Comparison of Phosphorus Forms in Wet and Dried Animal Manures by Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy and Enzymatic Hydrolysis

Zhongqi He, Barbara J. Cade-Menun, Gurpal S. Toor, Ann-Marie Fortuna, C. Wayne Honeycutt, and J. Thomas Sims

ABSTRACT

Both enzymatic hydrolysis and solution $^{31}$P nuclear magnetic resonance (NMR) spectroscopy have been used to characterize P compounds in animal manures. In this study, we comparatively investigated P forms in 0.25 M NaOH/0.05 M EDTA extracts of dairy and poultry manures by the two methods. For the dairy manure, enzymatic hydrolysis revealed that the majority of extracted P was inorganic P (50%), with 10% phytate-like P, 9% simple monoester P, 6% polynucleotide-like P, and 18% non-hydrolyzable P. Similar results were obtained by NMR spectroscopy, which showed that inorganic P was the major P fraction (64-73%), followed by 6% phytic acid, 14 to 22% other monoesters, and 7% phosphodiesters. In the poultry manure, enzymatic hydrolysis showed that inorganic P was the largest fraction (71%), followed by 15% phytate-like P and 1% other monoesters, and 3% polynucleotide-like P. NMR spectroscopy revealed that orthophosphate was 51 to 63% of extracted P, phytic acid 24 to 33%, other phosphomonoesters 6 to 12%, and phosphodiesters and DNA 2% each. Drying process increased orthophosphate (8.4% of total P) in dairy manure, but decreased orthophosphate (13.3% of total P) in poultry manure, suggesting that drying treatment caused the hydrolysis of some organic P to orthophosphate in dairy manure, but less recovery of orthophosphate in poultry manure. Comparison of these data indicates that the distribution patterns of major P forms in animal manure determined by the two methods were similar. Researchers can utilize the method that best fits their specific research goals or use both methods to obtain a full spectrum of manure P characterization.

Increased understanding of manure phosphorus (P) composition is needed for developing best management practices to optimize recycling of manure P while minimizing the adverse environmental effects of animal manure application. Solution $^{31}$P nuclear magnetic resonance (NMR) spectroscopy has been used to characterize P forms in environmental samples for decades (Newman and Tate, 1980; Condron et al., 1985; Leinweber et al., 1997; Pant et al., 1999; Zhang et al., 1999; Cade-Menun et al., 2002; Maguire et al., 2004; Cade-Menun, 2005; McGrath et al., 2005). This method allows P to be grouped into compound classes such as phosphonates, orthophosphate, orthophosphate monoesters, orthophosphate diesters, and polyphosphates (including pyrophosphate). Within these compound classes, many specific P compounds can be identified, such as phytate (myo-inositol hexakisphosphate, IP6) in the orthophosphate monoester region, and DNA in the orthophosphate diester region.

Enzymatic hydrolysis is another method used to identify organic P species in the environment. This method has been used to characterize hydrolyzable organic P in soils (Pant et al., 1994; Shand and Smith, 1997; Pant and Warman, 2000; Turner et al., 2002; He et al., 2004a, 2004b), manures (He et al., 2004c, 2006b, 2006c), and waters (Herbes et al., 1975; Toor et al., 2003). Unlike $^{31}$P NMR, enzymatic hydrolysis cannot classify all organic P in a sample. However, because organic P must first be hydrolyzed to inorganic P for plant uptake, enzymatic hydrolysis can provide an estimate of hydrolyzable, and thus bioavailable, organic P in manure and soil (Seeling and Jungk, 1996; Otani and Ae, 1999), which is not possible to obtain with NMR analysis. If appropriate phosphatases are used, specific forms of hydrolyzable organic P can be identified and quantified by the difference in inorganic P detected after incubation in the presence and absence of phosphatase (He and Honeycutt, 2001; Turner et al., 2002; Toor et al., 2003; He et al., 2004c, 2006b, 2006c). He et al. (2004b, 2006c) have developed a phosphatase hydrolysis method to characterize manure and soil P. In this enzymatic approach, organic P extracted from manure or soil is incubated at pH 5.0 (100 mM sodium acetate buffer) with either (i) acid phosphatase from potato; (ii) acid phosphatases from potato and nuclease P1; or (iii) phosphatases from potato and wheat germ plus nuclease P1. Each of the enzymes shows optimal activity at 37°C and near pH 5. The P differentially released by these three enzymes is designated as simple monoester P, polynucleotide-like P, and phytate-like P, respectively.

For any methods that attempt to characterize P, in sample extracts, concerns exist about the influence of sample preparation on extracted P forms. Manures are usually dried and ground for easier handling and to improve sample heterogeneity (Ajiboye et al., 2004; Vadas and Kleinman, 2006; Dail et al., 2007). However, drying samples before extraction has been shown to alter P extractability, e.g., reducing water-extractable P from poultry manure (Sistani et al., 2001), but increasing water-extractable P from dairy manure (Ajiboye et al., 2004; Vadas and Kleinman, 2006). These changes may occur through degradation of P forms or through changes in P bonding to the sample matrix (Turner, 2005). If P forms are degraded, this could influence the
results of both P NMR and phosphatase hydrolysis. However, if bonding mechanisms alone are altered, this may affect phosphatase hydrolysis but not P forms revealed by P NMR.

Both $^{31}$P NMR spectroscopy and phosphatase hydrolysis are valuable tools for P characterization. However, there have been no reports comparing data obtained by the two methods on a single extract of manure or soil, or comparing the effects of sample drying before extraction on the results from both methods. Thus, it is not clear to what degree the P forms identified by the two methods are similar, or may be affected by pre-extraction treatment. Therefore, in this work, we analyzed the P forms in NaOH-EDTA extracts of fresh and dried dairy and poultry manure by $^{31}$P NMR spectroscopy, quantitatively comparing the similarities and differences between the two methods. Such information is essential for cross comparison and application of the two sets of P data reported, and will aid researchers in choosing the method most applicable to their future research goals.

### MATERIALS AND METHODS

#### Manure Collection and Analysis

Fresh dairy manure (no bedding) was collected from a New York dairy farm by holding a bucket behind each cow (20–25 cows; ~2 kg per cow). The samples were mixed and a composite 20-kg sample was taken. The cows were fed a diet of total mixed ration that had 4.26 mg total P kg$^{-1}$ dry matter. Total P in the dried and ground manure was 6.88 g kg$^{-1}$. Fresh poultry manure (mixture of feces, spilled feed, without bedding) was collected from a Central Maine layer farm after a single annual cleaning. Five grab samples of manure were removed from inside the poultry house, composited, and homogenized (Dai et al., 2007).

A subsample of the fresh dairy and poultry manures was homogenized at room temperature. A part of the fresh homogenized manure sample was dried at 65°C, ground, and sieved through a 2-mm screen. Both fresh and dried samples were then stored at −20°C until use. Therefore, in this study, the terms “fresh” and “dried” refer to the initial different treatment conditions. The dry matter content of the poultry manure was 416 g kg$^{-1}$ (41.6%), while dairy manure was 150 g kg$^{-1}$ (15%). Total P, Ca, Mg, K, and Al contents in dried, ground, and sieved manures were determined, in duplicate, by microwave digestion with concentrated HNO$_3$ experiments (Cade-Menun et al., 2002), but may have been shorter than needed, particularly for the poultry manure (Cade-Menun et al., 2002). For each sample, the total NMR experiment lasted 3.5 to 4 h, collecting 2400 to 2800 scans.

#### Sodium Hydroxide-EDTA Extraction of Manures

One gram of each wet (dry weight equivalent) and dried manure sample was extracted with 15 mL of 0.25 M NaOH and 0.05 M Na$_2$EDTA at 22°C for 6 h on an end-over-end shaker (Cade-Menun and Preston, 1996). After extraction, the samples were centrifuged at 1500 × g for 20 min. A 1-mL aliquot was diluted to 10 mL by adding 0.17 mL of 2.5 M acetic acid, 1.44 mL of 400 mM acetic buffer (70.5% of sodium acetate and 29.5% of acetic acid, pH 5.0), and 7.39 mL of deionized water. The final pH of the diluted extracts was 5.0. The remainder of the supernatant (−10 mL) was freeze-dried and used for $^{31}$P NMR analysis.

Total recovery of P was 99 to 103% in dairy manure while in the poultry manure only 65 to 71% of P was removed with NaOH-EDTA extraction (Table 2). Therefore, we conducted an additional extraction of poultry litters with 1 M HCl. The poultry litter residues from NaOH-EDTA extraction were washed with 10 mL of deionized water and supernatant was discarded after centrifugation. Washed poultry litter residues were extracted with 1 M HCl for 16 h. After centrifuging, 1 mL of the HCl extracts was diluted to 10 mL by slowly adding 8 mL of 400 mM un-buffered sodium acetate and 1 mL of deionized water. The final pH of the diluted extracts was adjusted to 5.0. These pH-adjusted extracts were analyzed for P forms with NMR and enzymatic hydrolysis. The remainder of the sample was freeze-dried.

#### Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Freeze-dried NaOH-EDTA and HCl extracts were dissolved in 0.6 mL 10 M NaOH, 1.0 mL of deionized water, and 1.6 M deuterium oxide (D$_2$O), and were allowed to stand for 30 min with occasional vortexing. Samples were then centrifuged for 20 min at approximately 1500 × g, transferred to NMR tubes, and stored at 4°C before analysis within 24 h. Solution $^{31}$P NMR spectra were acquired at 202.5 MHz on a Bruker AVANCE 500 MHz spectrometer equipped with a 10-mm broadband probe, using a 90° pulse, 0.68-s acquisition, 4.32-s pulse delay, and 15-Hz spinning. The spectral width was 12135.9 Hz, and the number of points was 8192. Proton decoupling was not used. The delay time was based on prior T1 experiments (Cade-Menun et al., 2002), but may have been shorter than needed, particularly for the poultry manure (McDowell et al., 2006). Temperature was regulated at 20°C (Cade-Menun et al., 2002). For each sample, the total NMR experiment lasted 3.5 to 4 h, collecting 2400 to 2800 scans.

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Total contents of selected elements in dairy and poultry manures.</th>
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<tbody>
<tr>
<td><strong>Element</strong></td>
<td><strong>Dairy manure†</strong></td>
</tr>
<tr>
<td></td>
<td>mg kg$^{-1}$ dry matter</td>
</tr>
<tr>
<td>C</td>
<td>521 ± 4</td>
</tr>
<tr>
<td>P</td>
<td>6.88 ± 0.45</td>
</tr>
<tr>
<td>Ca</td>
<td>16.7 ± 1.6</td>
</tr>
<tr>
<td>Mg</td>
<td>6.4 ± 0.48</td>
</tr>
<tr>
<td>K</td>
<td>5.3 ± 0.38</td>
</tr>
<tr>
<td>Fe</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>Al</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Ca/P ratio</td>
<td>2.4</td>
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</table>

† Mean ± standard deviation (n = 2).‡ Mean ± standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>Recovery of P, Ca, Fe, and Al with NaOH-EDTA extraction of dairy manures.</th>
</tr>
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<tbody>
<tr>
<td><strong>Manure</strong></td>
<td><strong>P†</strong></td>
</tr>
<tr>
<td></td>
<td>mg kg$^{-1}$ dry matter</td>
</tr>
<tr>
<td><strong>Wet</strong></td>
<td>6798 ± 795</td>
</tr>
<tr>
<td><strong>Dry</strong></td>
<td>7074 ± 215</td>
</tr>
</tbody>
</table>

† Content of P was measured colorimetrically on the diluted extracts after autoclaving with H$_2$SO$_4$/K$_2$SO$_4$. Contents of metals were measured by ICP–AES on the freeze-dried samples redissolved in NaOH-EDTA for NMR analysis.‡ Mean ± standard deviation (n = 3).
Compounds were identified by their chemical shifts (ppm) relative to an external orthophosphate standard. The spectra were processed with NUTS software (Acorn NMR, Livermore, CA, 2000), using automated peak analysis and spectral integration, based on total peak area (Cade-Menun, 2005), after standardizing the orthophosphate peak to 6 ppm. Spectra were processed with 7 Hz and 1 Hz line broadening, using automated baseline-correction and peak-picking routines in the processing software. Peak assignments were based on previous work (Turner et al., 2003b; Cade-Menun, 2005).

The inorganic P forms detected in these samples included orthophosphate (6.0 ppm), pyrophosphate (−4.3 ppm), and polyphosphate (−8 to −22 ppm). Within the organic P forms, peaks for two phosphonates were detected: aminoethyl phosphonates at 20.1 ppm and phosphonolipids at 18.3 ppm (Cade-Menun, 2005). In the orthophosphate monoesters region, peaks were observed for phytate (myo-inositol hexakisphosphate) in most samples. The C2 peak of phytate was clearly visible in all spectra (Fig. 1), and was used to calculate total phytate concentration (Turner, 2004). Peaks for other orthophosphate monoesters were seen at 5.2, 5.0, 4.8, 4.7, 4.2, 3.6, 3.6, 3.3, and 2.9 ppm. These compounds may include other inositol phosphates, mononucleotide, and sugar phosphates, and may also include degradation products produced during extraction (Turner et al., 2003b), which were not specifically identified. Orthophosphate diester species were separated into phospholipids (1.7, 1.3, 1.1, 0.0, 0.5, 0.1, and −0.2 ppm), deoxyribonucleic acid (DNA) at −0.7 ppm, and other orthophosphate diesters (−1.0, −1.5 ppm).

Enzymes and Enzymatic Incubation

Acid phosphatases (EC 3.1.3.2), type IV-S from potato and type I from wheat germ, and phytase (EC 3.1.3.8) from Aspergillus fumigatus were purchased from Sigma (St. Louis, MO). Lyophilized nuclease P1 (EC 3.1.30.1) from Penicillium citrinum was purchased from Roche Diagnostics Corporation (Indianapolis, IN). One unit (U) of enzyme activity was defined as liberation of 1.0 μM of relevant product from appropriate substrates at appropriate incubation conditions based on the supplier’s information. The stock solutions of acid phosphatases and phytase were then dispensed in micro centrifuge vials in 1 mL each and stored at −20°C until use. Nuclease P1 was purchased in bottles containing 1 or 5 mg, and the buffer was directly added into the bottle to obtain an activity concentration of 960 U mL⁻¹. Enzyme solutions were stored per manufacturer’s instructions.

For enzymatic incubations, the diluted and pH-adjusted extracts were further diluted 4- to 15-fold with distilled water or sodium acetate buffer to keep a final sodium acetate buffer concentration of 100 mM at pH 5.0. All enzymatic incubations were performed at 37°C for 1 h in a refrigerator-shaker (250 rpm). Each incubation mixture (1.0 mL) contained 0.8 mL of extract and 0.2 mL of enzyme-buffer working solution (acid phosphatase type IV-S from potato 0.25 U, acid phosphatase from wheat germ 0.25 U, and nuclease P1 5 U mL⁻¹, separately or in combination) (He et al., 2004b). Controls omitting either the enzyme or substrates were included.

Soluble inorganic P in the incubation mixtures was determined by molybdate blue method, using 0.2 mL of each

![Fig. 1. Solution 31P NMR spectra of wet and dry dairy manure extracted with NaOH-EDTA, and wet and dry poultry manure extracted with NaOH-EDTA and HCl. Spectra are plotted with 7 Hz line broadening. Inserts, plotted with 1 Hz line broadening, show the expanded orthophosphate monoester region. The arrow indicates the diagnostic C2 peak for phytate, which was multiplied by 6 to determine total phytate concentration (Turner, 2004).](image)
mixture (He and Honeycutt, 2005). This method is modified from an early method (Dick and Tabatabai, 1977) by changing the measuring wavelength to 850 nm and addition of 2% sodium dodecyl sulfate. It is worth noting that this method is developed for accurate determination of orthophosphate, whereas other molybdenum blue methods determine a loosely defined “molybdenum-reactive P” which may include some labile organic P and condensed inorganic phosphate (Dick and Tabatabai, 1977; He et al., 2006a). Thus, in this work, soluble inorganic P determined in the enzymatic hydrolysis was also referred to as orthophosphate for the comparison with the $^{31}$P NMRA data. Three types of enzymatically hydrolyzable organic P forms were quantified based on previous substrate specificity studies (He et al., 2004b, 2006b): (i) simple monoester P, which was inorganic P released by potato phosphatase alone; (ii) polynucleotide P, which was the difference between inorganic P determined after incubation with potato phosphatase + nuclease P1, and potato phosphatase alone; and (iii) phytate-like P, which was defined as the net increase in inorganic P determined after incubation with potato phosphatase + nuclease P1 + wheat germ phosphatase, compared to inorganic P determined in step (ii). In the HCl extracts, only total monoester P was determined by incubating pH-adjusted extracts with potato phosphatase + wheat germ phosphatase, as a previous investigation demonstrated that monoester P (mainly phytate P) was the major organic P form in poultry litter (He et al., 2006b). Total P in each fraction was determined after persulfate-sulfuric acid digestion (He et al., 2006c). Non-hydrolyzable organic P was calculated as the difference between total P and the sum of other identified P forms as described above.

**Statistical Analyses**

Genstat 4.2, fifth edition (Lawes Agricultural Trust, Rothamsted, UK) was used to calculate means and standard deviations for the manure samples. The least significant difference (LSD) test using one-way analysis of variance in Genstat 4.2 was performed on the wet or dry manures to test for significant differences ($P < 0.05$) between P forms in these manures. A correlation coefficient was generated in the Correlation Analysis Tool of Microsoft Excel to compare the two sets of data of NaOH-EDTA extracts yielded by enzymatic hydrolysis and NMR analysis.

**RESULTS AND DISCUSSION**

**Recovery of Phosphorus with Sodium Hydroxide-EDTA and Hydrochloric Acid Extraction**

The NaOH-EDTA extracting solution removed up to 100% of total P from the dairy manure (Table 2). This corresponds with a prior report by Toor et al. (2005), in which 84% of P was removed by NaOH-EDTA extraction of dairy feces, and is higher than the P recoveries from cattle manure observed by Turner (2004) who reported data based on the pasture-fed dairy cattle. In contrast, NaOH-EDTA extraction of poultry manure removed 71% of total P in the wet sample and 65% of total P in the dry sample, and is lower than the 96% recovery from broiler litter noted by others (Turner, 2004; Maguire et al., 2004; McGrath et al., 2005). This low recovery of P in the poultry manure may result from the higher Ca content (152 g kg$^{-1}$) and higher Ca/P ratio (8) compared with dairy manure (Ca: 16.7 g kg$^{-1}$, Ca/P ratio 2.4; Table 2) in our study, or the broiler litter (Ca/P of 1.3) in Turner (2004). Increased Ca in poultry manure may form less soluble P compounds that were not extracted with NaOH-EDTA; P recoveries have been low in NaOH-EDTA extractions of calcareous soils (Turner et al., 2003a; Hansen et al., 2004). Other factors, such as spilled feed, where P may not have been extracted as effectively as manure by NaOH-EDTA, the method used here, different than that used in Turner (2004) which involved a 0.5 M NaOH + 50 mM EDTA solution, and a 1:20 solid/solution ratio, might have also contributed to the lower recovery. Therefore, we included an additional extraction procedure to extract the remainder of the P in the poultry manure by using 1 M HCl after NaOH-EDTA extraction. This resulted in extraction of the remaining P (35–36%) in the poultry manure (Table 3).

**Phosphorus Forms Identified by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy**

The NMR spectra for manures are shown in Fig. 1. In dairy manures, the majority of P occurred as orthophosphate (62–70%), non-phytate (other) orthophosphate monoesters (14–22%), phytic acid (5.4–6.0%), and phospholipids (5.9–6.5%; Table 4). Other orthophosphate diesters, DNA, other diesters, pyrophosphate, polyphosphate, and phosphonates were minor components (1–2% each).

Similarly, NaOH-EDTA extracts of poultry manures were dominated by orthophosphate (50–63%), phytic acid (24–33%), and other monoesters (7–12%; Table 4). Pyrophosphate, DNA, and other diesters were less than 3% of total extracted P. In contrast to dairy manures, no polyphosphate and phosphonates were detected in

**Table 3. Recovery of P and Ca with sequential NaOH-EDTA and HCl extraction of poultry manures.**

<table>
<thead>
<tr>
<th>Manure</th>
<th>P†</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet</td>
<td>13.472 ± 270</td>
<td>31.245 ± 1771</td>
</tr>
<tr>
<td>Dry</td>
<td>12.433 ± 368</td>
<td>34.246 ± 810</td>
</tr>
<tr>
<td>HCl extraction§</td>
<td>6.526 ± 643</td>
<td>8838</td>
</tr>
<tr>
<td>Dry</td>
<td>6.423 ± 492</td>
<td>5537</td>
</tr>
</tbody>
</table>

† Content of P was measured colorimetrically on the diluted extracts after autoclaving with H2SO4/K2S2O8. Content of Ca were measured by ICP–AES on the freeze-dried samples redissolved in NAOH-EDTA for NMR analysis. No Fe and Al were detected in all samples.

‡ Mean ± standard deviation ($n = 3$).

§ Combinations of triplicate HCl extracts were freeze-dried and not all of the freeze-dried HCl extracts were redissolved in NaOH-EDTA for NMR analysis. NaOH-EDTA extraction of the residual part of NaOH-EDTA extraction. Content of the redissolved NAOH-EDTA solutions of the freeze-dried HCl extracts was 2244 and 2219 mg kg$^{-1}$ for wet and dried poultry manures, respectively.
poultry manures. The HCl extracts of residues from both dry and wet poultry manure were 72 to 73% orthophosphate, 22 to 23% phytic acid, and 4 to 5% other monoesters (Table 4). No other P forms were detected.

Based on McDowell et al. (2006), it is possible that the delay time used for these NMR experiments was too short, particularly for the poultry manure samples, which had very high ratios of P/(Fe + Mn). However, increasing the delay times to 15 s or more was not practical with respect to machine time or cost. In addition, while McDowell et al. (2006) found that the delay from 0.1 to 15 s for a sheep dung sample improved spectral resolution and altered peak areas, the differences in relative percents was small (1–2%). Given that our spectral resolution and altered peak areas, the differences from 0.1 to 15 s for a sheep dung sample improved.

McDowell et al. (2006) found that increasing the delay to 15 s or more was not practical with respect to machine time or cost. In addition, while McDowell et al. (2006) found that the delay from 0.1 to 15 s for a sheep dung sample improved spectral resolution and altered peak areas, the differences in relative percents was small (1–2%). Given that our delay time (4.3 s) was much longer than the shortest delay time used by McDowell et al. (2006), we assumed that changes in peak areas for our samples with a longer delay time would be similar or smaller.

Drying dairy manures resulted in changes in P forms (Fig. 2). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\) (Fig. 2). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\). For example, drying significantly increased orthophosphate by 560 mg kg\(^{-1}\).

In contrast to dairy manure, drying poultry manure reduced P recovery as well as orthophosphate concentration. Given the high Ca concentration in poultry manure, drying appears to have precipitated orthophosphate into Ca-phosphates (Penn and Bryant, 2006), reducing its recovery with NaOH-EDTA. Although the results from HCl extraction of residues suggest that for both fresh and dry samples, orthophosphate and some phytate were insoluble in NaOH-EDTA, drying appears to cause a greater reduction in orthophosphate than in phytic acid, resulting in an apparent increase in phytic acid in poultry manure after drying.

### Table 4. Functional classes of P determined in NaOH-EDTA extracts of dairy manure and NaOH-EDTA and HCl extracts of poultry manure by solution \(^{31}\)P nuclear magnetic resonance (NMR) spectroscopy.

<table>
<thead>
<tr>
<th>Class</th>
<th>Inorganic phosphate</th>
<th>Organic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ortho- Pyro- Poly-</td>
<td>Monoesters Other</td>
</tr>
<tr>
<td>Dairy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet</td>
<td>62 ± 3.0a</td>
<td>0.7 ± 0.1a</td>
</tr>
<tr>
<td>Dry</td>
<td>70.6 ± 2.0b</td>
<td>1.2 ± 0.3a</td>
</tr>
<tr>
<td>LSD (5%)</td>
<td>5.76</td>
<td>0.51</td>
</tr>
<tr>
<td>Poultry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet</td>
<td>63.2 ± 4.2a</td>
<td>0.7 ± 0.0a</td>
</tr>
<tr>
<td>Dry</td>
<td>49.9 ± 7.6a</td>
<td>1.0 ± 0.3a</td>
</tr>
<tr>
<td>LSD (5%)</td>
<td>14.0</td>
<td>0.55</td>
</tr>
</tbody>
</table>

† Mean ± standard deviation (n = 3). Means followed by different letters are significantly different within each column for dairy or poultry at P < 0.05. No statistical data were available for HCl extracts as pooled extracts were used for the NMR analysis.

Using phosphatase hydrolysis, P in NaOH-EDTA extracts was classified into five groups (Table 5). For dairy manure, inorganic P was significantly greater in dry than wet manure, while organic P significantly decreased by drying. However, NaOH-EDTA solution extracted less inorganic P from dried poultry manure than from wet manure. No significant difference was observed in the organic P forms between the dry and wet samples of poultry manure although total organic P was slightly higher in dry (2700 mg kg\(^{-1}\)) than wet (2604 mg kg\(^{-1}\)) poultry manure. The majority of the hydrolyzable organic P was present in the monoester forms in both manures. However, dairy manure contained almost equal amounts of phytate and simple monoester P. Interestingly, polynucleotide-like P was significantly greater in wet compared with dry dairy manure, which clearly shows that the drying process hydrolyzed polynucleotide P. One possible explanation for this observation is that polynucleotide-like P originated from biomass of microbes present in manures before and/or after excretion whereas other P forms are more or less related to dietary P (Toor et al., 2005). These results are consistent with the P-NMR results, and also suggest that differing responses to drying are due to differences in manure chemistry.

Both manures contained significant enzymatically non-hydrolyzable P (1134–1842 kg\(^{-1}\)dry matter). Non-
hydrolyzable organic P in the NaOH-EDTA extracts of dairy manure accounted for 18 to 26% of total extracted P. However, the non-hydrolyzable P was 9 to 9.5% of total extracted P in poultry manure. Perhaps differing abilities of dairy cows and poultry to digest P caused this difference: hydrolyzable organic P in dairy feedstuff was most likely mineralized by inherent phytase present in the dairy cow rumen, which is lacking in monogastric poultry.

In the HCl extracts, enzymatic hydrolysis revealed that 6066 mg kg\(^{-1}\) (92%) of the wet poultry manure was inorganic P, 200 mg kg\(^{-1}\) (3%) was monoester P, and 310 mg kg\(^{-1}\) (4.7%) was non-hydrolyzable organic P (Table 5). In the HCl extracts of dry poultry manure, 5303 mg kg\(^{-1}\) (84%) was inorganic P, 973 mg kg\(^{-1}\) (16%) was monoester P, and no non-hydrolyzable organic P was detected. These data indicate that there was considerable inorganic and organic P in both wet and dry poultry manure after NaOH-EDTA extraction. A prior paper (Turner, 2004) recommended NaOH-EDTA extraction as a standard method for complete manure P characterization. Our data imply that researchers must be aware that NaOH-EDTA may not be effective to extract all P in high Ca manure as numerous factors discussed in the Recovery Section would affect the recovery. Therefore, the general applicability of the NaOH-EDTA procedure should be modified to account for the specific conditions of the poultry manures studied.

![Fig. 2. Effect of drying on P forms in dairy and poultry manures.](image-url)
Comparison and Scope of Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy and Enzymatic Hydrolysis to Identify Phosphorus Forms in Animal Manures

Although not all the P forms identified by the two methods were the same, there were four similar forms: orthophosphate or inorganic P, phytate-like P, other monoesters, and polynucleotide-like or DNA P. There was no significant difference in the amount of inorganic P in wet and dry dairy manure measured via the methods. However, there was a significant difference between the amount of inorganic P measured in wet and dry poultry manure (Fig. 3). Total organic P identified by NMR was significantly greater for all manure types than by enzymatic hydrolysis. Phytic acid in dairy manure showed inconsistent trends: no significant difference between the methods for wet manure, but lower phytic acid by enzymatic hydrolysis in dry manure. In poultry manure, enzymatic hydrolysis identified significantly less phytic acid than NMR for both wet and dry extractions. Similarly, enzymatic hydrolysis detected significantly less other monoesters in the extracts of both dairy and poultry manures than NMR. The lower contents of phytic acid and other monoesters with enzymatic hydrolysis suggest that not all these forms were hydrolyzed with added enzymes. The percentage of polynucleotide-like P determined by enzymatic hydrolysis was greater than DNA determined by NMR analysis. This may be attributed to the endonucleolytical cleavage of the P-O bonds present in both DNA and RNA by nuclease phosphatase. NP does not directly cleave the P-O bond in P,

Table 5. Phosphorus forms identified in NaOH-EDTA extracts of dairy manure and NaOH-EDTA and HCl extracts of poultry manure by enzymatic hydrolysis.

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<thead>
<tr>
<th>Class</th>
<th>Inorganic P</th>
<th>Total organic P</th>
<th>Phytate</th>
<th>Simple monoesters</th>
<th>Polynucleotide</th>
<th>Non-hydrolyzable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy Wet</td>
<td>59.3 ± 3.4a</td>
<td>25.3 ± 0.3a</td>
<td>10.1 ± 3.0a</td>
<td>8.8 ± 4.9a</td>
<td>6.4 ± 1.7a</td>
<td>18.0 ± 3.2a</td>
</tr>
<tr>
<td>Dairy Dry</td>
<td>67.9 ± 3.7b</td>
<td>7.0 ± 1.5b</td>
<td>3.8 ± 0.8b</td>
<td>2.9 ± 1.7a</td>
<td>0.3 ± 0.4b</td>
<td>26.0 ± 2.8b</td>
</tr>
<tr>
<td>Poultry Wet</td>
<td>71.2 ± 0.4a</td>
<td>19.4 ± 3.9a</td>
<td>15.3 ± 2.5a</td>
<td>1.0 ± 1.8a</td>
<td>3.0 ± 2.9a</td>
<td>9.5 ± 4.2a</td>
</tr>
<tr>
<td>Poultry Dry</td>
<td>69.2 ± 0.8b</td>
<td>21.8 ± 1.5a</td>
<td>14.5 ± 1.4a</td>
<td>3.0 ± 0.8a</td>
<td>4.3 ± 1.1a</td>
<td>9.0 ± 2.0a</td>
</tr>
</tbody>
</table>

‡ Mean ± standard deviation (n = 3).
§ Not applicable.

Overall, the distribution of various P forms determined by NMR or enzymatic hydrolysis was similar. Therefore, both methods were applicable for determining the P forms in the NaOH-EDTA extracts of animal manure. Statistical analysis of the two sets of data yielded by NMR analysis and enzymatic hydrolysis on all NaOH-EDTA extracts (Fig. 3) generated a correlation coefficient of 0.93. These results indicate that the trend of distribution between P forms measured via enzyme hydrolysis and 31P NMR analysis was similar. With 31P NMR analysis, most of the P forms were identified and all P was measured. However, this method requires additional sample preparation, and access to spectrometers, equipment not available in most laboratories. Analytical costs of NMR spectrometer time often limit the number of samples that can be run, resulting in few, if any, replicate analyses for many studies. On the other hand, enzymatic hydrolysis is a rapid method, requires less sample (<1 mL extracts), and multiple sample analysis (or replications) could be performed. More importantly, this method could provide information on hydrolyzable organic P, which is a better indicator of bioavailable organic P. The disadvantages of enzymatic hydrolysis appear to result from nonhydrolyzable organic P, leading to fewer identified P forms. Because the content of organic P forms is calculated by the difference from inorganic P determined in the presence and absence of certain enzymes, analytical errors in the determination of inorganic P may produce inaccurate or even negative concentrations (He et al., 2004a, 2004b).

The negative data seemed not to occur in 31P NMR analysis, although its signal noise and line broadening could obscure overlapping peaks, reducing the relative abundance of detected P forms. Therefore, each method had its advantages and disadvantages. A researcher may have to assess the pros and cons of each method and select the one best suited to their specific study needs.
CONCLUSIONS AND IMPLICATIONS

Combined 0.25 M NaOH/0.05 M EDTA extracted almost all of the P in either wet or dried dairy manure, but extracted only 71 and 65% of P in wet and dried poultry manures. Thus, caution must be taken when this extraction method is applied for complete manure P characterization or for comparison of different manure types. The P forms in the NaOH-EDTA extracts or HCl extracts could be identified and quantified by either enzymatic hydrolysis or solution 31P NMR analysis. Both methods revealed that 50 to 70% of extracted P was inorganic P, and orthophosphate monoesters, including phytic acid, were the major forms of organic P.

Fig. 3. Comparison of phosphorus forms in NaOH-EDTA extracts identified by enzymatic hydrolysis and solution 31P NMR analysis. Values followed by different letters within each manure treatment are significantly different at P < 0.05.
Comparison of the two sets of data indicated that the distribution patterns of major P forms in animal manure determined by the two methods were similar with a correlation coefficient of 0.93. Although it is acceptable to compare the information on P distribution obtained by the two methods, some discretion should be applied as NMR generally identified greater amounts of phytic acid and other monoesters than enzymatic hydrolysis. As each method had its advantages and disadvantages, and the P forms detected by the two methods are not always the same due to different identities and/or stability to hydrolysis, we recommend researchers use a method to best fit their specific research goals (i.e., use NMR for more information on P forms or enzymatic hydrolysis for information on bioavailable P fractions) or use both methods to obtain a full spectrum of P characterization.

REFERENCES


