SPECIAL SECTION ON FEED ADDITIVES AND CONTAMINANTS

Evaluation of Analytical Methods for the Determination of Moisture, Crude Protein, Crude Fat, and Crude Fiber in Distillers Dried Grains with Solubles

NANCY THIEX
South Dakota State University, Veterinary Science Department, Box 2170, ASC 136A, Brookings, SD 57007

A number of analytical methods for constituents commonly measured in distillers dried grains (DDG) are practiced in laboratories serving the agricultural sector. A large interlaboratory variability among results has been observed in the industry. Methods for moisture, crude fat, and crude fiber are empirical, thus part of this variability can be attributed to the use of different methods of analysis. A study was organized and supported by the American Feed Industry Association, the Renewable Fuels Association, and the National Corn Grain Association to evaluate the efficacy, applicability, and the intralaboratory variation of a number of methods for moisture, crude protein, crude fat, and crude fiber in DDG with solubles (DDGS). The moisture methods included in the study are AOAC 930.15, AOAC 934.01, AOAC 935.29, AOAC 2003.06, and National Forage Testing Association (NFTA) 2.2.2.5; the crude protein methods studied are AOAC 990.03 and AOAC 2001.13; the crude fat methods studied are AOAC 945.16, AOAC 954.02, AOAC 2003.05, and AOAC 2006.06; and the crude fiber methods studied are AOAC 978.10 and AOCS Ba 6a-05. A second study was undertaken to assess existing interlaboratory variation of the same methods in 23 laboratories. Based on the results of these studies, the sponsoring associations established recommended reference methods for use in commercial trade of DDGS. The reference methods selected are NFTA 2.2.2.5 for moisture, AOAC 990.03 and AOAC 2001.11 for crude protein, AOAC 945.16 for crude fat, and AOAC 978.10 for crude fiber.

Corn distillers dried grains (DDG) and corn DDG with solubles (DDGS) are co-products of fuel and beverage ethanol distilleries. They are obtained after the removal of ethanol by distillation from the yeast fermentation of a grain or a grain mixture by either separating the resultant coarse grain fraction of the whole stillage and drying, in the case of DDG, or by condensing and drying at least ¾ of the solids, in the case of DDGS. At an estimated 16 million tons for the 2007 year, DDG are the second-largest processed feed ingredient in the United States, second only to soybean meal (personal communication, Charles Staff, Distillers Grain Technology Council, Louisville, KY). Thus hundreds of DDG products are analyzed daily in the United States for nutritional components, quality control, marketing purposes, and ration formulation.

The absence of industry guidelines and recommendations on analytical test methods for the testing of DDGS has led to a high level of confusion related to analysis and subsequent interpretation of data for moisture, protein, fat, and fiber, all of which are critical feed qualities and trade parameters for DDGS. Most methods in use for the analysis of DDGS can be classified as empirical methods, meaning the results are defined by the method. Thus any change to the conditions of the method for the analyte of interest (time, temperature, particle size, reagent type, reagent concentration, etc.) would bias the results obtained. Because neither the industry nor the analytical community had standardized methods for the analysis of any given analyte in DDG, many different test conditions are in use among laboratories and often even within a single laboratory. This situation provides for results that vary significantly from laboratory-to-laboratory and thereby creates confusion for producers, marketers, nutritionists, regulatory bodies, and most importantly the customers/end users.

In the fall of 2005, the industry formed 2 working groups to collectively address the problem and cooperatively design a study that would lead to concrete recommendations on the most applicable test methods for DDGS. The 2 bodies groups formed were the Renewable Fuels Association (RFA) Testing Subcommittee, operating under the RFA Co-Products Committee, and the American Feed Industry Association (AFIA) DDGS Analytical Methods Sub-Working Group, operating under the AFIA DDGS Technical Issues Working Group. Members of the 2 groups are identified in Table 1.

The AFIA DDGS Analytical Methods Sub-Working Group was responsible for setting the direction of the study, saw to its completion, reported the final outcome back to

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Corresponding author's e-mail: nancy.thiex@sdstate.edu
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Table 1. Member stakeholders of the RFA testing subcommittee and the AFIA DDGS Analytical Methods Sub-working Group

Name	Company	RFA Body	AFIA Body
Shon Van Hulzen[a]	POET Management	✓	✓
Lance Forster	ADM	✓	✓
Charlie Staff	Distillers Grain Technology Council	✓	✓
Bob Dinneen	Renewable Fuel Association		✓
Thomas Robb	Abengoa Bioenergy	✓	
Thomas Sliffe	Perten Instruments		✓
Trace Yates	Tyson Foods, Inc.	✓	
Mark Host	FOSS North America		✓
Lars Reimann	Eurofins Scientific	✓	
Phil Smith	Tyson Foods, Inc.		✓

[a] Committee Chair for both the RFA and AFIA committees.

industry stakeholders, and made recommendations based on the data gathered to the members of the 2 associations. The RFA Co-Products Subcommittee provided input and insights from the perspective of the ethanol industry and also provided several members to serve on the AFIA group, which added several representatives from the feed industry as well as other stakeholder members. Funding for the study was provided by RFA, AFIA, and the National Corn Growers Association.

Experimental

Materials—Phase I and Phase II

Thirty DDGS materials collected from 6 locations (5 samples from each location) were selected by the AFIA DDGS Analytical Methods Sub-Working Group to be representative of the DDGS on the market nationwide. The 6 locations were:

(*1*) 2 locations from POET Companies corn dry mill plants (2 different processes);

(*2*) 2 locations from ADM corn dry mill plants (2 different processes);

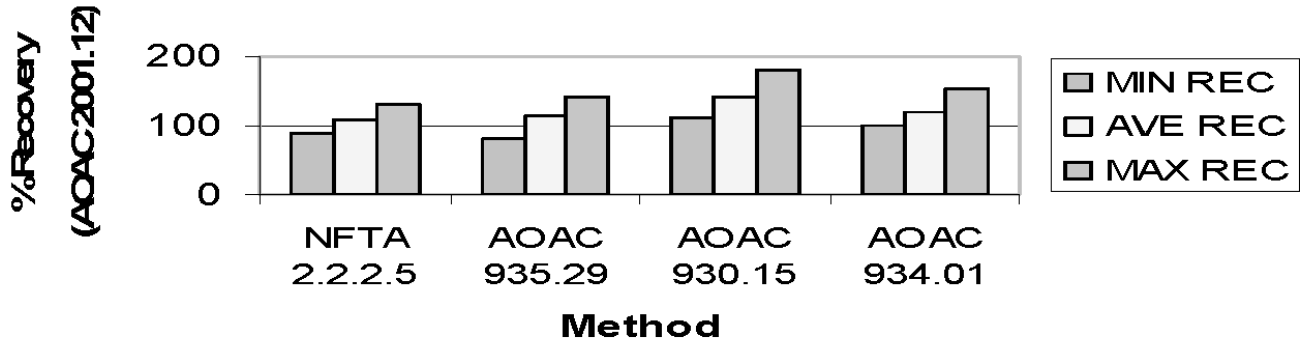

Figure 1. Average moisture recovery as a percentage of the Karl Fischer result for LOD methods.

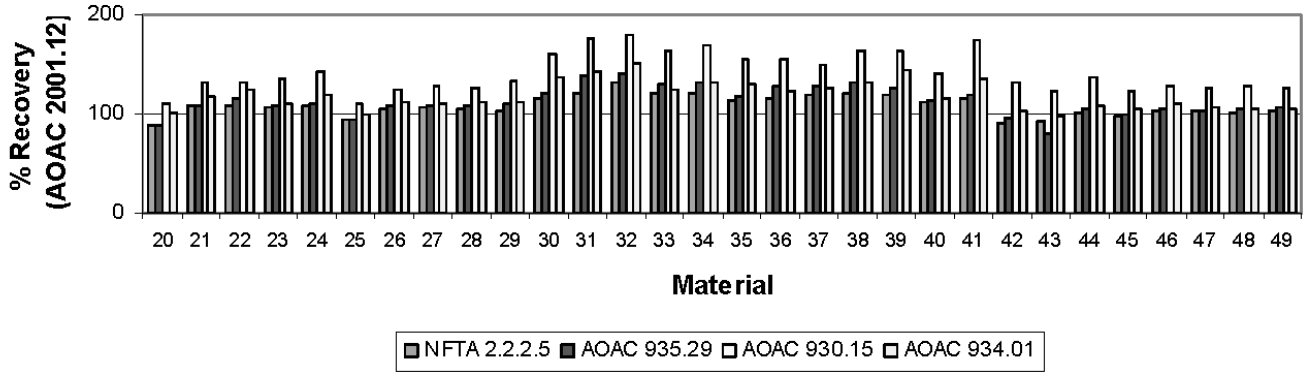

Figure 2. Moisture recovery for loss on drying methods by DDGS material.

Table 2. Moisture results[a] by method for 30 DDGS materials

	AOAC **2001.12**	NFTA **2.2.2.5**	AOAC **935.29**	AOAC **930.15**	AOAC **934.01**
Material	Moisture, %		LOD, %		
20	8.57	7.68	7.73	9.42	8.65
21	7.69	8.43	8.42	10.18	9.13
22	8.22	8.95	9.53	10.84	10.32
23	7.59	8.16	8.25	10.25	8.40
24	7.06	7.73	7.78	10.03	8.39
25	12.08	11.44	11.40	13.39	12.11
26	13.29	14.07	14.58	16.71	14.96
27	11.98	12.79	13.07	15.44	13.30
28	11.99	12.62	13.12	15.21	13.43
29	9.96	10.94	10.38	13.32	11.24
30	8.81	10.29	10.62	14.20	12.16
31	6.52	7.92	9.06	11.54	9.31
32	8.58	11.28	12.08	15.42	13.02
33	8.27	10.02	10.76	13.60	10.32
34	7.84	9.46	10.33	13.29	10.37
35	9.31	10.60	11.05	14.52	12.18
36	9.16	10.62	11.73	14.30	11.29
37	10.70	12.74	13.78	16.01	13.50
38	9.42	11.48	12.40	15.49	12.49
39	9.37	12.24	11.83	15.33	13.60
40	9.19	10.28	10.52	13.00	10.62
41	5.61	6.52	6.76	9.86	7.62
42	7.01	6.42	6.71	9.31	7.30
43	8.35	7.78	6.71	10.30	8.26
44	7.51	7.64	7.85	10.31	8.18
45	9.63	9.48	9.58	11.91	10.17
46	8.62	9.10	8.97	11.16	9.50
47	9.27	9.66	9.67	11.77	9.90
48	9.09	9.30	9.53	11.66	9.62
49	10.16	10.45	10.88	12.87	10.71
Mean[b]	9.03[e]	9.87[d, e]	10.17[d]	12.69[c]	10.67[d]
Avg. % recovery (KF)[f]	100	110	113	142	119
Max. % recovery (KF)		131	141	180	152
Min. % recovery (KF)		90	80	110	99
Avg. bias (KF)		0.84	1.14	3.66	1.64
High bias (KF)		2.87	3.50	6.84	4.23
Low bias (KF)		–0.89	–1.64	0.85	–0.09

[a] Each value is an average of triplicate measurements.

[b,c,d,e] Means with the same letter are not significantly different (P <0.0001).

[f] KF = Karl Fischer or AOAC **2001.12**.

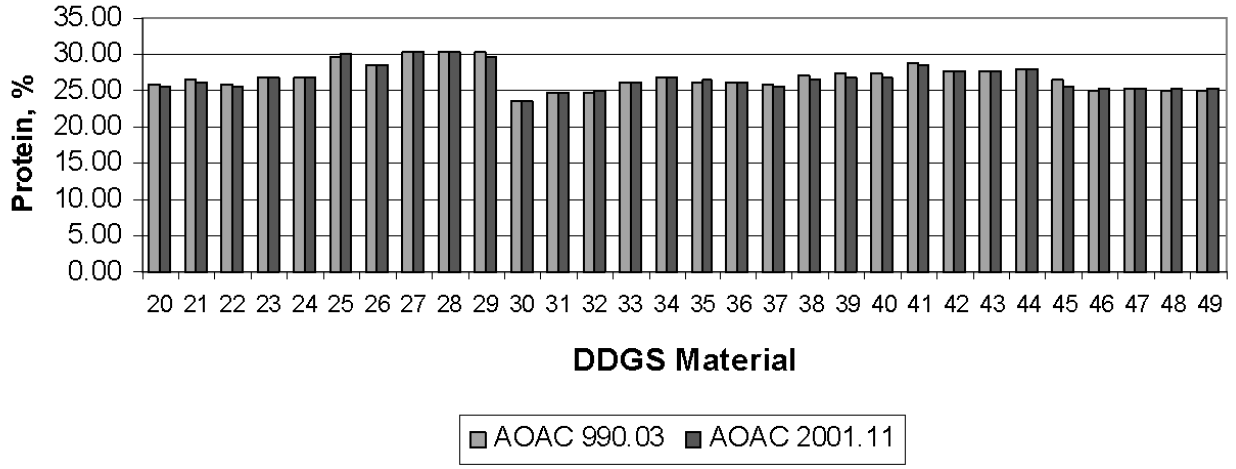

Figure 3. Protein results by method and DDGS material.

(*3*) One location from an alternative feedstock dry mill (Western Plains Energy in Oakley, KS); and

(*4*) One location from a beverage (portable) plant (Jim Beam).

All DDGS materials were shipped by the respective distilleries to South Dakota State University (SDSU) during May and June of 2006 for analysis. Particle size reduction for all materials was accomplished using a Retsch ZM100 Mill (Retsch, Haan, Germany) equipped with a 0.75 mm screen. Sample size reduction was done using a Fritsch Rotary Sample Divider (Fritsch GmbH, Idar-Oberstein, Germany). Materials were randomly assigned identification numbers 20 through 49.

Phase I Methods

Phase I of the project was designed to evaluate the accuracy (where possible), within-laboratory variation, and the bias among test methods for moisture, crude protein, crude fat, and crude fiber. The analytes had been targeted by the stakeholder body as critical in the trading and marketing of DDGS. The 30 DDGS materials were analyzed in triplicate for moisture, crude protein, crude fat, and crude fiber by each of the methods established by the stakeholder body as methods of interest. The chosen methods had to be methods endorsed or adopted by a methods validation association, preferably AOAC INTERNATIONAL, and be in routine use in feed laboratories. Methods evaluated in the study for DDGS materials were the following:

(**a**) *Moisture analysis*.—Loss on Drying at 95–100°C (vacuum oven), AOAC **934.01** (1); Moisture in Malt (103–104°C/5 h), AOAC **935.29** (2); Lab Dry Matter (105°C/3 h), NFTA **2.2.2.5** (3); Loss on Drying (135°C/2 h), AOAC **930.15** (4); Water/Dry Matter (Karl Fischer), AOAC **2001.12** (5).

(**b**) *Protein analysis*.—Crude Protein (Combustion), AOAC **990.03** (6); Crude Protein (Kjeldahl Copper), AOAC **2001.11** (7).

(**c**) *Fat analysis*.—Crude Fat (Randall/Soxtec/Ether-Submersion Method), AOAC **2003.05** (8); Fat (Acid Hydrolysis), AOAC **954.02** (9); Oil in Cereal (Pet Ether), AOAC **945.16** (10); Crude Fat (Randall/Soxtec/Hexanes–Submersion Method), AOAC **2003.06** (11).

(**d**) *Fiber analysis*.—Fiber in Animal Feed (Fritted Glass Crucible), AOAC **978.10** (12); ANKOM, American Oil Chemists' Society (AOCS) Ba **6a-05** (13).

Analysis by each method was conducted in triplicate at SDSU Olson Biochemistry Laboratories. Test portions for all 5 moisture methods were weighed on the same day for each material to ensure that observed differences in method precision and bias were due to actual method performance and to keep environmental influences to an absolute minimum. Due to the problems with commercial availability of filter bags for the AOCS Ba **6a-05** method, a modification of the method was tested to determine the bias introduced by the use of a commercially available filter bag.

The SAS/INSIGHT User's Guide (14) was used to determine if methods for the same constituent were statistically different.

Phase II Methods

Phase II of the study was designed to evaluate the interlaboratory variation. One of each of 5 types of DDGS from the Phase I project was prepared for use in Phase II. The sixth type (from the beverage plant) was eliminated due to the receipt of insufficient material. A gated riffle splitter was used to reduce the ground bulk sample into 4 equal portions, 3 of which were further reduced to 250 g portions using a Fristch Rotary Splitter, resulting in 24 laboratory samples. Homogeneity testing was conducted by randomly selecting one split sample from each of 3 batches of 8, resulting in

Table 3. Crude protein results[a] by method for 30 DDGS materials

Material	Crude protein, %		
	AOAC **990.03**	AOAC **2001.11**	Bias
20	25.99	25.70	0.29
21	26.40	26.22	0.18
22	25.79	25.48	0.32
23	26.68	26.72	–0.04
24	26.71	26.72	–0.01
25	29.71	29.89	–0.19
26	28.47	28.43	0.03
27	30.33	30.29	0.04
28	30.39	30.22	0.17
29	30.16	29.65	0.51
30	23.51	23.56	–0.05
31	24.73	24.80	–0.07
32	24.66	24.88	–0.22
33	26.11	26.27	–0.16
34	26.79	26.82	–0.03
35	26.28	26.35	–0.07
36	26.30	26.29	0.02
37	25.80	25.72	0.08
38	27.00	26.59	0.41
39	27.24	26.88	0.35
40	27.44	26.87	0.57
41	28.70	28.51	0.19
42	27.72	27.62	0.10
43	27.78	27.56	0.22
44	28.05	27.87	0.17
45	26.40	25.61	0.79
46	25.07	25.24	–0.16
47	25.22	25.22	0.00
48	25.02	25.23	–0.21
49	24.98	25.38	–0.40
Mean	26.85	26.75	0.09
Avg. SD	0.17	0.15	
Avg. RSD	0.64	0.58	
Min. bias			–0.40
Max. bias			0.79

[a] Each value is an average of triplicate measurements.

homogeneity comparisons on 3 split samples of each of 30 materials. Participating laboratories and contacts at each were provided by the stakeholder body. On October 10, 2006, each participant was shipped five 250 g laboratory materials labeled A through E, with a cover letter, instructions, and reporting forms. Results were received by SDSU until November 27, 2006.

Laboratories were asked to test moisture, protein, crude fat, and/or crude fiber in duplicate using any of the methods listed for Phase I and to specify the method(s) used, along with results. A Lab Ranking Test described by Youden and Steiner (15) was used to assess bias among laboratories participating for outlying laboratories. Outlying laboratories were considered as providing invalid data. Individual outlying results were removed by Cochran and Grubbs tests. Method repeatability (within-laboratory) and method reproducibility (among-laboratory) were calculated. Laboratories' identities were protected by assigning random numbers as IDs in place of laboratory name or contact.

Results and Discussion

Loss on Drying (Moisture) Phase I

Results of the Phase 1 moisture study can be found in Table 2. All of the loss on drying (LOD; oven) methods are empirical, estimating moisture based on LOD. When the materials are heated to evaporate water, other volatile substances that are present can be lost. If the temperature is excessive, additional weight loss can be assigned to the degradation of heat-sensitive substances. The Karl Fischer method (AOAC **2001.12**) is specific for water and is a reference that can be used to assess the empirical methods. When expressed as a % of the Karl Fischer result, the LOD methods can be evaluated based on % recovery of water (Figure 1). Using this procedure, NFTA **2.2.2.5** most closely approximated Karl Fischer, with an average recovery of 110% water and the most consistent recovery for the 30 materials in the study, with a recovery range of 90–131%. The second-closest approximation was method AOAC **935.29**, with an average recovery of 113% water and a range of 80–141%. The next was method AOAC **934.01**, with an average recovery of 119% water and a range of 99–152%. The poorest performance was by AOAC **930.15**, with an average recovery of 142% water and a range of 110–180%. Recoveries for individual materials were highly variable and are depicted in Figure 2. The bias was also used to evaluate the difference among the methods. Using AOAC **2001.12** (Karl Fischer) as the reference method, bias for the LOD methods is provided in Table 2. All average biases are positive, meaning that on the average, the LOD methods overestimate moisture. NFTA **2.2.2.5** has the smallest average bias of 0.84%. AOAC **935.29**, AOAC **934.01**, and AOAC **930.15** follow with average biases of 1.14, 1.64, and 3.66%, respectively.

The SAS GLM Procedure [Least Squares Difference (LSD); 14] was used to compare averages for the different moisture methods. NFTA **2.2.2.5** was not significantly different from AOAC **2001.12**. All other LOD methods are

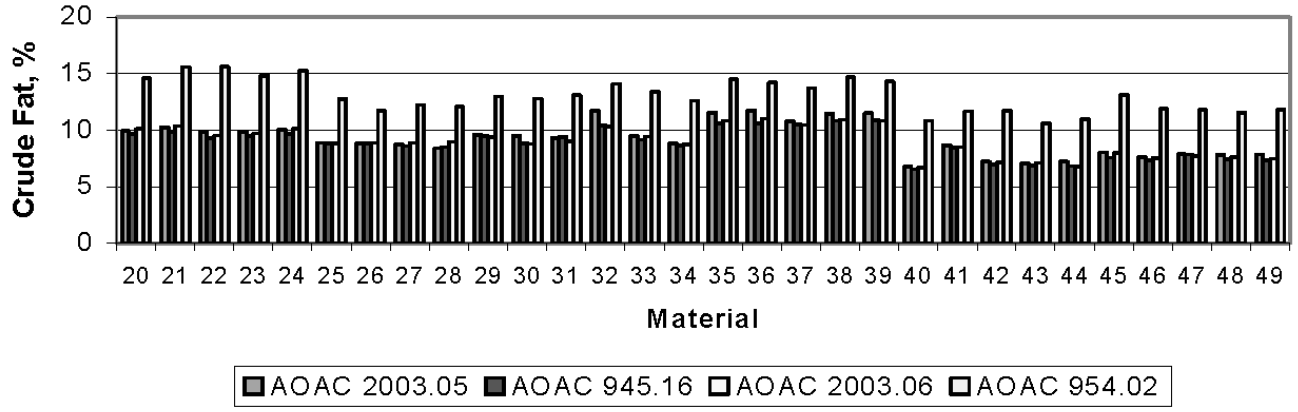

Figure 4. Crude fat results by DDGS material.

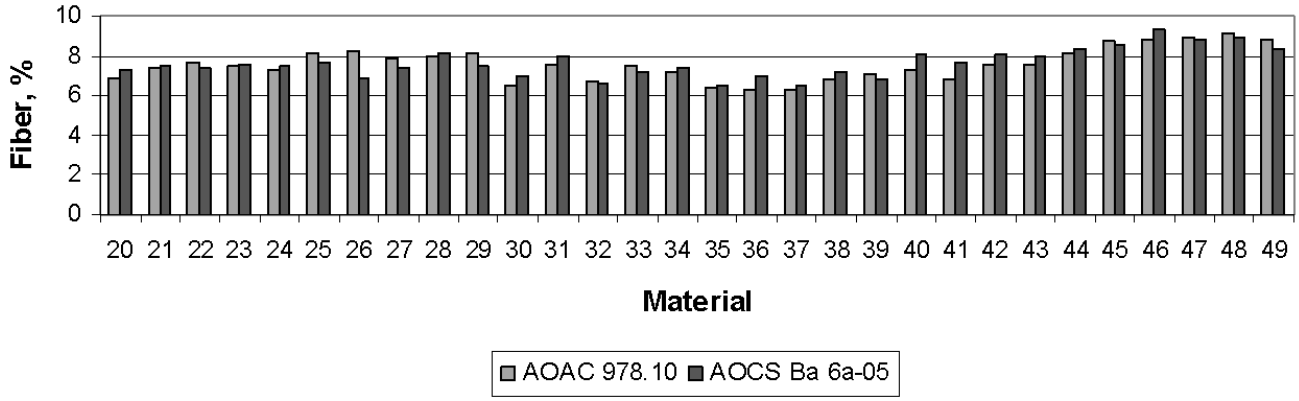

Figure 5. Crude fiber result by DDGS material.

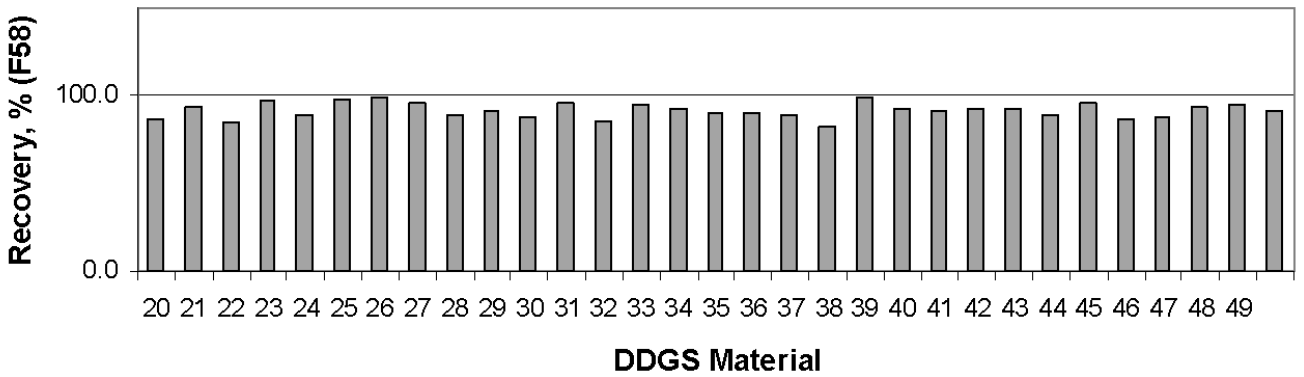

Figure 6. Recovery of crude fiber with the substitution of the F57 filter bag for the F58 filter bag.

Table 4. Crude fat results[a] by method for 30 DDGS materials

Material	Crude fat, %			
	AOAC **2003.05**	AOAC **945.16**	AOAC **2003.06**	AOAC **954.02**
20	9.94	9.69	10.11	14.58
21	10.24	9.88	10.36	15.56
22	9.81	9.31	9.56	15.61
23	9.85	9.51	9.72	14.83
24	10.07	9.68	10.11	15.26
25	8.88	8.85	8.83	12.76
26	8.85	8.83	8.90	11.71
27	8.78	8.60	8.89	12.24
28	8.39	8.52	8.97	12.09
29	9.61	9.45	9.41	12.97
30	9.49	8.86	8.80	12.81
31	9.31	9.41	9.00	13.10
32	11.71	10.43	10.31	14.09
33	9.53	9.17	9.44	13.40
34	8.84	8.64	8.72	12.61
35	11.54	10.63	10.83	14.48
36	11.75	10.64	11.02	14.21
37	10.78	10.50	10.44	13.72
38	11.44	10.84	10.93	14.70
39	11.51	10.87	10.83	14.30
40	6.78	6.57	6.71	10.83
41	8.68	8.44	8.53	11.70
42	7.24	6.98	7.13	11.74
43	7.08	6.86	7.12	10.60
44	7.22	6.84	6.79	10.95
45	8.05	7.59	7.99	13.13
46	7.63	7.33	7.57	11.90
47	7.90	7.85	7.74	11.80
48	7.83	7.44	7.61	11.53
49	7.86	7.35	7.48	11.81
Mean	9.22[b]	8.85[b]	9.00[b]	13.03[c]
Avg. SD	0.28	0.24	0.19	0.57
Avg. RSD	2.95	2.63	2.09	4.38

[a] Each value is an average of triplicate measurements.

[b,c] Means with the same letter are not significantly different (P <0.0001).

significantly different from AOAC **2001.12**. NFTA **2.2.2.5**, AOAC **935.29**, and AOAC **934.01** are not different from each other. NFTA **2.2.2.5** and AOAC **935.29** are not different from each other. AOAC **930.15** was found to be different from all other methods and should not be used for the determination of moisture in DDGS (Table 2).

DDGS are easily dried materials that contain volatile or degradable substances other than water. AOAC **2001.12** (Karl Fischer) should be used as the reference method to determine water in DDGS. The best estimate of water with an LOD method can be obtained by using NFTA **2.2.2.5**.

Crude Protein—Phase I

The methods for measuring protein content are the only methods not empirical. Both protein methods measure nitrogen, but by very different techniques. AOAC **990.03** measures nitrogen by combustion, oxidizing nitrogenous compounds followed by conversion to nitrogen and thermal conductivity detection of the nitrogen. AOAC **2001.11** measures nitrogen by the traditional Kjeldahl acid digestion, converting nitrogenous compounds to ammonia, which is distilled and titrated. With either procedure, crude protein is estimated as % N times 6.25.

Results of the Phase 1 protein study can be found in Table 3. Visual examination of the data indicates that the methods produce very similar results. Average % protein by AOAC **990.03** is 26.85 and average % protein by AOAC **2001.11** is 26.75. Average bias between the 2 methods is minimal at 0.09% protein, and is consistent across materials, ranging from –0.40 as a low to 0.79 as a high (Figure 3). Precision for the 2 methods is excellent and similar with average relative standard deviations (RSDs) for AOAC **990.03** and AOAC **2001.11** of 0.64 and 0.58, respectively.

The protein methods were not found to be different (P = 0.7988) by the SAS GLM Procedure (14). The protein methods under study (AOAC **990.03** and AOAC **2001.11**) are equivalent for DDGS materials and may be used as equivalent methods.

Crude Fat—Phase I

Crude fat methods are empirical; the "crude fat" fraction is defined by the solvent and the extraction conditions (time, temperature, etc.) and is not specific for the extraction of lipid material. In addition to lipids, crude fat methods can co-extract any other substances that are soluble under the conditions of the method, such as residual moisture, residual ethanol, pigments, carotenes, urea, and others. The methods are not specific to lipids, nor do the extraction conditions ensure that 100% of the lipid material will be extracted. The acid hydrolysis step is applicable for baked products, such as pet food, and facilitates the extraction of fatty acids from glycerides, glycol- and phosopholipids, and sterol esters that might otherwise be left unextracted. However, it can also facilitate coextraction of additional nonlipid materials. Because some DDGS materials have undergone heating steps, and because the acid hydrolysis method is widely used, it was

Table 5. Crude fiber results[a] for 30 DDGS materials

	Crude fiber, %				
Material	AOAC **978.10**	AOCS Ba **6a-05**	Modified AOCS Ba **6a-05**	Method bias (AOAC-AOCS)	Recovery, % Mod. AOCS/AOCS (F57/F58)
20	6.94	7.31	6.31	–0.37	86.3
21	7.34	7.47	6.98	–0.13	93.4
22	7.69	7.38	6.22	0.31	84.3
23	7.50	7.57	7.34	–0.07	97.0
24	7.33	7.48	6.68	–0.15	89.3
25	8.17	7.71	7.53	0.46	97.7
26	8.24	6.92	6.89	1.32	99.6
27	7.89	7.41	7.11	0.48	96.0
28	7.96	8.17	7.24	–0.21	88.6
29	8.17	7.43	6.78	0.74	91.3
30	6.48	6.99	6.18	–0.51	88.4
31	7.62	7.98	7.64	–0.36	95.7
32	6.70	6.61	5.64	0.09	85.3
33	7.46	7.18	6.80	0.28	94.7
34	7.16	7.34	6.75	–0.18	92.0
35	6.36	6.47	5.85	–0.11	90.4
36	6.34	7.02	6.31	–0.68	89.9
37	6.32	6.48	5.76	–0.16	88.9
38	6.83	7.20	5.90	–0.37	81.9
39	7.07	6.84	6.77	0.23	99.0
40	7.29	8.01	7.40	–0.72	92.4
41	6.77	7.70	7.00	–0.93	90.9
42	7.53	8.03	7.41	–0.50	92.3
43	7.62	7.98	7.38	–0.36	92.5
44	8.19	8.37	7.49	–0.18	89.5
45	8.71	8.57	8.18	0.14	95.4
46	8.88	9.28	8.07	–0.40	87.0
47	8.90	8.86	7.83	0.04	88.4
48	9.10	8.94	8.40	0.16	94.0
49	8.80	8.38	7.97	0.42	95.1
Average	7.58[b]	7.64[b]	6.99[c]	–0.06	91.6
SD	0.31	0.54	0.35		
RSD	4.02	7.08	5.13		

[a] Each value is an average of triplicate measurements, except Modified AOCS Ba **6a-05** for which each value is an average of duplicate measurements.

[b,c] Means with the same letter are not significantly different (P <0.0001).

Table 6. Summary of laboratories reporting data on loss on drying method and AOAC 2001.12

	No. of laboratories			Precision	
Moisture method	Submitting	Outlier labs[a]	Outlier results[b]	RSD_r, %	RSD_R, %
AOAC **934.01**	10	2(13, 15)	0	2.24–5.11	5.92–12.60
AOAC **935.29**	9	2(7, 14)	0	2.05–4.92	4.89–8.18
NFTA **2.2.2.5**	14	3(2, 5, 7)	7(2[c], 5 [2c,2d,e], 14[d])	1.94–3.06	2.57–6.27
AOAC **930.15**	21	4(5, 15, 19, 23)	4(5[2e], 15[2e])	1.83–2.86	3.69–11.94
AOAC **2001.12**	1	NA[f]	NA	0.68–5.55	NA

[a] Invalid data by Lab Ranking Test; laboratory numbers in parentheses.
[b] Outlier laboratory numbers in parentheses with number and types of outliers for each laboratory noted in superscripts.
[c] Data excluded by Cochran's test, P = 2.5% (1-tail).
[d] Data excluded by Single Grubbs' test, P = 2.5% (2-tail), 1.25% (1-tail).
[e] Data excluded by Double Grubb's test, P = 2.5% (2-tail), 1.25% (1-tail).
[f] N/A = Data not available.

Table 7. Range of results from study laboratories after outlier/invalid data removal

	Range of results (moisture, %)				
Moisture method	A	B	C	D	E
AOAC **934.01**	7.00–9.67	10.00–11.90	8.46–11.76	8.96–12.16	6.97–8.59
AOAC **935.29**	7.35–8.64	10.45–12.20	9.40–11.76	9.00–12.15	7.18–8.48
NFTA **2.2.2.5**	7.68–8.30	11.00–12.24	8.85–11.37	9.42–11.57	7.27–8.20
AOAC **930.15**	7.13–10.22	11.19–13.87	11.29–15.42	11.27–14.89	9.07–10.86
AOAC **2001.12**	7.11–7.31	10.54–10.76	7.87–8.04	7.96–8.61	6.24–6.30
Overall	7.00–10.22	10.00–13.87	7.87–15.42	7.96–14.89	6.24–10.86

Table 8. Summary of laboratories reporting data on crude protein methods

	No. of laboratories			Precision	
Protein method	Submitting	Outlier labs[a]	Outlier results	RSD_r, %	RSD_R, %
AOAC **990.03**	19	2(4, 23)	0	0.72–1.27	1.27–2.29
AOAC **2001.11**	8	0	0	0.61–1.05	0.96–1.35

[a] Invalid data laboratory numbers in parentheses.

Table 9. Range of results from study laboratories after outlier/invalid data removal

	Range of results (crude protein, %)				
Protein method	A	B	C	D	E
AOAC **990.03**	25.44–27.20	28.94–31.15	23.12–24.58	25.90–27.31	27.80–29.30
AOAC **2001.11**	25.15–27.20	29.37–30.78	22.83–23.71	25.50–26.72	26.90–28.38
Overall	25.15–27.20	28.94–31.15	22.83–24.58	25.50–27.31	26.90–29.30

Table 10. Summary of laboratories reporting data on crude fat methods

	No. of laboratories			Precision	
Fat method	Submitting	Outlier labs[a]	Outlier results[b]	RSD_r, %	RSD_R, %
AOAC **2003.05**	7	0	5(2 [4d], 12[c])	2.23–4.40	3.67–12.61
AOAC **945.16**	10	2(6, 18)	2(7 [d], 12[c])	1.76–2.07	2.72–3.35
AOAC **2003.06**	5	0	3(12 [3c])	1.24–5.37	3.59–6.90
AOAC **954.02**	12	3(7, 8, 23)	1(7 [c])	2.14–2.92	3.72–9.93

[a] Invalid data laboratory numbers in parentheses.
[b] Outlier laboratory numbers in parentheses with number and types of outliers for each laboratory noted in superscripts.
[c] Data excluded by Cochran's test, P = 2.5% (1-tail).
[d] Data excluded by Single Grubbs' test, P = 2.5% (2-tail), 1.25% (1-tail).

included in the study. Any modification of any of the fat methods yields a slightly different fraction and is, therefore, yet a different "crude fat" method. Therefore, care must be taken with any empirical method to follow the instructions exactly as directed.

Results of the Phase 1 fat study are presented in Table 4. Visual examination of the data indicates that 3 of the methods produce very similar results. Average % crude fat by AOAC **2003.05**, AOAC **945.16**, and AOAC **2003.06** are 9.22, 8.85, and 9.00, respectively (Figure 4). Precision for the 3 methods is also similar, with average RSDs of 2.95, 2.63, and 2.09, respectively. The fourth method uses an acid hydrolysis step. Results obtained by this method are higher, with an average % crude fat of 13.03 (almost 150% of the other 3 methods; Figure 4). This method is also more variable, with an average RSD of 4.38%.

The crude fat means were found to be different (P <0.0001) by the SAS GLM Procedure (LSD; 15). AOAC **954.02** was different from all other methods; and AOAC **2003.05**, AOAC **934.16**, and AOAC **2003.06** are not different, and could be used to yield similar results. Further study is needed to determine whether the acid hydrolysis step in AOAC **954.02** is needed to free all lipid material from the DDGS matrix, or if the acid hydrolysis results are erroneously high.

Crude Fiber—Phase I

Fiber methods are empirical; the crude fiber fraction is defined by the method. Any modification of the method yields a different fraction and is, therefore, a different crude fiber method. Care must be taken with any empirical method to follow the instructions exactly as directed. During the study, it was learned the F58 filter bag required for AOCS Ba **6a-05** was no longer commercially available. The bag that is available, the F57, has a larger porosity than the F58; the larger porosity allows more particles to pass and has the potential of yielding a lower fiber fraction. A modification of the AOCS method was investigated to determine the effect of substituting the F57 filter bag. The modified method was run in duplicate instead of triplicate, as with the original methods.

Visual examination of the data indicates that the methods produce very similar results. Average % crude fiber by AOAC **978.10** and AOCS Ba **6a-05** are 7.58 and 7.64, respectively. Average bias between the 2 methods is minimal at 0.06%. Method AOAC **978.10** has better precision than AOCS Ba **6a-05**, with an average RSD of 4.02 for AOAC **978.10** and 7.08 for AOCS Ba **6a-05** (Figure 5).

Table 11. Range of results from study laboratories after outlier/invalid data removal

	Range of results as crude fat, %				
Fat method	A	B	C	D	E
AOAC **2003.05**	9.92–11.31	8.60–11.33	8.59–12.11	10.69–13.05	6.63–8.15
AOAC **945.16**	9.64–10.25	8.20–9.17	8.77–9.25	10.35–11.28	6.70–7.33
AOAC **2003.06**	8.87–10.07	7.68–9.79	7.67–9.00	9.79–10.94	5.67–7.24
AOAC **954.02**	12.94–14.50	9.44–12.10	10.50–12.40	12.24–13.70	8.72–10.40
Overall	8.87–14.50	7.68–12.10	7.67–12.40	9.79–13.70	5.67–10.40
Overall[a]	8.87–11.31	7.68–11.33	7.67–12.11	9.79–13.05	5.67–8.15

[a] Nonhydrolysis methods.

Table 12. Summary of laboratories reporting data on crude fiber methods

	No. of laboratories			Precision	
Fiber method	Submitting	Outlier labs[a]	Outlier results[b]	RSD_r, %	RSD_R, %
AOAC **978.10**	10	4 (2, 6, 10, 11)	2 (10 [2c])	2.34–4.47	15.33–19.73
AOCS Ba **6a-05**	7	1 (9)	1 (17[c])	1.70–5.28	6.01–8.48

[a] Invalid data laboratory numbers in parentheses.
[b] Outlier laboratory numbers in parentheses with number and types of outliers for each laboratory noted in superscripts.
[c] Data excluded by Cochran's test, P = 2.5% (1-tail).

Results obtained with method AOCS Ba **6a-05** modified for use with the F57 bag were about 10% lower than those obtained with the F58 bag (Figure 6).

The crude fiber means were found to be different (P <0.0001) by the SAS GLM Procedure (LSD; 14). The AOCS Ba **6a-05** method modified for the F57 bag was different from both AOAC **978.10** and AOCS Ba **6a-05**. AOAC **978.10** and AOCS Ba **6a-05** are not different, and can be used interchangeably. However, AOAC **978.10** has better precision and the lack of commercial availability of the F58 filter bag is a concern.

Usage of the F57 bag is not in keeping with the specifications of the AOCS Ba **6a-05** Method. When the F57 filter bag is substituted for the F58 bag, the modification generally causes a low bias of about 10% (relative). After the completion of the study, it was pointed out by the authors and study directors for AOCS Ba **6a-05** that a smaller improper particle size reduction was used in this study. The method calls for grinding "samples through a centrifugal mill with a 2 mm screen or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may show particle loss from the filter bags and result in low values." In this study, a 10% loss was observed. It has been questioned if a coarser grind (larger particle size) with the F57 bag would provide comparable results to AOCS Ba **6a-05**, and subsequent work is planned to confirm this hypothesis. While this is a significant error, it is unlikely that laboratories will prepare a separate analytical sample for crude fiber for every laboratory sample received.

LOD (Moisture)—Phase II

A summary of the LOD and Karl Fischer data reported by participating laboratories is presented in Table 6. Approximately 20% of the laboratories submitted invalid data based on the Lab Ranking test (15). A client submitting the same material to the group of 23 laboratories that participated in this study could receive moisture test results (upon removal of invalid and outlier results) ranging as follows (Table 7): Material A, from 7.00 to 10.22% (about 8.6% true moisture); Material B, from 10.00 to 13.87% (about 12.1% true moisture); Material C, from 7.87 to 15.42% (about 8.8% true moisture); Material D, from 7.96 to 14.89% (about 9.3% true moisture); and Material E, from 6.24 to 10.86% (about 7.5% true moisture).

LOD (moisture) is one of the best examples of improperly performed analytical measurements; it is often not given proper attention by users of laboratory data or by laboratory personnel. The poorest performing (thus, least desirable) moisture method (AOAC **930.15**, 135°C/2 h) is the most widely used, while the best performing (thus, most desirable) moisture method (**2001.12**, Karl Fischer) is the least widely used. A serious industry-wide educational effort on LOD determinations is needed to improve the quality of analytical data.

Crude Protein—Phase II

A summary of the crude protein data reported by participating laboratories is presented in Table 8. Protein is an example of a well-performed analytical measurement. A client submitting a DDGS material to one of the 23 laboratories involved in the study would obtain very similar results, with RSD_R of 1.27–2.29% for the combustion technique and 0.96–1.35% for the Kjeldahl technique. Ranges of crude protein values, by material, were as follows (Table 9): Material A, from 25.15 to 27.20%; Material B, from 28.94 to

Table 13. Range of results from study laboratories after outlier/invalid data removal

	Range of results as crude fiber, %				
Fiber method	A	B	C	D	E
AOAC **978.10**	6.18–8.00	6.42–9.80	5.46–7.10	5.50–8.50	6.50–8.10
AOCS Ba **6a-05**	5.58–7.06	6.18–7.43	5.32–6.13	5.10–5.68	6.04–7.48
Overall	5.58–8.00	6.18–9.80	5.32–7.10	5.10–8.50	6.04–8.10

31.15%; Material C, from 22.83 to 24.58%; Material D, from 25.50 to 27.31%; and Material E, from 26.90 to 29.30%.

The results are excellent, especially considering that bias with the combustion technique is generally high and the bias with the Kjeldahl technique is generally low.

Crude Fat—Phase II

A summary of the crude fat data reported by participating laboratories is presented in Table 10. Crude fat results are highly variable; however, much of the variability was removed with the removal of the outlier laboratories. Measured ranges of crude fat values, by material, are presented in Table 11. The range is improved if acid hydrolysis results are omitted: Material A, from 8.87 to 14.50%, or from 8.87 to 11.31%, omitting AOAC **954.02**; Material B, from 7.68 to 12.10%, or from 7.68 to 11.33%, omitting AOAC **954.02**; Material C, from 7.67 to 12.40%, or from 7.67 to 12.11%, omitting AOAC **954.02**; Material D, from 9.79 to 13.70%, or from 9.79 to 13.05%, omitting AOAC **954.02**; and Material E, from 5.67 to 10.40%, or from 5.67 to 8.15%, omitting AOAC **954.02**.

Crude Fiber—Phase II

A summary of the crude fiber data reported by participating laboratories is presented in Table 12. The variability in crude fiber results is unexpected. The number of laboratories submitting invalid data by the Lab Ranking Test (15) is very high, with RSD_R ranges of 15.33–19.73% for AOAC **978.10** and 6.01–8.48% for AOCS Ba **6a-05**. Results for the crude fiber determinations by material are as follows (Table 13): Material A, from 5.58 to 8.00%, Material B, from 6.18 to 9.80%; Material C, from 5.32 to 7.10%; Material D, from 5.10 to 8.50%; and Material E, from 6.04 to 8.10%.

The variability underscores the importance of following empirical methods as written to avoid introduction of bias. As with fat, most of the variability is due to a small number of the laboratories and removal of their data serves to improve the spread of data.

Stakeholder Body Recommendations

On the basis of the results of the Phase I and Phase II studies, the AFIA DDGS Analytical Methods Sub-Working Group adopted the following recommendations to their industries (16):

(**a**) *AFIA recommendations for moisture*.—Although the Karl Fischer titration provides the most accurate measurement of water in feed, the labor (both time and training), reagent, and instrument costs make it less accessible for most laboratories. The committee recognizes these concerns and has used Karl Fischer as the means of determining the gravimetric (LOD) method that has the least amount of bias. Using this criteria, NFTA **2.2.2.5** Lab Dry Matter (105°C/3 h), was selected as the recommended method for the analysis of moisture in DDGS; this method also had acceptable coefficients of variation (CVs) in both the intra- and interlaboratory portions of the study.

The committee stresses that all gravimetric methods be considered, and used accordingly, as "LOD" methods and only serve as an estimation of the "true" moisture level. One of the gravimetric methods, AOAC **930.15**, Loss on Drying (moisture) for Feeds (135°C/3 h), was shown to dramatically overestimate the moisture content in DDGS and, therefore, use of this method is highly discouraged. However, use of this method is widespread, as demonstrated by the fact that 17 of the 25 laboratories reported values using AOAC **930.15**. Efforts to remove the method from use on DDGS should be pursued.

(**b**) *AFIA recommendations for protein*.—The protein methods investigated in this study were determined to be statistically equivalent and both had acceptable CVs for both the intra- and interlaboratory portions of the study. AOAC **990.03**, Protein (Crude) in Animal Feed—Combustion, and AOAC **2001.11**, Protein (Crude) in Animal Feed and Pet Food Copper Catalyst, can be thereby be used interchangeably to provide accurate and precise protein results on DDGS.

(**c**) *AFIA recommendations for fat*.—The 3 nonhydrolysis fat methods (AOAC **2003.05**, AOAC **945.16**, and AOAC **2003.06**) were determined to be statistically equivalent methods for the analysis of DDGS; however, in the interlaboratory portion of the study, AOAC **945.16**, Oil in Cereal Adjuncts (Petroleum Ether), had a significantly lower CVs than the other nonhydrolysis methods and has thereby proven to be a more robust method. Therefore it was chosen as the recommended test method for analysis of fat in DDGS.

(**d**) *AFIA recommendations for fiber*.—Both crude fiber methods evaluated, AOAC **978.10** and AOCS Ba **6a-05**, were considered to be not significantly different. However, the F58 filter bag, which is needed to comply with AOCS Ba **6a-05**, is no longer commercially available. The recommended replacement, the F57 filter bag, which is commercially available, has been shown to cause a 10% (relative) low bias and would be statistically equivalent. Based on lack of availability of the F58 filter bag, which is needed to perform AOCS Ba **6a-05**, the AOAC **978.20** Fiber (Crude) in Animal Feed and Pet Food (F.G. Crucible) is the recommended method for crude fiber analysis on DDGS.

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