1 Determination of phytate in high molecular weight, charged organic matrices by
2 two-dimensional size exclusion-ion chromatography

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8 **Abstract**

9 A two-dimensional chromatography method for analyzing phytate or other ionic
10 targets in matrices containing high molecular weight, charged organic species is
11 described. Prior to quantification by anion exchange chromatography, the sample
12 matrix was prepared by size exclusion chromatography, which removed the
13 majority of the matrix. Quantification of phytate on the AS11-HC was sensitive (0.25
14 μM , 0.17mg/l) and reproducible (4.6% RSD) allowing this method to provide
15 baseline separation of phytate from a manure extract within 14 minutes. The
16 method is simple, requiring only sample filtering, reproducible (between-run
17 variation 5% RSD) and linear from 0.38-76 μM (0.25-50 mg/l). The method is
18 suitable for routine determination of phytate in high organic matrices such as
19 manure extracts.

20 Key words:

21 Size Exclusion Chromatography; Ion Chromatography; Two-Dimensional
22 Chromatography; Matrix Removal; Phytate

23 **1. Introduction**

24

In ion chromatography (IC), interferences from sample matrices can inhibit sufficient separation and quantification of target analytes[1]. Generally, there are two approaches that the analyst can use to eliminate interfering matrices. Matrix elimination, either by selectively retaining the target and washing through the matrix, or retaining the problematic matrix components and collecting the target. Both are considered solid phase extraction (SPE) techniques, which are the hallmark of sample preparation[1]. For the removal of general organics, sample prep phases, which contain styrene-divinylbenzene resin or alkyl reversed phase material, are commonly used to remove aromatic or alkyl organics from the sample matrix. Charged organics, such as humic and fulvic acids are generally not well retained by these types of resins without neutralizing the sample pH. Without the removal of organics, IC columns quickly become fouled[2-4]. With both approaches however, SPE methods typically do not account for variation in samples or sample matrices. In this work, we examine the utility of size exclusion chromatography as an approach for matrix removal based on molecular weight.

The size exclusion chromatography approach for matrix removal described herein was developed in response to the need to isolate and measure myo-inositol hexakisphosphate, or phytate in a high dissolved organic matter extract (see supplementary material for more information). Characterizing phosphorus in natural surface waters, wastewaters, soils and manures is important for understanding the complexities of phosphorus losses to streams, rivers, and lakes that result in eutrophication of the freshwater aquatic environment. While phytate is only one fraction of organic phosphorus, its ubiquity in nature as the primary

plant phosphorus storage molecule makes it of particular interest. Additionally, phytate like most of the organic phosphorus fraction interacts with organic carbon making it challenging to isolate prior to analysis. Several extraction procedures, such as Mehlich-3 and basic-EDTA, have been developed which are intended to remove bound phosphorus from soil minerals and the organic carbon fraction[5, 6]. Many of the standard methods for analyzing phytate in these extracts, however, are either quite encumbering (solid phase extraction-liquid chromatography or nuclear magnetic resonance)[7, 8] or nonspecific (Inductively coupled plasma, colorimetry)[9-11]. In the case of phytate analysis, a size exclusion method provides a common procedure regardless of the sample matrix. Recently, ion chromatography (IC) has been gaining popularity as the analytical technique for speciating phosphorus[12-15]. Additionally, size exclusion chromatography has been shown as an effective method of fractionating large molecular weight humic acids[16, 17]. By expanding on this work, the same principles can be applied to fractionating sample matrices based on size prior to analysis. With the matrix removed, a heartcut injection onto a second column, which has a comparatively clean matrix allows reliable separation and quantification of phytate by electrical conductivity[18]. Here, we demonstrate large molecular weight exclusion for the quantification of phytate in a water extract of poultry manure as well as small molecular weight exclusion for eliminating interferences from matrix components following a poultry litter extraction.

2. Materials and methods

2.1 Materials

All chromatography separations were performed with a ThermoFisher-Dionex ICS5000+ chromatography system (Sunnyvale, CA, USA). The ICS5000+ system is equipped with 2 fully capable chromatography channels, which can be integrated together. The first channel used for matrix removal included an automated sample injector, a 4-channel gradient pump, 6-port high-pressure valve, 10 μ l injection loop, a (5 μ m 4.6x300 mm) SEC-300 size exclusion column, conductivity detector, and a 4-channel variable wavelength detector. The second channel included a high-pressure isocratic analytical pump, electrolytic eluent generator, 6-port high-pressure valve, (0.75x80 mm) MAC-200 anion concentrator column, 2x250 mm AS-11HC ion exchange column, electrolytic suppressor and conductivity detector. Instrument control and data acquisition was done using Chromeleon 7.2 software (ThermoFisher-Dionex, Sunnyvale, CA, USA).

All reagents and solutions used were of analytical grade. A ROES-75 Reverse osmosis water system capable of producing 16 M Ω water (APEC Water Systems, City of Industry, CA, USA), was added to the RFIC system and polished in-line using CIRA-10 electro dialytic water purification unit (Trovion, Campbell, CA, USA) prior to entering the high-pressure pump on both channels. Additionally, the size exclusion column used one mM HCl (pH 3.0) as the mobile phase. Standards were made using ACS grade phytic acid dipotassium salt.

2.2. Methods

Two common extraction procedures were performed on poultry manure sourced from a local farm, which does not fortify their poultry feed with phytase enzyme. All manure samples were dried, grinded and sieved as discussed in[19].

Samples were then extracted with deionized water following the method of Kleinman et al. [20]. Manure samples were also extracted by shaking 2.5 g of manure with 25 mL of Mehlich-3 solution (0.2 M CH₃COOH + 0.25 M NH₄NO₃ + 0.015 M NH₄F + 0.013 M HNO₃ + 0.001 M EDTA) for 5 min [6].

Separation of phytate was achieved using a 2-dimensional system by matrix removal in the first dimension, followed by ion exchange chromatography in the second. A 10 µl filtered, undiluted sample was injected on to the size exclusion column (Figure 1A), which was used to separate the matrix components based on molecular weight (Figure 1B). At 6.1 minutes, the valve between the first and second dimension was actuated to capture the effluent from the SEC-300 for 15-20 seconds using a MAC200 anion concentrator column (Figure 1C). After the 15-20 second interval, the valve was actuated again to inject the captured fraction onto the ion exchange column (Figure 1D). The total runtime for the analysis was 14 minutes under the operating conditions shown in Table 1.

The minimum width of the heartcut was determined using several different molecular weight standards (Polyacrylic acid M.W. 15K, 8K, 1200), and formic acid as the marker for the total permeation time). Once the permeation time between the 1200 M.W. marker and the formic acid was determined, subsequent injections of a 5.0 mg/l phytate standard were made to optimize phytate capture while minimizing the amount of SEC eluent (chloride) onto the IC column.

3. Results and discussion

The size exclusion column contains 5µm mono-dispersed hydrophilic polymethacrylate particles and is specifically designed for the separation of water-

soluble polymers and oligomers over a broad range of molecular weights. The polymethacrylate stationary phase has a small quantity of residual anionic charge. Therefore, to maximize molecular sieving it is critical to minimize any anionic repulsions from the residual charge. These undesired anionic interactions were overcome by adding 1 mM HCl. Additionally, the HCl served as a pH modifier to minimize the charge on the injected sample. It was determined that a pH of 3-3.5 was sufficient to minimize the charge of the smaller humics, which don't precipitate out of the sample under acidic conditions[21, 22]. Operating the SEC at a pH between 3 and 3.5 allowed more complete separation from the large molecular weight humic compounds as well as the small molecules, such as nitrate and acetate from the extraction solution. A pH of 3-3.5 ensured that the phytate contained minimal charge while operating within the acceptable range of the SEC (pH 2-12). The sieving mechanism of the SEC-300 provides a separation range of 100-50,000 Daltons. Many of the smaller humics, which did not precipitate out were in the 1000-5000 Dalton range and eluted prior to phytate (M.W. 660). Conversely, the Mehlich-3 extraction matrix was below the 100 Dalton range with the exception of EDTA eluted primarily at the column permeation time ($\approx$ 8.5 to 10.5 minutes). Total exclusion time under the experimental conditions above was $\approx$ 4 minutes.

Figure 2 illustrates the determination of phytate by anion exchange chromatography after matrix removal by size exclusion chromatography. The two-dimensional system allows for direct analysis of phytate while removing most of the sample matrix in-line, which negates the need for solid phase extraction and made the detection of phytate possible by electrical conductivity.

The system was optimized to remove a 15 to 20 second fraction (around 6.25 minutes) from the first dimension, loading it onto a concentrator, as seen in Figure 1. Furthermore, the use of an isocratic elution system on both dimensions allowed for higher throughput without the need for an equilibration step between samples. These findings were also supported by the use of blank injections between the standards and unknowns, which showed no detectable amount of phytate. Performance parameters for the determination of extracted phytate levels, as calculated from triplicate analysis of the water (WE), and Mehlich-3 (M3) extractions are summarized in Table 2. Phytate spike recoveries from the extractions were determined by adding a 100 μ l of 200 mg/l phytate stock solution to 10 ml of extraction solution prior to filtering. Samples were then filtered through a 0.45 μ m syringe filter and analyzed. The recovery of phytate added to water and Mehlich-3 extractions (n=10) to a final concentration of 30.3 μ M (20mg/l), was 99.2% (Relative standard deviation = 4.6 %). Detection by electrical conductivity was linear (Slope = 0.044 and R^2 = 0.997) over a concentration range of 0.38-76 μ M (0.25-50 mg/l) using 8 calibration standards (0.03, 0.25, 2.5, 5.0, 10, 20, 30, 50 mg/l), as seen in Figure 3. The LOD of the method was 0.05 μ M (0.03 mg/l) with an LOQ of 0.25 μ M (0.17mg/l).

To ensure analysis of phytate was reproducible, the same phytate standards were made up in 1.0 mM HCl and measured by directly injecting 175 μ l on the AS11-HC column, which was equal to the μ Eq loaded onto the MAC200 during a 30 second heartcut. In addition, phytate standards were also made up in water to ensure that there were no matrix effects by the size exclusion eluent. In both cases the retention

time and concentration of phytate standard was not statistically different from what was found in the 2-dimensional system.

In the first attempt using a 30 second heartcut, a standard capacity (2x250 mm) AS11 column was used resulting in poor phytate separation (a shoulder on the chloride tail) from the concentrated chloride eluent used in the first dimension. By instead using the AS11-HC, which has approximately 7 times the anion exchange capacity of the AS11, phytate could be completely resolved from the SEC chloride matrix.

4. Conclusions

Multi-dimensional chromatography is a well-known technique in the chromatography toolbox, which is particularly useful for matrix cleanup, or as an in-line sample prep method. Furthermore, such a method negates the need for sample preparation beyond filtering, and does not require any user intervention prior to detection. The method presented here is simple, economical, suitable for routine analyses of phytate or other ionic targets in complex matrices, and well within the capabilities of the average ion chromatography analyst. For these reasons, we see many opportunities to apply the principles of size exclusion chromatography as a matrix clean up procedure to a breadth of other matrices and ionic targets.

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Figure 1. Schematic of two-dimensional chromatography system.

*Color online only

Figure 1A: Loading manure matrix into the SEC loop (between valve “L” labels).

Figure 1B: Sample is injected onto the SEC column (matrix exclusion). Figure 1C:

Concentrator column on second valve collects the phytate fraction from the SEC

outlet (between valve “L” labels). Figure 1D: Phytate fraction (now on the

concentrator) is injected onto the anion exchange column prior to detection.

Figure 2. Analysis of phytate by SEC-IC.

*Color online only

Blue trace is UV at 220 nm at SEC outlet (first dimension) and black trace is conductivity on second dimension. Cut from first dimension occurs between 6.1 and 6.3 minutes (black trace starts). The large peak in the UV trace at about 9.5 minutes is the SEC void including the matrix components of the Mehlich-3 extract. All other known peaks are labeled for reference.

Figure 3. Linear regression plot.

*Color online only

The following standards (0.03, 0.25, 2.5, 5.0, 10, 20, 30, 50 mg/l) are shown including a matrix blank.

Figure 1

Figure 2

Figure 3

283 Table 1. Chromatography Conditions

Condition	SEC-300	AS11-HC
Flow rate (ml*min ⁻¹)	0.35	0.40
Column Temperature (°C)	30.0	30.0
Eluent concentration (mM)	1.0 (HCl)	65.0 (KOH)
Suppressor current (mA)	n.a.	65.0

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285
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Table 2. Sample analyses

Sample Type	Meas. Phytate Conc. (mg/l)	Conc. of 20 mg/l Std. recovered	%Recovery	%RSD
WE 1	2.87		102	
WE 2	2.99		106	
WE 3	2.61		92.4	
WE 1 + Spike	23.6	20.7	103	
WE 2 + Spike	23.1	20.1	101	
WE 3 + Spike	22.0	19.4	96.8	
WE Blk	<0.17			
WE Blk	<0.17			
WE Blk + Spike	3.38		99.4	
WE Blk + Spike	3.40		100	3.84
M3 1	26.5		111	
M3 2	21.6		90.8	
M3 3	23.3		97.7	
M3 1 + Spike	46.4	19.8	99.2	
M3 2 + Spike	41.5	19.9	99.4	
M3 3 + Spike	43.2	19.9	99.6	
M3 Blank	<0.17			
M3 Blank	<0.17			
M3 Blk + Spike	3.51		103	
M3 Blk + Spike	3.32		97.6	5.45

* (WE) Water extraction, (M3) Mehlich-3 extraction