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Lipid and NDF Analysis of Ethanol Byproduct Feedstuffs

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Summary

A newly developed biphasic feed lipid extraction procedure has increased accuracy relative to Goldfisch ether extraction, especially for condensed corn distillers solubles samples. A pre-NDF fat extraction must be completed prior to analyzing high fat feeds for NDF. Corn should be ground through a 1-mm screen on a Tecator Cyclotec sample mill to accurately determine corn NDF content.

Introduction

The ether extract procedure, a standard of lipid extraction for many years, may have limitations in accuracy with samples containing condensed corn distillers solubles (CCDS). Furthermore, fat content may decrease the accuracy of feed sample NDF determination, because fat may not be completely dissolved with the Van Soest procedure. Therefore, three experiments were conducted to optimize the performance of a new lipid analysis procedure for feedstuffs. Also, two studies were conducted to improve accuracy of determining corn NDF with the Van Soest beaker procedure.

Procedure

Experiment 1

Exp. 1 evaluated proper incubation time of distillers grains plus solubles (DGS) samples with a new biphasic lipid extraction procedure to optimize quantity of lipid extract compared to Goldfisch diethyl ether extraction. Five corn DGS samples were analyzed in duplicate for all incubation times. The biphasic extraction utilized 0.38 g of DGS DM incubated with 4 mL of a 1:1 ratio of hexane to diethyl ether in 16 x 125 mm screw-top test tubes for 0.1, 2, 4, 6, 8, 10, or 12 hours at 50°C. Four mL of solvent were sufficient to extract at least 0.5 g of lipid from the samples. After incubation, 3 mL of dilute hydrochloric acid water (1 drop concentrated hydrochloric acid/40 mL distilled water) were added to the tube to elevate the solvent and lipid extract layer above the remaining feed. The tube was recapped and vigorously shaken for 2 seconds to facilitate solvent removal from feed particles. The tubes were then centrifuged at 900 x *g* for 6 minutes to improve solvent phase separation. The upper lipid phase was transferred with a glass pipette to a pre-weighed test tube. An additional 2 mL of the solvent were added to the original tube, shaken, and transferred to the same corresponding tube with the same glass pipette. Previous unpublished research has shown that 2 extracts are sufficient for complete removal of lipid from the samples. Solvent was evaporated at 50° C under nitrogen, and lipid residue was weighed.

The diethyl ether procedure for lipid extraction using the Goldfisch fat extractor (Laboratory Construction Company, Kansas City, Mo.), utilized 1.2 g of DGS suspended in a thimble. Thirty five mL of diethyl ether were continuously refluxed through the sample for 4 hours. The solvent was then evaporated from the extract, and the lipid residue was weighed.

The PROC MIXED procedure of SAS with Tukey adjusted mean separation was utilized to analyze the effect of incubation time on biphasic lipid extract.

Experiment 2

Exp. 2 evaluated the effect of the hexane:diethyl ether ratio on efficiency of lipid extraction from dry DGS, modified DGS, wet DGS, dry rolled corn, corn germ meal, and CCDS samples. Five hexane:diethyl ether ratios were evaluated (1:0, 1:3, 1:1, 3:1, and 0:1) with a 9-hour biphasic incubation procedure similar to that employed in Exp. 1. Lipid extracts were prepared as fatty acid methyl esters for GC analysis with a methanolic boron trifluoride procedure, using heptadecanoic fatty acid as internal standard for 12- to 20-carbon fatty acid quantification.

Experiment 3

Exp. 3 compared CCDS lipid extraction from the Goldfisch diethyl ether procedure to the biphasic extraction with 1:1 ratio of hexane:diethyl ether or 100% diethyl ether. Three CCDS samples from previous UNL feedlot research trials were lyophilized and pulverized with a mortar and pestle. The three samples were analyzed in triplicate for each of four methods.

Method 1: The Goldfisch apparatus was the same as in Exp. 1. The solvent was evaporated, and the lipid residue was weighed in pre-weighed beakers. Hexane was then added to the extract to separate the lipids from the hexane insoluble materials and transferred to a test tube; hexane was evaporated under nitrogen at 50° C, and lipid was methylated for fatty acid analysis by GC. The hexane insoluble material (a clear material with physical properties similar to glycerol) was solubilized in isopropanol. This material was plated on a thin layer chromatography plate and analyzed for phospholipids, glycerol, and starch.

Methods 2 & 3: Samples were extracted using a biphasic extraction procedure with a 10-hour incubation procedure similar to that employed in Exp. 1, with either a 1:1 ratio of hexane:diethyl ether (Method 2) or diethyl ether alone (Method 3). The

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lipid fractions were methylated for GC fatty acid analysis.

Method 4: Samples were refluxed with the Goldfisch diethyl ether procedure as in Method 1. However, instead of evaporating the diethyl ether upon completion of the reflux period, the diethyl ether extract mixture was transferred to a screw top test tube. Three mL of the dilute hydrochloric acid solution from Exp. 1 were added to the tubes. Tubes were shaken, and the diethyl ether fraction was quantitatively transferred to an additional tube. Two additional mL of diethyl ether were added to the original tubes, and a second quantitative transfer was performed. The diethyl ether and water were evaporated from the respective tubes, and each tube was weighed to calculate diethyl ether and water-soluble CCDS fractions. The diethyl ether fraction was methylated for fatty acid analysis by GC.

Experiment 4

In the Van Soest NDF procedure, 0.5 g of sample (ground through a 1 mm screen in a Wiley Mill) was weighed into a tall-form 600 mL beaker, adding 100 mL of neutral detergent solution, refluxing for 1 hour, filtering the residue, and drying the filters. Three methods were evaluated to improve filtering capability and decrease fat contamination of DGS when measuring NDF. These methods included 1) the Van Soest method with an acetone residue rinse at filtering; 2) method 1 with 2 times the amount of neutral detergent solution; and 3) a biphasic fat extraction on the samples (same as Method 2 of Exp. 3), then rinsing the non-lipid residue into a beaker with 100 mL of neutral detergent solution and an acetone residue rinse. Sodium sulfite and alpha-amylase (20,350 LU/ mL) were used in all of the methods to digest protein and starch at 0.5 g and 0.5 mL per beaker, respectively. The samples used included varying levels of CCDS added to the DGS. These are represented as 0, 33, 67, 100, and 110% of the normal incorporation of CCDS to grains.

Table 1. Average lipid content of five DGS samples incubated for different times utilizing a new biphasic lipid extraction procedure[1].

Incubation time, hours	0.1	2	4	6	8	10	12
DGS lipid, % of DM[2]	11.1[a]	11.9[b]	12.0[b]	12.0[b]	12.1[b,c]	12.2[b,c]	12.3[c]

[1]DGS = lyophilized distillers grains plus solubles samples.
[2]Samples also were analyzed with the Goldfisch method and averaged 12.2% ether extract.
[a,b,c]Means with unlike superscripts are different at $P < 0.05$.

Table 2. Average lipid content of six feedstuffs incubated with different ratios of hexane:diethyl ether with a new biphasic lipid extraction procedure[1].

Hexane:Diethyl Ether	1:0	3:1	1:1	1:3	0:1
Gravimetric lipid, % of DM	12.4[a]	12.6[a]	12.7[a]	13.8[b]	14.2[b]
GC fatty acids, % of DM	11.0	11.3	11.4	11.2	11.3
GC:Gravimetric	0.90[a]	0.90[a]	0.90[a]	0.81[b]	0.79[b]

[1]GC = gas chromatography analysis of 12 to 20 carbon length fatty acids with heptadecanoic acid as internal standard.
[a,b]Means within a row with unlike superscripts are different at $P < 0.05$.

Table 3. Average lipid content of three lyophilized condensed corn distillers solubles samples with four different laboratory procedures[1].

Method	1	2	3	4
Gravimetric lipid, % of DM	23.4	17.6	20.0	17.5
GC fatty acids, % of DM	14.9	15.5	16.8	15.2
GC:Gravimetric	0.64[a]	0.88[b]	0.84[b]	0.87[b]

[1]Method 1 = Goldfisch extraction with diethyl ether; Method 2 = biphasic extraction with 1:1 hexane:diethyl ether; Method 3 = biphasic extraction with diethyl ether; Method 4 = Goldfisch extraction with subsequent biphasic extraction; GC = gas chromatography analysis of total fatty acids with heptadecanoic acid as internal standard.
[a,b]Means within a row with unlike superscripts are different at $P < 0.05$.

Experiment 5

To obtain accurate corn NDF values, the same corn hybrid (1-mm Wiley Mill grind) was used to compare NDF for dry rolled and high moisture processing types in addition to a steam-flaked corn sample. Sodium sulfite (0.5 g) was added, and alpha-amylase (0.5 mL; 20,350 LU/ mL) was administered during the hour reflux once, twice, or four times to digest corn starch.

Experiment 6

The effect of milling equipment on corn NDF content was evaluated. Four dry rolled corn samples were ground through a 1-mm screen on either a Wiley Mill (Thomas Scientific, Swedesboro, N.J.) or a Tecator Cyclotec sample mill (American Instrument Exchange, Haverhill, Mass.). Alpha-amylase was administered at the beginning of the reflux and 10 minutes prior to filtering (0.5 mL each). Sodium sulfite (0.5 g) was used in all corn NDF analyses.

Results

Experiment 1

Lipid extraction efficiency increased as incubation time increased from 0.1 to 12 hours in Exp. 1 (Table 1). The 0.1-hour extract was the least efficient of all levels evaluated ($P < 0.01$). Efficiency of the 12-hour incubation also was significantly greater than that observed at the intermediate incubation times ($P = 0.03$). However, efficiency at 12-hour incubation was not significantly different from that at 8- and 10-hour incubation. The extract at 10 hours yielded 12.2% lipid, which was

Table 4. Percentage NDF and fat for DGS samples with different condensed corn distillers solubles levels with three different methods for controlling fat.

	CCDS% of DGS DM				
Method[1]	0	33	67	100	110
1[a]	43.4	38.1	33.6	31.3	31.8
2[b]	41.6	37.9	34.8	30.7	30.7
3[c]	41.0	36.8	32.8	30.1	28.8
Fat[2]	7.1	9.2	10.8	12.8	13.9

[1]Method 1 = 100mL neutral detergent solution with acetone rinse at filtering; Method 2 = 200mL neutral detergent solution with acetone rinse at filtering; Method 3 = use residue remaining after biphasic fat extraction with 100 mL neutral detergent solution and acetone rinse at filtering.
[2] Lipid extract from pre-NDF fat extract of Method 3.
[a,b,c]Methods with unlike superscripts differ ($P < 0.01$).

Table 5. Dosage of alpha-amylase for determining NDF content for corn processing types.

	Sample[1]			
Alpha-amylase doses[2]/ reflux	DRC	HMC	SFC	Average
1	23.9	20.7	20.8	21.8[a]
2	14.2	12.4	12.0	12.9[b]
4	12.6	12.0	11.9	12.2[b]

[1]DRC = dry rolled corn for hybrid 1; HMC = high moisture corn for hybrid 1; SFC = steam flaked corn (not hybrid 1).
[2]Doses = number of doses with 0.5 mL alpha-amylase added (20,350 LU/ mL).
[a,b]Number of alpha-amylase doses with unlike superscripts differ ($P < 0.01$).

Table 6. Effect of milling equipment with 1-mm screen on NDF content of dry rolled corn samples with 2 doses of alpha-amylase.

	Dry rolled corn sample				
Milling equipment	1	2	3	4	Average
Wiley, corn % NDF	13.9	16.7	17.7	14.9	15.8[b]
Tecator Cyclotec, corn % NDF	10.6	10.4	9.7	9.5	10.1[a]

[a,b]Different grinds with unlike superscripts differ ($P < 0.01$).

similar to the amount yielded by the Goldfisch ether extract.

Experiment 2

Gravimetric quantification of the lipid extraction increased as proportion of diethyl ether increased in the solvent mixture (Table 2). Solvents with a diethyl ether concentration equal to or greater than the hexane concentration had increased lipid extract ($P < 0.01$). However, when the extracts were methylated and analyzed by GC, there were no differences in percent total fatty acids ($P > 0.30$) across solvent compositions. The ratio of GC-analyzed extract:gravimetric extract decreased as solvent diethyl ether content increased above hexane content. The ratio of 0.90 for the three highest proportions of hexane was greater than the ratio for the two lesser proportions of hexane (average ratio of 0.80; $P < 0.01$). The expected GC-analyzed:gravimetric ratio is approximately 0.90, because approximately 10% triglyceride glycerol content of the crude extract is not accounted for in the GC fatty acid analysis. Increased inclusions of diethyl ether extracted non-lipid material from the samples.

Experiment 3

Gravimetric CCDS lipid extraction was numerically greatest for the Goldfisch extraction method in Exp. 3 (Table 3). Biphasic lipid extraction with 1:1 hexane:diethyl ether (Method 2) was numerically similar to lipid extraction when water soluble impurities were removed with biphasic extraction from the Goldfisch extract (Method 4). CCDS lipid content with Methods 2 and 4 was 17.6% and 17.5%, respectively. CCDS non-lipid extract from the Goldfisch procedure ranged from 3 to 10% of sample and averaged 5.8% of CCDS DM. There were no significant differences in CCDS percent of GC-analyzed fatty acids. The ratio of GC: gravimetric extract was lowest for the Goldfisch procedure ($P = 0.01$) and similar for the other three procedures, indicating that non-lipid material was being extracted with the Goldfisch procedure. The percentage of CCDS DM in the water soluble fraction of Method 4 averaged 6.2%, which is similar to the difference in extraction between the Goldfisch and the 1:1 biphasic methods.

The water soluble impurities did not move from the origin when spotted on thin layer chromatography plates, indeed indicating the material was devoid of neutral lipid. In addition, enzymatic laboratory assays indicated there was very little phospholipid, glycerol or starch content in the water soluble material. We currently hypothesize the material is a yeast extract from the ethanol fermentation process; however, this has not been verified in the laboratory.

These data collectively indicate that a 10-hour incubation of samples with a 1:1 hexane:diethyl ether solvent for biphasic extraction of feedstuff lipids, especially from CCDS, is superior to Goldfisch ether extraction.

Experiment 4

As increased levels of solubles were added to the distillers grains, NDF content decreased (Table 4). This is to be expected as solubles contain very little NDF (2-8% of DM). Using 200 mL of neutral detergent solution did not aid in filtering (~15 minutes/ beaker) or decrease the fat coating on the filters compared to using the Van Soest method, as shown by little change in percent NDF ($P = 0.72$).

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However, when using the pre-NDF fat extraction, filtering was more efficient (~5 minutes) with no film on the filters. This procedure also decreased the analyzed NDF content compared to the other two methods ($P < 0.01$). Therefore, combining the biphasic fat procedure with NDF analysis provides an effective way to analyze both nutrients for high fat byproduct feeds.

Experiment 5

The NDF content for high moisture corn was lower than for dry rolled corn with the same corn hybrid, suggesting more starch breakdown (Table 5). With addition of more alpha-amylase, NDF values decreased ($P < 0.01$) and filtering became easier with a decrease in filtering time from 30 to 60 minutes down to 15 minutes. However, the NDF values were greater than 12% regardless of processing type, with observable granular, non-fibrous particles remaining in the filter.

Experiment 6

The four dry rolled corn samples had decreased NDF values (average = 10.1%, $P < 0.01$) and increased ease of filtering (5 minutes) when ground through the Tecator Cyclotec mill compared to the Wiley Mill (Table 6). When corn was ground through a Tecator Cyclotec, the NDF content was in the expected range (NRC, 1996).

Having accurate corn NDF values is important when evaluating the DGS produced from corn. The recommended NDF procedure is to grind the corn samples through a Tecator Cyclotec mill with a 1-mm screen and add 0.5 g sodium sulfite and 2 doses of 0.5 mL alpha-amylase during the reflux period, because this grinding method resulted in only observed fiber residue in the filter with no starch granules.

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