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REGULAR ARTICLE

# Distribution of inositol phosphates in animal feed grains and excreta: distinctions among isomers and phosphate oxygen isotope compositions

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**Abstract**
*Background and Aims* Phytate (*myo*-$IP_6$) is a common form of organic phosphorus in the environment. Little information is available, however, about the distribution of phytate and its degradation products. In this research, we aimed to identify the compositions of phytate in different natural P sources as well as to explore a reliable method to measure their isotope signatures so that the link between original phytate and P outputs in the environment could be established.
*Methods* A variety of feed ingredients for selected ruminant and non-ruminant animals and their excreta were analyzed using HPIC (high-performance ion chromatography) and their oxygen isotope ($\delta^{18}O_{PA\text{-}Pi}$) signatures were identified using IRMS (isotope ratio mass spectrometry) method.
*Results* The HPIC results show that $IP_6$ was dominant in all grains, followed by $IP_5$ and several $IP_4$ isomers, and an insignificant amount of $IP_3$. Similarly, $IP_6$ and $IP_5$ were also detected in all animal feeds and several excreta. More importantly, the distribution of different $IP_x$ species in a grain type was essentially the same. The $\delta^{18}O_{PA}$ values of phytate in grains varied from 20.5 to 24.2 ‰, while the $\delta^{18}O_{Pi}$ values of inorganic P in the same grains were heavier by 0.4-3.2‰. Similarly, the $\delta^{18}O_{PA}$ values of phytate in animal feeds and excreta were within the ranges of grain phytate.
*Conclusions* Overall, combination of results from IRMS and HPIC analyses provided important information on the distribution of $IP_x$ species in various sources and their distinct oxygen isotope ratios pointed towards the possibility of connecting the original phytate sources to degradation products in the environment.

**Keywords** Phytate · Inositol phosphate isomers · Animal feeds · Animal excreta · Phosphate oxygen isotopes

## Introduction

Phytate (the salt of *myo*-inositol1,2,3,4,5,6-hexakisphosphate or $IP_6$) is a naturally occurring organic phosphorus compound in plants and crops, constituting 1–5% of most cereals, legumes, and nuts by dry weight, and making up 50 to 80% of the total phosphorus in grains (Graf and Eaton 1990; Lott and Ockenden 1986; Raboy 2007). It serves as a reserve of inorganic phosphate ($P_i$) to support the emerging seedling and controls $P_i$ concentration during seed development (Lott et al. 2000; Nadeem et al. 2012; Strother 1980). Phytate is quite stable in a range of physicochemical conditions but can be hydrolyzed rapidly by phytate-degrading enzymes commonly known as phytases, which are widespread in the environment (Greiner 2007). Phytases can liberate phosphate groups by the cleavage of the P–O bond at different structural

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positions on the inositol ring and generate different inositol phosphate isomers ($IP_x$, x = 1,2,…,5) as well as $P_i$ (Greiner and Konietzny 2011).

Phytate hydrolysis by monogastric animals such as pig and poultry is limited due to the lack of ample phytases in their digestive tracts (McCuaig et al. 1972; Mullaney et al. 2007; Wodzinski and Ullah 1996). Many animals can only assimilate a small amount of P unless the excess inositol phosphates can be hydrolyzed either by the enzymes secreted by gut microorganisms of ruminant animals or phytases supplemented in the diet for monogastric animals (Lei and Porres 2007). As a result, variable amount of organic P ($P_o$) and $P_i$ are released into animal manures. The inositol phosphates in manures, once released to soils, can interact with polyvalent cations and form insoluble complexes (Celi et al. 1999; He et al. 2006) and delay the potential degradation. It means the application of manure on farms could increase phytate level (Leytem and Maguire 2007), leading to its accumulation in soils and nearby aquatic systems (McKelvie 2007). Recently, a series of laboratory incubation and soil transect studies have indicated that the degradation of phytate occurs faster in soils and sediments than previously thought (Doolette et al. 2010; Dou et al. 2009; Hill and Cade-Menun 2009; Monbet et al. 2009; Stout et al. 2016). This means that the role of phytate in the release of $P_i$ into runoff from agricultural soils and eventually to open waters should be more significant than commonly thought. Thus, to address environmental concerns surrounding P contamination, a prior knowledge on the source and fate of phytate and its degradation products including inositol phosphates and $P_i$ is required.

Understanding the distribution of phytate and its degradation products in animal feed in terms of their compositions and concentrations is a basic research question to be known priory to studying their fates in the environment. The greatest limitation of this investigation is the lack of suitable methods to track different $IP_x$ due to methodological challenges in extraction, separation, and purification from complex environmental samples. Although recent studies have improved $IP_x$ (primarily $IP_6$) separation and identification (Murthy 2007; Sjoberg et al. 2016; Turner et al. 2012), existing research methods are still incapable of connecting $IP_x$ sources and generating their transport pathways, which limits the understanding of the origins, routes of transformation, and potential degradation of $IP_x$ in the environment.

Stable isotopes have been used as a powerful tool to study sources, transfers, and cycling processes of various elements in the environment (Fry 2006). Because P has only one stable isotope, $^{31}P$, it cannot be used to understand P systematics like other nutrient elements (such as C and N) that have multiple stable isotopes. However, P occurs almost entirely as phosphate either as inorganic phosphate ($P_i$) or organic phosphate ($P_o$) in the environment and thus permits the use of stable isotope ratios of O in $P_i$ ($\delta^{18}O_P$) as a potential tracer of P (Jaisi et al. 2014). There are three major factors controlling the O isotope composition of $P_i$ in the environment: i) in abiotic processes, phosphate ion does not undergo any O isotopic composition with other oxyanions (Jaisi et al. 2011, 2014; Tudge 1960) for the timescale relvant to modern environment; ii) during biological processes, rapid O-isotope exchange between dissolved $P_i$ and water can produce temperature-dependent equilibrium isotope effects (Chang and Blake 2015; Longinelli and Nuti 1973); and iii) organophosphorus compounds can be hydrolyzed by extracellular enzymes and subsequently release $P_i$, and the released $P_i$ partially inherits O atoms from the parental compound, which follows disequilibrium (kinetic) isotope effects (Liang and Blake 2006a; Liang and Blake 2009; Wu et al. 2015). However, little information is available in terms of using oxygen isotopes to study organic P sources because of the technological limitation where measurement of $\delta^{18}O_P$ value is only possible for pure $P_i$ naturally present or derived from $P_o$ hydrolysis and organic P compounds having O atoms only in phosphate moieties (Sun et al. 2017; Wu et al. 2015). Most researches in this area have limited to pure organic P compounds (Liang and Blake 2006a, 2006b, 2009; Li et al. 2016; Sandy et al. 2013; Sun et al. 2017; von Sperber et al. 2015) and organic P from the natural environment has not yet been investigated.

In this research, we aimed to identify the composition of phytate from different natural P sources as well as to explore a reliable method to measure their isotope signatures so that the link between original phytate and P outputs in the environment could be established. We applied high-performance ion chromatography (HPIC) to separate and identify distinct $IP_x$ species and their isomers in soybean and corn grains, feeds, and manures and litters from different animals that could eventually end up in the soil and aquatic environments. After purification, the separated $IP_x$ species were hydrolyzed with a specific phytase and oxygen isotope composition of released $P_i$ was measured by isotope mass spectrometry.

## Materials and methods

*Chemical reagents and standards* Potassium phytate (K-phytate, a synthetic product with purity>95%) was purchased from Sigma-Aldrich, Missouri, U.S. Phytase from *Aspergillus niger* (Natuphos®) was kindly provided by BASF, New Jersey, U.S. Several inositol phosphate standards were used to identify and quantify $IP_x$ isomers. They are as follows: D-*myo*-inositol 1,2,4,5,6-pentakisphosphate, D-*myo*-inositol 1,2,3,4,6-pentakisphosphate, *myo*-inositol 1,3,4,6-tetrakisphosphate, *L*-*myo*-inositol 1,4,5-trisphosphate, D-*myo*-inositol 4,5-diphosphate and *myo*-inositol 2-monophosphate (Sigma-Aldrich, Missouri, U.S.); D-*myo*-inositol 1,3,4,5,6-pentakisphosphate, D-*myo*-inositol 1,2,5,6-tetrakisphosphate, D-*myo*-inositol 1,4,5,6-tetrakisphosphate, D-*myo*-inositol 1,3,4,5-tetrakisphosphate, D-*myo*-inositol 1,2,4,5-tetrakisphosphate, D-*myo*-inositol 1,2,6-triphosphate, D-*myo*-inositol 1,5,6-triphosphate, D-*myo*-inositol 1,2-diphosphate and D-*myo*-inositol 1-monophosphate (Cayman Chemical Company, Michigan, U.S.); and D-*myo*-inositol 1,2,3,5,6-pentakisphosphate, *myo*-inositol 1,2,3,6-tetrakisphosphate (Enzo Life Sciences, New York, U.S.). All assay reagents were prepared in double deionized water (dd-$H_2O$). Similarly, AG 1-X8 anion resin (100–200 mesh, chloride form) and AG 50W-X8 cation resin (200–400 mesh, hydrogen form,) were purchased from Bio-Rad, CA. All other chemicals and reagents were obtained from Sigma-Aldrich or Fisher Scientific.

*Collection of environmental sources of inositol phosphate and pre-processing* Five different soybean and corn samples were collected from different farms on the Delmarva Peninsula from September to October 2015. These grains were purchased directly by vendors and processed for animal feeds in the region. To compare the $IP_x$ distribution of different excreta from the same poultry farm, broiler cake manure and litter were collected from Georgetown Farm, DE, in October 2015. The only difference is that broiler litter not only contains the fresh excreta but also some bedding materials and feed spills. To compare the difference in the $IP_x$ distribution between monogastric and ruminant animals, five different animal excreta were also collected from farms around the Delmarva Peninsula from March to April 2017, including litter from broiler and manure from beef cattle, horse, sheep, and cow. Further, the feeds for those animals were collected at the same times. Broiler feed received 0.01 wt% of Quantum Blue® phytase, while the phytase supplement for the other animal feeds is currently unknown.

After collection, manure and litter samples were lyophilized. All the samples were ground and sieved (<2 mm) before further analyses. Total P content was analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) using an ICAP 7600 Duo view inductively coupled plasma optical emission spectrometer (Thermo Elemental, Madison, WI) after microwave digestion. Manure samples were digested by EPA Method 3051 (USEPA 1986) using a CEM MARs5 microwave digestion system (CEM, Matthews, NC). Grain and feed samples were digested using concentrated nitric acid and 30% hydrogen peroxide (Huang and Schulte 1985).

*Extraction and purification of inositol phosphate and inorganic P* Inositol phosphate isomers were extracted and purified based on the previous method (Chen 2004) after slight modification. Manure and litter samples (1.0–5.0 g) were weighed into 50 mL polypropylene centrifuge tubes and 25 mL of 0.5 M HCl was added. After vortexing for 5 min, the samples were shaken for 4 h at room temperature. Then, the extracts were centrifuged at 6200 *g* for 30 min, and the supernatants were collected and diluted with water (1:10). Two grams of AG 1-X8 anion resin, preconditioned in 20 mL deionized (DI) water, were added to a 10 mL plastic burette in a vertical column set up. The diluted sample extract was loaded in the resin column to trap inositol phosphate ions and rinsed with 20 mL 0.05 M HCl to remove contaminants. Inositol phosphates in the resin were then eluted with 40 mL of 2 M HCl. After evaporating at 50 °C in a water bath, the residues were dissolved in 1 mL of DI water, filtered through a 0.2 μm polyethersulfone filter, and then analyzed immediately by HPIC.

For soybean and corn grains, 1.0 g samples were first extracted with 20 mL of hexane for 2 h to remove fat. The extracts were then centrifuged at 6200 *g* for 30 min, and the supernatants were decanted. The remaining hexane was evaporated in a 50 °C water bath overnight. Soybean and corn extracts in the evaporated residues were further extracted by using 25 mL of 0.5 M HCl and purified with AG 1-X8 resin, and finally re-dissolved in water for HPIC analysis. Inorganic P in soybean and corn grains and animal feeds were extracted for 4 h with 0.5 M HCl (sample to solution ratio at 1:10)

directly after defatting with hexane 2 times. After centrifuging at 6200 *g* for 30 min, the supernatants were collected for further processing. Similarly, $P_i$ in manure samples was extracted with 0.5 M $NaHCO_3$ (at 1 g: 100 mL solid to solution ratio) for 16 h. The extraction was limited to $NaHCO_3$-P because this pool is considered readily available for plants and microorganisms (Hedley et al. 1982).

*Separation of inositol phosphate by high-performance ion chromatography* Among several chromatography techniques, high-performance ion chromatography (HPIC) is the most appropriate technique to separate various $IP_x$ species because this method can separate as many as 35 isomers (Chen and Li 2003). Recently, the Chen and Li (2003) method has been slightly modified to improve the separation of $IP_x$ species in environmental samples and shorten the separation time to 45 min (Oates et al. 2014). In this research, we used Chen and Li (2003) method for grain seeds and Oates et al. (2014) for animal feeds and manures, but both methods were tested for $IP_x$ standards. The HPIC system we used was a Dionex DX-500, equipped with an AS50 autosampler, AS50 thermal compartment, GP50 gradient pump, and an AD25 absorbance detector. The $IP_x$ compounds were separated on a Dionex CarboPac PA-100 guard column (50 mm × 4 mm) and a CarboPac PA-100 analytical column (250 mm × 4 mm, 10 μm) by a linear gradient elution program with 0.5 M HCl and $H_2O$, and then detected at 295 nm after mixing with a derivatization solution (1 g/L $Fe(NO_3)_3$ in 0.33 M $HClO_4$).

To identify different $IP_x$ isomers, an in-house reference standard containing various $IP_x$ isomers was prepared by hydrolyzing K-phytate with 2 M HCl at 140 °C following the Chen and Li (2003) method. To confirm and quantify the specific isomers, the authentic individual standards of different $IP_x$ purchased from Cayman and Enzo Life Sciences (see materials and methods) were used. Further, after separation by HPIC, the distinct pure $IP_6$ and $IP_5$ isomers from different samples were manually collected based on the different retention time and were used for oxygen isotope ratio analysis.

*Purification of inositol phosphates and inorganic P and measurement of oxygen isotope ratios* For $P_i$ extracted from grains and animal feeds, the dissolved organic matter was removed by using DAX 8 Superlite resin first; while for $P_i$ extracted from animal manures, pH was adjusted to neutral before the DAX treatment. For separated and collected phytate samples, O-isotope ratios ($\delta^{18}O_{PA}$) of intact phosphate moieties in the inositol ring could not be directly analyzed because of two other oxyanions, $Fe(NO_3)_3$ and $HClO_4$, present in the collected solution. In addition, the solution might contain other O contaminant sources (from unknown oxyanions) besides $PO_4$ moieties in $IP_x$ isomers, which also limits the direct measurement for the O-isotope ratios. Separation of all contaminant oxyanions is a nearly impossible and technically daunting task. In a previous study, we investigated the enzymatic degradation of pure phytate and measured O-isotope ratios of released phosphate groups ($\delta^{18}O_{PA\text{-}Pi}$) (Sun et al. 2017). Therefore purification of environmental and processed samples and precipitation as $Ag_3PO_4$ is the suitable method to measure O-isotope ratios. However, in this study, the collected solution from HPIC contains $Fe^{3+}$, which has strong inhibitory effect on enzymatic degradation of phytate because of the formation of a Fe-phytate precipitate (Greiner et al. 2009). Therefore, collected samples were neutralized with a small amount of 5 M NaOH and $Fe^{3+}$ was precipitated as $Fe(OH)_3$ overnight, and then removed by centrifugation at 6200 *g* for 15 min. The supernatant was transferred into 50 mL centrifuge tubes, and after adjusting pH to neutral, the solution was treated with 10 mL of 0.1 M sodium acetate buffer at pH 5.4 and incubated with 1 U *A. niger* phytase at 37 °C for 48 h. Released $P_i$ was quantified by using the phosphomolybdate blue method (Murphy and Riley 1962). The minimum amount of inorganic P extracted from grains, animal feeds, and excreta is 5 μmol to measure O-isotope ratio, while 1 μmol is sufficient for inositol phosphates.

All partially processed samples ($P_i$ directly extracted from environmental samples and $P_i$ released from pure phytate) were further purified using sequential precipitation and recrystallization methods for O-isotope ratio measurement (O'Neil et al. 1994; Blake et al. 1998; Jaisi and Blake 2014). Briefly, $P_i$ was precipitated as ammonium phosphomolybdate (APM) by adding ammonium molybdate solution, then the yellow APM precipitate was separated by filtration and dissolved in ammonium citrate solution. The solution was precipitated again as magnesium ammonium phosphate (MAP), separated by filtration and dissolved again in 0.5 N nitric acid. After pH adjustment, the solution was treated with AG 50W-X8 cation resin to remove cations. Finally, the purified $P_i$ was precipitated as $Ag_3PO_4$. After filtration, the

$Ag_3PO_4$ crystals were washed ten times with DI water and dried at 110 °C for 16 h to remove any trapped water. For the isotope analysis, 200–300 μg of $Ag_3PO_4$ was weighed into a silver capsule for each sample and pyrolyzed in a thermochemolysis/elemental analyzer (TC/EA) at 1460 °C. The liberated oxygen was converted to CO by reacting with glassy carbon and then $\delta^{18}O_P$ value in CO was measured in an isotope ratio mass spectrometer (IRMS; Thermo, Darmstadt, Germany).

For the measurement of water oxygen isotopes ($\delta^{18}O_W$), 0.3 mL water sample was injected into an airtight exetainer® (Labco Limited, UK) and equilibrated with 300 ppm $CO_2$ in He for 24 h at 26 °C (Upreti et al. 2015). The O-isotope ratios of $CO_2$ in the headspace were measured by IRMS. The $\delta^{18}O_W$ values were calculated from $\delta^{18}O_{CO2}$ values using the known fractionation factor ($\alpha_{CO2\text{-}H2O}$) at 26 °C (Cohn and Urey 1938).

All measured $\delta^{18}O_P$ values were calibrated against two silver phosphate standards, YR 1aR-2 and YR 3-2, with $\delta^{18}O_P$ values of −5.49 and + 33.63 ‰, respectively. Similarly, the $\delta^{18}O_W$ values were also calibrated against two USGS water standards with $\delta^{18}O_W$ values of −1.97 and − 9.15‰, respectively (USGS Reston Stable Isotope Laboratory, Reston VA). All samples and standards were run at least in triplicate. All isotope data are reported in permil (‰) values relative to the Vienna Standard Mean Ocean Water (VSMOW).

*Calculation of fractionation factor and isotope values of intact phosphate moieties in the inositol* During enzymatic degradation of phytate, nucleophilic attack on the P–O–C ester and cleavage of the P–O bond results in the inheritance of three O atoms from the phosphate moiety and incorporation of one O atom from ambient water into released $P_i$ (von Sperber et al. 2015; Wu et al. 2015). During the incorporation, there is a difference between oxygen isotope value of incorporated vs ambient water oxygen due to isotope fractionation. This fractionation factor ($F$) depends on the enzyme and substrate types and can be calculated using the following equation (Liang and Blake 2006a, 2009):

$$F = 4 \times \left[\delta^{18}O_{PA-Pi} - 0.75\delta^{18}O_{PA}\right] - \delta^{18}O_w \quad (1)$$

where $\delta^{18}O_{PA\text{-}Pi}$ is the oxygen isotope composition of $P_i$ released from phytate, $\delta^{18}O_W$ is the isotopic composition of ambient water, and $\delta^{18}O_{PA}$ is the original oxygen isotope composition of intact phosphate moieties in phytate.

To calculate the isotopic fractionation associated with the enzymatic degradation of phytate, a standard enzyme assay was performed. Briefly, 1 mM K-phytate standard was incubated with *A. niger* phytase following the same method for environmental samples. Released $P_i$ from K-phytate was purified and precipitated as $Ag_3PO_4$ and phosphate oxygen isotope values ($\delta^{18}O_{PA\text{-}Pi}$) were measured (as described above). The original oxygen isotope ratios of intact phosphate moieties ($\delta^{18}O_{PA}$) in the K-phytate standard were directly measured after pyrolyzing 75–100 μg freeze-dried powder in TC/EA.

## Results and discussion

*Composition of inositol phosphates in soybean and corn grains* Representative HPIC chromatograms of different $IP_x$ compounds extracted from soybean and corn samples are shown in Fig. 1a. Concentrations of these $IP_x$ isomers quantified based on authentic standards are summarized in Tables 1 and 2.

For all soybean samples, the predominant inositol phosphate among the total $IP_x$ identified was $IP_6$, with concentrations ranging from 8.64–12.26 μmol/g (Table 1 and Fig. 1). These results are comparable with the published values (Phillippy 2003) of $IP_6$ contents in soybean (5.79 μmol/g) and other bean species, which were found to vary from 5.01–13.05 μmol/g (Chen 2004). Among all observed $IP_5$ isomers, *D/L*-I(1,2,4,5,6)$P_5$ (*D/L* represents *D-* and/or *L-* isomers of $IP_x$) appeared as the dominant one with its concentration in the range of 1.61–2.14 μmol/g, consistent with Chen (2004). The concentrations of other $IP_5$ isomers decreased in the following order: *D/L*-I(1,2,3,4,5)$P_5$, I(1,2,3,4,6)$P_5$, and I(1,3,4,5,6)$P_5$. At least 7 $IP_4$ isomers were detected in all soybean samples analyzed (Table 2), of which *D/L*-I(1,2,4,5)$P_4$ and *D/L*-I(1,2,5,6)$P_4$ were the dominant isomers. Other minor $IP_4$ species detected were *D/L*-I(1,2,3,4)$P_4$/*D/L*-I(1,3,4,6)$P_4$ and *D/L*-I(1,3,4,5)$P_4$, while the least common $IP_4$ was identified as *D/L*-I(1,4,5,6)$P_4$. Additional two $IP_4$ isomers, *D/L*-I(1,2,4,6)$P_4$/*D/L*-I(1,2,3,5)$P_4$ and I(2,4,5,6)$P_4$, were also observed but not quantified due to the lack of commercially available standards. The $IP_3$ isomers were also present in the samples but in trace amounts including I(1,2,3)$P_3$/*D/L*-I(1,2,6)$P_3$/*D/L*-I(1,4,6)$P_3$ and *D/L*-I(1,5,6)$P_3$ (Fig. 1). No other low order $IP_x$ was detected. This might be due to the insufficient amount of these

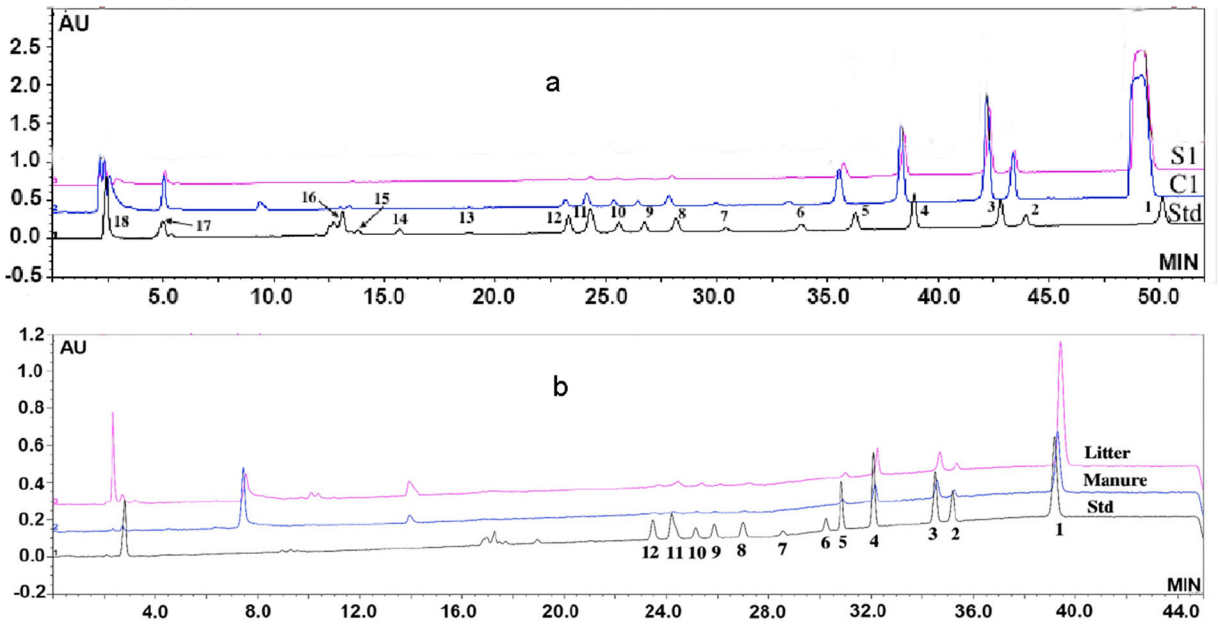


**Fig. 1** Representative HPIC chromatograms of inositol phosphates extracted from different natural samples. Separation methods were based on (**a**) Chen and Li (2003) and (**b**) Oates et al. (2014). The term Std refers to in-house standard with peaks numbered 1 to 17 are as follows: 1: $IP_6$; 2: $I(1,3,4,5,6)P_5$; 3: *D/L*-$I(1,2,4,5,6)P_5$; 4: *D/L*-$I(1,2,3,4,5)P_5$; 5: $I(1,2,3,4,6)P_5$; 6: *D/L*-$I(1,4,5,6)P_4$; 7: *D/L*-$I(2,4,5,6)P_4$; 8: *D/L*-$I(1,2,5,6)P_4$; 9: *D/L*-$I(1,3,4,5)P_4$; 10: *D/L*-$I(1,2,4,5)P_4$; 11: *D/L*-$I(1,2,3,4)P_4$, *D/L*-$I(1,3,4,6)P_4$; 12: *D/L*-$I(1,2,4,6)P_4$, *D/L*-$I(1,2,3,5)P_4$; 13: $I(4,5,6)P_3$; 14: *D/L*-$I(1,5,6)P_3$; 15: *D/L*-$I(2,4,5)P_3$; 16: $I(1,2,3)P_3$, *D/L*-$I(1,2,6)P_3$, *D/L*-$I(1,4,6)P_3$; 17: *D/L*-$I(1,2)P_2$; 18: $P_i$. Label S1 refers soybean grain 1, C1 refers corn grain 1, Litter refers broiler litter collected in 2015 and Manure refers broiler cake manure collected in 2015

isomers (i.e., below the detection limit) present in the samples. It could also be due to the limitation of the experimental method that $IP_1$, $IP_2$, and some other $IP_3$ isomers were not retained in the resin because the loading solution (0.05 M HCl) is itself a weak eluent (Chen 2004). In summary, at least 14 different inositol phosphates were identified in the soybean grains. For each soybean type, there was a marked difference in the contents of positional isomers, such as four $IP_5$ isomers (0.42–2.14 μmol/g). However, the amount of each

**Table 1** Quantitation of $IP_6$ and $IP_5$ isomers in different soybean and corn grains collected from agricultural farms

| Samples | $IP_x$ content (μmol/g) | | | | |
|---|---|---|---|---|---|
| | $I_{(1,2,3,4,5,6)}P_6$ | $I_{(1,2,3,4,6)}P_5$ | *D/L*-$I_{(1,2,3,4,5)}P_5$ | *D/L*-$I_{(1,2,4,5,6)}P_5$ | $I_{(1,3,4,5,6)}P_5$ |
| Soybean 1 | 11.12 ± 1.89 | 0.51 ± 0.08 | 0.70 ± 0.02 | 2.10 ± 0.02 | 0.39 ± 0.08 |
| Soybean 2 | 10.78 ± 0.47 | 0.44 ± 0.17 | 0.61 ± 0.11 | 2.14 ± 0.04 | 0.47 ± 0.03 |
| Soybean 3 | 11.16 ± 0.17 | 0.42 ± 0.04 | 0.56 ± 0.04 | 1.71 ± 0.08 | 0.43 ± 0.08 |
| Soybean 4 | 8.64 ± 0.64 | 0.45 ± 0.03 | 0.57 ± 0.08 | 1.61 ± 0.17 | 0.38 ± 0.04 |
| Soybean 5 | 12.26 ± 1.05 | 0.50 ± 0.02 | 0.55 ± 0.16 | 1.72 ± 0.49 | 0.41 ± 0.04 |
| Corn 1 | 6.27 ± 1.24 | 0.33 ± 0.10 | 0.40 ± 0.09 | 1.37 ± 0.26 | 0.35 ± 0.11 |
| Corn 2 | 6.70 ± 0.51 | 0.43 ± 0.02 | 0.52 ± 0.04 | 1.57 ± 0.04 | 0.39 ± 0.10 |
| Corn 3 | 5.95 ± 0.75 | 0.35 ± 0.07 | 0.43 ± 0.01 | 1.33 ± 0.01 | 0.33 ± 0.12 |
| Corn 4 | 7.82 ± 0.73 | 0.34 ± 0.01 | 0.41 ± 0.02 | 1.31 ± 0.10 | 0.30 ± 0.03 |
| Corn 5 | 7.78 ± 0.98 | 0.35 ± 0.02 | 0.42 ± 0.03 | 1.28 ± 0.01 | 0.30 ± 0.06 |

Standard deviation refers to duplicate analyses

**Table 2** Quantitation of $IP_4$ isomers from different soybean and corn grains (see Table 1 for other $IP_6$ and $IP_5$ species)

| Samples | $IP_4$ content (μmol/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *D/L*-$I_{(1,2,4,6)}P_4$/ *D/L*-$I_{(1,2,3,5)}P_4$ | *D/L*-$I_{(1,2,3,4)}P_4$/ *D/L*-$I_{(1,3,4,6)}P_4$ | *D/L*-$I_{(1,2,4,5)}P_4$ | *D/L*-$I_{(1,3,4,5)}P_4$ | *D/L*-$I_{(1,2,5,6)}P_4$ | $I_{(2,4,5,6)}P_4$ | *D/L*-$I_{(1,4,5,6)}P_4$ |
| Soybean 1 | trace | 0.11 ± 0.03 | 0.23 ± 0.09 | 0.15 ± 0.04 | 0.27 ± 0.08 | trace | 0.08 ± 0.01 |
| Soybean 2 | trace | 0.10 ± 0.05 | 0.27 ± 0.09 | 0.23 ± 0.08 | 0.33 ± 0.06 | trace | 0.06 ± 0.01 |
| Soybean 3 | trace | 0.09 ± 0.01 | 0.21 ± 0.02 | 0.11 ± 0.01 | 0.25 ± 0.04 | trace | 0.03 ± 0.01 |
| Soybean 4 | trace | 0.08 ± 0.02 | 0.25 ± 0.02 | 0.12 ± 0.02 | 0.21 ± 0.05 | trace | 0.02 ± 0.01 |
| Soybean 5 | trace | 0.12 ± 0.04 | 0.32 ± 0.01 | 0.12 ± 0.07 | 0.32 ± 0.04 | trace | 0.05 ± 0.01 |
| Corn 1 | trace | 0.04 ± 0.02 | 0.10 ± 0.01 | 0.07 ± 0.01 | 0.13 ± 0.03 | trace | 0.03 ± 0.01 |
| Corn 2 | trace | 0.08 ± 0.01 | 0.18 ± 0.05 | 0.09 ± 0.01 | 0.19 ± 0.01 | trace | 0.04 ± 0.01 |
| Corn 3 | trace | 0.07 ± 0.01 | 0.12 ± 0.01 | 0.08 ± 0.01 | 0.15 ± 0.04 | trace | 0.03 ± 0.01 |
| Corn 4 | trace | 0.05 ± 0.01 | 0.11 ± 0.05 | 0.06 ± 0.01 | 0.14 ± 0.05 | trace | 0.03 ± 0.01 |
| Corn 5 | trace | 0.06 ± 0.01 | 0.11 ± 0.02 | 0.06 ± 0.01 | 0.13 ± 0.01 | trace | 0.03 ± 0.01 |

Standard deviation refers to duplicate analyses

individual $IP_x$ was very similar for all types of soybeans studied.

For corn grains, $IP_6$ was observed as the major inositol phosphate, ranging from 5.95 to 7.82 μmol/g. Among $IP_5$ isomers detected, *D/L*-I(1,2,4,5,6)$P_5$ was the major one, while the other three remaining $IP_5$ isomers showed very comparable concentrations. Seven $IP_4$ peaks were observed in all five corn samples (Fig. 1) but with slightly different amounts (Table 2). We were unable to identify additional lower order $IP_x$ isomers in corn. All the $IP_x$ in corns were relatively low amounts compared to those in soybean samples; however, the relative distribution among different $IP_x$ isomers was quite consistent.

Based on the HPIC results and total P content, concentrations of $IP_x$ species in different samples were calculated and shown in Table 3. Total P content in soybean grains ranged from 5376 to 5920 mg/kg, with inositol P contributing 39–53% of total P. Among $IP_x$, $IP_6$ constituted 29–42% of total P while $IP_5$ and $IP_4$ were at 10 and 2%, respectively. Corn grains had relatively lower content of total P (2465–2821 mg/kg) compared to those of soybeans; however, the $IP_x$ contribution was higher (60–75% of total P). These results are comparable with a past publication (Lazarte et al. 2015) and also consistent with the proportion of phytate-P of the total P in grains (Lott et al. 2000; Selle et al. 2010).

*Inositol phosphate and isomer compositions in animal feeds and excreta* Animal feed samples collected in 2017 contain various $IP_x$ species; however, the concentrations varied among different animals (Table 4). For example, the concentrations of $IP_6$ in the broiler, sheep, and beef cattle feeds were high and ranged from 9.18 to 9.96 μmol/g, which are close to those of soybean and corn grains, indicating a high component of soybean and corn in these animal feeds. In contrast, sheep and cow feeds contained relatively low content of $IP_6$ (4.67 and 2.01 μmol/g). This is expected due to the composition of ingredients, which besides soybean and corn, contains hay—a low phytate component (Ray et al. 2012). The $IP_5$ isomers were also detected in all five feed samples, of which *D/L*-I(1,2,4,5,6)$P_5$ was present in the dominant form, followed by *D/L*-I(1,2,3,4,5)$P_5$ and I(1,3,4,5,6)$P_5$. Small amounts of I(1,2,3,4,6)$P_5$ were also detected in three of five feed samples. As shown in Table 5, total P contents in animal feeds ranged from 3542 to 5953 mg/kg, while inositol phosphates accounted for 12–45%. Among all feed types analyzed, cow feed contained the lowest concentration of inositol phosphates (12%) while beef cattle feed had the highest (45%).

For the excreta samples collected in 2017, compositions and concentrations of $IP_x$ isomers were found to be slightly different (Table 4). A relatively large amount of $IP_6$ was detected in broiler litter (3.63 μmol/g), while a small amount of $IP_6$ was present in other manure samples (0.032–0.310 μmol/g). Among five animal manures analyzed, broiler litter was the only one that contained low order $IP_x$ besides $IP_6$ with *D/L*-I(1,2,4,5,6)$P_5$ as the predominant species followed by *D/L*-I(1,2,3,4,5)$P_5$ and I(1,3,4,5,6)$P_5$.

**Table 3** Total P and $IP_x$ content in different soybean and corn grains

| Samples | Total P (mg/kg) | $IP_x$ content (mg P/kg)/(percentage, %) | | | |
|---|---|---|---|---|---|
| | | $IP_6$ | $IP_5$ | $IP_4$ | $IP_x$ total |
| Soybean 1 | 5478.9 | 2068.3/(37.8) | 576.3/(10.5) | 103.3/(1.9) | 2747.9/(50.2) |
| Soybean 2 | 5919.6 | 2005.1/(33.9) | 567.4/(9.6) | 122.7/(2.1) | 2695.2/(45.5) |
| Soybean 3 | 5376.3 | 2075.8/(38.6) | 483.5/(9.0) | 85.9/(1.6) | 2645.1/(49.2) |
| Soybean 4 | 5505.1 | 1607.0/(29.2) | 466.4/(8.5) | 85.9/(1.7) | 2159.4/(39.2) |
| Soybean 5 | 5468.8 | 2280.4/(41.7) | 492.4/(9.0) | 114.7/(2.1) | 2887.4/(52.8) |
| Corn 1 | 2652.7 | 1166.2/(44.0) | 379.8/(14.3) | 46.7/(1.8) | 1592.7/(60.0) |
| Corn 2 | 2821.0 | 1246.2/(44.2) | 450.8/(16.0) | 71.6/(2.5) | 1768.6/(62.7) |
| Corn 3 | 2467.3 | 1106.7/(44.9) | 379.2/(15.4) | 56.2/(2.3) | 1542.2/(62.5) |
| Corn 4 | 2767.6 | 1454.5/(52.6) | 364.3/(13.2) | 49.2/(1.8) | 1868.0/(67.5) |
| Corn 5 | 2465.8 | 1447.1/(58.7) | 363.5/(14.7) | 48.3/(2.0) | 1858.9/(75.4) |

$IP_5$ is the sum of $I_{(1,2,3,4,6)}P_5$, *D/L*-$I_{(1,2,3,4,5)}P_5$, *D/L*-$I_{(1,2,4,5,6)}P_5$ and $I_{(1,3,4,5,6)}P_5$; $IP_4$ is the sum of *D/L*-$I_{(1,2,3,4)}P_4$/ *D/L*-$I_{(1,3,4,6)}P_4$, *D/L*-$I_{(1,2,4,5)}P_4$, *D/L*-$I_{(1,3,4,5)}P_4$, *D/L*-$I_{(1,2,5,6)}P_4$ and *D/L*-$I_{(1,4,5,6)}P_4$

For the broiler excreta collected in 2015, the concentrations of $IP_6$ and $IP_5$ from broiler cake manure were slightly lower than those from broiler litter. This difference could be due to the fact that the broiler litter not only contains the fresh broiler excreta but also some bedding materials and feed spills. However, the compositions of inositol phosphates in two broiler litter samples collected in 2015 and 2017 were strikingly similar. This result likely arises from the fixed formulation of ingredients in poultry feeds, and this industrial practice is encouraging for source tracking research because the composition of the inositol phosphate species in particular feed system could remain similar in different years.

Results of total P and inositol phosphate content in animal feeds and manures are shown in Table 5. Briefly,

**Table 4** Quantitation of $IP_6$ and $IP_5$ isomers in different animal feeds and manures

| Samples | $IP_x$ content (μmol/g) | | | | |
|---|---|---|---|---|---|
| | $I_{(1,2,3,4,5,6)}P_6$ | $I_{(1,2,3,4,6)}P_5$ | *D/L*-$I_{(1,2,3,4,5)}P_5$ | *D/L*-$I_{(1,2,4,5,6)}P_5$ | $I_{(1,3,4,5,6)}P_5$ |
| Broiler feed[a] | 9.18 ± 0.53 | 0.10 ± 0.03 | 0.15 ± 0.05 | 0.55 ± 0.15 | 0.08 ± 0.03 |
| Sheep feed[b] | 4.67 ± 0.29 | – | 0.01 ± 0.01 | 0.10 ± 0.02 | 0.02 ± 0.01 |
| Horse feed[b] | 9.67 ± 0.54 | 0.07 ± 0.03 | 0.12 ± 0.07 | 0.56 ± 0.11 | 0.08 ± 0.03 |
| Beef cattle feed[b] | 9.96 ± 0.12 | 0.09 ± 0.02 | 0.14 ± 0.01 | 0.70 ± 0.15 | 0.14 ± 0.02 |
| Cow feed[c] | 2.01 ± 0.23 | – | 0.03 ± 0.01 | 0.16 ± 0.04 | 0.02 ± 0.01 |
| Broiler litter[a] | 3.63 ± 0.85 | – | 0.27 ± 0.06 | 0.58 ± 0.05 | 0.12 ± 0.02 |
| Sheep manure[b] | 0.31 ± 0.01 | – | – | – | – |
| Horse manure[b] | 0.02 ± 0.01 | – | – | – | – |
| Beef cattle manure[b] | 0.10 ± 0.01 | – | – | – | – |
| Cow manure[c] | 0.03 ± 0.01 | – | – | – | – |
| Broiler manure 2015[d] | 2.77 ± 0.54 | – | 0.17 ± 0.06 | 0.54 ± 0.12 | 0.14 ± 0.08 |
| Broiler litter 2015[d] | 3.69 ± 0.79 | 0.03 ± 0.01 | 0.34 ± 0.18 | 0.57 ± 0.19 | 0.12 ± 0.04 |

[a] Sample collected from a chicken house at the university research farm, Newark, DE in 2017;

[b] Sample collected from a university research farm (Webb Farm), Newark, DE in 2017;

[c] Sample collected from the university dairy cow farm, Newark, DE in 2017;

[d] Sample collected from a chicken house at the university research farm, Georgetown, DE in 2015

Standard deviation refers to duplicate analyses

**Table 5** Total P and $IP_x$ content in different animal feeds and manures

| Samples | Total P (mg/kg) | $IP_x$ content (mg P/kg)/(percentage, %) | | |
|---|---|---|---|---|
| | | $IP_6$ | $IP_5$ | $IP_x$ total |
| Broiler feed[a] | 5404 | 1707.5/(31.6) | 135.8/(2.5) | 1843.3/(34.1) |
| Sheep feed[b] | 2828 | 868.6/(30.7) | 20.6/(0.7) | 889.2/(31.4) |
| Horse feed[b] | 5954 | 1798.6/(30.2) | 128.8/(2.2) | 1927.4/(32.4) |
| Beef cattle feed[b] | 4442 | 1852.6/(41.7) | 165.5/(3.7) | 2018.1/(45.4) |
| Cow feed[c] | 3543 | 373.9/(10.6) | 33.3/(0.9) | 407.2/(11.5) |
| Broiler litter[a] | 13,795 | 675.2/(4.9) | 150.4/(1.1) | 825.5/(6.0) |
| Sheep manure[b] | 6174 | 57.7/(0.9) | – | 57.7/(0.9) |
| Horse manure[b] | 4033 | 3.2/(0.1) | – | 3.2/(0.1) |
| Beef cattle manure[b] | 4176 | 18.6/(0.5) | – | 18.6/(0.5) |
| Cow manure[c] | 5526 | 6.0/(0.1) | – | 6.0/(0.1) |
| Broiler cake manure 2015[d] | 12,450 | 515.2/(4.2) | 131.8/(1.0) | 647.0/(5.2) |
| Broiler litter 2015[d] | 11,799 | 686.3/(5.9) | 164.3/(1.4) | 850.6(7.2) |

[a] Samples collected from a chicken house at the university research farm, Newark, DE in 2017;

[b] Samples collected from a university research farm (Webb Farm), Newark, DE in 2017;

[c] Samples collected from the university dairy cow farm, Newark, DE in 2017;

[d] Samples collected from a chicken house at the university research farm, Georgetown, DE in 2015

broiler excreta had a very high total P (11,799–13,795 mg/kg), while the other manures contained about half or one-third of that, which is comparable with results from previous studies (Leytem and Maguire 2007; Peperzak et al. 1959). The $IP_6$ represented very insignificant amounts of P in ruminant animal manures: only 0.1% of total P from cow, 0.5% from beef cattle, and 0.9% from sheep. Inositol phosphate distributions between input to the animal (feeds) and output (manures) were significantly different, which is due to the fact that these ruminant animals have the capacity to digest most inositol phosphates present in the feed, leaving little $IP_x$ remaining in their manure wastes (Vohra and Satyanarayana 2003). For the excreta from monogastric animals, horse manure contained only 0.08% inositol phosphate compared with total P, while broiler excreta contained a large amount of inositol phosphates. It is noticeable that $IP_x$ contributed 5.2–7.2% of total P in studied broiler excreta. However, percentages of $IP_x$ in broiler cake manure and litter were less than those in broiler feed in this study, and amounts of detected $IP_x$ in broiler cake manure and litter were also lower than results from previous publications (He et al. 2009; Hill and Cade-Menun 2009). The discrepancy could, however, originate from different proportions of dietary phytase, which is often added to poultry and other monogastric animal feeds to increase P bioavailability (Lei and Porres 2007). Previous studies have found that phytase supplements could reduce the concentration of $IP_6$ in pig feces up to 64% (Skoglund et al. 1998). For turkey and broiler manures, phytase addition in diet could decrease $IP_6$ concentration by 16–38% (Leytem and Maguire 2007; Maguire et al. 2006). Nevertheless, the digestibility of total P or phytate for a given feed ingredient may be affected by diet composition, age of the animal, amount of P present in the diet, or endogenous excretion of P (Shen et al. 2002) besides experimental methods used for extraction and quantitation. The broiler feed used in this study was supplemented with 0.01 wt% of Quantum Blue phytase but the supplemental phytase for other animal feeds are unknown. However, we tested the phytase activity in the horse feed extract by incubating with pure phytate. The result showed that phytate was partly hydrolyzed indicating the presence of phytase in horse feed. However, whether the phytase activity measured is native to feed ingredients or supplemented cannot be identified. Nonetheless, this finding may explain, at least in part, why only a small amount of inositol phosphates were detected in the horse manure. Further, although monogastric animals lack phytate-degrading enzymes, a

limited specific activity of phytase could still be observed in the guts of pigs and poultry even without supplemental phytase in the diet (Dersjant-Li et al. 2015). The other possibility could be methodological limitation such as the acid extraction method used in this study is not as effective as the alkaline extraction (NaOH-EDTA) used for NMR analysis (Ray et al. 2012) and interpretational such as the mis-assignment of resonance peaks and the broad signal in NMR spectra of NaOH-EDTA extracts could overestimate phytate concentrations (Doolette et al. 2011; Smernik and Dougherty 2007). Last, manure storage conditions also affect phytate content. For example, wet storage has been found to decrease the phytate content in poultry litter by 22% (McGrath et al. 2005), potentially due to higher microbial activity supported by high moisture content. In contrast, drying poultry manure result in no or limited conversion of phytate to $P_i$ (He et al. 2007).

*Oxygen isotope composition of phytate in soybean and corn grains* The calculated isotopic fractionation (using eq. 1) associated with phytate degradation by *A. niger* phytase as well as phytate isotope composition in soybean and corn grains are summarized in Table 6. The $\delta^{18}O_{PA\text{-}Pi}$ values of released $P_i$ from K-phytate were 17.27 (±0.13)‰, and the $\delta^{18}O_{PA}$ values of intact K-phytate, measured directly after pyrolysis, were 20.53 (±0.22‰). With the known $\delta^{18}O_W$ values of water (−7.02 ± 0.04‰) used in the experiment, the isotope fractionation factor of *A. niger* phytase calculated is 14.51(±0.84)‰. This value is consistent with past published result (Sun et al. 2017). Due to separation/analytical issues, $\delta^{18}O_{PA}$ values of intact phytate from environmental samples could not be measured directly; however, they can be calculated from $\delta^{18}O_{PA\text{-}Pi}$ value of released $P_i$, the isotope fractionation factor, and $\delta^{18}O_W$ value of water used in the experiments according to eq. (1). Based on this calculation, $\delta^{18}O_{PA}$ values of different grains were found to be slightly different: the values from soybeans ranged from 20.48 to 23.87‰, while the values were slightly heavier from corns (22.30–24.20‰). Since some pure $IP_5$ isomers were separated and collected from environmental samples, separate incubation of these isomers with *A. niger* phytase allowed independent comparison of their isotope values with those generated from $IP_6$. Interestingly, for some collected pure $IP_5$ isomers, the calculated oxygen isotope values were found to be the same as those of $IP_6$. For example, $\delta^{18}O_{PA}$ value of *D*/*L*-I(1,2,3,4,5)$P_5$ from corn 3 sample was 24.37‰ compared to 24.20‰ in $IP_6$ of the same corn sample. The difference is insignificant because it is within the range of measurement precision (±0.3‰). This result suggests that different $IP_x$ isomers from the same grain, irrespective of whether the parent phytate ($IP_6$), or the other degradation products (like $IP_4$, $IP_5$), carry the same oxygen isotope signatures. This unique result serves as an important proxy and greatly facilitates tracking lower order $IP_x$ isomers and phytate-derived $P_i$ in the environment.

Inorganic P present in soybean and corn grains was extracted separately and their $\delta^{18}O_{Pi}$ values were measured to compare to those of $\delta^{18}O_{PA}$ values of phytate in the same grain type. As shown in Table 6, the $\delta^{18}O_{Pi}$ values of $P_i$ in the grains ranged from 23.13 to 25.86‰, which are 3–6‰ heavier than those $\delta^{18}O_{PA\text{-}Pi}$ released from phytate hydrolysis. Interestingly, the $\delta^{18}O_{Pi}$ values were also 0.4–3.2‰ heavier than calculated $\delta^{18}O_{PA}$ values of phytate, except one soybean sample (for which the difference was small, ~0.5 ‰). These differences in isotope values of $P_i$ vs phytate can be illustrated by the mechanism by which these two P pools co-occur in grains. The predominant pathway for the biosynthesis of phytate in plant grains starts from the substrate glucose-6-phosphate, which first forms inositol 3-phosphate (I(3)$P_1$). This intermediate substrate is converted to phytic acid via stepwise and ordered phosphorylation of the inositol ring with five additional phosphate moieties by the action of a number of phosphokinase enzymes (Rasmussen et al. 2010; Stevenson-Paulik et al. 2005). Thus, one potential reason for the difference in phosphate oxygen isotope compositions between $P_i$ and phytate in grains could be that the kinases preferentially use $P_i$ with lighter oxygen isotope values for phosphorylation, which leads to the remaining $P_i$ in grains becoming isotopically heavier. Comparison of $IP_6$ with other $IP_x$ and $P_i$ could provide additional insights into the difference in isotope compositions among different grains. While it is premature to generalize from our limited data that are insufficient for statistical analysis, it is, nonetheless, likely that relative abundances of $P_i$, $IP_6$, and $IP_{x\ (x<6)}$ could also play a role in controlling their isotope compositions due to size of each pool and mechanism of how one pool is formed at the expense of other P pools. While this is beyond the scope of the current work, further research in this direction is needed to generate a mechanistic understanding of processes that imprint starting isotope signature of different P pools in grains.

**Table 6** Isotopic compositions of coexisting inorganic P ($P_i$) and phytate in different grains

| Samples | $\delta^{18}O_{Pi}$(‰) | $\delta^{18}O_{PA\text{-}Pi}$ (‰) | $\delta^{18}O_W$ (‰) | $\delta^{18}O_{PA}$ (‰) |
|---|---|---|---|---|
| K-Phytate | – | 17.27 ± 0.13 | −7.02 ± 0.04 | 20.53 ± 0.22 |
| Soybean 1 | 23.88 ± 0.37 | 17.47 ± 0.25 | −7.31 ± 0.02 | 20.89 |
| Soybean 2 | 24.56 ± 0.02 | 18.90 ± 0.04 | −7.14 ± 0.05 | 22.74 |
| Soybean 3 | 23.31 ± 0.32 | 19.78 ± 0.06 | −7.00 ± 0.06 | 23.87 |
| Soybean 4 | 23.69 ± 0.49 | 17.24 ± 0.13 | −6.98 ± 0.05 | 20.48 |
| Soybean 5 | 23.13 ± 0.65 | 18.25 ± 0.43 | −7.09 ± 0.09 | 21.86 |
| Corn 1 | 23.27 ± 0.43 | 18.56 ± 0.42 | −7.18 ± 0.03 | 22.30 |
| Corn 2 | 25.86 ± 0.24 | 19.82 ± 0.37 | −6.75 ± 0.06 | 23.84 |
| Corn 3 | 24.93 ± 0.13 | 19.95 ± 0.31 | −7.31 ± 0.02 | 24.20 |
| Corn 4 | 25.06 ± 0.09 | 18.90 ± 0.18 | −6.69 ± 0.01 | 22.59 |
| Corn 5 | 24.29 ± 0.70 | 19.90 ± 0.14 | −6.48 ± 0.01 | 23.86 |

Symbols $\delta^{18}O_{Pi}$, $\delta^{18}O_{PA}$, $\delta^{18}O_W$, and $\delta^{18}O_{PA\text{-}Pi}$ correspond to oxygen isotope ratios of $P_i$ in the seed, intact phytate, and water used in the extraction, and $P_i$ released from phytate during hydrolysis, respectively. The fractionation factor ($F = 14.51 \pm 0.84$) was used to calculate $\delta^{18}O_{PA}$ from $\delta^{18}O_{PA\text{-}Pi}$. All isotope values are expressed in permil relative to VSMOW. Standard deviation refers to triplicate analyses

*Oxygen isotope compositions of phytate in animal feeds and manures* For animal feeds and manures, the $\delta^{18}O_{PA\text{-}Pi}$ values of the released $P_i$ from phytate were in a narrow range from 18.46‰ to 19.71‰ and the calculated $\delta^{18}O_{PA}$ values ranged from 22.00‰ to 24.09‰ (Table 7). These isotope compositions are within the ranges of soybean and corn grain phytates (Table 6). It is noticeable that the isotope values of phytate were consistent between broiler feed and broiler litter from the same research farm in 2017 (24.09‰ and 23.52‰), validating that the method of calculating the original phytate oxygen isotope composition based on the constant isotope fractionation factor is reliable.

Unlike soybean and corn grains, the $\delta^{18}O_{Pi}$ values of $P_i$ from animal feeds were in the range of 16.78–22.13‰. This means that isotope compositions of original $P_i$ in the feeds are lighter than those of $P_i$ from soybean and corn grains. The major ingredients of animal feeds are ground corn and soybean grains. However, P minerals are often added to meet the nutritional demands of animals, for which the $\delta^{18}O_P$ values are in the ranges of 15.5–25.3‰ (Davies et al. 2014). We have tested the inorganic P contents and found that animal feeds contain much more $P_i$ than grains. A large amount of mineral P in animal feeds will consequently change the isotope compositions depending on the isotope values of mineral P added compared to that of $P_i$ in the original grains.

Interestingly, the $\delta^{18}O_{Pi}$ values of $NaHCO_3$ extractable P from all animal manures (12.66–17.10‰) were much lighter than those in the feeds. Once within the animal gut, inositol phosphates are hydrolyzed either by supplemented phytases or those present in gut microbiota (Zeller et al. 2015). During this process, the released $P_i$ partially retains O from the original inositol phosphates (Wu et al. 2015). However, the phosphate oxygen isotope signature of released $P_i$ could be erased because of a rapid oxygen isotope exchange with ambient water catalyzed by pyrophosphatase following an equilibrium isotope effect (Chang and Blake 2015). We calculated the equilibrium phosphate isotope values following a temperature dependent equation (Chang and Blake 2015) using the water oxygen isotope values ($\delta^{18}O_w$) reported in dairy cattle plasma (−4.7 to −6.2‰) (Abeni et al. 2015) and animal body temperatures. The results showed the $\delta^{18}O_{Pi}$ values of the bioavailable $P_i$ in manures were driven towards the equilibrium values (13.2−15.3‰), indicating that the $P_i$ (which should be most bioavailable) was enzymatically cycled within the animal body before excreted as manure.

*Oxygen isotope composition of organic phosphorous compounds* Very few studies have reported the oxygen isotope compositions of organic P compounds ($\delta^{18}O_{Po}$). This is because the presence of non-phosphate O atoms in the molecule prevents accurate measurement of oxygen isotope composition of phosphate O atoms. The most commonly used method is liberating the phosphate

**Table 7** Isotopic compositions of coexisting inorganic P ($P_i$) and phytate in animal feeds and manures. The feed and manure were obtained from the same animal operation

| Samples | $\delta^{18}O_{Pi}$(‰) | $\delta^{18}O_{PA\text{-}Pi}$ (‰) | $\delta^{18}O_W$ (‰) | $\delta^{18}O_{PA}$ (‰) |
|---|---|---|---|---|
| Broiler feed [a] | 16.78 ± 0.32 | 19.40 ± 0.61 | −6.91 ± 0.04 | 24.09 |
| Broiler litter [a] | 15.37 ± 0.23 | 19.56 ± 0.42 | −6.84 ± 0.04 | 23.52 |
| Sheep feed [b] | 17.18 ± 0.37 | 18.46 ± 0.43 | −6.66 ± 0.13 | 22.00 |
| Sheep manure [b] | 12.66 ± 0.18 | – | – | – |
| Beef cattle feed [b] | 18.58 ± 0.07 | 19.43 ± 0.04 | −6.80 ± 0.07 | 23.34 |
| Beef cattle manure [b] | 14.05 ± 0.13 | – | – | – |
| Horse feed [b] | 22.13 ± 0.41 | 18.87 ± 0.01 | −6.80 ± 0.22 | 22.59 |
| Horse manure [b] | 16.52 ± 0.17 | – | – | – |
| Cow feed [c] | 21.78 ± 0.35 | 19.71 ± 0.47 | −6.86 ± 0.05 | 23.73 |
| Cow manure [c] | 13.03 ± 0.27 | – | – | – |
| Broiler cake manure 2015 [d] | 17.10 ± 0.10 | 19.62 ± 0.30 | −6.82 ± 0.15 | 23.72 |
| Broiler litter 2015 [d] | 15.61 ± 0.15 | 18.71 ± 0.54 | −6.80 ± 0.04 | 22.37 |

Symbols $\delta^{18}O_{Pi}$, $\delta^{18}O_{PA}$, $\delta^{18}O_W$, and $\delta^{18}O_{PA\text{-}Pi}$ correspond to oxygen isotope ratios of $P_i$ in the seed, intact phytate, water used in the extraction, and $P_i$ released from phytate during hydrolysis, respectively. The fractionation factor ($F = 14.51 \pm 0.84$) was used to calculate $\delta^{18}O_{PA}$ from $\delta^{18}O_{PA\text{-}Pi}$

[a] Sample collected from a chicken house at the university research farm, Newark, DE in 2017;

[b] Sample collected from a university research farm (Webb Farm), Newark, DE in 2017;

[c] Sample collected from the university dairy cow farm, Newark, DE in 2017;

[d] Sample collected from a chicken house at the university research farm, Georgetown, DE in 2015. Standard deviation refers to triplicate analyses

group from its parent organic P molecule first and then measuring $\delta^{18}O_{Pi}$ of the released $P_i$. For example, Liang and Blake (2006b) studied four different $P_i$ extraction methods from organic P compounds and found that dry combustion completely erased the original $\delta^{18}O_{Po}$ isotope signature but acid and enzyme-catalyzed hydrolysis partially retain the original $\delta^{18}O_{Po}$ values. Among four methods studied, UV-radiation (UVR) catalyzed degradation was found to have the least alteration of original oxygen isotope values (Liang and Blake 2006b). However, UVR hydrolysis of organic P is also accompanied by incorporation of oxygen from ambient water into the released $P_i$ (Sandy et al. 2013; Wu et al. 2015). In mineral-catalyzed degradation of an organic P compound, similar incorporation of water oxygen atoms is reported (Jaisi et al. 2016; Li et al. 2016). Thus, this methodological challenge requires an establishment of a common method to allow comparison among data on $\delta^{18}O_{Po}$ values derived from measured $\delta^{18}O_{Pi}$ values. In fact, most studies report oxygen isotope signatures of $P_i$ generated by enzyme hydrolysis of organic P (Liang and Blake 2006a; von Sperber et al. 2014, 2015). Since pure organic P compounds were hydrolyzed to liberate $P_i$ in these studies, a correlation between $\delta^{18}O_{Pi}$ and $\delta^{18}O_{Po}$ values could be proposed. In our recent study, we found that the isotope fractionation factors were constant for specific enzymes in phytate degradation and independent of reaction rate or time of degradation (Sun et al. 2017). In addition, constant isotope values of phosphate groups released from phytate over time suggest that all phosphate moieties attached to the inositol, irrespective of their structural positions, have the same oxygen isotope composition. Based on these two important findings, the compositions of specific enzymatic degradation products and oxygen isotope values could provide useful information for tracing the original source of phytate and tracking its dephosphorylated products in the environment. Further, this is the first report of phosphate oxygen isotopes being determined from natural organic P samples by using an enzyme to hydrolyze the purified compounds and calculating based on the certain isotope fractionation factor of the specific enzyme. Results from our study suggest that enzymatic degradation could be a reliable method to trace original organic P compounds by measuring the $\delta^{18}O_{Pi}$ values of

generated $P_i$, which provides important information on source tracking research. It further builds on the systematic analyses of compound-specific isotopes of organic P compounds present in the environment.

*Implication of tracking phytate and its products in the environment* Phytate is one of the most common and recalcitrant forms of $P_o$ in the environment and thus plays a major role in terrestrial P cycling. However, gaps in scientific information to connect sources and products have limited our understanding of the origins and routes of transformation. This limitation has barred calculating their residence times in the environment. In this research, different sources of crops (soybean and corn grains), animal feeds, and animal manures were analyzed. Inositol phosphates in these environmental samples were extracted and purified and then analyzed by HPIC. The $IP_x$ species were found to be the major P forms in soybean and corn with $IP_6$ being the most dominant $IP_x$ species, followed by $IP_5$, $IP_4$, and a small amount of $IP_3$. Animal feeds contained a certain amount of $IP_6$ and $IP_5$. Even though phytase is often added to animal feeds, similar $IP_x$ species were still observed in animal manures, especially from monogastric animals. Optimizing the P nutritional requirements for different animal species is the first step in reducing P in their excreta, which is tied to the digestibility of grain-based P sources. Thus, a link between animal feeds and animal wastes provides new insights into particular $IP_x$ isomers that could be formed or digested in animal guts. For example, the ratios of $IP_6$: $IP_5$ among animal feeds are around 14–42, while they decrease sharply among animal excreta samples (3.9–4.5), suggesting the relative abundance of $IP_5$ in animal excreta compared with feed samples. This indicates that the $IP_6$ is significantly hydrolyzed by enzymes while hydrolysis of $IP_5$ is inefficient.

During phytate degradation by *A. niger* phytase, $P_i$ was liberated from the inositol ring, of which 75% of O was directly inherited from $IP_6$, allowing calculation of its original oxygen isotope composition. Unfortunately, current methods in the literature are neither capable of distinguishing sources of $IP_x$ nor differentiating natural from anthropogenic loading and their roles in P cycling. Thus, the combination of isotope tools with HPIC raises the possibility of their applications in tracing phytate P sources and tracking its products in the environment. Research outcomes in this study are the first and essential step in the systematic analysis of different $IP_x$ sources that are eventually loaded in the environment and connecting sources and products to generate a quantitative understanding of the impact of different $IP_x$ in the environment.

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