IZiNCG practical tips

A Polyacrylamide Gel Electrophoresis (PAGE) Method for Assay of Phytic Acid Species

The PAGE method illustrated here was first was described in Losito et al. (2009). The basic principle of chromatography is that different compounds will migrate at different rates down the gel and thus be separated. A video demonstration can be found at http://www.jove.com/details.php?id=3027, as described in Loss et al. (2011). As described in the video, this method was initially developed for the isolation and identification of compounds related to phytic acid called "inositol pyrophosphates". These compounds have seven or eight phosphate esters per molecule, as compared with phytic acid (inositol hexaphosphate or IP6), which has six phosphate esters per molecule. PAGE is typically used to chromatographically separate and detect molecules of much higher molecular weight than inositol phosphates, such as proteins and nucleic acid fragments. To provide a gel of sufficient density to retain these lower molecular weight inositol phosphates, this method uses a much higher concentration of 33.3% acrylamide.

1. Required Reagents

The following chemicals are widely available from many commercial sources.

1. 40% Acrylamide/Bisacrylamide (19:1)
2. Ammonium Persulfate (APS)
3. Tetramethylethylenediamine (TEMED)
4. Tris(hydroxymethyl)aminomethane (TRIS) Base
5. Boric Acid
6. Ethylenediaminetetraacetic acid (EDTA)
7. Toluidine Blue
8. Sodium Hydroxide
9. Orange G

2. Experimental Standards

Standards are readily available from many commercial sources.

1. 1.0 mM IP6 prepared using phytic acid dodecasodium salt hydrate prepared from rice (Sigma, St. Louis MO, USA).
2. 1.0 mM D-*myo*-inositol-1,2,3,4,5-pentakisphosphate or IP5 (Cayman Chem., Ann Arbor MI, USA)
3. 1.0 mM D-*myo*-inositol-1,2,3,4-tetrakisphosphate or IP4 (Sigma, St. Louis MO, USA).

3. PAGE Fractionation: An Overview

PAGE methods are commonly used in biochemistry, biotechnology, forensics and genetics research. As a result, the equipment for PAGE techniques are widely available and designed for ease of use.

The basic operation of PAGE includes the following steps:

1. An acrylamide “slab” gel is cast between two glass plates. Gel dimensions vary greatly depending on the equipment used and the application. In the one used to generate the data for Table 1, the gels were 1.5 cm thick, 16 cm wide and 20 cm long. Prior to polymerization of the acrylamide solution into a gel, a comb is inserted at the top. After polymerization, the comb is removed and wells are formed into which extraxt supernatant aliquots are pipetted. “Pre-cast” gels are commercially available for a number of applications, but are not commonly available for this application, which requires an atypically high concentration of 33.3% acrylamide.
2. The PAGE apparatus is assembled so that the gel contacts both electrophoresis buffers in the top and bottom chambers.
3. Predefined aliquots of standard and samples are pipetted into the wells at the top of gel. Each sample or standard should also contain the appropriate amount of loading dye to visualize location within the gel. Electric current is applied and passes through the gel. In this application, compounds in the sample migrate from the cathode to the anode.
4. Once adequate migration has occurred, (adequate migration defined here as being that which provides at a minimum “base-line” separation of IP6 and IP5), the electric current is disengaged and the different compounds are visualized with Toluidine Blue.
5. Sample bands can be quantitated with imaging software, such as Image J (NIH).

4. Sample Extraction

Dry food samples should be finely milled, for example to pass a 40-mesh screen. Aliquots of sample (e.g. 50 mg) are placed in a 1.5 mL microtube and extracted in 500 mL of 0.2 M HCl:5% Na_2SO_4 at 4°C overnight with shaking, followed by centrifugation (12,000 RPM, 20 min, 4°C). The supernatant then can be analyzed for phytic acid and its breakdown products, as described below. Filtering of extracts is not necessary. If desired, supernatants can be pipetted off into a second microtube for storage prior to analysis to 4°C for short periods or -20°C for longer.

5. Reagent Preparation

Running Buffer: The running buffer is 0.5X Tris-Borate-EDTA (TBE). Pre-prepared concentrated TBE can be obtained from commercial sources. To prepare in house, make 1 L of 10X TBE as follows: 108 g TRIS Base; 55 g Boric Acid; 40 mL 0.5 M EDTA pH 8.0; bring up to 1 L with distilled, deionized H_2O. Then dilute this 10X TBE to 0.5X (1:20) with distilled-deionized H_2O.

1. Acrylamide Pre-Mix: A stock solution of pre-mix is often made that for multiple gels. For the PAGE apparatus used here, each gel requires about 37.7 mL of acrylamide mix. Acrylamide pre-mix for a minimum of 10 gels can be prepared as follows: 317 mL of 40% Acrylamide/Bisacrylamide (19:1), 38 mL of 10X TBE, and 22 mL distilled, deionized H_2O.
2. Tracking 6X Orange G tracking dye: 10 mM TrisHCl pH 7.0, 1 mM EDTA, 30% glycerol, and 0.1% Orange G.
3. Stain Solution: 20% methanol, 2% glycerol, and 0.05% Toluidine Blue
4. Destain Solution: 20% methanol and 2% glycerol

6. Experimental Methodology

1. Cast gel by pouring 50 mL of pre-mix into a 100 mL cylinder and add 340 µL of 10% APS and 37 µL of TEMED. The solution should be immediately poured between the glass plates, avoiding air bubbles. Then the comb is inserted at the top to form the sample wells. Completion of polymerization varies, but often takes 30-60min.

2. Once gel has polymerized, transfer the glass plate & gel assembly into the apparatus. Add the appropriate amount of 0.5X TBE running buffer to the upper and lower chambers and carefully remove the comb.
3. Pre-run empty gel at 200 volts for 30 min at approximately 4°C using refrigerated circulating water bath or place in a refrigerator or cold room.
4. Prepare the sample mixes to be added to gel, typically 10-20 µL of standards and samples, appropriate aliquots of 2N NaOH to neutralize to ~pH 7, and 10 µL of 6X Orange G tracking dye.
5. Pipette sample mixes into pre-determined wells in the gel, avoiding air bubbles and overflow of wells.
6. Run the electrophoresis apparatus at 250 V constant current overnight (16-20 hours) at 4°C.
7. After electrophoresis, carefully remove the gel from the glass plates and transfer to a new container.
8. Stain gel with Stain Solution for 10 min at room temperature with gentle rocking. Then de-stain gel with Destain Solution for 30 min with two changes of the solution to visualize bands.
9. Photograph the gel using a white-light transilluminator. Quantitate bands using software such as Image J.

7. Results

In this demonstration, standards or extracted supernatants were assayed as shown in **Figure 1**. The PAGE apparatus used here was a Protean II gel apparatus (Bio-Rad Labs Inc., Hercules CA). Following running the gels at 250 volts overnight at 4oC, phytic acid (IP6) has a Rf (retention factor) value relative to Orange G of ~1.4, meaning that IP6 moved 1.4 times as far down the gel as did Orange G. Gels are typically run until the Orange G migrated 6 to 8 cm down the gel. Figure 1B also illustrates that this method provides the separation needed of IP6, IP5 and IP4. Figure 1B Lanes 1 and 2 contain the standards IP6 and IP5, respectively, whereas Lane 4 represents a fractionation of inositol phosphates contained within the seed of the barley line "low phytic acid 2". Seed of this line contains a mixture of IP6, IP5 and IP4 and inspection of Figure 1B Lane 4 illustrates that all three were "baseline" separated.

Figure 1. An example of a PAGE gel and fractionation of the standards IP6 and IP5 and samples. The upper 12 cm of a 20 cm-long gel are illustrated. Gels are typically run until the Orange G migrated 6 to 8 cm down the gel (A) the gel after electrophoresis but before Toluidine Blue staining. (B) The same gel after Toluidine Blue staining.

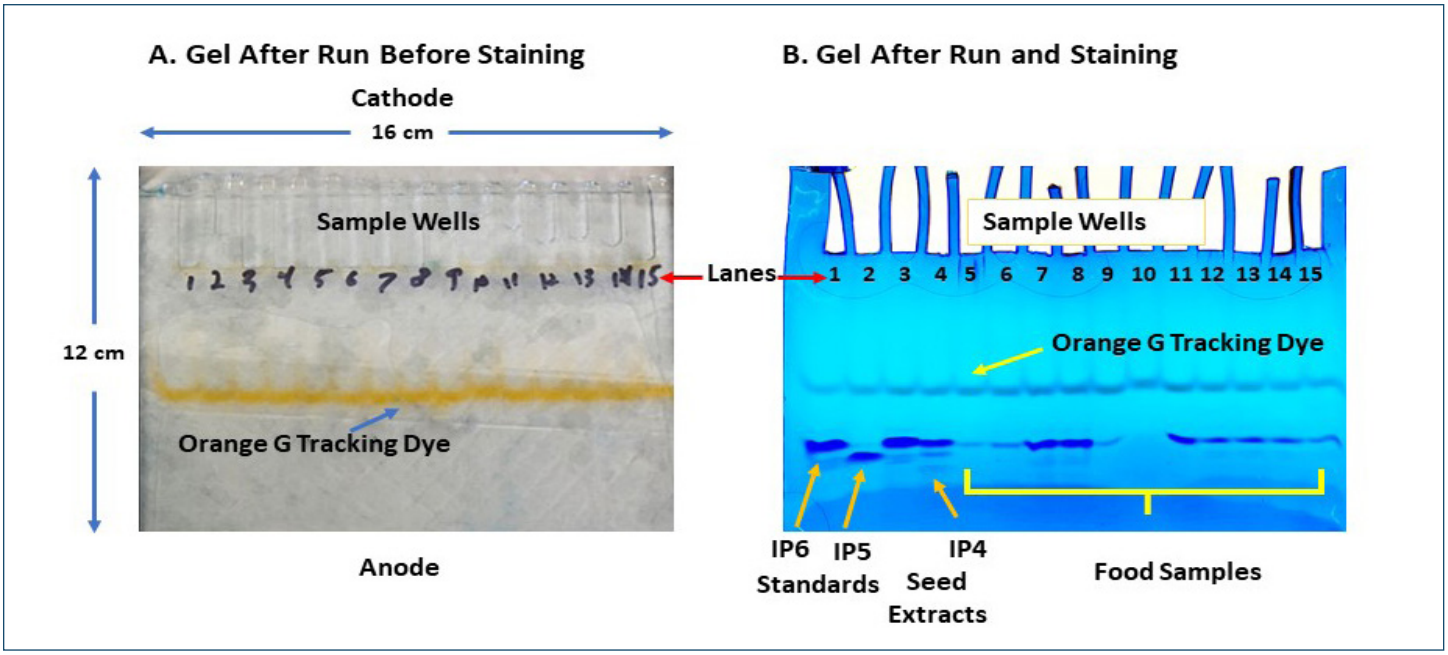


Figure 2 illustrates the procedure to quantitate the amount of compound in a given band using the open-access gel analysis software Image J (https://imagej.net/Fiji). The image of the gel is converted to 32-bit grayscale, then rectangular sections of the gel containing each band are digitally selected using the "rectangle tool" (Step 2) and plotted (Steps 3). The "area under the curve" is then calculated using the "gel analysis" function in Image J. The identity of the band (IP4, IP5 or IP6) is determined via comparison of mobility with standards. The amount of a given compound in a band is then determined via use of a standard curve (Steps 4 and 5). The IP5 and IP6 standards demonstrates the linearity of the standard curve within this range (Figure 2). The lower limit of detection for these inositol phosphates is about 0.5 µg per band, equivalent to about 25 mg inositol phosphate/100 g sample. This PAGE method's sensitivity permits a laboratory in a low-income country to obtain data that are sufficiently precise to determine the phytic acid content of foods or whole diets relative to the four levels defined by EFSA (2014).

Figure 2. PAGE analysis of inositol phosphates in seeds and foods. The gel was run overnight, stained with Toluidine Blue and photographed. As part of Image J analysis, gel image is then converted to 32-bit grayscale and lane sections containing bands of interest are selected with the "rectangle tool." Inositol phosphate standards were: (1) 2.3 µg IP6; (2) 2.9 µg IP5; (3) 6.6 µg IP6; (4) 5.8 µg IP5; (5) 9.9 µg IP6; (6) 8.7 µg IP5. For this test, Lane 7 contains 20 µL of an extract of red bean flour (500 mg flour extracted in 5 mL of 0.4 M HCl).

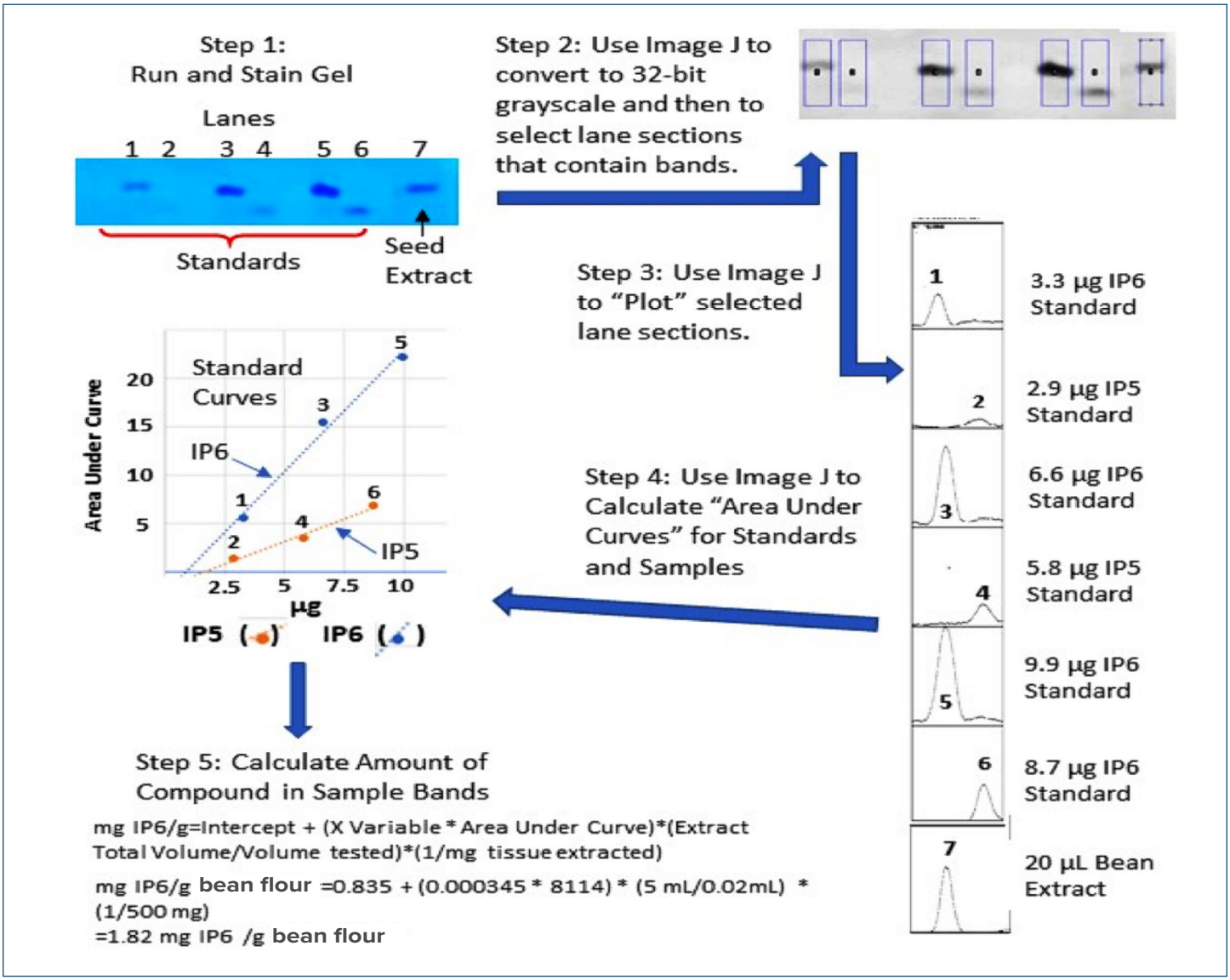
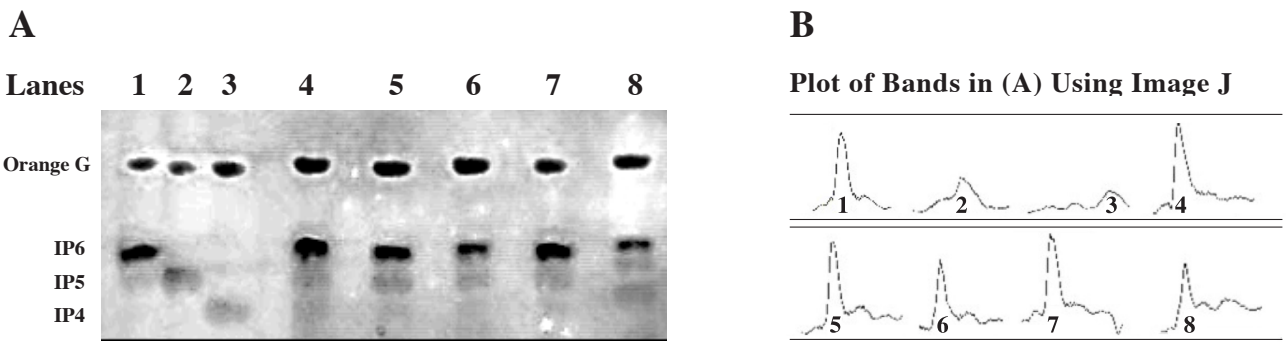


Figure 3 illustrates a PAGE analysis of phytic acid and inositol phosphates in food and flour samples. Following fractionation of sample extracts using PAGE, the gel was then stained with Toluidine Blue to reveal the inositol phosphate bands, the gel image then converted to 32-bit grayscale (which is illustrated), and the phytic acid or inositol phosphate represented by a given band in the gel quantitated using ImageJ. Thus using this method one could estimate the impact of the content of a given diet's inositol phosphates on zinc bioavailability.

Figure 3. PAGE analysis of phytic acid and inositol phosphates in food and flour samples. The gel image was first converted to 32-bit grayscale and lane sections were plotted and quantitated. Panel A: Lanes 1, 2, and 3 are 20 µL of 1 mM standards (IP6, IP5 and IP4, respectively). Lanes 4 through 8 are 20 µL of extracts of whole mung bean (Lane 4), red kidney bean (Lane 5), raw tofu (Lane 6), barley cv. Harrington (Lane 7) and barley *low phytic acid* 2 (Lane 8). Panel B: Plot bands in Panel A using Image J.



The results in **Figure 3** illustrate the critically important baseline separation of IP6, IP5 and IP4 (Fig. 3A lanes 1, 2 and 3, respectively) provided via this PAGE method. The relative differences in levels of IP6, IP5 and IP4 between samples is also apparent; IP5 and IP4 levels were on the order of 5% to 10% of the total inositol phosphate in a sample. When the bands in Figure 3 were quantitated, the values were comparable to those obtained via use of the HPLC method of Lehrfeld (1989) (**Table 1**). For example, for individual samples the data for phytic acid obtained via PAGE in this limited test ranged from 75% to 121% of that obtained via HPLC, across samples on average there was only a 1% difference between PAGE and HPLC.

Table 1 . Comparison of the phytic acid (IP6), inositol pentaphosphate (IP5) and inositol tetraphosphate (IP4) in selected plant-based foods and flours as obtained with HPLC and the Polyacrylamide Gel Electrophoresis (PAGE) assay.

	HPLC[a]			PAGE[b]		
Food sample or Flour	**IP6**	**IP5**	**IP4[c]**	**IP6**	**IP5[c]**	**IP4[c]**
	mg/100 g			**mg/100 g**		
White mung beans	614	66	ND	741	ND	ND
Red kidney beans	604	51	ND	612	138	ND
Tofu, raw	547	33	ND	411	89	ND
Harrington barley	724	141	16	666	ND	ND
Low phytic acid 2 barley	307	166	174	325	38	253

[a] The HPLC method used was a modification of that described in (13).
[b] The PAGE method used was a modification of that described in (14).
[c] ND=not detected. The lower limit of detection for inositol phosphates was 6 mg/100g for the HPLC assay and 25mg/100 g for the PAGE assay.

7. Cited Literature

Losito O, Szijgyarto Z, Resnick AC, Saiardi A. Inositol pyrophosphates and their unique metabolic complexity: analysis by gel electrophoresis. PLoS One. 2009;4:e5580.

Loss O, Azevedo C, Szijgyarto Z, Bosch D, Saiardi A. 2011. Preparation of quality inositol pyrophosphates. JoVE. 2011;55:e3027.

European Food Safety Authority (EFSA). Scientific opinion on Dietary Reference values for zinc. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *ESFA J*. 2014;12(10):3844.

Lehrfeld J. High-performance liquid chromatography analysis of phytic acid on a pH-stable, microporous polymer column. *Cereal Chemistry.* 1989;66:510-515.

This document was prepared by Victor Raboy, USDA-ARS, Aberdeen, Idaho, USA (retired).