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Determination of Phytic Acid and Inositol Pentakisphosphates in Foods by High-Performance Ion Chromatography

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A high-performance anion exchange chromatographic method was adapted for the quantitative determination of phytic acid and inositol pentakisphosphate isomers (excluding enantiomers) in foods. Because of the cost and limited availability of inositol phosphate standards, a phytic acid sodium salt standard was used for the calculation of an average relative response factor for the quantification of inositol pentakisphosphate isomers, and the purity of phytic acid sodium salt standard was also accurately established. The detection limits (S/N = 3) for phytic acid and inositol pentakisphosphates were in the range of 1.5−3.4 μM (0.1−0.2 μg/100 μL). This method has been successfully applied to the determination of phytic acid and inositol pentakisphosphates in a variety of beans and nuts after extraction with 0.5 M HCl and cleanup with solid phase extraction cartridges. The results demonstrated that there was a strong correlation between either the phytic acid content or the total content of phytic acid together with inositol pentakisphosphates and the total dietary fiber content in the group of all raw dry beans and in the group of raw dry black beans but not in the group of raw dry red kidney beans, which was probably due to the insufficient number of the raw dry red kidney bean samples.

KEYWORDS: Phytic acid; inositol pentakisphosphate; high-performance ion chromatography; food; bean; nut

INTRODUCTION

Phytic acid, $InsP_6$, is a naturally occurring component and the principal storage form of phosphorus in cereals, legumes, and nuts, which are also rich in dietary fiber. Historically, $InsP_6$ was considered solely as an antinutrient mainly due to its ability to bind essential dietary minerals including calcium, iron, and zinc, as well as proteins and starch, and to consequently reduce their bioavailability in humans (*1*−*3*). In recent years, a variety of epidemiological and animal studies have shown that $InsP_6$ also has beneficial functions such as reducing the risk of cancers, heart disease, diabetes, and renal calculi (*1*−*4*), some of which were earlier solely attributed to dietary fiber. During food processing and storage, $InsP_6$ can be partially dephosphorylated to yield a large number of *myo*-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphate ($InsP_5$−$InsP_1$) isomers, and in some cases free *myo*-inositol, by soaking, germinating, fermenting, cooking, frying, canning, and autoclaving, etc. (*5*). Therefore, foods, especially processed foods, may contain a mixture of different isomeric forms of inositol phosphates ($InsP_n$) in varying amounts, which may interact with each other and other food components. Several $InsP_3$ and $InsP_4$ isomers exhibit vital physiological functions, such as intracellular messengers (*6*), antiinflammatory effects (*7*), and prevention of diabetes complications (*8*). The position of the phosphate groups on the inositol ring is of great significance to their physiological properties. In addition, the degradation of $InsP_6$ in foods is of nutritional importance because, with the removal of phosphate groups from the inositol ring, the mineral binding strength of $InsP_n$ decreases, resulting in an increased bioavailability of dietary minerals. Bioavailability studies also revealed that neither $InsP_4$ nor $InsP_3$ in isolated form inhibited mineral absorption (*9*). When present in a mixture of $InsP_n$, $InsP_4$ and $InsP_3$ might contribute to the negative effect on mineral absorption by interactions with the more phosphorylated $InsP_n$, i.e., $InsP_6$ and $InsP_5$, through mineral binding (*10*). After all, $InsP_6$ and $InsP_5$, either singly or in combination, are still the primary mineral binding entities (*1*). Hence, in the nutritional studies, it is very necessary and important to establish an accurate and reliable method for the separation and determination of different $InsP_n$ isomers, mainly $InsP_6$−$InsP_3$, and in particular $InsP_6$−$InsP_5$ in foods in order to make a valuable evaluation on their physiological functions.

At present, the existing official AOAC method 986.11 (*11*), which can indirectly quantify $InsP_6$ via determination of inorganic phosphorus by spectrophotometry after conventional anion exchange column extraction and acidic digestion, may systematically overestimate the $InsP_6$ content in some foods because it cannot distinguish between $InsP_3$−$InsP_5$, if present, and $InsP_6$, as well as some food components such as nucleotides, can also give elevated $InsP_6$ values (*3*, *12*−*14*). During the past

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two decades, numerous novel analytical techniques (*13*−*15*) have been applied to the determination of $InsP_6$ or other $InsP_n$ in various matrices, and ion pair chromatography (*16*−*21*) as well as high-performance ion chromatography (*22*−*31*) are the most commonly used methods, most of which are capable for simultaneous separation and determination of $InsP_6$ and other $InsP_n$. The ion pair chromatographic procedures, first developed by Sandberg and Ahderinne (*16*), can separate $InsP_3$−$InsP_6$ based only on the number of phosphate groups in the inositol ring without differentiating isomeric forms of $InsP_n$. The high-performance ion chromatographic approaches, first established by Phillippy et al. (*22*, *23*), have proven to be the isomer specific analytical techniques, with the capability to separate not only $InsP_n$ with different numbers of phosphate groups but also the different isomeric forms (excluding enantiomers) of $InsP_n$ with the same number of phosphates. However, nucleotides, such as ADP and ATP, may interfere with the determination of $InsP_n$ using both types of analytical techniques (*3*). Up to now, because not all of the $InsP_n$ isomer standards are commercially available, the purity information is not provided for some commercial $InsP_n$ isomer standards, and more importantly, all of the $InsP_n$ isomer standards are very hygroscopic (*32*) and only in microgram levels from commercial sources except for $InsP_6$, it is always an important challenge to identify all of the chromatographic peaks that have been separated in any particular study and accurately quantify individual $InsP_n$ isomers, even $InsP_6$ since a certified reference material for $InsP_6$, which can give the exact purity information, is not currently available (*33*). The estimation of $InsP_3$−$InsP_5$ can be conducted by using the $InsP_6$ standard in combination with the correction factors or relative response factors of $InsP_3$−$InsP_5$ to $InsP_6$ (*16*, *19*−*21*, *24*−*26*, *28*, *30*). Because $InsP_3$−$InsP_5$ pure standards are needed in most methods, while the accurate purity information is usually lacking, even using the similar treatment methods, the results obtained in different studies are conflicting. Recently, the present authors developed a high-performance ion chromatography, in which all 35 possible $InsP_2$−$InsP_6$ isomers (excluding enantiomers) were separated into 27 peaks (so far the highest number of $InsP_2$−$InsP_6$ peaks that can be separated) for the first time, and the elution order of all $InsP_2$−$InsP_6$ isomers was definitively established, several of which were based on the investigation of $InsP_n$ hydrolysis products (*34*). By using this method, in the present study, an average relative response factor of $InsP_5$ to $InsP_6$ was determined by further research of $InsP_6$ hydrolysis, and the accurate analysis of $InsP_6$ purity was also carried out, both of which led to the establishment of a quantitative method for accurate determination of $InsP_6$ and approximate determination of $InsP_5$ isomers (excluding enantiomers). This method has been successfully applied to analyze some selected foods and to study the correlations, if any, between the $InsP_6$ content or the sum of $InsP_6$ and $InsP_5$ and TDF content in raw dry beans.

MATERIALS AND METHODS

Chemicals. All of the chemicals used in this study have been described in a previous paper (*34*). In this study, $InsP_6$ reagent refers to Aldrich phytic acid (dodecasodium salt hydrate) standard unless otherwise specified.

Instrumentation. The detailed information about the ion chromatographic instrument and method was given earlier (*34*). Prior to the chromatographic analysis, aliquots of the sample solutions were filtered through 0.22 μm Millipore Millex-GV membrane filters (Bedford, MA). The spectrophotometric experiments were carried out by using a Beckman DU-7 spectrophotometer (Irvine, CA).

Peak Identification. An in-house reference standard solution for the identification of chromatographic peaks was prepared, and the peak identification was accomplished as described earlier (*34*).

Average Relative Response Factor of $InsP_5$ to $InsP_6$. A 4 mL portion of $InsP_6$ reagent solution (apparent concentration, 2.5 mg/mL) was diluted to 10 mL with water and injected into the chromatographic system. In addition to the $InsP_6$ peak, two small peaks for DL-Ins(1,2,3,4,5)P_5 and DL-Ins(1,2,4,5,6)P_5, which were impurities, were detected. All of the peak area responses were measured. A series of $InsP_6$ hydrolysis solutions only containing $InsP_5$ and $InsP_6$ were prepared as well: portions (4 mL) of $InsP_6$ reagent solutions (apparent concentration, 2.5 mg/mL) were transferred into glass tubes with Teflon-lined screw caps, and 0.71 mL of TFA or 0.79 mL of HCl was added into each tube (to a final concentration of 2 M TFA at pH ca. 1 or 2 M HCl at pH ca. 0.5−0.6). Capped tightly, these solutions were heated in an oven at the different temperatures (100 °C, 110 °C for both kinds of solutions, and 120 °C for only TFA solution) for 1 h. After the solutions were cooled, all of the tubes were put in a 40 °C water bath, and the contents were dried under nitrogen. In each tube, the residues were dissolved with 10 mL of water. The final $InsP_6$ hydrolysis solutions were injected into the chromatographic system after filtering. Only the $InsP_6$ and four $InsP_5$ peaks were found in the chromatograms, and all of these peak area responses were measured. Because during the hydrolysis the amount of $InsP_6$ dephosphorylated is equimolecular to the amount of $InsP_5$ isomers newly formed, the average relative response factor of $InsP_5$ to $InsP_6$ (F) was calculated as the total newly increased peak area responses of $InsP_5$ isomers divided by the difference between the peak area responses of $InsP_6$ with and without hydrolysis.

Purity of $InsP_6$ Reagent. In this paper, the purity of $InsP_6$ reagent is expressed as the weight percentage content of inositol hexakisphosphoric acid (i.e., as free acid) in the commercial reagent (dodecasodium salt hydrate). It can be determined by analyzing the $InsP_6$-associated phosphorus amount using spectrophotometry along with ion chromatography. The molybdate solution, sulfonic acid reagent solution, phosphate standard solution, and phosphate standard curve, which were all used in spectrophotometry, were prepared according to AOAC method 986.11 (*11*).

$InsP_6$ Solution (Apparent Concentration, 8 mg/mL). An amount of 0.8000 g of $InsP_6$ reagent was accurately weighed and dissolved in 100 mL of water.

Determination of Total Phosphorus in $InsP_6$ Reagent (P_t). A 2 mL portion of $InsP_6$ reagent solution (apparent concentration, 8 mg/mL) was transferred into a glass tube with a Teflon-lined screw cap, and 0.5 mL of H_2SO_4 (98%, w/v) was added. The solution was mixed well, and the tube was tightly capped. This solution was heated in a 150 °C oven overnight. After it was cooled, the solution was completely transferred into a 50 mL volumetric flask and diluted to volume with water; 5 mL of the resulting solution was transferred into a 50 mL volumetric flask, and the following procedure was the same as the "preparation of phosphate standard curve" in AOAC method 986.11 (*11*). An experimental blank solution was also prepared and determined using the whole procedure. The difference between these two results was the total amount of phosphorus in the $InsP_6$ reagent (P_t).

Determination of Phosphorus Associated with Free Phosphate in $InsP_6$ Reagent (P_f). An aliquot of 5 mL of $InsP_6$ reagent solution (apparent concentration, 8 mg/mL) was transferred into a 50 mL volumetric flask, and the following procedure was the same as the "preparation of phosphate standard curve" in AOAC method 986.11 (*11*).

$InsP_5$ Impurities in $InsP_6$ Reagent. A portion of $InsP_6$ reagent solution (apparent concentration, 1 mg/mL) was injected into the chromatographic system. Besides the $InsP_6$ peak, only two small peaks for DL-Ins(1,2,3,4,5)P_5 and DL-Ins(1,2,4,5,6)P_5 were found. The total peak area response of the $InsP_5$ peaks (PA_5) divided by the peak area response of $InsP_6$ (PA_6) was defined as the peak area responses ratio of $InsP_5$ to $InsP_6$. This ratio divided by the average relative response factor of $InsP_5$ to $InsP_6$ (F) was equal to the mole ratio of $InsP_5$ to $InsP_6$ in the $InsP_6$ reagent:

$$\frac{PA_5/PA_6}{F} = \frac{yW_i}{M_5} \times \frac{M_6}{xW_i} = \frac{y}{x} \times \frac{M_6}{M_5} \quad (1)$$

where W_i is the total weight of $InsP_6$ reagent injected into the chromatographic system (mg), x and y are the contents of $InsP_6$ (as free acid) and $InsP_5$ (as free acid) in $InsP_6$ reagent (w/w, %), respectively,

and M_6 and M_5 are the formula weights of $InsP_6$ (as free acid) and $InsP_5$ (as free acid), which are 660.3 and 580.1, respectively. Actually, x is the purity of the $InsP_6$ reagent.

Calculation of Purity of $InsP_6$ Reagent. The total amount of phosphorus in $InsP_6$ reagent (P_t) is the sum of phosphorus associated with free phosphate (P_f), $InsP_5$, and $InsP_6$:

$$P_t = P_f + \frac{xW_r}{M_6} \times 6 \times M_p + \frac{yW_r}{M_5} \times 5 \times M_p \qquad (2)$$

where W_r is the total weight of $InsP_6$ reagent used for the determination of total phosphorus in $InsP_6$ reagent (16 mg) and M_p is the formula weight of phosphorus, 30.97. According to eqs 1 and 2, the purity of the $InsP_6$ reagent, x, can be easily calculated.

SPE Study. The experimental conditions for SPE were established by studying the recoveries of $InsP_6$ and $InsP_5$ with Alltech SAX Extract-Clean columns (200 mg/4.0 mL, Deerfield, IL) and an in-house SPE testing solution.

In-House SPE Testing Solution. Aliquots (2 mL) of $InsP_6$ reagent solutions (apparent concentration, 5 mg/mL) were transferred into glass tubes with Teflon-lined screw caps, and 0.4 mL of HCl (37%, w/v) was added into each tube. Capped tightly, the tubes were divided into two groups. The two groups of solutions were heated in an oven at 100 °C and 140 °C for 1 h, respectively. After the solutions were cooled, all of the tubes were put in a 40 °C water bath, and the contents were dried under nitrogen. In each tube, the residues were dissolved with 10 mL of water. The two groups of solutions were mixed in a ratio of 1 to 1 (v/v).

SPE Recovery Test. A portion (5 mL) of the SPE testing solution was transferred to a 25 mL volumetric flask, and 2.5 mL of 0.5 M HCl was added and diluted to volume with water. This solution was loaded into an Alltech SAX Extract-Clean column (200 mg/4.0 mL), which had been conditioned with 10 mL of water. After 10 mL of water was used for cleanup, 2 or 4 M HCl was applied for eluting $InsP_n$. The eluates were dried under nitrogen in a 40 °C water bath. The residues were dissolved in 3 mL of 0.04 M HCl, filtered, and injected into the chromatographic system. The recoveries of $InsP_6$ and $InsP_5$ were calculated by comparing the relevant peak areas of the testing solution after and before SPE treatment.

Preparation of Samples. All of the six nuts and 15 raw dry beans, stored in sealed packages, were purchased at supermarkets in the Washington, DC, metropolitan area and Fargo, North Dakota, from September 2002 to April 2003. Among the nuts were almonds, pecans, and walnuts, which were raw, whereas cashews, macadamias, and peanuts were roasted; all of them were ready-to-eat. Among the raw dry beans, there were six brands of black beans (black beans A−F), four brands of red kidney beans (red kidney beans A−D), and one brand of Great Northern beans, mung beans, navy beans, pink beans, and pinto beans, respectively.

Raw Dry Beans. The raw dry beans were ground in a coffee grinder (Type 203, Krups, Germany) to pass a 60 mesh screen for the analysis of moisture, TDF, and $InsP_n$. The moisture and TDF determinations were performed according to AOAC method 925.10 (*35*) and Li (*36*), respectively. The analysis of $InsP_n$ was carried out by the following procedure: ground samples (ca. 0.7−0.8 g) were accurately weighed into 50 mL polysulfone centrifuge tubes with screw caps in quadruplicate, and 15 mL of 0.5 M HCl was added to each tube. After the samples were vortexed for 1 min and sonicated in a water bath for 1−2 min, the samples were extracted by continuous shaking for 3 h. The extracts were centrifuged at 39200*g* for 25 min. The supernatant (2.5 mL) was diluted to 25 mL with water. The resulting solution was applied to an Alltech SAX Extract-Clean column (200 mg/4.0 mL), which had been conditioned with 10 mL of water, washed with 10 mL of water, and eluted with 12 mL of 2 M HCl. The eluates were dried under nitrogen in a 40 °C water bath. The residues were dissolved in 3 mL of 0.04 M HCl (to avoid possible precipitation formed by residual metal ions with $InsP_n$), filtered, and injected into the chromatographic system.

Cooked Beans. One brand of black beans (black beans A) and one brand of red kidney beans (red kidney beans A), together with all other varieties of raw dry beans, were soaked and cooked as follows: 100 g of each kind of bean was added into 500 mL of boiling deionized water in a beaker, and the solution was boiled for 2 min and then left to stand for 1 h at room temperature. Cooking was resumed with occasional stirring. After 15 min, the beans were tested for doneness at a 5 min interval until they could be crushed easily between the fingers, usually after 20−30 min. The cooked beans were left to cool for ca. 2 h, drained, and freeze-dried for 48 h and then ground in a coffee grinder to pass a 60 mesh screen. The analysis of moisture and $InsP_n$ was the same as that for raw dry beans.

Nuts. The nuts were ground in a coffee grinder. The ground samples (ca. 1.5−1.6 g) were accurately weighed into 50 mL Teflon centrifuge tubes with screw caps in quadruplicate; 20 mL of hexane was added, and the contents were vortexed vigorously and then shaken continuously for 1 h with periodic sonication for every 15 min. The solutions were centrifuged at 1600*g* for 10 min, and the supernatants were decanted. The extraction steps were repeated once. The remaining hexane was evaporated by sitting the tubes in the hood overnight. The residues were transferred into 50 mL polysulfone centrifuge tubes with screw caps with 15 mL of 0.5 M HCl, and the following treatment procedure for $InsP_n$ analysis was the same as that for raw dry beans.

Calculations. Each sample was weighed and extracted in quadruplicate, and aliquots of each final solution were injected into the chromatographic system in duplicate. The peak area measurements for all calculations were adopted, and the means and standard deviations were calculated from the four averages of the duplicates ($n = 4$).

Spike Recovery Study. The accuracy of the method was evaluated through a spike recovery study using one brand of raw dry black beans (black beans F) and peanuts after the fat extraction step as the testing samples; 20 mL of 5 M HCl was added to a 200 mL volumetric flask and diluted to volume with the SPE testing solution. Instead of 15 mL of 0.5 M HCl, 15 mL of this solution was added into the samples for extraction. The following treatment steps were the same as mentioned above. Also, 2.5 mL of this solution was transferred into a glass tube and dried under nitrogen in a 40 °C water bath. The residues were dissolved with 3 mL of 0.04 M HCl. The resulting solution was injected into the chromatograph after filtering, and the spiked amounts of $InsP_6$ and $InsP_5$ in the testing samples were determined. On the basis of the analytical results of samples with and without spiking, as well as the spiked amounts, the spike recovery of $InsP_6$ and $InsP_5$ in food samples was calculated.

RESULTS AND DISCUSSION

Until now, although many ion pair chromatographic and high-performance ion chromatographic assays have been proposed for the quantitative determination of $InsP_6$ and/or other $InsP_n$, strictly speaking, no single one has been well-recognized as accurate and reliable, which is partially caused by two reasons: a sufficient amount of individual standards for the analytical method development and validation is not commercially available except for $InsP_6$, and the accurate purity information is usually lacking for commercial standards including $InsP_6$. Because most analytical laboratories have no facilities to produce a sufficient amount of pure $InsP_n$ standards, using the relative response factors (or correction factors) of $InsP_n$ to $InsP_6$ for the quantification of $InsP_n$ is a very reasonable choice. In many methods (*20*, *24*−*26*, *28*, *30*), the individual $InsP_n$ standards were employed for the calculation of relative response factors of $InsP_n$ to $InsP_6$ by comparing the relevant peak responses of $InsP_n$ with $InsP_6$. However, neither purity information nor handling method for these standards was provided since the $InsP_n$ standards except $InsP_6$ were extremely hygroscopic (*32*) and in microgram levels from commercial sources; therefore, the calculation results were not reliable. By using the $InsP_6$ hydrolysis solution, Sandberg et al. proposed a method (*16*): the fractions of $InsP_6$ hydrolysis solution separated by ion pair chromatography were collected and then analyzed for inositol by gas chromatography and phosphorus by spectrophotometry after acid digestion, respectively; the correction factors were

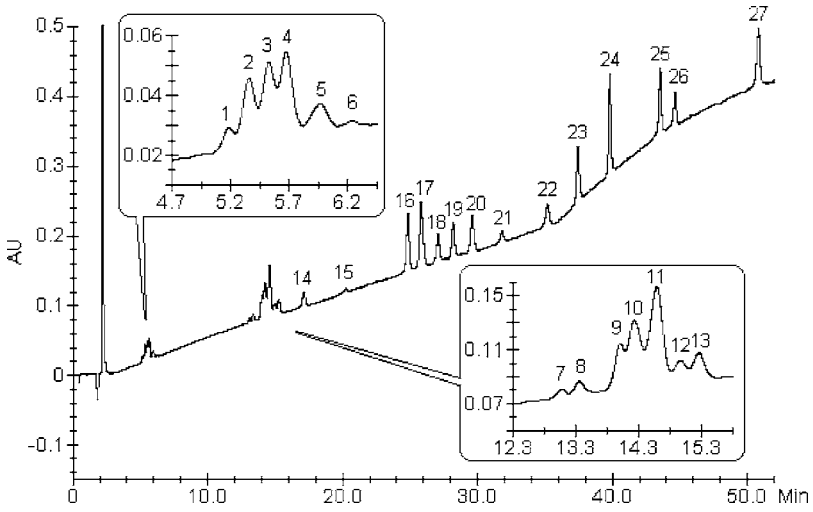

Figure 1. Chromatogram of the in-house reference standard solution. Peaks: 1, Ins(1,3)P_2; 2, DL-Ins(3,4)P_2, DL-Ins(1,5)P_2; 3, DL-Ins(1,2)P_2; 4, DL-Ins(2,4)P_2, DL-Ins(1,4)P_2; 5, DL-Ins(4,5)P_2, Ins(2,5)P_2; 6, Ins(4,6)P_2; 7, Ins(1,3,5)P_3; 8, Ins(2,4,6)P_3; 9, DL-Ins(1,3,4)P_3; 10, DL-Ins(1,2,4)P_3, DL-Ins(2,3,5)P_3; 11, Ins(1,2,3)P_3, DL-Ins(1,2,6)P_3, DL-Ins(1,4,6)P_3; 12, DL-Ins(1,4,5)P_3; 13, DL-Ins(2,4,5)P_3; 14, DL-Ins(1,5,6)P_3; 15, Ins(4,5,6)P_3; 16, DL-Ins(1,2,4,6)P_4, Ins(1,2,3,5)P_4; 17, DL-Ins(1,2,3,4)P_4, Ins(1,3,4,6)P_4; 18, DL-Ins(1,2,4,5)P_4; 19, DL-Ins(1,3,4,5)P_4; 20, DL-Ins(1,2,5,6)P_4; 21, Ins(2,4,5,6)P_4; 22, DL-Ins(1,4,5,6)-P_4; 23, Ins(1,2,3,4,6)P_5; 24, DL-Ins(1,2,3,4,5)P_5; 25, DL-Ins(1,2,4,5,6)P_5; 26, Ins(1,3,4,5,6)P_5; and 27, InsP_6. With regard to enantiomers not separated, both possible isomers are denoted according to this rule (DL-Ins).

calculated by comparison of peak areas of InsP_n with InsP_6 obtained by ion pair chromatography in relation to the amount of inositol or phosphorus in the collected fractions. Although this method is theoretically strict, in practice, it is very easy to introduce various errors due to its complicated multistep procedures. Brooks et al. calculated the relative response factors of InsP_3−InsP_5 by preparing InsP_6 hydrolysis solutions containing different concentrations of InsP_6−InsP_3 and analyzing the chromatograms by multiple regression but did not provide the details (*21*). A crucial premise was not mentioned that no other less phosphorylated InsP_n, i.e., InsP_2 or InsP_1, should be present in the hydrolysis solutions, and the employed preparation method for hydrolyzing InsP_6 (*18*) could not ensure that the hydrolysis solutions only contained InsP_6−InsP_3 without InsP_2−InsP_1 either. Recently, the present authors proposed a high-performance ion chromatographic method (*34*) that separated all 35 InsP_6−InsP_2 isomers (excluding enantiomers) into 27 peaks (**Figure 1**). By using this method, in the present study, the InsP_6 hydrolysis solutions will be prepared under the specific conditions and used for the calculation of relative response factors of InsP_n to InsP_6, if possible, by comparing the relevant peak area responses of InsP_n with InsP_6. Afterward, because the quantification accuracy of InsP_n will wholly rely on the accuracy of InsP_6 purity, an accurate and simple method for the determination of InsP_6 purity of the commercial reagent (dodecasodium salt hydrate) will be established. Both of them will be fundamental for the accurate quantification of InsP_n.

Average Relative Response Factor of InsP_5 to InsP_6. Theoretically, it is feasible to calculate the relative response factor for each InsP_5 isomer (excluding enantiomers) to InsP_6 using a system of linear equations if there are only InsP_5 peaks with different profiles in addition to the InsP_6 peak in the chromatograms of the InsP_6 hydrolysis solutions. However, in all of the hydrolysis solutions prepared in 2 M TFA at 100−120 °C or 2 M HCl at 100−110 °C, the profiles of InsP_5 isomers are the same [after subtracting the peak area responses of existing DL-Ins(1,2,4,5,6)P_5 and DL-Ins(1,2,3,4,5)P_5 as the impurities in the InsP_6 reagent from those of corresponding peaks]: the peak of DL-Ins(1,2,4,5,6)P_5 is the largest one, followed by the peaks of DL-Ins(1,2,3,4,5)P_5, Ins(1,3,4,5,6)P_5, and Ins(1,2,3,4,6)P_5 in turn. (The InsP_6 hydrolysis solution in 2 M HCl heated at 120 °C for 1 h was not adopted because several small InsP_4 peaks appeared in the chromatogram.) From the mathematical viewpoint, the resulting system of linear equations is so "unstable" or "sensitive" that a minor error of any peak area measurement may result in a serious calculation error for the relative response factor of the individual InsP_5 isomer to InsP_6. In fact, the calculated results also confirmed this. In this case, the calculation of the relative response factor of the individual InsP_5 isomer to InsP_6 was not conducted. When the total newly increased peak area responses of InsP_5 isomers, obtained from the hydrolysis solutions mentioned above, are divided by the difference between the peak area responses of InsP_6 with and without hydrolysis, the results, which can be considered as the average relative response factor of InsP_5 to InsP_6 (*F*), were very similar. Therefore, it can be used to replace the individual relative response factors of InsP_5 isomers to InsP_6 for the approximate quantification of InsP_5. The calculated results obtained using the hydrolysis solutions in 2 M TFA or 2 M HCl were listed in **Table 1**. Finally, the *F* value was calculated as 0.66 with the relative standard deviation of 4.0% ($n = 5$), which means that by using this specific chromatographic method, InsP_5 can yield 66% of the peak area response that equimolecular InsP_6 can produce. In the following sections, the *F* value was chosen as 0.66 for all of the relevant calculations.

Table 1. Average Relative Response Factor (*F*)

heating temperature (°C)	TFA	HCl
100	0.634 (5.0%)[a]	0.629 (9.0%)
110	0.657 (13.0%)	0.678 (17.5%)
120	0.688 (15.8%)	

[a] Figures in parentheses represent the decomposition percentages of InsP_6 during hydrolysis.

In addition, the satisfactory reproducibility of *F* values was further confirmed by using the InsP_6 hydrolysis solutions in 2 M formic acid or 2 M acetic acid, which were heated at 100 °C for 1 h and had the similar InsP_5 distribution profiles, and the obtained *F* values were 0.64 and 0.66, respectively.

In principle, the average relative response factor of $InsP_4$ to $InsP_6$ can be determined using the same method as well. However, practically, it is very difficult to prepare the hydrolysis solution containing a sufficient amount of $InsP_4$ isomers for peak area measurement without $InsP_3$ isomers. Also, the average relative response factor of $InsP_5$ to $InsP_6$, which actually is an estimated value, needs to be used for the calculation of average relative response factor of $InsP_4$ to $InsP_6$, most likely introducing more errors. As a result, only the average relative response factor of $InsP_5$ to $InsP_6$ was established in this study.

Purity of $InsP_6$ Reagent. In general, the current commercially available $InsP_6$ reagents are usually sodium salts, which contain a certain amount of moisture besides some possible impurities including other unspecified metallic ions and less phosphorylated $InsP_n$, especially $InsP_5$. In addition, there are some other factors that may influence the purity of $InsP_6$ reagent (*33*). It is very necessary to establish the $InsP_6$ reagent purity prior to use as a standard. Brooks et al. used a microplate adaptation of the AOAC method 986.11 (*11*) for determining the purity of the $InsP_6$ reagent obtained from Sigma (*33*). The possible drawbacks of this method are that the microchemistry experimental skills are needed for ensuring the accuracy of results, and the insensitive detection assays are used to analyze the reagent and subsequently preclude the probability of other $InsP_n$ present in reagent, which is not appropriate. More importantly, this method is only applicable when other less phosphorylated $InsP_n$ does not exist in the $InsP_6$ reagent. In fact, using the proposed chromatographic method with a higher detection sensitivity (*34*), several commercial $InsP_6$ reagents (the accurate purity information was not provided) including that obtained from Sigma were all found to contain several $InsP_5$ isomers. Therefore, it is very important to develop a more accurate method for establishing the purity of the $InsP_6$ reagent containing $InsP_5$ impurities. Among all of the reagents tried in this study, Aldrich $InsP_6$ reagent, containing the least amount of $InsP_5$ (judged from the ratio of PA_5 to PA_6, 0.00231, which is the minimum) and no other less phosphorylated $InsP_n$, was considered to be the purest one and thus employed for the purity study.

In the proposed method, a simplified modification of the AOAC method 986.11 (*11*), which is much easier to operate as compared with the method of Brooks et al. (*33*), was used for measuring the total phosphorus content in $InsP_6$ reagent, as well as the phosphorus content associated with the free phosphate as an impurity of $InsP_6$ reagent. The results showed that the phosphorus content associated with the free phosphate only accounted for 0.023% (w/w) of this $InsP_6$ reagent. Moreover, on the basis of the eqs 1 and 2, the content of $InsP_6$ (as free acid) in this $InsP_6$ reagent (w/w), x, was calculated as 58.1 $\pm$ 0.6% (mean $\pm$ standard deviation, $n = 3$), and the content of $InsP_5$ isomers (as free acids) in this $InsP_6$ reagent (w/w), y, was 0.18 $\pm$ 0.02% ($n = 3$), respectively.

Interferences. Using the proposed chromatographic method, the common anions, including nitrate, sulfate, carbonate, formate, acetate, citrate, and glucose-1-phosphate, do not interfere with the detection (*34*). Also, the retention times of ADP and ATP are around 2.6 and 8.3 min, respectively, and thus, they do not interfere with the $InsP_n$ detection.

Analytical Characteristics. Under the experimental conditions, Aldrich phytic acid solution (40%, w/w), which contains a number of $InsP_6$−$InsP_1$ isomers, was diluted in different ratios and used for studying the analytical characteristics of this method. The $InsP_6$ and $InsP_5$ concentrations in the diluted solutions were quantified using the proposed method. $InsP_6$ and $InsP_5$ showed good linearity between the concentrations and the peak area responses. The detection limits (signal-to-noise ratio 3:1) were also calculated (**Table 2**). The precisions were evaluated by performing eight replicated injections of a diluted Aldrich phytic acid solution where the concentrations of Ins(1,2,3,4,6)P_5, DL-Ins(1,2,3,4,5)P_5, DL-Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5, and $InsP_6$ were 62, 109, 111, 87, and 249 μM, respectively. The relative standard deviations of the peak areas were 2.8, 2.1, 2.1, 3.5, and 1.4% for Ins(1,2,3,4,6)P_5, DL-Ins(1,2,3,4,5)P_5, DL-Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5, and $InsP_6$, respectively.

Table 2. Linearity and Detection Limits for $InsP_5$ and $InsP_6$

analyte	concentration range (μM)	correlation coefficient ($n = 6$)	detection limit (μM, S/N = 3)
Ins(1,2,3,4,6)P_5	3.4–310	0.9997	3.4
DL-Ins(1,2,3,4,5)P_5	3.6–543	0.9998	2.3
DL-Ins(1,2,4,5,6)P_5	3.7–556	0.9999	2.1
Ins(1,3,4,5,6)P_5	2.9–436	0.9998	2.1
$InsP_6$	8.3–1250	0.9997	1.5

SPE Study. The sample preparation techniques used in most methods for $InsP_n$ analysis mainly include extraction of $InsP_n$ from samples with varying concentrations of acids, mainly HCl, and purification of crude acid extracts with SPE, followed by removal by evaporation of the excess acid used as the eluent in the SPE operation (*1*, *13*, *14*). Among them, SPE is the most important step that is used mainly for the preconcentration of $InsP_n$ on ion exchange resins and elimination of most interfering substances. Because the acid concentration in the crude extracts is usually so high that most $InsP_n$ cannot be retained on the ion exchange resin and thus needs be reduced, usually, the acid needs to be either removed by evaporation (*16*, *20*, *28*) or diluted with water (*17*, *18*, *23*) prior to SPE operation. In this study, 0.5 M HCl was used as the extractant. Initially, the acid extract was evaporated to complete dryness, and the contents were redissolved with water followed by the SPE operation. However, the recovery results obtained from food samples were poor with wide variations, which were probably a result of the wall-induced condensation reactions of $InsP_n$ with other coextracted components as well as the incomplete redissolution of the dried extract from the walls of a glass vessel (*37*). So, in this study, the HCl extract was diluted with water before the SPE operation.

Several anion exchange resins were compared, among which the Bio-Rad Poly-Prep prefilled chromatography column (0.8 cm $\times$ 4 cm, Hercules, CA), packed with AG1-X8 (200−400 mesh) anion exchange resin, and the Alltech SAX Extract-Clean column (200 mg/4.0 mL) were the most suitable for purification of crude acid extracts. Both of the resins are polystyrene−divinylbenzene-based anion exchangers with the quaternary amine functional groups and can be used in the strong acid solution. Actually, the Alltech column is the disposable cartridge with frits and thus easy to use. Also, its ion exchange capacity (the apparent capacity, 0.3 mequiv) is less than that of the Bio-Rad column (the apparent capacity, 2.4 mequiv) and consequently is helpful to reduce the volume of the acid that is needed to elute $InsP_n$, which was confirmed by the experimental results later. Therefore, the Alltech SAX Extract-Clean column was adopted in this study. To obtain the reliable results, a SPE testing solution was prepared, in which all $InsP_n$ isomers were present (*34*) and the distributions of $InsP_6$ and $InsP_5$ isomers were similar to the real samples: the concentrations of Ins(1,2,3,4,6)P_5, DL-Ins(1,2,3,4,5)P_5, DL-Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5, and $InsP_6$ were 47, 78, 80, 43, and 391 μM, respectively. Under the experimental conditions, when the SPE testing solution in 0.05

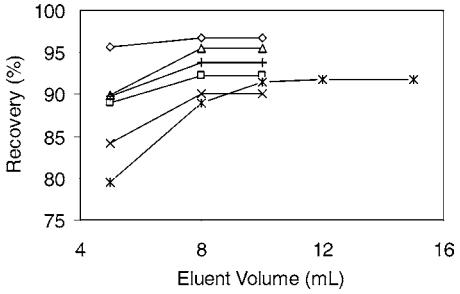

Figure 2. Effect of HCl (2 M) volume on the recoveries of analytes: ◇, Ins(1,2,3,4,6)P_5; □, DL-Ins(1,2,3,4,5)P_5; △, DL-Ins(1,2,4,5,6)P_5; ×, Ins(1,3,4,5,6)P_5; +, Σ$InsP_5$; and *, $InsP_6$.

M HCl was loaded into the cartridge, inorganic phosphate, all $InsP_1$ and $InsP_2$ isomers, as well as part amounts of all $InsP_3$ isomers could not be retained because the loading solution itself (0.05 M HCl) was a weak eluent. The first 5 mL of 2 M HCl could elute the remaining amounts of all $InsP_3$ isomers, nearly 100% of all $InsP_4$ isomers (judged by comparison of the relevant peak area responses), and the major amounts of $InsP_5$ isomers and $InsP_6$. **Figure 2** demonstrates that 12 mL of 2 M HCl can elute not less than 90% of $InsP_6$ and $InsP_5$ isomers. Also, it was found that 4 or 2 M HCl had no difference for eluting $InsP_n$ from the resin. Because it would take a longer time to dry the eluate with nitrogen when 4 M HCl was used, finally, 2 M HCl was chosen, and the eluent volume was adopted as 12 mL. By measuring the maximum volume of a given concentration of $InsP_6$ solution (pH 10.8) that can be completely retained on the cartridge, the practical anion exchange capacity of the cartridge was approximately estimated as 0.15 mequiv, which was sufficient for extraction of $InsP_n$, mainly $InsP_6$−$InsP_4$, in diluted acid crude extracts of food samples. In addition, the experimental results revealed that $InsP_n$ isomers did not decompose when they were dried under nitrogen in a 40 °C water bath, which was similar to the result of some earlier studies that the solutions containing $InsP_6$ could be safely vacuum evaporated at a temperature not exceeding 40 °C (*16*, *17*). In summary, the combination of this SPE operation and the proposed chromatographic method is applicable to quantitative determination of $InsP_6$ and $InsP_5$, as well as qualitative analysis of $InsP_4$ and $InsP_3$, if present in sufficient amounts. Because $InsP_2$ isomers, if any, in food samples could not be retained on the cartridge and thus could not be analyzed, one possible and reasonable choice is to modify the gradient elution program of the chromatographic method so that in a shortened elution time only $InsP_3$−$InsP_6$ could be separated. However, the experimental results showed that if the elution time was shortened by steeping the elution gradient, the separations of isomers of $InsP_3$ and $InsP_4$ and, more importantly, some late eluting $InsP_4$ isomers and $InsP_5$ isomers were poor or even unacceptable. The current long gradient elution program was proven a must for the whole analytical method.

Analysis of Real Samples. In this study, 28 samples including 15 raw dry beans, seven cooked beans, and six nuts, were analyzed, and the chromatograms of some samples are illustrated in **Figure 3**. The analytical results of raw dry and cooked beans are also summarized in **Table 3**. Among $InsP_n$, only $InsP_6$ and $InsP_5$ were detected in all of the beans. There was a wide variation in the $InsP_6$ or $InsP_5$ content among different brands of raw dry black beans or red kidney beans. $InsP_6$ content (per kg, adjusted by moisture) in raw dry beans ranged from 5.87 mmol in mung beans to 14.86 mmol in black beans D and in the selected cooked beans from 5.21 mmol in

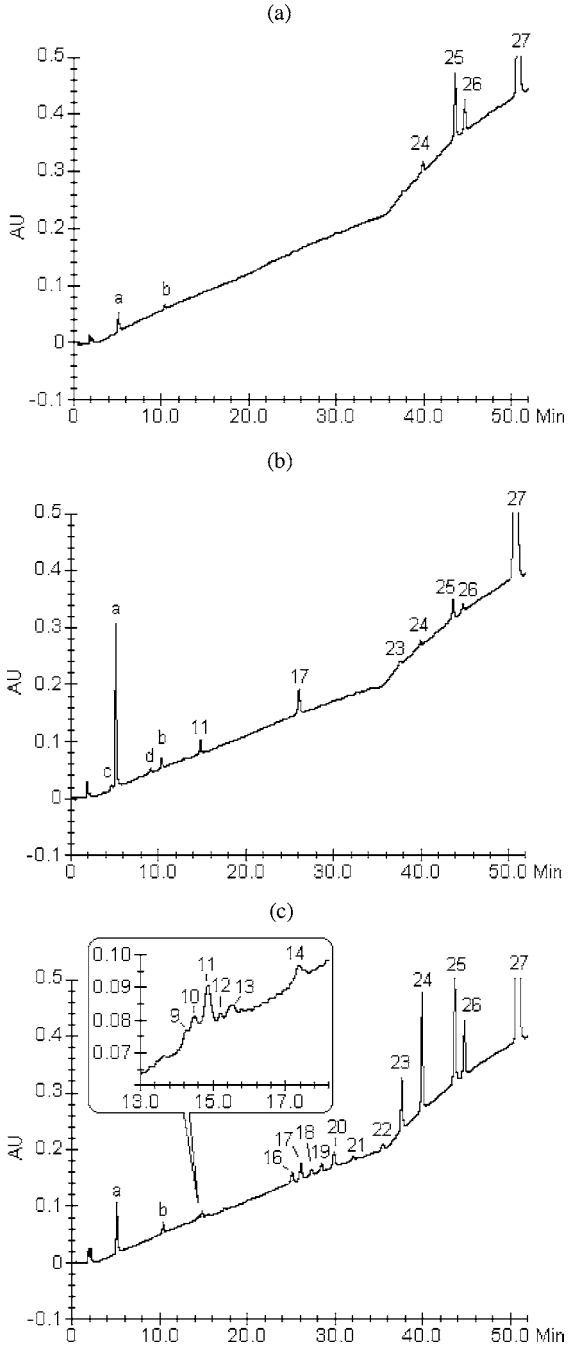

Figure 3. Chromatograms of raw dry black beans F (**a**), almonds (**b**), and dry roasted peanuts (**c**). The peak numbers are as in **Figure 1**. Peaks a–d, unidentified substances in foods.

mung beans to 9.75 mmol in black beans A. $InsP_6$ was the predominate inositol phosphate of the total $InsP_n$ determined in raw dry beans, ranging from 63.9% in red kidney beans C to 97.5% in pinto beans, and in the selected cooked beans, ranging from 81.2% in mung beans to 88.2% in black beans A. Of the four possible $InsP_5$ isomers (excluding enantiomers), DL-Ins(1,2,4,5,6)P_5 was dominant in raw dry beans, followed by Ins(1,3,4,5,6)P_5, DL-Ins(1,2,3,4,5)P_5, and Ins(1,2,3,4,6)P_5, if present, which indicated that there probably were some common profiles at least for raw dry beans. The determination results of $InsP_6$ in raw dry and cooked beans are very comparable to the literature values, which were summarized by Phillippy recently (*3*), and the sums of $InsP_5$ isomers were somewhat less than the literatures values (*3*), which partially was the result of the proposed relatively accurate quantitative method for $InsP_5$

Table 3. Analytical Results of Beans

sample	moisture[a] (g/100 g)	TDF[a,b] (g/100 g)	$InsP_n$ content[b,c] (mmol/kg)				
			Ins(1,2,3,4,6)P_5	DL-Ins(1,2,3,4,5)P_5	DL-Ins(1,2,4,5,6)P_5	Ins(1,3,4,5,6)P_5	$InsP_6$
black beans A	9.83	18.50	ND[d]	ND	0.40 ± 0.05	0.12 ± 0.01	13.05 ± 0.95
cooked	7.23		ND	0.17 ± 0.03	0.83 ± 0.02	0.30 ± 0.01	9.75 ± 0.08
B	12.99	19.35	ND	ND	0.54 ± 0.01	0.20 ± 0.02	14.46 ± 0.45
C	11.12	12.57	0.16 ± 0.03	0.41 ± 0.05	1.88 ± 0.21	0.79 ± 0.09	6.66 ± 0.70
D	11.39	18.63	ND	ND	0.72 ± 0.04	0.30 ± 0.01	14.86 ± 0.48
E	11.34	13.63	ND	0.19 ± 0.02	1.75 ± 0.02	0.72 ± 0.03	7.40 ± 0.21
F	9.81	20.12	ND	0.24 ± 0.01	1.90 ± 0.04	0.83 ± 0.04	11.01 ± 0.57
Great Northern beans	10.90	17.37	ND	ND	0.48 ± 0.04	0.13 ± 0.02	13.08 ± 0.33
cooked	8.65		0.14 ± 0.01	0.23 ± 0.02	1.02 ± 0.02	0.42 ± 0.04	9.19 ± 0.08
mung beans	11.40	10.25	ND	0.10 ± 0.00	0.77 ± 0.02	0.31 ± 0.03	5.87 ± 0.31
cooked	11.24		ND	0.13 ± 0.02	0.77 ± 0.02	0.31 ± 0.01	5.21 ± 0.07
navy beans	11.41	19.47	ND	ND	0.47 ± 0.04	0.13 ± 0.01	12.60 ± 0.37
cooked	8.27		ND	0.13 ± 0.02	0.85 ± 0.05	0.37 ± 0.03	9.16 ± 0.39
pink beans	9.58	18.49	ND	ND	0.46 ± 0.02	0.14 ± 0.02	13.07 ± 0.23
cooked	8.57		ND	0.22 ± 0.03	0.97 ± 0.03	0.38 ± 0.01	9.40 ± 0.19
pinto beans	11.10	16.23	ND	ND	0.31 ± 0.03	ND	12.08 ± 0.43
cooked	4.05		ND	0.12 ± 0.02	0.82 ± 0.03	0.33 ± 0.01	9.17 ± 0.33
red kidney beans A	10.51	17.99	ND	ND	0.27 ± 0.01	0.10 ± 0.01	9.19 ± 0.18
cooked	6.88		ND	0.10 ± 0.01	0.63 ± 0.03	0.25 ± 0.01	7.24 ± 0.08
B	13.02	18.88	ND	ND	0.34 ± 0.02	0.11 ± 0.01	12.68 ± 0.28
C	12.84	19.86	ND	0.18 ± 0.02	3.24 ± 0.14	1.38 ± 0.05	8.48 ± 0.53
D	9.56	16.26	ND	0.28 ± 0.01	1.71 ± 0.03	0.72 ± 0.02	12.29 ± 0.27

[a] Mean of duplicate analyses ($n = 2$). [b] Adjusted by moisture. [c] Mean ± standard deviation ($n = 4$). [d] ND, not detected.

isomers in this study. The $InsP_6$ content in the cooked beans averaged 76.2%, ranging from 70.3% in Great Northern beans to 88.8% in mung beans, of the content in the raw dry beans, which was similar to that reported in an earlier study (*38*), although the cooking methods were somewhat different. Also, the total content of $InsP_n$ (actually of $InsP_6$ and $InsP_5$) in the cooked beans averaged 83.3%, ranging from 79.6% in navy beans to 90.9% in mung beans, of the content in the raw dry beans.

Because the dietary fiber and $InsP_6$ are abundant in plant foods and both of them have the same or similar physiological functions, either positive or negative (*39*−*41*), there is naturally a concern whether there is a relationship, more specifically a correlation, between the content of TDF and $InsP_6$ or all $InsP_n$ in foods. Plaami et al. (*42*) found that in cereal-based foods, the total $InsP_3$−$InsP_6$ content was parallel to the TDF values: the correlation coefficients between $InsP_3$−$InsP_6$ and TDF content were 0.75 and 0.77 in breakfast cereals and breads, correspondingly; there was a strong correlation also between $InsP_6$ and TDF content of breakfast cereals (correlation coefficient, 0.77) but not in the bread group (correlation coefficient, −0.197) due to the decomposition of $InsP_6$ during processing. Because the different $InsP_n$ isomers usually were produced by the degradation of $InsP_6$ during the food processing, including $InsP_2$ and $InsP_1$, which was not considered by Plaami et al. (*42*), to study the possible correlations between the content of $InsP_6$ or all $InsP_n$ and TDF, it is more easier to obtain the reliable results to choose the raw foodstuffs, for example, the raw dry beans as the study objects. From **Figure 4**, it can be found that there is a strong correlation between the TDF content and the $InsP_6$ or total $InsP_n$ levels (actually $InsP_6$ and $InsP_5$ for these samples) in the group of all 15 raw dry beans, and the correlation coefficients are 0.713 and 0.773, respectively. There is also a good correlation between the TDF content and the $InsP_6$ or total $InsP_n$ levels (actually $InsP_6$ and $InsP_5$) in the group of six raw dry black beans (the correlation coefficients are 0.862 and 0.916, respectively) but not in the group of four raw dry red kidney beans (the correlation coefficients are −0.521 and −0.244, respectively), which was probably due to the insufficient number of the red kidney bean samples.

In this study, six nuts are all ready-to-eat, among which cashews, macadamias, and peanuts were roasted when purchased. The removal of a high content of fat, which roughly ranged from 42% (w/w) in peanuts to 72% (w/w) in macadamias, was necessary before the extraction of $InsP_n$. All of the nuts contained $InsP_6$, and all four $InsP_5$ isomers (excluding enantiomers) with the exception of pecans (**Table 4**). All seven $InsP_4$ peaks and only one small $InsP_3$ peak [for Ins(1,2,3)P_3, DL-Ins(1,2,6)P_3, and DL-Ins(1,4,6)P_3, which were coeluted in this system] were detected in the chromatogram for cashews. All $InsP_4$ peaks, except the peaks for DL-Ins(1,4,5,6)P_4 and Ins(2,4,5,6)P_4, as well as one small $InsP_3$ peak for DL-Ins(2,4,5)P_3, were detected in the chromatogram for macadamias. **Figure 3c** shows that all seven $InsP_4$ peaks and six $InsP_3$ peaks can be detected in the chromatogram for peanuts. Among the unroasted nuts, no $InsP_4$ or $InsP_3$ isomer was found in pecans and walnuts. On the other hand, the analytical results revealed that almonds are very unique (**Figure 3b**): the $InsP_6$ content in almonds is very high; in addition to all four $InsP_5$ peaks, only one $InsP_4$ peak [for DL-Ins(1,2,3,4)P_4 and Ins(1,3,4,6)P_4] and one $InsP_3$ peak [for Ins(1,2,3)P_3, DL-Ins(1,2,6)P_3 and DL-Ins(1,4,6)P_3] were detected. In the other nuts, if $InsP_4$ was present, usually several $InsP_4$ isomers could be detected simultaneously. In terms of peak area responses in almonds, the $InsP_4$ peak was even higher than any of the four $InsP_5$ peaks although it cannot be quantified, and the $InsP_3$ peak was not small (considering that the $InsP_3$ recovery during the SPE step was far less than 100%, the actual $InsP_3$ content should be even higher). In this case, the $InsP_4$ isomer(s) in almonds may be formed via specific (or selective) phosphorylation or dephosphorylation pathways. In other words, there are probably some specific enzymes (kinase or phytase) in raw almonds. If this hypothesis is correct, almonds might be a good research object for studying the metabolism (biosynthesis or degradation) of $InsP_6$ in foods.

To verify the accuracy of this method, a spike recovery study was performed and the results shown in **Table 5** were very satisfactory.

To obtain precise, accurate, and reliable analytical results using this proposed method, there are some important points

Table 4. $InsP_6$ and $InsP_5$ Content of Nuts (mmol/kg)[a]

sample	Ins(1,2,3,4,6)P_5	DL-Ins(1,2,3,4,5)P_5	DL-Ins(1,2,4,5,6)P_5	Ins(1,3,4,5,6)P_5	$InsP_6$
almonds	0.05 ± 0.01	0.06 ± 0.01	0.23 ± 0.01	0.07 ± 0.00	14.28 ± 0.14
cashews, whole[b]	0.38 ± 0.02	0.91 ± 0.04	3.65 ± 0.18	1.61 ± 0.04	7.56 ± 0.30
macadamias[b]	0.18 ± 0.01	0.35 ± 0.02	0.45 ± 0.04	0.19 ± 0.01	3.98 ± 0.21
peanuts, dry roasted[b]	0.64 ± 0.02	1.22 ± 0.03	1.48 ± 0.04	0.63 ± 0.03	6.78 ± 0.14
pecan halves	ND[c]	0.10 ± 0.01	0.15 ± 0.01	0.05 ± 0.01	6.85 ± 0.43
walnuts	0.05 ± 0.01	0.43 ± 0.06	0.24 ± 0.01	0.09 ± 0.01	10.14 ± 0.38

[a] Mean ± standard deviation ($n = 4$). [b] Food was roasted when purchased. [c] ND, not detected.

Table 5. Spike Recovery Results of Samples (%)[a,b]

sample	Ins(1,2,3,4,6)P_5	DL-Ins(1,2,3,4,5)P_5	DL-Ins(1,2,4,5,6)P_5	Ins(1,3,4,5,6)P_5	$InsP_6$
black beans F[c]	102.1 ± 2.7 (0.90)	99.0 ± 0.8 (1.50)	104.8 ± 2.8 (1.55)	98.2 ± 1.4 (0.82)	94.8 ± 2.3 (7.56)
peanuts, dry roasted[d]	95.1 ± 5.0 (0.39)	98.8 ± 5.9 (0.66)	96.8 ± 7.9 (0.68)	89.6 ± 6.2 (0.36)	98.9 ± 7.8 (3.31)

[a] Mean ± standard deviation ($n = 4$). [b] Added amount in parentheses, mmol/kg sample. [c] Sample weight, 0.7 g. [d] Sample weight, 1.6 g.

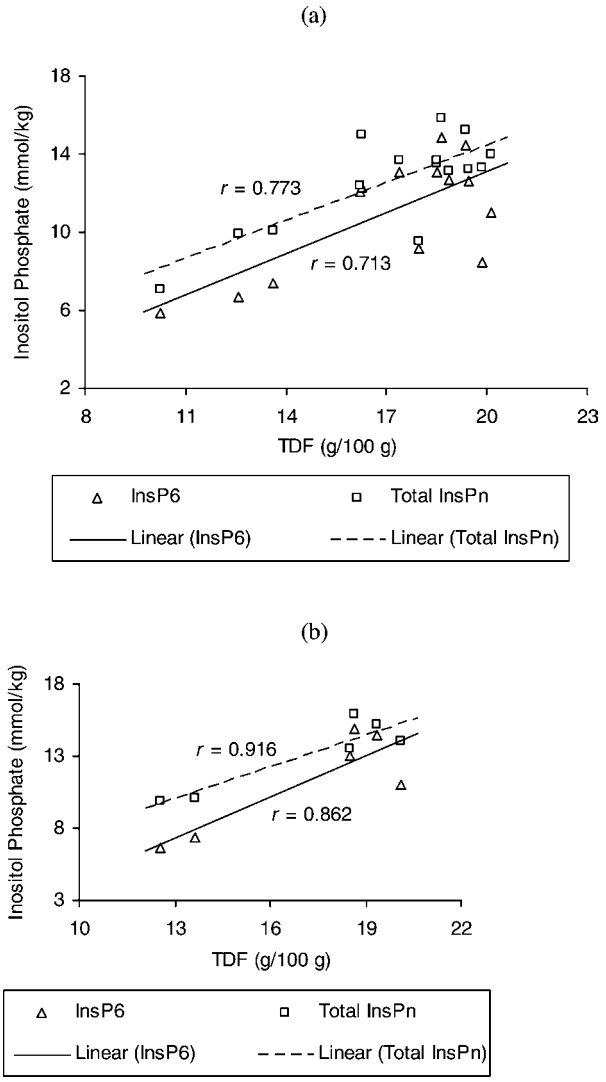

Figure 4. Correlations between $InsP_6$ or all $InsP_n$ (actually $InsP_6$ and $InsP_5$) and TDF content of all 15 raw dry beans (**a**) and all six raw dry black beans (**b**).

that need special attention. It has been reported that nonspecific adsorption of $InsP_6$ occurs with stainless steel columns, and consequently, a first injection of a sample containing $InsP_6$ may show no peaks (*18*). Because the materials used in this analytical system including columns and tubes are PEEK except the stainless steel injection needle of the autosampler, the nonspecific adsorption is not very serious, although it also takes place with other $InsP_n$ besides $InsP_6$. To diminish and eliminate this adsorption, every day before any analysis, two injections of 1:400 (v/v) diluted commercial Aldrich phytic acid solution (40%, w/w) need be carried out by using a short isocratic elution program (eluent, 500 mM HCl:H_2O = 90:10; flow rate, 1 mL/min). Each chromatographic run takes about 11 min. Then, the gradient elution program is used for analysis after equilibration, and the $InsP_n$ peak responses are very reproducible. Meanwhile, 400 μL of water is employed for flushing the injection needle after each injection, and no carryover effect is observed. Also, to avoid the possible clog in the postcolumn reaction coil, the $Fe^{3+}/HClO_4$ solution needs be filtered with 0.45 μm Millipore HA filters before use. This solution can be stored at room temperature for at least 1 month.

In this chromatographic system, the elution program containing a strong acid is actually an on-line cleanup program for the anion exchange separation columns as well: cations will not be retained, and most anions will be converted to neutral molecules or cations and thus elute easily, which will be very helpful for the column protection in the analysis of real samples. Also attributed to the pretreatment method for the real samples, no change of the column separation performance is observed after more than 320 injections of real sample solution, indicating that the whole analytical method is very advantageous for the column protection.

ABBREVIATIONS USED

$InsP_6$, *myo*-inositol hexakisphosphate; $InsP_5$, *myo*-inositol pentakisphosphate; $InsP_4$, *myo*-inositol tetrakisphosphate; $InsP_3$, *myo*-inositol trisphosphate; $InsP_2$, *myo*-inositol bisphosphate; $InsP_1$, *myo*-inositol monophosphate; $InsP_n$, *myo*-inositol phosphates; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; TDF, total dietary fiber; TFA, trifluoroacetic acid; SPE, solid phase extraction; PEEK, polyether ether ketone.

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