# Determination of Inositol Phosphates in Dried Distillers Grains with Solubles

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## Introduction

Inositol hexakisphosphate (phytic acid or Ins-$P_6$, Figure 1A) is the principal storage form of phosphorus in many plant-based materials, including vegetables, legumes, cereals, and seeds. In these materials, Ins-$P_6$ exists in its ionic form, phytate. Phytate accounts for 50–80% of the phosphorus in seed-based feedstuff. At physiological pH, phytate carries a negative charge and can thus chelate di- and trivalent cations and form stable complexes with proteins and starches. In a complexed form (Figure 1B), phytate's phosphorus and bound minerals become nutritionally unavailable. Therefore, dried distillers grains with solubles (DDGS), a corn-based animal feed, are supplemented with inorganic phosphorus to meet the dietary needs of livestock animals.[1]

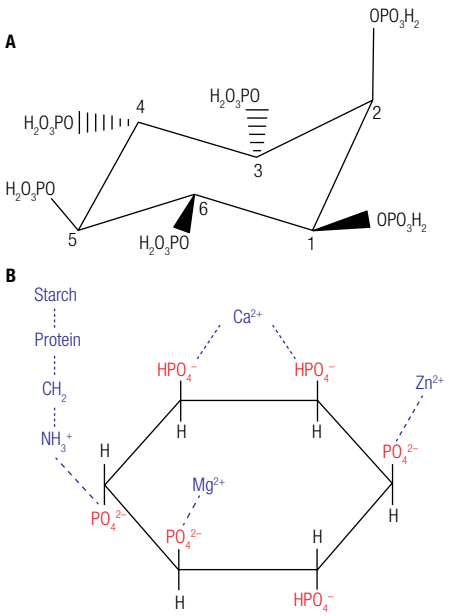


Figure 1. Inositol hexakisphosphate (A) and complexed forms of phytate (B).

Factors that contribute to the nutritional variation in DDGS include the original corn source, fermentation variables, and processing conditions such as drying and pelleting.[2] Some studies suggest that the available phosphorus in DDGS varies primarily with the extent of phytate hydrolysis during fermentation and processing.[3] Studies also suggest that increasing heat during processing reduces the amount of intact phytate and enhances the bioavailability of phosphorus in DDGS.[2,4,5] During fermentation and processing, phosphate groups are released from phytate by enzymatic and nonenzymatic hydrolysis mechanisms to produce $InsP_5$–$InsP_1$, *myo*-inositol, and free phosphate. Moreover, commercial phytase enzymes are routinely added to feed to increase phosphorus bioavailability by significantly reducing the phytate levels from DDGS.[6,7] Therefore, a method to determine the level of phytate hydrolysis products in DDGS is useful in determining phosphorus bioavailability to livestock animals.

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Common challenges with determination of inositol phosphates (InsPs) include sample preparation and isomer separation/identification. The most frequently used sample preparation procedure includes extraction with HCl (0.5 M) and sample cleanup/concentration on a strong anion-exchange resin, such as AG 1×8 resins or SAX cleanup columns.[8-11] Using this procedure, the InsP isomers are trapped and then released from the resin with a more concentrated acid (up to 2 N HCl) that must be removed before column chromatography. In this work, elimination of the acid and other sample components is achieved using two Thermo Scientific™ Dionex™ OnGuard™ II cartridges. The Dionex OnGuard II RP cartridge removes interfering hydrophobic compounds and the AG/H cartridge removes excess chloride ions in the extraction solution that would otherwise overload the column. These two Dionex OnGuard II cartridges used in series simplify sample preparation by eliminating the traditional capture/release procedures and sample drying.

Inositol phosphate isomers can be separated and identified by several techniques, including anion-exchange/conductivity detection (a basic eluent system)[10, 12–14] and anion-exchange/post column derivatization with UV detection (an acidic eluent system).[13–17] In a 1998 publication, Skogland et al. compared six anion-exchange columns using both acidic and basic eluent systems for the separation of a reference sample. They showed that InsP isomers elute in an increasing number of phosphate groups when using an acidic eluent system, thus making it the optimal choice for the separation and identification of up to 25 $InsP_{2-6}$ isomers.[13] In addition, only compounds that react with $Fe(NO_3)_3$ (postcolumn reaction) and have an absorbance near 290 nm will be detected using the acidic system, resulting in a detection method with greater selectivity for inositol phosphates when compared to conductivity detection. Furthermore, most interfering compounds elute near the void.

In a 2003 publication, Chen and Li also compared basic and acidic eluent systems and demonstrated the separation of 27 peaks on a Thermo Scientific™ Dionex™ CarboPac™ PA100 column using an in-house reference standard that contained 35 InsP isomers.[14] In this work, the Chen and Li acidic gradient eluent conditions were modified to improve the separation between InsPs and fermentation residuals on the Dionex CarboPac PA100 column. This modified method separated 25 inositol phosphate peaks in an in-house prepared reference standard and a DDGS sample in <45 min.

## Goal

To develop a rapid and reliable sample preparation procedure and ion chromatography (IC) method to determine the phytate hydrolysis products in DDGS.

## Equipment, Software, and Consumables

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system, capable of supporting high-pressure IC, including:
  - SP Single Pump or DP Dual Pump
  - DC Detector/Chromatography Compartment
- Thermo Scientific Dionex AS-AP Autosampler
- VWD-3400RS Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation Four Channel Variable Wavelength Detector (Without Flow Cell) (P/N 5074.0010)
- Analytical Flow Cell for VWD-3000 Series, PEEK, 11 µL Volume, 10 mm Pathlength (P/N 6074.0200)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.1
- Vial Kit, 0.3 mL Polypropylene with Caps and Septa (P/N 055428)
- Vial Kit, 1.5 mL Polypropylene with Caps and Septa (P/N 079812)
- Vial Kit, 10 mL Polystyrene with Caps and Septa (P/N 055058)
- Nitrogen, 4.5 Grade (99.995%) or better
- Knitted Reaction Coil, 750 µL (P/N 042631)
- Manifold, 3-way mixing tee (P/N 024313)
- Falcon™ 50 mL Conical Centrifuge Tubes (Fisher Scientific P/N 14-432-22)
- Thermo Scientific™ Nunc™ Serological Pipettes (P/N 159633)
- Titan™ 2 Syringe Filter Disk, 30 mm, 0.2 µm PES membrane (Thermo Scientific P/N 42225-PS)
- 1000 mL Rapid Flow Filter Unit, 0.2 µm nylon membrane (Fisher Scientific P/N 164-0020)
- End Line Filter (P/N 045987)
- Dionex OnGuard Sample Prep Workstation (P/N 039599)
- Dionex OnGuard Needle, 18 Gauge, 1.25/Luer (P/N 039996)
- Dionex OnGuard Sample Reservoir, 5 cc (P/N 041233)
- Dionex OnGuard Valve, Luer Stopcock (P/N 040896)
- Dionex OnGuard II RP Cartridges, 2.5 cc (P/N 057084)
- Dionex OnGuard II Ag-H Cartridges, 2.5 cc (P/N 057410)

## Thermo Scientific Dionex AXP Auxiliary Pump Configuration

- Dionex AXP Auxiliary Pump (P/N 063973)
- Thermo Scientific Dionex EGC Carbonate Mixer Kit (P/N 079943)
- Bottle, 2 L, Plastic (P/N 062510)
- Black PEEK tubing, 0.25 mm, 0.010 in. i.d. (P/N 042690)
- Green PEEK tubing, 0.75 mm, 0.030 in. i.d. (P/N 044777)
- Yellow PEEK tubing, 0.075 mm, 0.003 in. i.d. (P/N 049715)

## Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistance or better
- Hydrochloric Acid, HCl, Optima™ (Fisher Scientific P/N A466-250)
- Iron (III) Nitrate Nonahydrate, $Fe(NO_3)_3 \cdot 9\ H_2O$, 99.99% (Fisher Scientific P/N AC20249)
- Perchloric Acid, $HClO_4$, 70%, Certified ACS (Fisher Scientific A229-1LB)
- Combined Seven Anion Standard II (P/N 057590)
- Sodium Oxalate (White Crystals or Powder), Fisher BioReagents (P/N BP353-500)
- Thermo Scientific Dionex Sulfate Standard, 1000 µg/mL (P/N 037160)
- Phosphate Standard, 1000 µg/mL in water (low TOC, <50 ppb) (Fisher Scientific P/N ICC-005)
- Phytic Acid Mix Solution, 40% in $H_2O$, Technical Grade (Fluka P/N 80180, Sigma-Aldrich®)
- Ins(4,5)-$P_2$ (sodium salt); D-*myo*-Inositol-4,5-diphosphate (Cayman Chemical P/N 10008418)
- Ins(1,2)-$P_2$ (sodium salt); D-*myo*-Inositol-1,2-diphosphate (Cayman Chemical P/N 10008439)
- Ins(1,4)-$P_2$ (sodium salt); D-*myo*-Inositol-1,4-diphosphate (Cayman Chemical P/N 10008438)
- Ins(1,5)-$P_2$ (sodium salt); D-*myo*-Inositol-1,5-diphosphate (Cayman Chemical P/N 10008441)
- Ins(2,4)-$P_2$ (sodium salt); D-*myo*-Inositol-2,4-diphosphate (Cayman Chemical P/N 10008419)
- Ins(1,3)-$P_2$ (sodium salt); D-*myo*-Inositol-1,3-diphosphate (Cayman Chemical P/N 10008443)
- Ins(1,4,6)-$P_3$ (sodium salt); D-*myo*-Inositol-1,4,6-triphosphate (Cayman Chemical P/N 10008427)
- Ins(1,2,6)-$P_3$ (sodium salt); D-*myo*-Inositol-1,2,6-triphosphate (Cayman Chemical P/N 10007780)
- Ins(1,2,5,6-)$P_4$ (sodium salt); D-*myo*-Inositol-1,2,5,6-tetraphosphate (Cayman Chemical P/N 10008444)
- Ins(1,2,4,5,6)-$P_5$ (sodium salt); D-*myo*-Inositol-1,2,4,5,6-pentaphosphate (Cayman Chemical P/N 10008452)
- Ins(1,2,3,4,5,6)-$P_6$ (dodecasodium salt hydrate); Phytic acid sodium phytate dodecasodium salt hydrate (Fisher Scientific P/N 50-121-7886)

## Sample

DDGS (Sample was generously donated)

| Chromatographic Conditions | |
|---|---|
| Columns: | Dionex CarboPac PA100 Guard, 4 × 50 mm (P/N 043054)<br>Dionex CarboPac PA100 Analytical, 4 × 250 mm (P/N 043055) |
| Eluent A: | DI water |
| Eluent B: | 0.5 M HCl |
| Gradient: | -15–0 min, 5% B;<br>0–8 min, 5–10% B;<br>8–25 min, 10–35% B;<br>25–35 min, 35–100% B;<br>35–42 min, 100% B;<br>42–42.1 min, 100–5% B |
| Flow Rate: | 1.0 mL/min |
| Injection Volume: | 100 µL (full loop) |
| Column Temp: | 30 °C |
| Backpressure: | 2500–2700 psi |
| Autosampler Temp: | 10 °C |
| Run Time: | 42 min |
| **Postcolumn Conditions** | |
| Postcolumn Reagent (PCR): | 1% $Fe(NO_3)_3 \cdot 9\ H_2O$ in 0.33 M $HClO_4$ |
| PCR Flow Rate: | 0.4 mL/min |
| Detection: | UV absorbance, 290 nm |
| Dionex AXP Auxiliary Pump Backpressure: | 1800 psi |
| Noise: | 0.9 mAU |

These conditions apply to Figures 2 and 3.

## Preparation of Solutions and Reagents

### DI Water Preparation

Degas the DI water by filtering through a 1 L 0.2 µm Thermo Scientific™ Nalgene™ filter unit, then sonicate under vacuum for 10–15 min.

### Hydrochloric Acid, 0.5 M

In a well-ventilated hood, prepare the eluent in a 2 L glass volumetric flask by adding 1500 mL of degassed DI water followed by 85.8 mL of concentrated HCl then carefully swirl to mix. Fill to the mark with degassed DI water and transfer the solution to a 2 L eluent bottle. In a separate 1 L glass volumetric flask, add 800 mL of degassed DI water followed by 42.9 mL of concentrated HCl then carefully swirl to mix. Fill to the mark with degassed DI water and transfer the solution to a 1 L glass bottle with cap for storage in a designated acid cabinet. Use this solution to prepare standards and samples.

### PCR: 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ in 0.33 M Perchloric Acid

Prepare the PCR solution and transfer it to its eluent reservoir in a well-ventilated hood using secondary containment in case of an accidental spill. Wear the proper personal protection equipment (lab coat, goggles, and gloves) as described in the Material Safety Data Sheet.[18] It is important that reagents be pure and free of organic and inorganic impurities, including metals. These impurities can cause insoluble metal complexes with phytate.

- Filter and degas 2 L of DI water.
- Fill a 2 L glass volumetric flask with 1.6 L of degassed DI water. Use a glass pipette to slowly add 56.8 mL of 70% perchloric acid then carefully swirl to mix.

  *Caution: Pay special attention to the acidic solution when transferring it to the volumetric flask. If a small amount of the acid is dropped in the secondary containment while transferring it to the eluent reservoir, dilute the spilled acid with 3× the volume of water to a concentration of less than 5% then discard the spill into the waste stream designated for perchloric acid. Organic absorbents, such as paper towels or Kimwipes, must not be used to soak up any spilled material.*
- When the transfer is complete, immediately rinse the pipette three times with water and collect the rinse in a glass beaker. Dispose the rinse in a designated waste stream.
- Weigh 2 g of $Fe(NO_3)_3 \cdot 9H_2O$ on a weigh paper and add it to the perchloric acid solution in the volumetric flask. Swirl to mix and fill to the mark with the remaining degassed DI water.
- Transfer the PCR solution to a 2 L eluent reservoir. Attach an end line filter unit to the end of the eluent line. If using the Thermo Scientific Dionex PC10 Postcolumn Delivery System to deliver the PCR, which holds a 1 L eluent reservoir, store the remaining 1 L of PCR solution in a plastic 2 L eluent bottle under $N_2$ until ready to use. The eluent line outlet of the stored PCR solution must be plugged prior to pressurizing with $N_2$ to prevent the reagent from flowing through the lines.

### Phytic Acid Stock Standard Solution, 10,000 mg/L

Dry 1 g of phytic acid dodecasodium salt hydrate in an oven at 100 °C for 3 h. Cool to room temperature. Place a 20 mL polypropylene vial on the balance and tare it. Weigh 0.13996 g of the dried phytic acid into the vial and tare the balance. Add 10 g of DI water, cap, and mix to dissolve the solid. Store the solution at 4 °C and store the remaining dried material in a desiccator.

### Stock 1000 mg/L Phytic Acid Stock Standard Solution

Place a 125 mL polypropylene bottle on the balance and tare it. Weigh 0.13996 g of the dried phytic acid into the bottle and tare the balance. Add 100 g of DI water, cap, and mix to dissolve the solid. Store the solution at 4 °C.

### Phytic acid purity determination

The Ins-$P_6$ commercial reagent is known to contain impurities.[19] Therefore, to determine the Ins-$P_6$ purity, a 1000 mg/L stock solution prepared from the oven-dried solid was analyzed for less phosphorylated InsP isomers and free phosphate using the IC method described here. The Ins(1,2,3,4,5,6)-$P_6$ standard was 92.5% pure, based on the relative peak area, and contained 6.64% $InsP_5$, 0.44% $InsP_4$, and 0.34% phosphate.

### Phytic Acid Calibration Standards

Taking into consideration the phytic acid purity, aliquot the appropriate amount of the 1000 mg/L stock solution into a 20 mL polypropylene vial and dilute to 10 g with DI water. For example, to prepare the 300 mg/L calibration standard, pipette 3 mL of the 1000 mg/L stock solution into a 20 mL polypropylene vial and dilute to 10 g with DI water. For this study, eight calibration levels were prepared at 300, 200, 100, 50, 25, 10, 5, and 2.5 mg/L.

### $InsP_4$ and $InsP_5$ Calibration Standards

Remove the cap, place the reagent vial containing 100 µg of the InsP standard on the balance, and tare it. Add 0.5 mL of DI water to make a 200 µg/mL stock solution. Use 250 µL of this solution as the highest calibration level. Use the remaining 250 µL of the stock solution to make six serial dilutions by diluting in half each time. This procedure will give seven calibration standards: 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL.

Use the 100 mg/mL solution to determine the purity of the commercial standards using the same method used for the Ins-$P_6$ commercial reagent. In this work, the Ins(1,2,4,5,6)-$P_5$ and Ins(1,2,5,6)-$P_4$ commercial standards were 96.7 and 92.1% pure, respectively. Standard purity was considered when calculating the actual concentration for each isomer calibration level.

### In-house Reference Standard Solution

#### Stock solution

Use a modified version of the 1997 Skogland procedure to prepare the in-house reference standard solution.[10] Set up a 250 mL glass round-bottom flask with a reflux condenser and stir bar. Weigh 1 g of phytic acid on a weigh paper and add to the 250 mL glass round-bottom flask. Add 100 mL of 0.5 M HCl to the round-bottom flask, connect the reflux condenser, and use a mineral oil bath to heat the solution to a reflux (~95–100 °C) for a total of 68 h. Use a hot plate with stirring capabilities to mix the solution while it is heating. After 68 h, remove the reaction from the heat and cool the solution to room temperature.

#### Working solution

Place a 125 mL polypropylene bottle on the balance and tare it. Pipette 5 mL of the hydrolyzed stock solution into the bottle and dilute to 100 mL with DI water by weight, cap, and shake to mix. Prepare a Dionex OnGuard Ag/H cartridge according to Table 4 in the Thermo Scientific Dionex OnGuard II Cartridges Product Manual.[20] Pass a 10 mL portion of the diluted solution through a Dionex OnGuard Ag/H cartridge and a 0.45 µm syringe filter unit connected in series. Discard the first 6 mL and collect the remaining 4 mL into a 10 mL autosampler vial. Store the stock solution and the diluted solution at 4 °C.

## System Configuration

### Column Preparation

Before switching to an acidic eluent, rinse the columns to remove the basic solution in which they are stored. To rinse the columns, prime the pump with DI water and start the flow at 1 mL/min through the PEEK tubing leading to the column. Install a Dionex CarboPac PA100 guard column (4 × 50 mm) and flush with DI water for at least 20 min before connecting it to the Dionex CarboPac PA100 analytical column (4 × 250 mm). Flush the guard and analytical columns with DI water for at least 60 min before applying the acidic eluent used in this work. After rinsing the columns with DI water, rinse them again with 50% B (0.5 M HCl) for 30 min followed by 5% B for 30 min.

*Caution: Once the PCR is in line, do not allow the postcolumn flow to be on without the analytical pump turned on; to do so can cause potential contamination of the column from the PCR solution. To avoid backflow onto the column, always turn on the analytical pump first followed by the postcolumn reagent. When shutting down the system, always turn off the postcolumn pump first, then the analytical pump.*

### Postcolumn Reactor Configuration

The system configuration of the IC with postcolumn delivery system is shown in Figure 3 of Dionex Technical Note 26.[21]

Three different postcolumn delivery systems were configured to compare ease of use and performance. The three pumps tested to deliver PCR were the second pump on the DP module, a Dionex PC10 postcolumn delivery system, and a Dionex AXP auxiliary pump. The background noise of the three systems was compared. The Dionex AXP auxiliary pump and Dionex PC10 postcolumn delivery system provided comparable noise (<1 mAU), whereas the DP unit had slightly higher baseline noise (>1.5 mAU). In this study, the Dionex AXP auxiliary pump was used to generate the data rather than the Dionex PC10 postcolumn delivery system (P/N 050601) because of the ease of use, low noise, and the ability to communicate with Chromeleon CDS software.

#### Dionex AXP auxiliary pump configuration

The Dionex AXP auxiliary pump offers a low-pulsation flow produced by the reciprocating single-piston pump with an internal pulse damper.[22] To further reduce pump pulsations, install the Dionex EGC carbonate mixer and yellow PEEK tubing after the pump.

Connect the Dionex AXP auxiliary pump directly to the computer using the USB port and turn on the power. Add the Dionex AXP auxiliary pump to the system configuration. Reboot the computer if the system cannot recognize the communication port. This will reset communication between the computer and the Dionex AXP auxiliary pump. Once configured, the Dionex AXP auxiliary pump is controlled by Chromeleon CDS software for automated operation. When compared to the Dionex PC10 postcolumn delivery system, the main benefits of this pump are that it supports direct communication with the software and can tolerate higher backpressures.

Place the reservoir containing the PCR at the same level or slightly higher than the pump. Connect the PCR to the Dionex AXP check valve inlet. Prime the pump by connecting a plastic disposable 20 mL syringe to the priming adapter. Run the Dionex AXP auxiliary pump at a flow rate of 3–5 mL/min while pulling on the syringe to collect at least 20 mL. Disconnect the syringe from the priming adapter and connect ~250 cm of yellow PEEK tubing to create 1800 psi of backpressure. Check that the pump is accurately delivering the PCR by measuring the volume at 1 mL/min for 5 min.

To further dampen pump noise, install a Dionex EGC carbonate mixer between the Dionex AXP auxiliary pump and the 3-way mixing tee. Disconnect the yellow PEEK tubing and connect the Dionex EGC carbonate mixer to the pump using the black PEEK tubing. Set the pump flow rate to 5 mL/min and collect the effluent in a waste container. Run the pump until the mixer is filled and there is a consistent flow of PCR exiting the mixer outlet (~5 min). Turn off the pump and reinstall the 250 cm piece of yellow PEEK tubing. Connect the yellow PEEK tubing to the Dionex EGC carbonate mixer inlet and connect the black PEEK tubing to the Dionex EGC carbonate mixer outlet, which is then connected to the 3-way mixing tee.

### Autosampler Configuration

Due to limited standard available for Ins(1,2,5,6)-$P_4$ and Ins(1,2,4,5,6)-$P_5$ (100 µg), set the Dionex AS-AP Autosampler loop overfill to 2×. This allows the minimum amount of sample in the full loop injection mode to be used while maintaining sufficient reproducibility.

## Sample Preparation

### Inositol Phosphate Extraction

1. Place a 50 mL polypropylene centrifuge tube on the balance and tare it.
2. Add 4 g of DDGS to the centrifuge tube and record the weight. To ensure a representative DDGS sample is analyzed, mix the solid material thoroughly, then take portions from three different areas of the DDGS sample.
3. Add 20 mL of 0.5 M HCl to the centrifuge tube and record the total weight. Cap the tube and vigorously shake to mix.
4. Sonicate in a water bath for 15 min.
5. While waiting, prepare the Dionex OnGuard II Ag/H and RP cartridges in accordance with Table 4 in the Thermo Scientific Dionex OnGuard II Cartridges Product Manual.[20]
6. When the sonication is complete, centrifuge the tube at 5000 rpm and 4 °C for 25 min.

### Extract Filtration

1. Assemble a 20 mL disposable syringe with filter disk and remove the plunger. Using a 10 mL disposable pipette, aspirate the liquid from the centrifuge tube without collecting any solids.

   *Note: Do not decant because this will cause too many solid particles to enter the syringe filter and immediately clog it.*

2. Transfer the liquid to the syringe connected to a 0.2 µm syringe filter disk. Using the plunger, push the sample through into a clean 50 mL centrifuge tube until at least 10 mL of filtered material is collected. Make sure the filtrate is not cloudy because this will clog the Dionex OnGuard II cartridges in the following steps. If cloudy, repeat the filtration step with a new filter disk. When filtration is complete, set aside a 1.25 mL aliquot if performing a recovery study.

### Dionex OnGuard Sample Treatment

A Dionex OnGuard II sample prep station can be used to prepare up to 12 samples in parallel.[23]

After both cartridges have been prepared in accordance with Inositol Phosphate Extraction Step 5, connect the Ag/H cartridge to the outlet of the RP cartridge. Connect a 20 mL syringe with the plunger removed to the inlet of the RP cartridge and pour the sample collected in Extract Filtration Step 2 into the syringe. The sample size will be minimally 8.75 mL (i.e., 10 mL were collected with 1.25 mL reserved for a recovery study). Pass the sample through the cartridges at 2 mL/min. Discard the first 6 mL and collect the next 2 mL.

*Note: If performing a Dionex OnGuard II cartridge recovery study, discard the first 6 mL, then remove the RP cartridge and manually push approximately 1.25 mL through the Ag/H cartridge into a 1.5 mL autosampler vial; set this aliquot aside. Reconnect the RP cartridge to the Ag/H cartridge and collect the next 2 mL.*

### Final Sample Dilution and Filtration

Perform a final 1:4 dilution for all samples by transferring 1 g of the sample into a 20 mL polypropylene vial followed by 3 g of DI water. Filter the diluted sample through a 0.2 µm polyethersulfone filter disk into a 10 mL autosampler vial. Keep samples in the autosampler tray at 10 °C and analyze as soon as possible. If samples are not immediately analyzed, store them at 4 °C for up to three days.

## Results and Discussion

### Separation and Detection

Separation of $InsP_2$–$InsP_6$ was achieved on a Dionex CarboPac PA100 column (4 × 250 mm) in <42 min using an HCl gradient followed by postcolumn derivitization with ferric nitrate and UV detection at 290 nm. Under these acidic conditions, inositol phosphates elute in order of increasing number of phosphate groups, which simplifies identification compared to basic eluent systems. Twenty-five InsP peaks were identified and labeled according to Chen and Li.[14] In this study, the gradient was modified from that of Chen and Li to improve the resolution between the $InsP_2$ region and oxalate, which is a naturally occurring analyte found in plants. The $InsP_2$ standards were run individually and as a mix to verify elution order. In addition to providing better separation of the $InsP_2$ region from other matrix analytes, the method was optimized to reduce the analysis time from 52 to 42 min.

There is a report of methanesulfonic acid (MSA) being used as an eluent for the separation inositol phosphates prior to postcolumn derivatization with ferric nitrate and UV detection.[24] This was found to reduce the baseline drift during the gradient. However, MSA was not evaluated in this work.

### Standards

An in-house standard was prepared by refluxing phytic acid in HCl for nearly three days. Hydrolysis reactions proceed at different rates and yield different relative amounts of InsP isomers according to pH, temperature, and reaction time. Figure 2 shows a chromatogram of the in-house standard solution diluted 1:10 with DI water; the peaks are labeled with their identities reported in Table 1.

Pretreatment of the in-house standard solution with the Dionex OnGuard II Ag/H cartridge provided improved peak shapes for the early eluting peaks (<10 min) by removing the excess chloride and therefore preventing column overload. When compared to a commercially available 40% phytic acid solution, the in-house solution contains a more even distribution of InsPs, allowing for improved method development and peak identification.

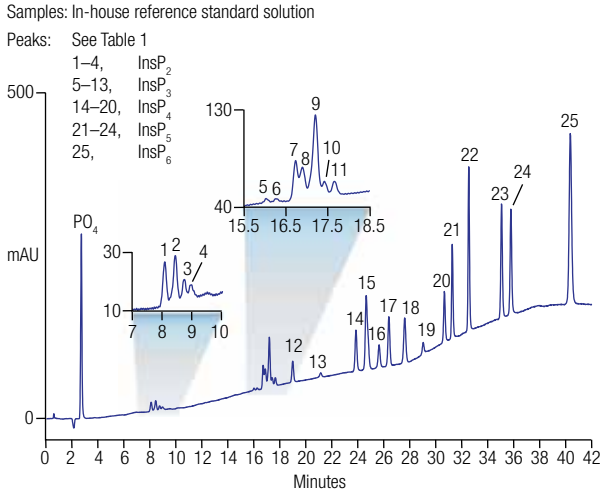


Figure 2. In-house reference standard solution.

Table 1. InsP peak identification

| Peak No. | Retention Time (min) | InsP Isomer |
|---|---|---|
| 1 | 7.9 | Ins(1,3)-$P_2$ |
| 2 | 8.3 | Ins(1,2)-$P_2$ |
| 3 | 8.6 | Ins-(2,4)-$P_2$, Ins(1,4)-$P_2$ |
| 4 | 8.8 | Ins-(4,5)-$P_2$, Ins(2,5)-$P_2$ |
| 5 | 15.9 | Ins(1,3,5)-$P_3$ |
| 6 | 16.1 | Ins(2,4,6)-$P_3$ |
| 7 | 16.6 | Ins(1,3,4)-$P_3$ |
| 8 | 16.8 | Ins(1,2,4)-$P_3$, Ins(2,3,5)-$P_3$ |
| 9 | 17.1 | Ins(1,2,3)-$P_3$, Ins(1,2,6-)$P_3$, Ins(1,4,6)-$P_3$ |
| 10 | 17.3 | Ins(1,4,5)-$P_3$ |
| 11 | 17.5 | Ins(2,4,5)-$P_3$ |
| 12 | 18.9 | Ins(1,5,6)-$P_3$ |
| 13 | 21.0 | Ins(4,5,6)-$P_3$ |
| 14 | 23.8 | Ins(1,2,4,6)-$P_4$, Ins- (1,2,3,5)-$P_4$ |
| 15 | 24.6 | Ins(1,2,3,4)-$P_4$, Ins(1,3,4,6)-$P_4$ |
| 16 | 25.5 | Ins(1,2,4,5)-$P_4$ |
| 17 | 26.3 | Ins(1,3,4,5)-$P_4$ |
| 18 | 27.5 | Ins(1,2,5,6)-$P_4$ |
| 19 | 28.9 | Ins(2,4,5,6)-$P_4$ |
| 20 | 30.6 | Ins(1,4,5,6)-$P_4$ |
| 21 | 31.2 | Ins(1,2,3,4,6)-$P_4$ |
| 22 | 32.5 | Ins(1,2,3,4,5)-$P_4$ |
| 23 | 35.0 | Ins(1,2,4,5,6)-$P_4$ |
| 24 | 35.8 | Ins(1,3,4,5,6)-$P_4$ |
| 25 | 40.3 | Ins(1,2,3,4,5,6)-$P_6$ |

### Interference Studies

Most common anions either are not detected because they do not react with the PCR or elute near the void and therefore do not interfere with the determination of inositol phosphates. However, oxalate and sulfate are detected with this method and elute at ~6.9 and ~12.9 min, respectively. These analytes are often present in DDGS samples and can interfere with the determination of some inositol phosphates. A small unknown peak at 28.7 min elutes in the $InsP_4$ region but can be removed by treatment with the Dionex OnGuard II RP cartridge and therefore is unlikely to be an InsP isomer.

### Linearity, limit of detection (LOD), and limit of quantification (LOQ)

To determine method linearity, calibration standards were injected in duplicate over the calibration ranges listed in Table 2. The LOD and LOQ were estimated for Ins(1,2,5,6)-$P_4$, Ins(1,2,4,5,6)-$P_5$, and InsP(1,2,3,4,5,6)-$P_6$ using a DDGS sample. The LOD and LOQ were defined as 3× the signal-to-noise ratio (S/N) and 10× the S/N, respectively. The system baseline noise for the LOD and LOQ was 0.9 mAU, based on measuring the peak-to-peak noise between 14 and 15 min over six injections of prepared DDGS samples (three separate preparations injected two times each). This method estimated sample LODs and LOQs at 1 and 3.2 mg/L, respectively, for the three $InsP_{4\text{-}6}$ isomers tested. Table 2 summarizes the calibration, LOD, and LOQ data.

### Sample Analysis

Inositol phosphates were extracted from the DDGS sample and then treated using Dionex OnGuard II RP and Ag/H cartridges. Figure 3 shows the sample with and without Dionex OnGuard II cartridge treatment. The Dionex OnGuard II RP cartridge removed hydrophobic compounds—primarily present between 0 and 5 min—and the Dionex OnGuard Ag/H II cartridge was used to remove chloride. Removal of chloride in the sample

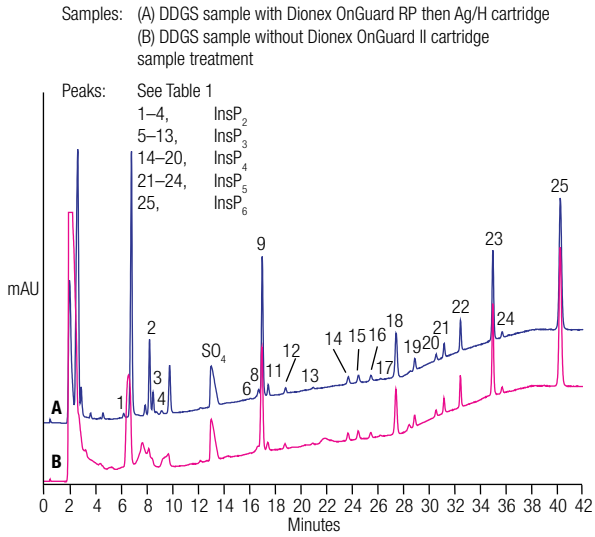


Figure 3. DDGS sample with and without Dionex OnGuard II cartridge sample treatment.

eliminated column overloading and enabled improved detection, peak shape, and resolution. Twenty-two of the 25 InsPs peaks detected in the in-house standard were present in the sample. Due to naturally occurring phytases in plants and microorganisms (i.e., yeast or bacteria used to ferment corn) and the possible addition of commercial phytase enzymes, a variety of phytases can be present in a DDGS sample. The major dephosphorylated isomers present in the sample (Peaks 2, 9, 18, and 23) are indicative of a naturally occurring 3-phosphatase that liberates chemically bound phosphorus in the form of phosphate starting at the 3 position.[1,14]

### Dry matter (DM) analysis

A sample was measured for DM content by drying 2 g of DDGS in an oven at 135 °C for 2 h. The sample contained 10.5% moisture (a DM value of 89.5%). This measurement is consistent with data reported in previous studies.[2]

Table 2. Linearity, LODs, and LOQs.

| InsP Isomer | Calibration Range (mg/L) | Linearity[1] ($r^2$) | LOD[2] (mg/L) | LOQ[3] (mg/L) |
|---|---|---|---|---|
| Ins(1,2,5,6)-$P_4$ | 3.1–200 | >0.999 | 1.0 | 3.2 |
| Ins(1,2,4,5,6)-$P_5$ | 3.1–200 | >0.999 | 1.0 | 3.2 |
| $InsP_6$ | 10.0–300 | >0.999 | 1.0 | 3.2 |

[1] Six calibration levels, each level injected in duplicate

[2] LOD calculated as 3 × S/N

[3] LOQ calculated as 10 × S/N

### Quantitation of InsPs

Individual isomers—Ins(1,2,5,6)-$P_4$, Ins(1,2,4,5,6)-$P_5$, and Ins-$P^6$—were determined in two separate sample preparations and averaged using Equation 1. To compare the amounts to the literature values, InsP amounts were determined on a DM basis. The samples contained 0.07, 0.14, and 0.20% of Ins(1,2,5,6)-$P_4$, Ins(1,2,4,5,6)-$P_5$, and Ins-$P_6$, respectively (Table 3).

Equation 1:

$$\% \text{ Ins P on a DM basis} = C \times \frac{M2 \times M4 \times DM}{M1 \times M3 \times 1000} * 100$$

C = InsP concentration (mg/L) converted to (g/L)

M1 = mass of the DDGS sampled (4.0 g)

M2 = mass of DDGS sample plus acid (24.0 g)

M3 = mass portion of the filtrate used (1.0 g)

M4 = (dilution) mass filtrate plus water (4.0 g)

DM = dry matter value (0.895)

### Dionex OnGuard Cartridge Recovery Studies

Because Ins-$P_6$ recovery was primarily affected by the Dionex OnGuard II cartridge treatment, it was critical to perform recovery studies on Ins-$P_6$ at different steps of sample treatment. The order of the cartridges and the sample pH were critical in obtaining acceptable recovery. Phytic acid has 12 protons, six of which are strongly acidic with pKa values <2.[1] To keep the phytic acid fully protonated, the sample must not be diluted with water before using the Dionex OnGuard II cartridges. The recovery of phytate was measured after each Dionex OnGuard cartridge treatment step and overall recovery was 95.3%. In addition, because the Ag/H cartridge changes the pH of the solution and therefore allows for deprotonation of the phosphate groups, a loss of up to 22% was observed when the Dionex OnGuard II cartridge order was reversed (Ag/H then RP).

### Accuracy and Precision

Interday precision was evaluated by preparing a DDGS sample over three days (three sample preparations injected in duplicate for a total of six injections each) then calculating the overall precision. The average amount of Ins-$P_6$ in the three samples was 2.0 mg/g on a DM basis with an RSD of 2.8 (Table 4). Intraday retention time and peak area precisions were measured for all 22 peaks present in the sample over six injections (three sample preparations injected in duplicate). Retention time RSDs were <0.15 and peak area precision was <10% for the InsP peaks with most peak area RSDs <5.

Method accuracy was evaluated by calculating the recovery of Ins-$P_6$ after spiking directly into the dry grain at 50 and 100% of the expected amount. Samples were prepared and injected in duplicate. Recoveries for Ins-$P_6$ spiked at 50 and 100% of the expected amount were 103–106% and 98–101%, respectively (Table 5).

Table 3. Quantitation of individual InsP isomers.

| InsP Isomer | Preparation No. | Peak Area (mAU*min) | Peak Area RSD | Amount Found (mg/L) | Calculated Amount (mg/g, DM)[a] | DM (%) | Average DM (%) |
|---|---|---|---|---|---|---|---|
| Ins(1,2,5,6)-$P_4$ | 1 | 18.9 | 1.3 | 33.0 | 0.68 | 0.07 | 0.07 |
| | 2 | 17.4 | 0.3 | 30.4 | 0.66 | 0.07 | |
| Ins(1,2,4,5,6)-$P_5$ | 1 | 28.9 | 0.1 | 67.7 | 1.5 | 0.15 | 0.14 |
| | 2 | 25.9 | 0.4 | 60.7 | 1.3 | 0.13 | |
| $InsP_6$ | 1 | 62.2 | 0.1 | 97.6 | 2.1 | 0.21 | 0.20 |
| | 2 | 59.5 | 4.1 | 93.7 | 2.0 | 0.20 | |

[a] DDGS sample analyzed for DM content, 89.5%

Table 4. Repeatability.

| Sample | Day | Amount ± SD (mg/L)[1] | Calculated Amount (mg/g, DM)[2] | Overall Average Amount (mg/g) | Overall Precision RSD (%) |
|---|---|---|---|---|---|
| DDGS | 1 | 93.5±0.7 | 2.0 | 2.03 | 2.8 |
| | 2 | 97.7±0.2 | 2.1 | | |
| | 3 | 93.7±3.6 | 2.0 | | |

[1] Analyzed over three days, each preparation injected two times

[2] DDGS sample analyzed for DM content, 89.5%

Table 5. $InsP_6$ recovery in spiked samples.[a]

| n = 2 | Amount Added (mg/L) | Average Amount Found[a] (mg/L) | Average Amount Recovered (mg/L) | Recovery (%) |
|---|---|---|---|---|
| Unspiked Sample | — | 93.0[b] | — | — |
| +50% | 45.4 | 140 | 46.9 | 103 |
| | 45.8 | 142 | 48.6 | 106 |
| +100% | 89.6 | 181 | 87.6 | 98 |
| | 90.4 | 185 | 91.6 | 101 |

[a] Samples prepared in duplicate, each preparation injected two times

[b] Unspiked amount average calculated from three sample preparations, each injected two times

## Conclusion

This study demonstrates the determination of 25 inositol phosphate isomers—$InsP_2$–$InsP_6$—in an in-house prepared reference solution with the method applied to a DDGS sample. Separation of the InsP isomers was achieved using a Dionex CarboPac PA100 column (4 × 250 mm) in <42 min with an HCl gradient, followed by a postcolumn reaction with ferric nitrate in dilute perchloric acid and then UV detection. The Dionex OnGuard II RP and Ag/H cartridges simplified the sample preparation procedure when compared to the most commonly used capture/release methods. The amounts of InsPs determined in the DDGS sample were consistent with literature values. Spike recovery experiments also indicated that the method was accurate.

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