Quantifying phytate in dairy digesta and feces: Alkaline extraction and high-performance ion chromatography¹

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ABSTRACT

Development of an analytical method with appropriate combination of extraction and quantification approaches for undigested phytate in ruminant feces and digesta will advance knowledge of phytate degradation in ruminants and help to reduce phosphorus excretion. Established quantification methods give satisfactory results for feedstuffs and nonruminant manure but recovery of phytate is incomplete for ruminant feces and digesta because of their complex sample matrix and low ratio of phytate to inorganic P. The objective was to develop a robust, accurate, sensitive, and inexpensive method to extract and quantify phytate in feeds, ruminant feces, and digesta. Diets varying in phytate content were fed to dairy heifers, dry cows, and lactating cows to generate digesta and fecal samples of varying composition to challenge extraction and quantification methods. Samples were extracted with 0.5 M HCl or 0.25 M NaOH + 0.05 M EDTA. Acid extracts were mixed with 20% NaCl, alkaline extracts were acidified to final pH <2, and then both extracts were clarified with C₁₈ cartridges and 0.2-μm filters. High-performance ion chromatography (HPIC) was used to quantify phytate. In feed samples, the measured phytate was comparable in alkaline and acid extracts (2,965 vs. 3,085 μg/g of DM). In digesta and fecal samples, alkaline extraction yielded greater estimates of phytate content than did acid extraction (40.7 vs. 33.6 and 202.9 vs. 144.4 μg/g of DM for digesta and fecal samples, respectively). Analysis of alkaline extracts by HPIC is usually not possible because of sample matrix interferences; acidification and C₁₈-cartridge elution of alkaline extracts prevented this interference. Pure phytate added to dry samples before extraction was almost

completely recovered (88 to 105%), indicating high extraction efficiency, no adverse effect of extract clean-up procedures, and accurate quantification of phytate. The proposed method is rapid, inexpensive, robust, and combines the extraction power of NaOH-EDTA with the precision and sensitivity of HPIC quantification, allowing accurate quantification of phytate in feeds, ruminant digesta, and fecal samples.

Key words: high-performance ion chromatography, dairy feces, phytate

INTRODUCTION

The quantification of phytate in feed, digesta, and feces from dairy cows is important in nutritional and environmental research. Environmental pollution due to P runoff from livestock farms is a growing global concern, leading to efforts to develop and implement nutritional strategies to minimize the P excretion without impairing production (Knowlton et al., 2004; VandeHaar and St-Pierre, 2006). Improved understanding of the fate of dietary P in the digestive tract may support improved P utilization by livestock to decrease their environmental impact.

One challenge limiting application of dietary P management is variation in P availability or digestibility in feedstuffs (Bravo et al., 2003; Mjoun et al., 2008; Cherry et al., 2010). Phytate is the major form of P in grains and many byproduct feed ingredients (Eeckhout and De Paepe, 1994; Ravindran et al., 1994). Ruminants can utilize phytate-P because ruminal microorganisms express the enzyme phytase, which hydrolyzes phytate into simpler organic forms of P or inorganic P. Because it is the major form of P in the concentrate portion of dairy rations, however, incomplete hydrolysis of phytate in the rumen may limit P availability. Although a body of research from previous decades documents complete hydrolysis of phytate in the rumen of lactating cows (Nelson et al., 1976; Clark et al., 1986; Morse et al., 1992), high-producing dairy cows consume more DM and their diets contain less forage as compared with the animals used in early studies (Phipps et al., 2000). The resulting higher dietary phytate intake and higher

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passage rate of digesta due to high DMI may result in incomplete degradation of phytate P in the rumen. Other factors that have been shown to cause variation in the hydrolysis of phytate in the rumen are the type of grain, processing of feedstuffs, and supplementation with exogenous enzymes (Park et al., 1999; Bravo et al., 2003; Kincaid et al., 2005).

In interpreting earlier work on phytate digestion by ruminants, limitations in methods used to quantify phytate P in feces must also be considered. Classical methods are based on the precipitation of phytate as an insoluble iron-phytate complex but these methods are too insensitive for low phytate samples (Wheeler and Ferrel, 1971). Also, the complexity of feces with its undigested feed residues, partially digested microbial residues, and endogenous cellular material challenges traditional methods of phytate quantification. Within this matrix, phytate is complexed with minerals and other nutrients. If recovery of fecal phytate is incomplete due to methodological limitations, fecal phytate may be underestimated in these earlier studies.

The initial step for the quantification of phytate P is the extraction of that form of P from the sample. Quantitative extraction of phytate from digesta and feces is more difficult than from feed because this phytate is always bound with cations, protein, or other nutrients to a greater extent than it is in feed and in monogastric manure samples, leading to erroneous estimation or damage to the analytical instruments, or both. Factors affecting recovery of P-containing compounds in different extraction schemes include type of extractant, sample matrix, and sample handling. Of 2 commonly used extractants, HCl gives satisfactory recovery of total and phytate P in feed and monogastric digesta and feces (Skoglund et al., 1997; Leytem et al., 2008) but recovery of P is poor in the complex matrix of ruminant feces (McDowell et al., 2008). Alkaline extraction, most commonly with 0.25 M NaOH + 0.05 M EDTA, gives almost complete recovery of total and various forms of P in feed and fecal samples (Turner, 2004; Toor et al., 2005; McDowell et al., 2008) but is incompatible with the high-performance ion chromatography (HPIC) quantification method because the alkaline extract causes column damage and interference from the matrix of the extracted sample. Thus, phytate in alkaline extracts is quantified using nuclear magnetic resonance (^{31}P NMR), but this method has shortcomings, including long analysis time, relatively high cost, and insensitivity to low-phytate samples such as ruminant feces. High performance ion chromatography is compatible with acid extraction but recovery of total and phytate P from ruminant feces is not complete.

Development of robust, inexpensive, and reproducible techniques for accurate quantification of undigested phytate in complex animal manure and digesta samples is essential to advance knowledge of the effects of diet on phytate and P excretion in ruminants (Turner et al., 2002). Consequently, the objectives were to identify and evaluate an extraction method that adequately recovers phytate from feed, ruminant digesta, and fecal samples and is fully compatible with HPIC quantification.

MATERIALS AND METHODS

Sample Collection

For this method development project, dairy heifers ($n = 2$) and cows ($n = 2$ dry and 2 lactating) were fed 2 or 3 different diets in sequential periods. Diets were designed to generate coordinated feed, digesta, and fecal samples of widely varying composition to challenge extraction and quantification methods. The experiment was carried out according to procedures approved by the Virginia Tech Animal Care and Use Committee.

Animals were fed experimental diets (Table 1) in 10-d periods; 7 d of diet adaptation and 3 d for sample collection. On d 8 to 10, fecal grab samples were collected twice daily, pooled by diet, and frozen (-20°C). Lactating and dry cows were ruminally cannulated, allowing simultaneous collection of omasal samples using a vacuum pump (Huhtanen et al., 1997). Total mixed rations, concentrate mixes, and individual grain and forage samples were collected once per period and stored frozen (-20°C). The diet sequence was fixed and diets were specific to a class of animals (heifers, dry cows, or lactating cows) because the focus was on generating a diverse sample set rather than evaluating the effect of animal, period, or diet.

Sample Preparation and Conventional Analysis

All samples were oven dried at 55°C to constant weight and ground with a Wiley mill (1-mm screen; Arthur H. Thomas Co., Philadelphia, PA). Dried samples were analyzed in duplicate for NDF, ADF, and ADL (Van Soest et al., 1991), starch (Hall, 2009), total Kjeldahl N, Ca, P, Mg, Al, Fe, and K (AOAC, 1984). Fiber analysis was sequential and NDF was quantified using amylase and sodium sulfite. Samples were ground further through a 0.2-mm sieve (Z Grinder; ZM 100 USA, Retsch, Haan, Germany) and stored in air-tight containers for further analysis. A subset of fecal samples was thawed at room temperature (20°C) and extracted wet (described below).

Table 1. Ingredient composition of experimental diets

Ingredient (% of DM)	Mixed ration 1	Mixed ration 2	Mixed ration 3	Mixed ration 4	Mixed ration 5	Mixed ration 6 ¹	Hay
	Lactating cows	Lactating cows	Lactating cows	Heifers	Heifers	Dry cows	Dry cows
Grass hay	—	9.03	—	71.3	71.3	64.0	100
Alfalfa haylage	—	20.1	—	—	—	—	—
Alfalfa silage	9.24	—	11.6	—	—	—	—
Corn silage	35.9	27.3	48.2	8.83	8.67	21.6	—
Barley straw	—	—	1.89	—	—	—	—
Grain mix 1 ²	54.8	—	—	—	—	—	—
Grain mix 2 ³	—	43.6	—	—	—	—	—
Grain mix 3 ⁴	—	—	38.4	—	—	—	—
Grain mix 4 ⁵	—	—	—	19.9	—	—	—
Grain mix 5 ⁶	—	—	—	—	20.0	—	—
Grain mix 6 ⁷	—	—	—	—	—	14.4	—

¹Dry cows were pen fed, and reported proportions of dietary ingredients are approximate.

²Grain mix 1 contained 60.3% corn, 28.0% soybean meal, 3.66% soybean hulls, 4.33% tallow, 1.67% limestone, 1.00% sodium bicarbonate, 0.33% salt, 0.33% urea, 0.17% potassium magnesium sulfate, 0.13% monoammonium phosphate, 0.04% Se, 0.03% mineral premix, 0.03% vitamin ADE, and 0.01% vitamin E.

³Grain mix 2 contained 64.4% corn, 33.1% cottonseed meal, 1.81% limestone, 0.50% trace mineral mix, and 0.16% vitamin ADE.

⁴Grain mix 3 contained 13.27% cottonseed meal, 13.27% soybean meal, 11.75% corn distillers whiskey (dehydrated), 7.5% corn, 15.33% hominy, 15.16% wheat middlings, 11.63% citrus pulp (dehydrated), 2.07% Megalac Plus (Church & Dwight Co. Inc., Princeton, NJ), 1.60% cane molasses (dehydrated), 0.63% tallow, 2.11% limestone, 2.33% Pro-Lak (H. J. Baker & Bro. Inc., Westport, CT), 0.42% urea, 0.63% salt, 0.42% magnesium oxide, 0.42% potassium magnesium sulfate, 1.26% sodium bicarbonate, 0.07% Se (600), 0.06% trace mineral premix, 0.04% vitamin ADE, and 0.04% vitamin A.

⁵Grain mix 4 contained 50.25% corn, 49.3% soybean meal, 0.35% trace mineral mix, and 0.10% vitamin ADE.

⁶Grain mix 5 contained 61.64% corn, 37.91% cottonseed meal, 0.35% trace mineral mix, and 0.10% vitamin ADE.

⁷Grain mix 6 contained 39.9% corn, 29.9% soybean meal, 12.5% soybean hulls, 11.5% wheat middlings, 3.03% limestone, 1.00% salt, 0.50% magnesium oxide, 0.50% magnesium sulfate, 0.28% Se (0.06%), 0.13% trace mineral premix, 0.13% vitamin ADE, and 0.27% vitamin E.

Extraction, Extract Clean Up, and Phytate Analysis

Chemical Extraction. Samples were subjected to 1 of 4 extraction protocols: 0.5 M HCl, 1 M HCl, 0.25 M NaOH-0.05 M EDTA, and sequential extraction (0.5 M HCl extraction, followed by 0.25 M NaOH-0.05 M EDTA extraction). For the extraction of dry samples, 1 g of dried ground sample was shaken with 20 mL of extractant on a horizontal shaker continuously for 4 h at room temperature. Then, samples were centrifuged at $30,000 \times g$ for 15 min at 4°C and supernatants decanted into acid-washed vials. Samples were extracted in triplicate, and for each replicate extract, duplicate 5-mL aliquots of supernatant were analyzed for total P (AOAC, 1984). The 0.5 M HCl and 0.25 M NaOH-0.05 M EDTA extracted P more efficiently than did the other 2 extraction methods, and these 2 were used for further experimentation focused on phytate quantification by HPIC.

Clean Up of Acid Extracts. Acidified extracts were categorized by sample matrix (high protein and starch feed samples vs. high fiber fecal and digesta samples) to determine the appropriate clean-up procedure. To remove proteins and starch, grain and TMR sample extracts were mixed 1:1 with a 20% (wt/vol) NaCl solution and stored overnight at 4°C. Salt-treated extracts were centrifuged at $30,000 \times g$ for 20 min at

4°C and the supernatant was decanted into acid-washed vials. After centrifugation of salt-treated extracts, clear supernatants were diluted with deionized distilled water at 1:5 to bring the phytate concentration within the detection limit of the HPIC, and passed through a 0.2- μ m ion chromatography (IC) membrane (filter) into Dionex sample vials (Dionex Corp., Sunnyvale, CA) for HPIC analysis.

Forage, omasal, and fecal sample extracts, neither salt treated nor diluted, were centrifuged at $30,000 \times g$ for 20 min at 4°C and supernatant was decanted into acid-washed vials. After centrifugation, clear supernatants were passed through a methanol-conditioned C₁₈ cartridge (Waters Corp., Milford, MA) and then through a 0.2- μ m IC membrane (filter) into Dionex sample vials, discarding the first 2 mL of supernatant. The C₁₈ cartridge has a hydrophobic silica-based binding phase capable of adsorbing analytes even with poor hydrophobicity from solutions.

Clean Up of Alkaline Extracts. Alkaline extracts were again categorized by sample matrix (high-protein and -starch feed samples vs. high-fiber fecal and digesta samples) to determine the appropriate clean-up procedure. Alkaline extracts of grain and TMR samples were acidified with HCl-HF acid solution (6 M HCl and 1.2 M HF) by adding 500 μ L of HCl-HF acid solution to 5 mL of sample extract. Acidified extracts

were stored overnight at 4°C and then centrifuged at $30,000 \times g$ for 20 min at 4°C. After centrifugation of acidified extracts, clear supernatants were collected and diluted with deionized distilled water at 1:5 to bring the phytate concentration within the detection limit of the HPIC. The diluted extracts were then passed through a methanol-conditioned C_{18} cartridge before passing through a 0.2- μ m IC membrane (filter) into Dionex sample vials for IC analysis. The first 2 mL of supernatant were discarded.

Forage, omasal, and fecal sample extracts were acidified with the HCl-HF acid solution as above and then centrifuged at $30,000 \times g$ for 20 min at 4°C; supernatant was decanted into acid-washed vials. Supernatants of each sample were passed through a methanol-conditioned C_{18} cartridge (Waters Corp.) and then through a 0.2- μ m IC membrane (filter) into Dionex sample vials, discarding the first 2 mL of supernatant.

Spike Recovery. To evaluate extraction efficiency and accuracy of IC analysis, spike recovery tests were performed on selected low-phytate samples. Dried ground forage and omasal samples (1 g of DM in triplicate) were spiked using 1 mL of phytate solution (100 μ g of phytate-P/mL), whereas fecal samples (higher in phytate than forage and omasal samples) were spiked using 1 mL of higher-concentration phytate solution (300 μ g of phytate-P/mL). Spiked samples were subjected to the same extraction, extract clean up, and analysis protocols described above. Samples were allowed to sit for 1 h after spike addition to improve the assimilation of spiking solution into the sample matrix.

HPIC Analysis. Chromatographic quantification of phytate in pretreated samples was achieved by HPIC (Dionex ICS 3000 with an HPLC pump, a Dionex 4 $\times$ 50 mm IonPac AG7 guard column, and a 4 $\times$ 250 mm IonPac AS7 analytical column; Dionex Corp.) using postcolumn reaction detection. Treated sample extracts were injected and phytate (inositol hexaphosphate) was eluted at 7.2 min with isocratic elution (0.25 M HNO_3) at a flow rate of 1 mL/min. Eluted phytate was mixed with 0.1% $Fe(NO_3)_3$ in 2% $HClO_4$ solution in a postcolumn reactor. The phytate products were measured at 290 nm on a UV-visible (UV-VIS) detector. Calibration standards (3, 15, and 30 μ g of P/mL) were prepared from phytic acid, sodium salt hydrate (Sigma, St. Louis, MO).

Statistical Analysis

Total P extraction efficiency data were analyzed using PROC GLIMMIX of SAS (SAS Institute, 2002). The model included extractant, sample type, and their interaction as fixed effects. The model evaluating the effect of feces drying on total P extraction efficiency

of different extractants included extractant, physical property, and their interaction as fixed effects. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Sample Properties

As intended, selected samples varied widely in their protein, starch, and fiber content (Tables 2 and 3). Feed CP content varied from 7.8 to 54.8%, with standard deviation of 18.6%. The variability in starch content of feed samples was $\pm 19.9\%$ standard deviations. The fiber and lignin content of feed samples also varied between classes with high standard deviation. Within class, the variability in sample properties was less in omasal and fecal samples in comparison with the feed samples. Sample CP and starch composition may influence efficiency of different extractants for total P or phytate because phytate forms stable complexes with protein and starch (Fontaine et al., 1946; Bourdillon, 1951). Furthermore, in plant feed sources, the distribution of phytate varies with the type of plant and with the proportion of other nutrients (Lin et al., 2005). Therefore, different extractants may be needed for different sample types and also for samples within a type that vary in composition.

Content of mineral elements varied widely among sample types (feed, omasal contents, and feces) as well as within sample type (Tables 2 and 3). Among the major elements, K concentration was the highest, followed by Ca and Mg, in feed and omasal samples; Ca was predominant in fecal samples. Among trace elements, Al and Fe were higher in omasal and fecal samples than in feed samples. He et al. (2009) suggested that the appropriate extractants for P depends on sample characteristics, including the association of P with Ca, Al, or Fe because adverse effects of these on the solubility of P or on P extraction efficiency have been reported for a variety of extractants (McDowell and Stewart, 2005; McDowell et al., 2008; He et al., 2009). McDowell and Stewart (2005) reported that Al and Fe were the major cations having an adverse effect on P solubility in dairy cattle feces, but in another study, McDowell et al. (2008) reported Ca as the major cation affecting total P extraction in feces of dairy cattle. Ajiboye et al. (2007) reported Ca as the most prevalent cation regulating P solubility in biosolids and hog, dairy, beef, and poultry manure.

Total P concentration in dried samples varied widely within sample type and across sample class (Table 4). The variability in total P concentration of fecal and omasal samples can be attributed to combined effects of variation in feed ingredients, individual animal varia-

Table 2. Nutrient content of feed samples

Sample	Nutrient (% of DM)								Nutrient (mg/kg of DM)	
	CP	Starch	NDF	ADF	ADL	Ca	K	Mg	Al	Fe
Grain										
Soybean meal sample 1	52.1	7.6	21.6	6.34	3.01	0.35	2.59	0.31	545	230
Soybean meal sample 2	54.8	7.1	39.3	6.98	0.59	0.47	2.59	0.36	37	120
Soybean meal sample 3	50.7	6.9	29.8	8.73	1.58	0.43	2.51	0.37	752	564
Corn	9.5	69.5	10.6	2.71	0.12	0.01	0.39	0.12	14	32
Cottonseed meal	50.2	4.3	46.7	22.2	13.7	0.28	1.84	0.81	58	237
Grain mix 3	22.3	23.8	26.8	10.7	5.57	1.57	1.16	0.62	128	352
Grain mix 6	19.1	36.1	31.4	15.5	2.09	1.49	1.17	0.70	255	495
Forage										
Alfalfa hay	17.4	1.1	54.9	40.9	14.0	1.07	2.30	0.27	592	763
Grass hay	10.8	1.7	70.5	41.4	7.74	0.40	2.64	0.19	363	366
Corn silage sample 1	7.8	33.6	42.4	22.1	1.13	0.26	1.32	0.22	29	163
Corn silage sample 2	8.2	38.9	36.4	19.1	1.95	0.19	1.14	0.15	51	142
Mixed ration										
Mixed ration 1	15.3	29.3	31.7	16.2	3.72	0.80	1.4	0.29	237	312
Mixed ration 2	19.7	29.7	32.1	17.8	2.93	0.73	1.36	0.33	225	324
Mixed ration 3	15.6	24.6	38.7	20.2	4.15	1.36	1.37	0.59	1,112	1,047

tion in digestion, and P utilization efficiency related to the production stage of animals. In feed samples, total P concentration ranged from 1.6 to 14.8 (SD = 3.6) mg/g of DM. Commonly used feed ingredients contain total P ranging from 2.3 to 11.6 mg/g of DM (NRC, 2001). Total P concentration in feces ranged from 5.6 to 12.0 mg/g of DM (Table 4), similar to values reported in the literature (4.6 to 14 mg/g of DM; Dou et al., 2003; Ajiboye et al., 2007; McDowell et al., 2008). Total P concentration in omasal samples was higher than in feed and feces (12.9 to 21.6 mg/g of DM) because of the incorporation of inorganic P into the flow of P from the rumen through the release of recycled salivary P into the rumen (Valk et al., 2002; Pugaard et al., 2011).

Also, absorption of organic compounds, such as VFA from the rumen, concentrated P in the omasal contents.

Efficiency of Extraction of Total P

The efficiency of total P extraction from dried samples varied with extractant, sample type, and the interaction of extractant and sample type (Table 5). Alkaline extraction (NaOH-EDTA) was highly effective in extracting P from all sample types, and the extraction efficiency was generally lower with 0.5 M HCl than with other extractants (Table 5). The P in omasal contents (largely inorganic) was efficiently extracted by all extractants. The most commonly used acid and alkaline

Table 3. Nutrient content of digesta and feces samples

Sample type	Diet ¹	Nutrient (% of DM)								Nutrient (mg/kg of DM)	
		CP	Starch	NDF	ADF	ADL	Ca	K	Mg	Al	Fe
Omasal content											
Dry cow	Mixed ration 6	17.6	0.9	36.9	19.8	8.70	0.52	3.90	0.27	1,515	935
Dry cow	Grass hay only	14.2	0.9	44.2	25.2	12.0	0.65	8.90	0.28	521	359
Lactating cow	Mixed ration 1	31.9	1.9	49.3	28.3	13.4	1.02	3.24	0.37	441	538
Lactating cow	Mixed ration 2	29.3	1.4	55.8	30.3	18.3	0.80	2.37	0.41	316	467
Lactating cow	Mixed ration 3	22.6	1.0	36.7	19.7	9.40	1.02	3.11	0.42	233	362
Feces											
Heifer	Mixed ration 4	19.3	5.8	41.6	22.6	6.09	1.42	1.43	0.80	714	808
Heifer	Mixed ration 5	15.2	16.4	51.5	32.2	6.84	0.89	1.30	0.56	468	701
Dry cow	Mixed ration 6	15.8	1.1	59.6	33.3	9.89	1.69	1.23	0.67	1,902	1,608
Dry cow	Grass hay only	11.2	0.7	59.3	35.7	18.1	1.06	0.85	0.46	952	776
Lactating cow	Mixed ration 1	16.7	1.2	50.6	27.9	8.47	2.28	0.80	0.66	946	1,340
Lactating cow	Mixed ration 2	17.5	9.7	54.7	31.4	7.60	2.14	0.68	0.71	743	1,110
Lactating cow	Mixed ration 3	16.5	1.4	53.5	30.7	7.78	2.33	0.71	0.84	492	903

¹Diets are described in Tables 1 and 2.

Table 4. Total P content and P extraction efficiency of feed, digesta, and feces samples with HCl, NaOH-EDTA, and sequential extraction

Sample	Total P ($\mu\text{g/g}$ of DM)	Extraction efficiency (% of total P)			
		0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
Grain					
Soybean meal sample 1	7,157	66.8	77.9	97.6	82.8
Soybean meal sample 2	8,467	71.3	76.3	95.6	84.0
Soybean meal sample 3	7,900	65.4	78.8	95.1	83.8
Corn	3,065	89.4	91.0	90.8	94.5
Cottonseed meal	14,794	82.0	88.8	96.4	89.5
Grain mix 3	7,289	89.0	95.5	104.2	98.2
Grain mix 6	5,233	76.5	81.1	93.0	86.4
Forage					
Alfalfa hay	2,915	92.0	89.4	92.6	85.3
Grass hay	2,395	99.6	103.9	103.1	89.1
Corn silage sample 1	1,632	91.8	93.3	89.5	87.4
Corn silage sample 2	2,327	77.4	84.9	95.7	75.8
Mixed ration					
Mixed ration 1	4,296	94.4	98.9	107.7	99.7
Mixed ration 2	4,988	90.8	92.7	101.7	92.9
Omasal content					
Dry cow fed mixed ration 6	19,662	95.4	97.7	100.8	100.6
Dry cow fed grass hay only	21,582	95.8	94.9	96.7	98.2
Lactating cow fed mixed ration 1	12,864	93.0	91.9	97.2	97.3
Lactating cow fed mixed ration 2	16,309	95.9	95.9	100.0	96.6
Lactating cow fed mixed ration 3	18,394	92.4	94.3	96.5	95.8
Feces					
Heifer fed mixed ration 4	12,022	84.2	88.1	97.0	89.6
Heifer fed mixed ration 5	9,034	78.6	74.7	93.3	79.0
Dry cow fed mixed ration 6	7,019	69.5	76.4	97.8	80.1
Dry cow fed grass hay only	6,143	62.9	67.3	84.9	67.4
Lactating cow fed mixed ration 1	5,623	73.7	72.6	92.7	75.3
Lactating cow fed mixed ration 2	7,438	75.4	84.6	100.0	91.6
Lactating cow fed mixed ration 3	8,955	82.0	87.4	92.9	85.5

extractants are HCl and NaOH-EDTA, respectively, and water and NaHCO_3 are often added for sequential extraction procedures (Dou et al., 2000). Little direct comparison of different extractants used individually to extract total P in feed, digesta, and fecal samples from dairy cows has been reported. In a recent study, McDowell et al. (2008) used acid and alkaline extractants (0.012 M HCl and 0.25 M NaOH-0.05 M EDTA) to extract P from feces of dairy cows and observed higher total P recovery in alkaline extracts than in HCl extracts.

Effectiveness of Acid Extraction in Different Types of Samples. Acid extraction was highly effective for omasal samples (Table 5) because of the dominance of salivary (inorganic) P in that sample type. In contrast, HCl extracted only 75 to 79% of the P in feces, similar to the 61 to 86% of total P extracted from dairy fecal samples with dilute HCl (0.012 M) by McDowell et al. (2008). With repeated extractions of dairy feces with 1 M HCl recovery of P was maximized at 90% (Dou et al., 2000). In the current study, the 2 concentrations of HCl (0.5 and 1 M) were equally

Table 5. Phosphorus extraction efficiency from 4 classes of feeds with HCl, NaOH-EDTA, and sequential extraction

Feed class ¹	Extraction efficiency (% of total P)			
	0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
Grains and mixed rations	80.6 $\pm$ 0.92 ^{gf}	86.8 $\pm$ 0.92 ^{de}	98.1 $\pm$ 0.93 ^a	93.3 $\pm$ 0.95 ^{ab}
Forages	90.2 $\pm$ 1.38 ^{bcd}	93.2 $\pm$ 1.41 ^{abc}	95.2 $\pm$ 1.38 ^{ab}	87.14 $\pm$ 1.41 ^{cde}
Omasal contents	94.5 $\pm$ 1.23 ^{ab}	95.0 $\pm$ 1.23 ^{ab}	98.2 $\pm$ 1.23 ^a	98.6 $\pm$ 1.23 ^a
Dry feces	75.2 $\pm$ 1.05 ^h	78.7 $\pm$ 1.04 ^{gh}	94.1 $\pm$ 1.04 ^{ab}	84.1 $\pm$ 1.04 ^{ef}

^{a-h}Values with different superscripts are significantly different ($P < 0.05$).

¹Classes are groupings of the feeds described in Tables 1 and 2. Values reported are LSM $\pm$ SEM.

effective in omasal samples and equally ineffective in feces (Table 5). The HCl extractant was more effective in feed samples than in fecal samples, perhaps because P is mostly present as salts of K, Mg, and Ca in feed samples but is present also as P complexed with Al and Fe in feces (McDowell et al. 2008). Hydrochloric acid extractable P is thought to be associated with Ca, whereas NaOH-EDTA-extractable P is considered to be associated with Al and Fe (McDowell and Stewart, 2005). Our observation of greater recovery of total P from feces in alkaline extracts than in acid extracts (discussed below) would support this hypothesis. Within feed samples, the HCl extraction was more effective in forage samples (average 90.2%) than in grains and TMR (average 80.6%). In most studies, 1 M HCl is used as a step of sequential extraction of total P, and the 0.5 M concentration is used for phytate quantification studies using HPIC.

Effectiveness of Alkaline Extraction in Different Samples. The 0.25 M NaOH-0.05 M EDTA extractant extracted almost all P from feces (94.1% of total P; Table 5), as has been reported by others (Toor et al., 2005; McDowell et al., 2008). The efficiency of extraction of P from feeds with the alkaline extractant was also essentially complete (95.2% for forage; 98.1% for grains and TMR) and higher than reported by McDowell et al. (2008; 60–87% recovery of total P in extracts of grain, forage, and TMR).

Polyvalent cations in the sample affect P extraction efficiency and should be considered when choosing the extractant. The Ca content of feeds may challenge alkaline extraction (Chapuis-Lardy et al., 2004; McDowell et al., 2008) because NaOH is less efficient in extracting Ca than is HCl and Ca-P complexes influence the solubility of P (Turner and Leytem, 2004; Ajiboye et al., 2007). Likewise, the Al and Fe content of feces affects recovery of P with alkaline extraction. The use of EDTA to chelate the polyvalent cations can help resolve these problems. In cattle manure, improved recovery of total P was reported with the use of NaOH-EDTA instead of NaOH alone (80 vs. 32%; Turner, 2004). Very low recovery of total P in 0.25 M NaOH-0.05 M EDTA extract of cattle feces was reported by McDowell and Stewart (2005); this poor recovery was attributed to high concentrations of Al and Fe in their

samples (8,500 and 4,300 mg/kg of DM, respectively); so high that these minerals could not be fully chelated by EDTA. Samples in the current study were comparatively low in Al and Fe (888 and 1,035 mg/kg of DM, respectively; Tables 2 and 3).

Sequential Extraction. For both feed and fecal samples, lower P extraction efficiency was observed with sequential extraction than with alkaline extraction (0.25 M NaOH-0.05 M EDTA) alone (Table 5). Sequential extraction (acid followed by alkaline, with or without water and NaHCO_3) is the most common approach to fractionating P forms in soil, animal manure, and other organic amendments when the research purpose is to address the issue of environmental pollution from P runoff (Sharpley and Moyer, 2000; Hansen et al., 2004; McDowell and Stewart, 2005). In this study, we used HCl extraction, followed by NaOH-EDTA extraction as the sequential extraction scheme and omitted the H_2O and NaHCO_3 steps because our interest was to select the best 2 extractants for complete recovery of total P rather than identifying different fractions of P.

Effect of Drying Fecal Samples on Extraction Efficiency

Drying did not affect the efficiency of P extraction from feces (Table 6). The variability in extraction efficiency within extractant was higher in wet feces compared with dry feces, possibly due to the errors inherent in mixing and weighing wet fecal samples. Also, the interaction of the extractant and sample matrix may differ between dry and wet samples.

In most studies, observed changes with drying are usually transformation between different P forms rather than changes in total extractable P. For instance, McDowell et al. (2008) did not find any significant effect of drying on total P recovery from dairy feces but reported increased molybdate-reactive P (mostly inorganic phosphate) in NaOH-EDTA extracts of dry feces relative to wet feces; the opposite was observed in water extracts. Ethylenediaminetetraacetate nullifies any effect of drying on total P extractability because it acts as a chelating agent on humic compounds. In contrast, McDowell and Stewart (2005) observed that, when used as a step of sequential extraction, NaOH

Table 6. Effect of drying on extraction efficiency of total P in feces

Feces group	Extraction efficiency (% total P)			
	0.5 M HCl	1 M HCl	NaOH-EDTA	Sequential
Dry feces	75.2 ± 2.04 ^d	78.7 ± 2.01 ^{cd}	94.1 ± 2.01 ^a	84.1 ± 2.01 ^{bc}
Wet feces	72.4 ± 2.04 ^d	80.3 ± 2.03 ^{cd}	90.1 ± 2.01 ^{ab}	91.1 ± 2.7 ^{ab}

^{a-d}Values with different superscripts are significantly different ($P < 0.05$).

Table 7. Hydrochloric acid (0.5 *M*)- and 0.25 *M* NaOH-0.05 *M* EDTA-extractable phytate P in feed samples¹

Class	Feed	HCl	NaOH-EDTA
		Phytate P ($\mu\text{g/g}$ of DM)	Phytate P ($\mu\text{g/g}$ of DM)
Grain	Soybean meal 1	4,729	3,831
Grain	Soybean meal 2	5,282	4,237
Grain	Soybean meal 3	3,630	4,047
Grain	Corn	2,567	2,577
Grain	Cottonseed meal	10,871	10,520
Grain	Dry cow pellet	3,384	3,662
Grain	Lactating cow pellet	4,457	4,311
Forage	Alfalfa silage	6	15
Forage	Grass hay	22	39
Forage	Corn silage sample 1	3	10
Forage	Corn silage sample 2	2	9
Mixed ration 2	For lactating cow, containing cottonseed meal, corn silage, and grass hay	2,377	2,855
Mixed ration 3	For lactating cow, containing cottonseed meal, soybean meal, and alfalfa and corn silage	1,230	2,434

¹Diets are described in Tables 1 and 2.

recovered less total P from dry cattle feces than from fresh feces, probably because of increased hydrophobicity as a result of rearrangement of humic compounds. Chapuis-Lardy et al. (2004) observed that oven drying at 65°C reduced water-extractable inorganic P. Due to the inconsistent pattern of extraction efficiency in dry and wet feces for different extractants and the difficulty in storing wet feces, most studies use dry feces for convenience and decreased variation. Our results support that preference.

Phytate Analysis by HPIC

Based on the total P recovery data 2 extractants, 0.5 *M* HCl and 0.25 *M* NaOH-0.05 *M* EDTA, were selected

for phytate quantification. The NaOH-EDTA extraction was chosen because it yielded almost complete recovery of total P and the 0.5 *M* HCl extraction was used to reflect easily soluble P, likely available for absorption by the animal. Given the lack of improvement in total P recovery with sequential extraction and the increased opportunity for analytical error, sequential extraction was not explored further.

Phytate concentration in HCl and NaOH-EDTA extracts of feed ranged from <5 to 10–11,000 $\mu\text{g/g}$ of DM (Table 7), with values similar to previously reported for comparable human foods and livestock feeds (Eeckhout and De Paepe, 1994; Talamond et al., 2000; McDowell et al., 2008). Phytate was also detectable in HCl and NaOH-EDTA extracts of omasal and fecal samples but

Table 8. Hydrochloric acid (0.5 *M*)- and 0.25 *M* NaOH-0.05 *M* EDTA-extractable phytate P in digesta and feces samples¹

Sample type	Diet	HCl	NaOH-EDTA
		Phytate P ($\mu\text{g/g}$ of DM)	Phytate P ($\mu\text{g/g}$ of DM)
Omasal content			
Dry cow	Mixed ration 6	80	103
Dry cow	Grass hay only	11	5
Lactating cow	Mixed ration 1	ND ²	ND
Lactating cow	Mixed ration 2	3	7
Lactating cow	Mixed ration 3	31	48
Feces			
Heifer	Mixed ration 4	176	231
Heifer	Mixed ration 5	270	369
Dry cow	Mixed ration 6	65	138
Dry cow	Grass hay only	39	68
Lactating cow	Mixed ration 1	57	95
Lactating cow	Mixed ration 2	167	196
Lactating cow	Mixed ration 3	237	324

¹Diets are described in Tables 1 and 2.

²ND = not detected.

Table 9. Effect of extraction and extract clean up on the recovery of phytate spiked to samples¹

Sample	Phytate recovery (% of quantity spiked)	
	HCl	NaOH-EDTA
Alfalfa silage	29.7	88.0
Omasal content		
Mixed ration 2 fed lactating cow	42.8	87.5
Mixed ration 3 fed lactating cow	97.2	90.4
Feces		
Mixed ration 5 fed heifer	81.5	105.5
Mixed ration 3 fed lactating cow	85.0	100.2

¹Diets are described in Tables 1 and 2.

at lower concentrations (<5 to 80–100 µg/g of DM and <50 to <400 µg/g of DM respectively; Table 8).

Spike Recovery. In the spike recovery tests, the recovery of added phytate from spiked samples was almost complete with 0.25 M NaOH-0.05 M EDTA extraction (88–106% of spiked phytate; Table 9). Recovery of spiked phytate was not complete in many samples with HCl extraction. Poor recovery of spiked phytate with HCl extraction was observed primarily with low-phytate samples, likely due to a complex interaction among phytate, extractant, and sample matrix. Complete recovery (95–105% of spiked phytate) of phytate spiked to food samples was reported with HCl extraction and HPIC analysis (Skoglund et al., 1997; Brooks and Lampi, 2001; Chen, 2004), although in those studies, inositol hexaphosphate (**IP6**) spikes were added after extraction, so evaluated just analytical efficiency rather than extraction efficiency. The current study used preextraction spikes (spikes added to dried ground samples before extraction). The full recovery of phytate spiked to samples preextraction suggests both the absence of any adverse effect of the alkaline extraction on phytate and also the applicability of 0.25 M NaOH-0.05 M EDTA extraction for phytate quantification using HPIC.

Utility of HPIC Versus NMR. High-performance ion chromatography and HPLC have previously been used to quantify phytate in monogastric feces and digesta (Leytem et al., 2008), although NMR analysis is sometimes used. Leytem et al. (2008) found HPLC (acid extraction) to yield comparable results to NMR (alkaline extraction) when quantifying IP6 in broiler ileal digesta. For ruminant feces, ³¹P NMR is typically used to analyze NaOH-EDTA extracts for various P-containing compounds (McDowell and Stewart, 2005; Toor et al., 2005; McDowell et al., 2008). McDowell et al. (2008) used 0.012 M HCl as extractant in feed and feces from dairy cows before ³¹P NMR analysis, but mandatory pH adjustment to an alkaline pH before

NMR analysis caused precipitation of certain P fractions with polyvalent cations, leading to underestimation of some P forms.

Although ³¹P NMR provides a full spectrum of different P forms in 1 run with just a single extraction, this technique is relatively insensitive and more expensive than HPLC or HPIC. Another disadvantage of NMR is that identification of inositol phosphates is difficult due to poor resolution in the orthophosphate monoester region of NMR spectra; this compromises the separation of inositol phosphate esters from other orthophosphate monoesters, leading to overestimation of IP6. This problem can be overcome by using deconvolution software but this requires considerable expertise. Due to its greater sensitivity, relative simplicity, and lower cost, HPIC is preferred over ³¹P NMR for quantification of different P forms in a wide range of samples.

Special Problems with Soybean Meal. Although NaOH-EDTA was at least as effective as 0.5 M HCl in extracting phytate from most samples, soybean meal (**SBM**) appears to pose a special problem (Table 7). When quantified using HPIC, the phytate concentration in NaOH-EDTA extracts of SBM samples was lower than in HCl extracts. This may be attributed to the acidification of 0.25 M NaOH-0.05 M EDTA extracts before chromatographic analysis rather than to inefficiencies of the alkaline extraction per se. Acidification of alkaline extracts is necessary to make extracted samples compatible with chromatographic analysis, but in SBM it may cause the formation of insoluble complexes between phytate and polyvalent cations or soy proteins, or both. We speculate that the NaOH-EDTA extractant releases protein not only from phytate-protein complexes in the sample but also from complexes of protein with other nonphytate compounds. Also the alkaline extract would theoretically be able to interrupt metal-ligand coordination within proteins and break their H bonds and physical molecular forces, possibly even covalent bonds. To the eye, the supernatant of the alkaline extracts were obviously not true solutions with simple salts but instead contained colloids. When the alkaline extracts of SBM were acidified in preparation for HPIC, the protonated (H⁺) sites on proteins in the extract likely formed insoluble complexes with the (negatively charged) phytate in the extract. Precipitate was visually obvious in the alkaline extracts following acidification and we hypothesize that the precipitate comprised insoluble protein-phytate complexes, removing phytate from the solution.

This is a problem specific to SBM because, in soy, more phytate is associated with protein than with Ca and Mg (Brooks and Morr, 1982). Tzeng et al. (1990) reported that proteins and metal ions compete with

each other to form complexes with phytic acid in acidic media. Hartman (1979) reported that in pH range of 2 to 4.5, phytate removal from SBM extract was inefficient due to strong phytate-protein complexes; phytate can be displaced from this complex only in the presence of excess divalent cations.

Thus, the apparently low recovery of phytate from SBM with alkaline extraction in our study was likely due to problems imposed with the acidification step of the postextraction treatment. Therefore, although it gave incomplete recovery of total P (Tables 4 and 5), we conclude that HCl extraction is still preferred for SBM samples unless alkaline extracts can be made compatible with HPIC in some way other than acidifying the extract. More research is needed with other high-protein feeds to evaluate this hypothesis.

CONCLUSIONS

A method is described for analysis of phytate in ruminant digesta and fecal samples consisting of extraction of samples, extract clean up, and HPIC analysis. Alkaline extraction was able to extract P almost completely from all sample types; acid extraction was adequate for feeds and omasal samples but not for feces. The described extract clean-up procedures successfully removed the interference from sample matrix, allowing accurate measurement of low concentrations of phytate in fecal and digesta samples from dairy cattle with HPIC. Soy samples may pose special problems when alkaline extracted because of protein-phytate interactions in the extract. The described method combines the extraction power of NaOH-EDTA with the precision and sensitivity of HPIC quantification to measure phytate in feeds, ruminant digesta, and feces. This method is simple, rapid, accurate, and robust and may help advance knowledge of dynamics of digestion of phytate and P in ruminants.

REFERENCES

- Ajiboye, B., O. O. Akinremi, Y. Hu, and D. N. Flaten. 2007. Phosphorus speciation of sequential extracts of organic amendments using nuclear magnetic resonance and X-ray absorption near-edge structure spectroscopies. *J. Environ. Qual.* 36:1563–1576.
- AOAC. 1984. Official Methods of Analysis. 14th ed. Association of Official Analytical Chemists. Washington, DC.
- Bourdillon, J. 1951. A crystalline bean seed protein in combination with phytic acid. *J. Biol. Chem.* 189:65–72.
- Bravo, D., F. Meschy, C. Bogaert, and D. Sauvant. 2003. Phosphorus availability of oilseed meals determined by the apparent faecal digestibility technique. *Anim. Feed Sci. Technol.* 108:43–60.
- Brooks, J. R., and C. V. Morr. 1982. Phytate removal from soy protein isolates using ion exchange processing treatments. *J. Food Sci.* 47:1280–1282.
- Brooks, S. P. J., and B. J. Lampi. 2001. Problems associated with measuring phytate in infant cereals. *J. Agric. Food Chem.* 49:564–569.
- Chapuis-Lardy, L., J. Fiorini, J. Toth, and Z. Dou. 2004. Phosphorus concentration and solubility in dairy feces: Variability and affecting factors. *J. Dairy Sci.* 87:4334–4341.
- Chen, Q. 2004. Determination of phytic acid and inositol pentakisphosphates in foods by high-performance ion chromatography. *J. Agric. Food Chem.* 52:4604–4613.
- Cherry, N. M., B. D. Lambert, and J. P. Muir. 2010. Ruminant and total tract phosphorus release from feedstuffs in cattle measured using the mobile nylon bag technique. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 94:665–669.
- Clark, W. D. Jr., J. E. Wohlt, R. L. Gilbreath, and P. K. Zajac. 1986. Phytate phosphorus intake and disappearance in the gastrointestinal tract of high producing dairy cows. *J. Dairy Sci.* 69:3151–3155.
- Dou, Z., J. D. Ferguson, J. Fiorini, J. D. Toth, S. M. Alexander, L. E. Chase, C. M. Ryan, K. F. Knowlton, R. A. Kohn, A. B. Peterson, J. T. Sims, and Z. Wu. 2003. Phosphorus feeding levels and critical control points on dairy farms. *J. Dairy Sci.* 86:3787–3795.
- Dou, Z., J. D. Toth, D. T. Galligan, C. F. Ramberg Jr., and J. D. Ferguson. 2000. Laboratory procedures for characterizing manure phosphorus. *J. Environ. Qual.* 29:508–514.
- Eeckhout, W., and M. De Paepe. 1994. Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Anim. Feed Sci. Technol.* 47:19–29.
- Fontaine, T. D., W. A. Pons, and G. W. Irving. 1946. Protein-phytic acid relationship in peanuts and cottonseed. *J. Biol. Chem.* 164:487–507.
- Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. *J. AOAC Int.* 92:42–49.
- Hansen, J. C., B. J. Cade-Menun, and D. G. Strawn. 2004. Phosphorus speciation in manure-amended alkaline soils. *J. Environ. Qual.* 33:1521–1527.
- Hartman, G. H. Jr. 1979. Removal of phytate from soy protein. *J. Am. Oil Chem. Soc.* 56:731–735.
- He, Z., C. W. Honeycutt, T. S. Griffin, B. J. Cade-Menun, P. J. Pel-lechia, and Z. Dou. 2009. Phosphorus forms in conventional and organic dairy manure identified by solution and solid state P-31 NMR spectroscopy. *J. Environ. Qual.* 38:1909–1918.
- Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. *J. Anim. Sci.* 75:1380–1392.
- Kincaid, R. L., D. K. Garikipati, T. D. Nennich, and J. H. Harrison. 2005. Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. *J. Dairy Sci.* 88:2893–2902.
- Knowlton, K. F., J. S. Radcliffe, C. L. Novak, and D. A. Emmerson. 2004. Animal management to reduce phosphorus losses to the environment. *J. Anim. Sci.* 82(E-Suppl.):E173–E195.
- Leytem, A. B., P. Kwanyuen, P. W. Plumstead, R. O. Maguire, and J. Brake. 2008. Evaluation of phosphorus characterization in broiler ileal digesta, manure, and litter samples: ³¹P-NMR vs. HPLC. *J. Environ. Qual.* 37:494–500.
- Lin, L., I. Ockenden, and J. N. A. Lott. 2005. The concentrations and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid 1-1 (lpa1-1) corn (*Zea mays* L.) grains and grain parts. *Can. J. Bot.* 83:131–141.
- McDowell, R. W., Z. Dou, J. D. Toth, B. J. Cade-Menun, P. J. A. Kleinman, K. Soder, and L. Saporito. 2008. A comparison of phosphorus speciation and potential bioavailability in feed and feces of different dairy herds using ³¹P nuclear magnetic resonance spectroscopy. *J. Environ. Qual.* 37:741–752.
- McDowell, R. W., and I. Stewart. 2005. Phosphorus in fresh and dry dung of grazing dairy cattle, deer, and sheep: Sequential fraction and phosphorus-31 nuclear magnetic resonance analyses. *J. Environ. Qual.* 34:598–607.

- Mjoun, K., K. F. Kalscheur, A. R. Hippen, and D. J. Schingoethe. 2008. Ruminal phosphorus disappearance from corn and soybean feedstuffs. *J. Dairy Sci.* 91:3938–3946.
- Morse, D., H. H. Head, and C. J. Wilcox. 1992. Disappearance of phosphorus in phytate from concentrates in vitro and from rations fed to lactating dairy cows. *J. Dairy Sci.* 75:1979–1986.
- Nelson, T. S., L. B. Daniels, J. R. Hall, and L. G. Shields. 1976. Hydrolysis of natural phytate phosphorus in the digestive tract of calves. *J. Anim. Sci.* 42:1509–1512.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Park, W.-Y., T. Matsui, C. Konishi, S.-W. Kim, F. Yano, and H. Yano. 1999. Formaldehyde treatment suppresses ruminal degradation of phytate in soyabean meal and rapeseed meal. *Br. J. Nutr.* 81:467–471.
- Phipps, R. H., J. D. Sutton, D. E. Beever, and A. K. Jones. 2000. The effect of crop maturity on the nutritional value of maize silage for lactating dairy cows 3. Food intake and milk production. *Anim. Sci.* 71:401–409.
- Puggaard, L., N. B. Kristensen, and J. Sehested. 2011. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *J. Dairy Sci.* 94:1420–1429.
- Ravindran, V., G. Ravindran, and S. Sivalogan. 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant-origin. *Food Chem.* 50:133–136.
- SAS Institute. 2002. SAS User's Guide: Statistics. Version 9.2. SAS Inst. Inc., Cary, NC.
- Sharpley, A., and B. Moyer. 2000. Phosphorus forms in manure and compost and their release during rainfall. *J. Environ. Qual.* 29:1462–1469.
- Skoglund, E., N.-G. Carlsson, and A.-S. Sandberg. 1997. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. *J. Agric. Food Chem.* 45:431–436.
- Talamond, P., S. Doulbeau, I. Rochette, J.-P. Guyot, and S. Treche. 2000. Anion-exchange high-performance liquid chromatography with conductivity detection for the analysis of phytic acid in food. *J. Chromatogr. A* 871:7–12.
- Toor, G. S., B. J. Cade-Menun, and J. T. Sims. 2005. Establishing a linkage between phosphorus forms in dairy diets, feces, and manures. *J. Environ. Qual.* 34:1380–1391.
- Turner, B. L. 2004. Optimizing phosphorus characterization in animal manures by solution phosphorus-31 nuclear magnetic resonance spectroscopy. *J. Environ. Qual.* 33:757–766.
- Turner, B. L., and A. B. Leytem. 2004. Phosphorus compounds in sequential extracts of animal manures: Chemical speciation and a novel fractionation procedure. *Environ. Sci. Technol.* 38:6101–6108.
- Turner, B. L., M. J. Papházy, P. M. Haygarth, and I. D. McKelvie. 2002. Inositol phosphates in the environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 357:449–469.
- Tzeng, Y.-M., L. L. Diosady, and L. J. Rubin. 1990. Production of canola protein materials by alkaline extraction, precipitation, and membrane processing. *J. Food Sci.* 55:1147–1151.
- Valk, H., L. B. J. Šebek, and A. C. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *J. Dairy Sci.* 85:2642–2649.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597.
- VandeHaar, M. J., and N. St-Pierre. 2006. Major advances in nutrition: Relevance to the sustainability of the dairy industry. *J. Dairy Sci.* 89:1280–1291.
- Wheeler, E. L., and R. E. Ferrel. 1971. A method for phytic acid determination in wheat and wheat fraction. *Cereal Chem.* 48:312–316.