	21.9	10.2	189	11.7	6.20	196	25.5	13.0	202	2.47	1.23	193	5.45	2.82
Alfalfa 3 (low lignin)	194	13.4	6.90	173	2.55	1.47	175	11.0	6.25	180	1.70	0.94	182	1.97	1.08
Birdsfoot trefoil	193	12.2	6.33	159	6.43	4.06	162	12.0	7.39	161	1.13	0.70	175	1.69	0.97
Red clover	226	22.3	9.85	131	4.81	3.67	143	0.78	0.54	136	5.52	4.05	147	18.4	12.5
Big bluestem	479	50.6	10.6	534	3.54	0.66	551	3.68	0.67	531	11.5	2.16	572	28.5	4.98
Brachiaria (Mulato)	268	15.1	5.65	260	11.7	4.52	255	12.4	4.86	231	16.8	7.25	268	4.37	1.63
Corn silage 1	162	19.3	12.0	179	9.48	5.31	198	8.06	4.07	187	1.06	0.57	205	11.1	5.43
Corn silage 2 (BMR§)	170.3	18.8	11.0	243	10.5	4.34	266	21.6	8.14	204	10.8	5.27	274	12.5	4.56
Coastal panic grass	460	28.7	6.24	484	3.32	0.69	487	1.98	0.41	499	9.48	1.90	500	11.4	2.28
Festulolium	113	11.3	9.96	80.3	3.68	4.58	94.9	11.5	12.1	99.1	13.6	13.7	109	8.08	7.39
Meadow fescue	129	14.9	11.5	127	3.89	3.07	135	7.57	5.60	130	7.92	6.10	139	4.16	2.98
Miscanthus	577	8.96	1.55	592	2.90	0.49	607	15.8	2.61	603	4.24	0.70	626	14.3	2.29
Oat straw	448	25.1	5.61	505	7.71	1.53	531	8.49	1.60	527	13.0	2.47	528	27.6	5.23
Orchardgrass 1	176	17.5	9.90	161	11.0	6.81	171	4.74	2.77	175	7.78	4.45	175	1.30	0.74
Orchardgrass 2	176	15.2	8.64	156	17.1	11.0	171	4.31	2.53	166	7.00	4.23	186	6.87	3.70
Quackgrass	174	11.5	6.64	138	12.1	8.78	164	10.5	6.37	152	2.69	1.77	167	10.2	6.14
Reed canarygrass	134	33.2	24.7	119	2.55	2.14	140	6.72	4.79	130	0.85	0.65	156	2.20	1.42
Rice straw	408	7.60	1.86	397	8.27	2.08	406	12.7	3.14	399	0.78	0.20	440	11.0	2.49
Smooth bromegrass	151	16.2	10.8	185	7.50	4.06	229	1.84	0.80	174	5.66	3.25	216	16.8	7.77
Sorghum × sudangrass	292	15.2	5.22	314	10.6	3.38	324	0.78	0.24	303	6.58	2.17	349	6.66	1.91
Switchgrass	560	49.1	8.77	595	0.49	0.08	602	20.8	3.46	589	0.78	0.13	612	3.06	0.50
Timothy	147	21.8	14.9	128	9.48	7.43	161	1.98	1.23	145	0.64	0.44	155	8.24	5.33
Eastern gamagrass	329	35.5	10.8	318	3.46	1.09	328	9.40	2.87	315	7.71	2.45	341	0.82	0.24
Method avg.	264	20.3	8.76	262	7.04	3.79	276	8.94	4.00	265	5.90	2.85	285	9.27	3.71

† SS, Na_2SO_3 included in neutral detergent solution.

‡ DM, dry matter.

§ BMR, brown-midrib.

Table 3. Observed means of undigested neutral detergent fiber at 120 h on an organic matter basis (uNDF120om), SDs, and CVs for 24 forages.

	Method														
	Flask SS†			F57 SS			F57 no SS			F58 SS			F58 no SS		
Forage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
	— g kg^{-1} DM‡ —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%
Alfalfa 1	129	36.9	28.6	108	7.87	7.28	113	1.55	1.38	119	1.22	1.03	123	0.32	0.26
Alfalfa 2	200	23.5	11.8	180	5.97	3.32	183	18.5	10.1	191	4.64	2.44	191	3.54	1.85
Alfalfa 3 (low lignin)	181	32.4	17.9	166	0.17	0.10	171	5.04	2.95	173	4.34	2.50	173	7.54	4.35
Birdsfoot trefoil	187	20.0	10.7	147	10.0	6.80	158	8.18	5.17	158	4.96	3.16	163	8.02	4.94
Red clover	162	16.4	10.1	102	8.57	8.40	112	5.94	5.30	113	5.03	4.44	116	4.57	3.94
Big bluestem	351	35.7	10.2	368	6.24	1.70	381	4.28	1.12	369	13.0	3.53	388	5.12	1.32
Brachiaria (Mulato)	161	15.1	9.38	149	7.96	5.35	156	1.98	1.27	154	1.94	1.26	159	2.78	1.75
Corn silage 1	115	15.8	13.7	111.8	1.01	0.90	117	1.90	1.62	126	5.90	4.67	132	2.39	1.81
Corn silage 2 (BMR§)	108	10.8	10.0	92.3	7.05	7.63	108	5.32	4.92	97.4	1.47	1.50	115	2.12	1.85
Coastal panic grass	314	33.2	10.6	358	6.47	1.81	361	13.2	3.66	375	4.16	1.11	363	12.15	3.35
Festulolium	62.3	19.0	30.5	48.0	9.55	19.9	53.3	4.70	8.81	58.1	2.34	4.04	55.9	3.21	5.75
Meadow fescue	85.4	17.7	20.7	73.0	4.02	5.51	76.6	1.71	2.24	79.1	1.60	2.02	84.7	0.51	0.61
Miscanthus	484	32.3	6.66	515	4.80	0.93	522	9.05	1.73	534	1.75	0.33	535	8.47	1.58
Oat straw	312	41.1	13.2	339	6.73	1.99	366	2.58	0.71	353	2.16	0.61	365	3.51	0.96
Orchardgrass 1	129	25.1	19.5	119	10.6	8.92	132	0.55	0.42	134	5.93	4.43	136	0.55	0.40
Orchardgrass 2	99.2	17.3	17.4	92.4	3.64	3.94	96.8	4.26	4.40	97.6	4.21	4.31	102	3.01	2.95
Quackgrass	97.3	24.1	24.8	84.6	6.96	8.23	96.0	0.19	0.20	96.0	0.63	0.65	101	0.28	0.28
Reed canarygrass	90.4	28.4	31.5	72.7	2.53	3.48	84.5	4.79	5.67	84.5	1.57	1.85	88.4	6.71	7.59
Rice straw	245	21.3	8.69	209	7.16	3.42	223	14.6	6.55	222	0.36	0.16	232	13.2	5.72
Smooth bromegrass	101	21.2	21.1	89.5	6.49	7.25	95.7	6.78	7.09	102	0.42	0.41	103	6.10	5.92
Sorghum × sudangrass	172	23.9	13.9	177	7.82	4.41	185	1.20	0.65	181	0.78	0.43	186	0.73	0.39
Switchgrass	410	30.8	7.52	449	6.61	1.47	448	18.8	4.20	462	0.64	0.14	458	4.39	0.96
Timothy	87.0	27.5	31.6	66.2	5.36	8.09	75.4	1.89	2.50	77.2	4.32	5.60	79.2	1.18	1.50
Eastern gamagrass	194	25.8	13.3	182	3.90	2.14	194.3	2.04	1.05	185	3.97	2.15	200	6.08	3.05
Method avg.	187	24.8	16.4	179	6.15	5.12	188	5.79	3.49	189	3.22	2.20	194	4.44	2.63

† SS, Na_2SO_3 included in neutral detergent solution.

‡ DM, dry matter.

§ BMR, brown-midrib.

Mean uNDF240om values for the five methods showed a consistent effect of including Na_2SO_3 in the neutral detergent solution on indigestibility estimates (Fig. 1). Inclusion of Na_2SO_3 reduced indigestibility an average of 11.5 g kg^{-1}. The effect of filter pore size on 240-h residue proportion was consistent, with an average reduction in residue proportion of 0.76 g kg^{-1} for every 1-μm increase in filter pore size (Fig. 1). The consistency between ANKOM and conventional flask systems in regards to filter pore size provides some evidence that the digestion process for the two systems behaved similarly. The slope comparison of methods that used Na_2SO_3 ($y = -0.80x + 123$) vs. methods that did not use Na_2SO_3 ($y = -0.75x + 126$) had a p value of 0.06 (Fig. 1).

Digestions in conventional flasks were significantly different from F58 without Na_2SO_3 for uNDF30 (Table 5). F57 without Na_2SO_3 was significantly different from F57 with Na_2SO_3, F57 with Na_2SO_3 was different from F58 without Na_2SO_3, and F58 with Na_2SO_3 was significantly different from F58 without Na_2SO_3 for uNDF30 (Table 5) ($p < 0.05$). At the uNDF120om time point, F57 with Na_2SO_3 was significantly different from F57 without Na_2SO_3, F58 without Na_2SO_3, and F58 with Na_2SO_3 ($p < 0.05$) (Table 6).

Digestions in conventional flasks were significantly different in uNDF240om from ANKOM F57 bag methods and F58 with Na_2SO_3 (Table 7). All ANKOM methods differed from one another in uNDF240om except F57 without Na_2SO_3 compared with F58 with Na_2SO_3. The uNDF240om ranged from 50 to 500 g kg^{-1} across forages (Table 4).

Least squares means of the SD between methods (Table 8) showed the largest difference between F57 with Na_2SO_3 and F58 without Na_2SO_3. The smallest reported difference was between the F57 without Na_2SO_3 and F58 with Na_2SO_3. Rank correlations established if methods ranked forage samples in a comparable order (Table 9). Spearman correlations were all high and significant for both uNDF240om values and rates of digestion.

Digestion Rate

Forages with high uNDF240om values tended to have the smallest rates of digestion, and legumes had the largest rates of aNDFom digestion. The numerically maximum method SD for kd among forages was with the F57 without Na_2SO_3 method for alfalfa 2, and the numeri-cally maximum method CV for kd among forages was

with the F57 without Na_2SO_3 method for alfalfa 3 (low lignin). Conventional flask had the numerically greatest average SD and CV for all forages (Table 10). All methods had SDs and CVs at zero or close to zero for one or more forages. The F57 with Na_2SO_3 method had the numerically smallest average SD and CV. None of the method comparisons for kd were significantly different (Table 11).

Grasses vs. Legumes

Of the 24 forages in this study, five were legumes and 19 were grasses. Comparisons made between methods of uNDF240om for grasses show that F57 with Na_2SO_3 was consistently different from the other methods, and F58 without Na_2SO_3 was different from all other methods except the conventional flask method (Table 12). Comparisons between the methods of uNDF240om for legumes show the conventional flask method was different from both ANKOM F57 methods (Table 13).

Least squares means comparisons for both grass and legume forages showed differences between the conventional flask method and all other methods for

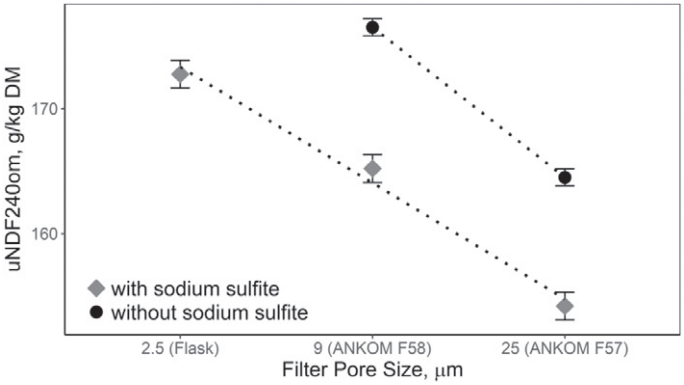

Fig. 1. Observed means of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) and filter pore size for a glass microfiber filter (2.5 μm), F58 ANKOM bag (8–10 μm), and F57 ANKOM bag (25 μm), with and without Na_2SO_3 included in the neutral detergent solution. Standard errors were 15.1, 16.8, 16.2, 17.4, and 16.6 g kg^{-1} dry matter for the conventional flask method, F57 without Na_2SO_3, F57 with Na_2SO_3, F58 without Na_2SO_3, and F58 with Na_2SO_3, respectively. An ANOVA model with uNDF240om as the dependent variable with pore size and forage as main effects had a slope of $y = -0.80x + 123$ and coefficient of correlation of –0.993 for the three methods containing Na_2SO_3.

Table 4. Observed means of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om), SDs, and CVs for 24 forages.

	Method														
	Flask SS†			F57 SS			F57 no SS			F58 SS			F58 no SS		
Forage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
	— g kg^{-1} DM‡ —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%	— g kg^{-1} DM —		%
Alfalfa 1	119	27.8	23.3	107	12.4	11.6	107	7.51	7.02	115	1.87	1.63	120	3.49	2.91
Alfalfa 2	186	9.72	5.23	178	12.0	6.75	179	7.12	3.98	189	2.11	1.12	186	7.13	3.84
Alfalfa 3 (low lignin)	175	25.9	14.8	155	5.85	3.77	166	2.82	1.70	173	3.10	1.79	168	1.48	0.88
Birdsfoot trefoil	178	18.3	10.3	150	13.1	8.72	150	10.5	6.99	146	7.93	5.42	160	5.15	3.22
Red clover	133	47.0	35.3	101	16.5	16.3	111	8.80	7.94	111	5.31	4.80	114	5.56	4.89
Big bluestem	312	12.5	3.99	294	9.75	3.32	318	0.81	0.26	310	9.21	2.97	344	2.72	0.79
Brachiaria (Mulato)	150	14.1	9.39	121	10.8	8.91	131	0.21	0.16	132	5.26	3.98	140	4.55	3.26
Corn silage 1	105	6.17	5.85	86.1	9.24	10.7	97.8	12.2	12.5	103	3.71	3.61	117	7.82	6.72
Corn silage 2 (BMR§)	103	15.2	14.8	67.2	4.62	6.88	73.9	0.01	0.01	75.2	6.02	8.01	90.9	13.6	14.9
Coastal panic grass	292	13.9	4.77	302	5.45	1.81	315	5.42	1.72	315	4.62	1.47	331	3.11	0.94
Festulolium	58.9	14.4	24.4	41.2	8.51	20.7	38.0	2.75	7.25	49.9	0.62	1.25	49.6	2.52	5.08
Meadow fescue	74.9	10.6	14.2	57.1	10.4	18.3	61.9	6.55	10.6	70.9	1.58	2.22	78.9	3.25	4.11
Miscanthus	471	23.6	5.01	464	11.5	2.48	485	12.8	2.64	488	4.92	1.01	509	1.76	0.35
Oat straw	282	23.0	8.16	281	15.0	5.33	313	3.72	1.19	295	7.91	2.68	337	7.95	2.36
Orchardgrass 1	115	14.7	12.8	106	14.5	13.6	117	0.91	0.77	112	0.83	0.75	130	1.98	1.53
Orchardgrass 2	90.9	11.0	12.1	71.0	5.71	8.03	77.0	1.75	2.28	80.5	5.30	6.59	82.7	3.86	4.67
Quackgrass	91.6	15.5	16.9	72.3	11.3	15.6	81.9	4.94	6.03	78.8	5.90	7.49	85.9	4.28	4.98
Reed canarygrass	82.1	16.5	20.2	63.0	13.8	21.9	77.8	0.49	0.63	74.3	4.77	6.43	79.2	1.63	2.06
Rice straw	233	16.4	7.03	171	6.53	3.82	185	1.17	0.63	180	4.60	2.56	196	5.76	2.94
Smooth bromegrass	95.1	17.8	18.7	71.2	8.70	12.2	76.5	12.0	15.7	81.6	4.72	5.78	89.1	6.57	7.38
Sorghum × sudangrass	161	13.7	8.50	141	8.54	6.07	155	8.45	5.47	158	1.99	1.26	170	14.4	8.48
Switchgrass	388	24.7	6.36	398	9.36	2.35	406	12.7	3.12	415	2.02	0.49	421	0.55	0.13
Timothy	77.3	16.1	20.9	56.5	10.9	19.3	64.0	7.72	12.1	63.8	2.65	4.16	68.4	2.97	4.35
Eastern gamagrass	172	8.32	4.84	149	11.6	7.82	163	2.86	1.76	149	1.68	1.13	172	2.50	1.46
Method avg.	173	17.4	12.8	154	10.3	9.84	165	5.59	4.68	165	4.11	3.27	177	4.78	3.84

† SS, Na_2SO_3 included in neutral detergent solution.

‡ DM, dry matter.

§ BMR, brown-midrib.

Table 5. Least squares means comparisons of the difference between undigested neutral detergent fiber at 30 h on an organic matter basis (uNDF30om) values for conventional flask digestion, ANKOM F57 or F58 bag digestion, and with or without Na_2SO_3 in the neutral detergent solution.

Method comparisons	Estimate†	*t* ratio	*p* value†
	g kg^{-1} dry matter		
Conventional flask SS and F57 no SS‡	–11.3	–2.67	0.0588
Conventional flask SS and F57 SS	2.61	0.62	0.9725
Conventional flask SS and F58 no SS	–20.8	–4.92	<0.0001
Conventional flask SS and F58 SS	–0.78	–0.19	0.9997
F57 no SS and F57 SS	13.9	3.28	0.0090
F57 no SS and F58 no SS	–9.54	–2.25	0.1604
F57 no SS and F58 SS	10.5	2.48	0.0946
F57 SS and F58 no SS	–23.4	–5.54	<0.0001
F57 SS and F58 SS	–3.39	–0.80	0.9301
F58 no SS and F58 SS	20.0	4.74	<0.0001

† Least squares means separation performed by Tukey's honestly significant difference test on an ANOVA with uNDF30om as the dependent variable and method and forage as main effects.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 6. Least squares means comparisons of the difference between undigested neutral detergent fiber at 120 h on an organic matter basis (uNDF120om) values for conventional flask digestion, ANKOM F57 or F58 bag digestion, and with or without Na_2SO_3 in the neutral detergent solution.

Method comparisons	Estimate†	*t* ratio	*p* value†
	g kg^{-1} dry matter		
Conventional flask SS and F57 no SS‡	–1.38	–0.46	0.9904
Conventional flask SS and F57 SS	7.34	2.48	0.0996
Conventional flask SS and F58 no SS	–7.19	–2.43	0.1120
Conventional flask SS and F58 SS	–2.59	–0.88	0.9057
F57 no SS and F57 SS	8.72	2.94	0.0295
F57 no SS and F58 no SS	–5.82	–1.96	0.2876
F57 no SS and F58 SS	–1.22	–0.41	0.9940
F57 SS and F58 no SS	–14.5	–4.91	<0.0001
F57 SS and F58 SS	–9.93	–3.35	0.0083
F58 no SS and F58 SS	4.60	1.55	0.5297

† Least squares means separation performed by Tukey's honestly significant difference test on an ANOVA with uNDF120om as the dependent variable and method and forage as main effects.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 7. Least squares means comparisons of the difference between undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) values for conventional flask digestion, ANKOM F57 or F58 bag digestion, and with or without Na_2SO_3 in the neutral detergent solution.

Method comparisons	Estimate†	*t* ratio	*p* value†
	g kg^{-1} dry matter		
Conventional flask SS and F57 no SS‡	8.24	3.36	0.0080
Conventional flask SS and F57 SS	18.5	7.55	<0.0001
Conventional flask SS and F58 no SS	–3.77	–1.54	0.5386
Conventional flask SS and F58 SS	7.55	3.08	0.0196
F57 no SS and F57 SS	10.2	4.18	0.0004
F57 no SS and F58 no SS	–12.0	–4.90	<0.0001
F57 no SS and F58 SS	–0.69	–0.28	0.9986
F57 SS and F58 no SS	–22.2	–9.08	<0.0001
F57 SS and F58 SS	–10.9	–4.46	0.0001
F58 no SS and F58 SS	11.3	4.62	0.0001

† Least squares means separation performed by Tukey's honestly significant difference test on an ANOVA with uNDF240om as the dependent variable and method and forage as main effects.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 8. Least squares means of SDs of the difference between undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) values between methods for conventional flask digestion, ANKOM F57 or F58 bag digestion, and with or without Na_2SO_3 in the neutral detergent solution. Standard error of least squares means is 1.45 for the linear regression model with uNDF240om SD between methods as the dependent variable with method comparison and forage as main effects.

Method comparisons	Least squares means†
	g kg^{-1} dry matter
Conventional flask SS and F57 no SS‡	11.3
Conventional flask SS and F57 SS	14.2
Conventional flask SS and F58 no SS	11.2
Conventional flask SS and F58 SS	10.2
F57 no SS and F57 SS	7.44
F57 no SS and F58 no SS	8.49
F57 no SS and F58 SS	4.04
F57 SS and F58 no SS	15.7
F57 SS and F58 SS	7.94

† Least squares means corrected by Tukey's honestly significance test.

‡ SS, Na_2SO_3 included in neutral detergent solution.

rates of digestion (Tables 14 and 15). Particle size reduction in legumes has been reported to be initiated earlier and occurred faster in legumes compared with grasses (Bowman and Firkins, 1995), which would contribute to faster rates of digestion (Table 15).

Ruminal Fluid Refreshing

Comparisons between ANKOM F57 with ruminal inoculum plus buffer refreshing every 2 d, ANKOM F57 without ruminal inoculum plus buffer refreshing, and the conventional flask method without ruminal inoculum plus buffer refreshing are shown in Table 16. The conventional flask method was not significantly different in uNDF240om from F57 with ruminal inoculum plus buffer refreshing. The conventional flask method had significantly smaller uNDF240om values compared with F57 without ruminal inoculum plus buffer refreshing (Table 16). Ruminal fluid plus buffer refreshing every 48 h did result in significantly smaller uNDF240om values compared with no ruminal fluid plus buffer refreshing, for the F57 bags. Least squares means of uNDF240om values averaged over 24 forages were 196, 173, and 166 g kg^{-1} dry matter (SE $\pm$ 2.95) for ANKOM F57 without ruminal fluid plus buffer refreshing, conventional flask method, and ANKOM F57 with ruminal fluid plus buffer refreshing, respectively.

DISCUSSION

System Comparisons

Our objective was to compare F57 and F58 filter bags in the ANKOM system with the conventional flask system for in vitro long digestions. We followed ostensibly standard procedures for both methods, which resulted in the use of different in vitro buffers. It was not feasible to conduct all in vitro studies at the same site using the same ruminal fluid inoculum. However, since different donor cows with different diets were used for ANKOM and conventional flask systems, direct comparison of digestion values between these two systems was confounded with ruminal fluid source. Although this can affect direct comparisons of digestion values between ANKOM and conventional flask systems, it is less likely to affect differences within method replicates.

Table 9. Spearman correlation coefficients (*r*) of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) and rates of digestion for five methods compared over 24 forage samples.

Method correlations	*r* of uNDF240om	*r* of rates of digestion
Conventional flask SS and F57 no SS†	0.977***	0.697***
Conventional flask SS and F57 SS	0.989***	0.729***
Conventional flask SS and F58 no SS	0.983***	0.693***
Conventional flask SS and F58 SS	0.981***	0.755***
F57 no SS and F57 SS	0.986***	0.968***
F57 no SS and F58 no SS	0.978***	0.970***
F57 no SS and F58 SS	0.983***	0.977***
F57 SS and F58 no SS	0.982***	0.956***
F57 SS and F58 SS	0.994***	0.964***

*** Significant at the 0.001 probability level.

† SS, Na_2SO_3 included in neutral detergent solution.

Implications of Undigested Neutral Detergent Fiber Data on an Organic Matter Basis

Five of the ten uNDF30om least squares means comparisons were different ($p < 0.05$) (Table 5), and three of the ten uNDF120om least squares means comparisons were different (Table 6) without any clear trend. Eight of the ten least squares means comparisons were different for uNDF240om values, excluding the conventional flask to ANKOM F58 without Na_2SO_3 and ANKOM F57 without Na_2SO_3 to F58 with Na_2SO_3 (Table 7). The lack of significance in the uNDF240om select comparisons was likely from the opposite effects of Na_2SO_3 and pore size. Reduced pore size increased uNDFom by allowing less residual NDF to escape, whereas the Na_2SO_3 decreased uNDFom by decreasing nitrogenous concentrations in residues and potentially reducing lignin concentrations (Hintz and Mertens, 1996). Digestion times >240 h might be required to clarify interactions further.

Rates of Digestion

Where we observed significant differences between methods for uNDF30om, uNDF120om, and uNDF240om values (Tables 5–7), we did not observe differences between the rates of digestion (Table 11). Variation between uNDFom values at four time points (0, 30, 120, 240 h) was consistently either larger or smaller depending on the method, which essentially shifted the digestion curve up or down

but kept the rate similar. Spearman rank correlations (Table 9) of digestion rates were all high and significant between methods, showing that rates were ordered similarly for each forage. Minor variations observed between uNDFom values did not affect rate estimates.

Differences among Grass and Legume Forages

Our research analyzed a diverse range of forages that had, at times, drastically different digestion behaviors. Compared with the flasks, ANKOM bags generally produced larger uNDF240om values for grasses that tended to have greater uNDF240om values such as oat straw, coastal panic grass [*Panicum amarum* Elliott (Hitchc. & Chase) P.G. Palmer], big bluestem, switchgrass, and miscanthus (*Miscanthus* ×*giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize) (Table 4) but produced smaller uNDF240om values for grasses that tended to have smaller uNDF240om values such as festulolium [*Festulolium braunii* (K. Richt.) Stace], timothy (*Phleum pratense* L.), and quackgrass [*Elytrigia repens*

Table 10. Rates of digestion observed means, SDs, and CVs for 24 forages.

	Method														
	Flask SS†			F57 SS			F57 no SS			F58 SS			F58 no SS		
Forage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
	— % h^{-1} —		%	— % h^{-1} —		%	— % h^{-1} —		%	— % h^{-1} —		%	— % h^{-1} —		%
Alfalfa 1	5.11	1.76	34.4	10.5	2.55	24.2	10.7	2.69	25.1	10.5	0.21	2.03	10.9	1.41	13.0
Alfalfa 2	6.56	2.12	32.4	9.35	0.64	6.81	8.70	3.25	37.4	8.40	0.71	8.42	11.5	0.92	8.03
Alfalfa 3 (low lignin)	8.25	2.28	27.7	8.25	0.49	6.00	11.3	3.54	31.3	10.7	1.06	9.96	9.00	0.71	7.86
Birdsfoot trefoil	7.92	0.82	10.3	8.90	0.85	9.53	9.50	0.42	4.47	8.45	1.91	22.6	8.30	0.85	10.2
Red clover	3.03	0.15	4.88	6.85	0.78	11.4	7.25	0.92	12.7	7.35	0.21	2.89	7.10	1.56	21.9
Big bluestem	3.38	1.15	34.1	1.85	0.07	3.82	1.95	0.07	3.63	2.05	0.07	3.45	2.15	0.21	9.87
Brachiaria (Mulato)	4.98	0.01	0.11	4.35	0.07	1.63	4.80	0.28	5.89	5.55	0.64	11.5	4.65	0.07	1.52
Corn silage 1	6.09	1.09	17.9	4.15	0.07	1.70	4.00	0.57	14.1	4.35	0.07	1.63	4.35	0.21	4.88
Corn silage 2 (BMR‡)	5.56	0.44	7.92	2.25	0.07	3.14	2.10	0.28	13.5	3.25	0.49	15.2	2.45	0.35	14.4
Coastal panic grass	3.06	0.52	16.9	2.35	0.07	3.01	2.50	0.14	5.66	2.20	0.00	0.00	2.55	0.21	8.32
Festulolium	7.51	0.20	2.68	8.65	0.21	2.45	7.65	0.49	6.47	7.80	0.99	12.7	7.25	0.64	8.78
Meadow fescue	7.78	0.18	2.31	6.95	0.78	11.2	6.85	0.64	9.29	7.50	0.57	7.54	7.45	0.07	0.95
Miscanthus	3.69	0.21	5.71	2.50	0.14	5.66	2.55	0.07	2.77	2.65	0.35	13.3	2.65	0.49	18.7
Oat straw	3.71	0.14	3.75	2.55	0.07	2.77	2.50	0.14	5.66	2.45	0.21	8.66	3.00	0.57	18.9
Orchardgrass 1	6.62	0.02	0.26	7.00	0.14	2.02	7.05	0.35	5.01	6.15	0.64	10.4	7.60	0.00	0.00
Orchardgrass 2	6.51	0.16	2.46	6.50	0.71	10.9	6.35	0.07	1.11	6.45	0.64	9.87	6.00	0.42	7.07
Quackgrass	6.45	0.25	3.93	7.35	0.21	2.89	6.70	0.57	8.44	6.95	0.07	1.02	6.60	0.28	4.29
Reed canarygrass	7.87	0.96	12.1	7.55	0.49	6.56	7.35	0.35	4.81	7.55	0.21	2.81	6.60	0.00	0.00
Rice straw	3.52	0.04	1.01	2.80	0.00	0.00	2.90	0.00	0.00	2.85	0.07	2.48	2.50	0.14	5.66
Smooth bromegrass	7.63	0.00	0.02	4.90	0.00	0.00	3.95	0.35	8.95	5.65	0.49	8.76	4.50	0.28	6.29
Sorghum × sudangrass	4.45	0.46	10.2	3.50	0.14	4.04	3.55	0.07	1.99	4.05	0.21	5.24	3.40	0.14	4.16
Switchgrass	2.60	0.57	22.0	1.90	0.14	7.44	1.95	0.07	3.63	2.15	0.07	3.29	2.00	0.00	0.00
Timothy	7.13	0.25	3.57	7.15	0.07	0.99	6.10	0.28	4.64	6.50	0.14	2.18	6.45	0.21	3.29
Eastern gamagrass	4.30	0.67	15.6	3.80	0.14	3.72	3.85	0.07	1.84	3.85	0.21	5.51	3.70	0.00	0.00
Method avg.	5.57	0.60	11.4	5.50	0.37	5.49	5.50	0.65	9.10	5.64	0.43	7.14	5.53	0.41	7.42

† SS, Na_2SO_3 included in neutral detergent solution.

‡ BMR, brown-midrib.

Table 11. Least squares means comparisons of the difference between digestion rates for conventional flask digestion, ANKOM F57 or F58 bag digestion, and with or without Na_2SO_3 in the neutral detergent solution.

Method comparisons	Estimate†	*t* ratio	*p* value†
	% h^{-1}		
Conventional flask SS and F57 no SS‡	0.06	0.29	0.9985
Conventional flask SS and F57 SS	0.07	0.32	0.9977
Conventional flask SS and F58 no SS	0.04	0.19	0.9997
Conventional flask SS and F58 SS	−0.07	−0.30	0.9983
F57 no SS and F57 SS	0.01	0.04	1.0000
F57 no SS and F58 no SS	−0.02	−0.09	1.0000
F57 no SS and F58 SS	−0.13	−0.58	0.9779
F57 SS and F58 no SS	−0.03	−0.13	0.9999
F57 SS and F58 SS	−0.14	−0.62	0.9723
F58 no SS and F58 SS	−0.11	−0.49	0.9884

† Least squares means corrected by Tukey's honestly significance test for a linear regression model with rate of digestion as the dependent variable and method and forage as main effects.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 12. Least squares means and comparison of the difference between estimates of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) between methods for grass forages (n = 19). Standard error of least squares means is 2.72 for the grass forage type in a linear regression with uNDF240om as the dependent variable, method and legume or grass fixed effects, an interaction between method and legume or grass, and legume or grass nested within forage.

Method	LS mean†	Flask SS‡	F57 SS	F57 no SS	F58 SS
	g kg^{-1} dry matter				
Flask SS	177	–	–	–	–
F57 SS	159	18.1***	–	–	–
F57 no SS	170	6.27	11.8***	–	–
F58 SS	170	6.49	–11.6***	0.22	–
F58 no SS	184	–7.05	–25.2***	–13.3***	13.5***

*** Significant at the 0.001 probability level.

† Least squares means separation performed by Tukey's honestly significant difference test.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 13. Least squares means and comparison of the difference between estimates of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) between methods for legume forages (n = 5). Standard error of least squares means is 5.30 for the legume forage type in a linear regression with uNDF240om as the dependent variable, method and legume or grass fixed effects, an interaction between method and legume or grass, and legume or grass nested within forage.

Method	LS mean†	Flask SS‡	F57 SS	F57 no SS	F58 SS
	g kg^{-1} dry matter				
Flask SS	158	–	–	–	–
F57 SS	138	19.9*	–	–	–
F57 no SS	143	15.7*	4.22	–	–
F58 SS	147	11.6	–8.36	–4.15	–
F58 no SS	150	8.70	–11.2	–6.99	2.85

* Significant at the 0.05 probability level.

† Least squares means separation performed by Tukey's honestly significant difference test.

‡ SS, Na_2SO_3 included in neutral detergent solution.

(L.) Nevski] (Table 4). This effect might have to do with a buildup of gas; however, gas retention in bags should have more influence early in digestion, and more influence on forages that digest quickly, which would affect rate of digestion more than extent.

Legumes such as birdsfoot trefoil (*Lotus corniculatus* L.) and red clover were largely digested by 30 h, whereas some grasses such as oat straw or rice straw (*Oryza sativa* L.) continued digesting well after 120 h. Legumes that digested more quickly had greater relative digestion rates (Table 15) than grasses that digested more uniformly over the 240 h (Table 14). One plausible reason for legume digestion behavior could be particle size after grinding. When forages are mechanically ground, grasses tend to shred, producing long thin pieces, whereas legumes typically

Table 14. Least squares means and comparison estimates of rate of digestion between methods for grass forages (n = 19). Standard error of least squares means is 0.14 for the grass forage type in a linear regression with rate of digestion as the dependent variable, method and legume or grass fixed effects, an interaction between method and legume or grass, and legume or grass nested within forage.

Method	LS mean†	Flask SS‡	F57 SS	F57 no SS	F58 SS
	g kg^{-1} dry matter				
Flask SS	5.40	–	–	–	–
F57 SS	4.63	0.77***	–	–	–
F57 no SS	4.46	0.95***	–0.18	–	–
F58 SS	4.73	0.67***	–0.10	–0.28	–
F58 no SS	4.52	0.89***	0.12	–0.06	–0.22

*** Significant at the 0.001 probability level.

† Least squares means separation performed by Tukey's honestly significant difference test.

‡ SS, Na_2SO_3 included in neutral detergent solution.

Table 15. Least squares means and comparison estimates of rate of digestion between methods for legume forages (n = 5). Standard error of least squares means is 0.28 for the legume forage type in a linear regression with rate of digestion as the dependent variable, method and legume or grass fixed effects, an interaction between method and legume or grass, and legume or grass nested within forage.

Method	LS mean†	Flask SS‡	F57 SS	F57 no SS	F58 SS
	g kg^{-1} dry matter				
Flask SS	6.18	–	–	–	–
F57 SS	8.77	–2.59***	–	–	–
F57 no SS	9.49	–3.31***	0.72	–	–
F58 SS	9.06	–2.88***	–0.29	0.43	–
F58 no SS	9.35	–3.17***	–0.58	0.14	0.29

*** Significant at the 0.001 probability level.

† Least squares means separation performed by Tukey's honestly significant difference test.

‡ SS, Na_2SO_3 included in neutral detergent solution.

shatter to smaller more cubical shapes (Van Soest, 1994; Paulson et al., 2008). Smaller legume particles that escape digestion bags early in the digestion run would have a larger effect with reducing uNDF30om for ANKOM methods compared with the flask system. Although legumes usually have greater lignin content than grasses, they are low in esterified hydroxycinnamic acids and high in syringyl and guaiacyl units. This implies that legumes have less condensed types of lignins, which affect cell wall degradation and protection and render explanations of legume degradation different than grass forages (Raffrenato, 2011). There are likely other factors such as stage of maturity and agronomic conditions like water stress, heat, and light that also influence the creation of lignin linkages and subsequent degradation (Raffrenato, 2011). More research is required to understand how and why degradation rates vary by forage types. Even considering these differences among the methods in their behaviors

Table 16. Least squares means comparisons of undigested neutral detergent fiber at 240 h on an organic matter basis (uNDF240om) with ruminal fluid plus buffer refreshing during in vitro digestions for conventional flask digestion vs. ANKOM F57 bag digestion, all with Na_2SO_3 in the neutral detergent solution.

Method comparisons	Estimate†	*t* ratio	*p* value
	g kg^{-1} dry matter		
Conventional flask and F57 no ruminal inoculum plus buffer refreshing	−23.4	−5.60	<0.0001
Conventional flask and F57 with ruminal inoculum plus buffer refreshing	6.38	1.53	0.2821
F57 no ruminal inoculum refreshing and F57 with ruminal inoculum plus buffer refreshing	29.8	7.13	<0.0001

† Least squares means separation performed by Tukey's honestly significant difference test of an ANOVA model with uNDF240om as the dependent variable and method and forage as main effects.

toward legumes and grasses, the high correlations between the ranking of forages and Spearman rank correlations (Table 9) indicated that all five methods ranked samples in the same relative order for uNDF240om. Each method behaved similarly with each forage.

Residual Neutral Detergent Fiber as a Function of Pore Size

Pore size explained variation within the varying levels of uNDF240om between methods. The conventional flask method included glass fiber mats with a pore size of 2.5 μm for filtration. The F57 bags have a pore size of 25 μm, and F58 bags have an 8- to 10-μm pore size. There was a relationship (Fig. 1) between pore size in the filtration method and residual NDF at 240 h. Methods with smaller pore size filters tend to have greater average uNDF240om values than methods with larger pore size filters. We expected a similar finding because potentially undigested NDF may be retained with finer filters, whereas potentially indigestible and digestible NDF may inadvertently escape from a coarser filter. Filtering difficulties tend to increase with decreased size of filter pores. The three methods with Na_2SO_3 were negatively correlated to uNDF240om with a correlation of −0.993. This relation indicates that uNDF linearly increases as filtration pore size decreases.

Potentially counterbalancing the effect of pore size, ANKOM F57 and F58 bags trap some gas produced during digestion, which could potentially reduce digestion when compared with the conventional flask method, and F58 bags appeared to trap more gas more than F57 bags. Buildup of fermentation gas in bags with small pores can restrict microbial access to the substrate (Valente et al., 2015) or potentially cause floating of bags above the in vitro solution, or depression of pH (Kitessa et al., 1999). Expelling of gas, which was done at 2-d intervals in this study, should decrease any adverse effects that entrapped gases may have on sample digestibility (Adesogan, 2005). Expelling gas was a brief event that could result in the expulsion of some small particles, although there is no evidence to prove or disprove this, and any such expulsion is unlikely to affect results significantly.

Differences within and between Methods

ANKOM F58 bags were successfully used for long-term in vitro digestions; however, the outer layer of material on the bags tended to dislodge to some extent and bunch up before 30 h of digestion and presented a ragged appearance on the bag surface. After digestion and neutral detergent extraction, weight of dried blank (empty) F57 bags in this study averaged 99.8% (SD = 0.18, n = 130) of the original undried weight. The weight of dried blank F58 bags after neutral detergent extraction averaged 96.9% (SD = 0.51, n = 130) of the original undried weight. Weight loss of F57 blank bags was similar for 30, 120, and 240 h of digestion, and weight loss of F58 bags also was consistent over digestion times. Although F58 blank bags were more variable in weight loss after digestion and neutral detergent extraction, this did not appear to translate to more variable results from F58 bags than from F57 bags. It may be prudent to use more blank bags for F58 digestion runs than required for F57 bags.

Table 4 shows the SD between uNDF240om of their replicated runs. The conventional flask method had the largest difference within runs compared with the ANKOM bag methods. When comparing average uNDF240om between methods by the SD (Table 8), all comparisons' SDs were less than the SD observed within the conventional flask method's replicated runs in Table 4. Considering that within-method variation was, at times, larger than between-method variation indicates that uNDF240om values from separate methods could provide as reliable results as comparing uNDF240om values from the same method and laboratory. The degree of variation within the conventional flask replicates underpins the concept that uNDF240om values should represent a relative index of forage quality rather than a precise measure of extent.

Changing Ruminal Fluid

To accurately simulate the rumen, one would surmise that regular refreshing of ruminal inoculum plus buffer would be advantageous because rumen microorganism populations change over 240 h in the rumen. ANKOM has the advantage of easily changing ruminal fluid plus buffer during in vitro analyses because of system design. Changing ruminal inoculum plus buffer is more complicated with the

conventional flask system. Changing ruminal inoculum plus buffer did alter uNDF240om for F57 ANKOM bags. Results (Table 16) showed that ANKOM F57 without ruminal fluid plus buffer refreshing left the most residual NDF, followed by the flask method, and finally ANKOM F57 with ruminal fluid plus buffer refreshing. Results indicate that refreshing ruminal fluid plus buffer reduces uNDF240om levels more than the observed pore size effect. ANKOM F57 bags' larger porosity compared with the flask method would suggest a smaller uNDF240om value, but without refreshing ruminal fluid plus buffer, the uNDF240om value increased considerably for the ANKOM F57 bags. Comparisons between the ruminal fluid refreshing plus buffer methods showed that the conventional flask and F57 with ruminal fluid plus buffer refreshing were not different from one another, whereas both other comparisons were different (Table 16). F57 bags receiving refreshed ruminal fluid plus buffer also had any gas buildup pressed out during fluid changes. This reduction in gas formation may have increased digestion but was confounded with the ruminal fluid plus buffer refreshing that also increased digestion. Our findings contradict the widely practiced concept that deems it unnecessary to change ruminal fluid throughout long in vitro laboratory tests (Palmonari et al., 2017). We hypothesize that if the conventional flask method could operationalize regular ruminal inoculum plus buffer refreshing for long digestions, uNDF240om may further decrease.

CONCLUSIONS

This study established that uNDF240om values were significantly different from one another among methods, but rates of digestion were not significantly different, although ANKOM and conventional flask methods were confounded with different ruminal fluid inocula. Method pore size for final filtration was an important contributing factor in determining the extent of digestion at 240 h. ANKOM F58 was as precise as ANKOM F57 and the conventional flask method for long in vitro digestions and displayed a greater level of precision when considering SDs between replicated runs, although some apparent degradation of F58 bags was observed. Producers should consider in vitro laboratory analyses as a relative index of forage quality that can be useful for comparing forages with each other or comparing the same forage over time. Using the same laboratory technique, whether it be the conventional flask, ANKOM F57, or ANKOM F58, for in vitro analysis will give similar results for rates of digestion.

Conflict of Interest

The authors declare that there is no conflict of interest.

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