Rapid Analysis of Inositol Phosphates

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Fast and simple analytical methods for the determination of inositol bis- to hexakisphosphates or only inositol hexakisphosphate in foods and feces are presented. The methods are both faster and simpler with regard to analytical detection and sample pretreatment as compared to previously reported methods. The samples are pretreated using extraction and centrifugal ultrafiltration and analyzed using high-performance ion chromatography (HPIC) with gradient or isocratic elution. The analytes are detected using ultraviolet detection after postcolumn reaction. The methods are efficient, highly selective, and appropriate for analyzing inositol phosphates in food and feces samples. The between- and within-day variances were generally below 8 and 5% (relative standard deviation), respectively, for the presented HPIC method with gradient elution.

Keywords: *Inositol phosphates; high-performance ion chromatography; HPIC; food*

INTRODUCTION

Recent years have seen an increasing need for rapid, simple, and robust methods that can be used to analyze the content of *myo*-inositol phosphates in foods, especially because the sample load often is very high. The focus of interest in inositol phosphates in the field of food science is the ability of phytate (inositol hexakisphosphate) to chelate metals. Because of its chelating ability, phytate can be regarded as an antinutrient, lowering the bioavailability of minerals, such as iron, zinc, and calcium, in humans (*1*−*5*). In cereals phytate contains 60−90% of the phosphorus. During food processing phytate can be degraded to lower *myo*-inositol phosphates, phosphorus, and, in some cases, free *myo*-inositol (*6*). Methods for analyzing *myo*-inositol hexakisphosphate have frequently been reviewed (*7*−*10*). The most reliable methods for the quantitative and qualitative analysis of *myo*-inositol phosphates are high-performance ion chromatographic (HPIC) methods. In 1985, Phillippy and Johnston (*11*) presented the first ion chromatographic method for the determination of *myo*-inositol hexakisphosphate. Since then, several methods able to separate *myo*-inositol phosphates and their isomers using HPIC have been described (*12*−*16*). These methods use gradient elution. In our previous studies (*15*, *16*) we describe HPIC methods for the separation and quantitative determinations of *myo*-inositol hexakisphosphate to *myo*-inositol monophosphate ($InsP_1$−$InsP_6$) and their isomers in foods, feeds, and intestinal contents. The methods include extraction of the samples with HCl, separation of the *myo*-inositol phosphates from the crude extract by anion-exchange chromatography (i.e., solid phase extraction, SPE), separation on high-performance ion-exchange columns with gradient elution in two combined HPIC systems, and detection using either postcolumn reaction and UV detection, chemically suppressed conductivity detection, or pulsed amperometric detection. These and similar methods are particularly promising for studies in the field of physiology, when the isomeric form of the inositol phosphates is decisive and the levels of analytes are very low. For studies of food, feed, and feces samples the analyte levels are commonly in the micromolar to millimolar range. The aim of the present paper was to speed the analysis by using centrifugal ultrafiltration in the preparation of the samples instead of separating the *myo*-inositol phosphates from the crude extract by SPE. The *myo*-inositol phosphates are subsequently separated from each other on a high-performance ion-exchange column with a gradient or isocratic elution and detected according to the method of Skoglund et al. (*15*). We are also evaluating if it is possible to extract samples with a smaller volume of HCl (5 mL instead of 20 mL) than previously used to be able to detect lower amounts of inositol phosphates. The results of the development and validation of the analytical procedure are demonstrated by analyses of food and feces samples and compared with analysis using a reference method. The reference method is described by Sandberg and Ahderinne (*17*) and Sandberg et al. (*18*) and has previously been compared to the AOAC method (*4*).

MATERIALS AND METHODS

Chemicals. D-*myo*-Inositol 1,4-bisphosphate potassium salt, L-*myo*-inositol 5,6-bisphosphate, cyclohexylammonium salt, D-*myo*-inositol 1,4,5-trisphosphate, hexasodiumsalt, D-*myo*-inositol 1,5,6-trisphosphate, D-*myo*-inositol 3,4,5,6-tetrakisphosphate, D-*myo*-inositol 1,3,4,5-tetrakisphosphate, and *myo*-inositol 1,3,4,6-tetrakisphosphate, ammonium salts, were obtained from Sigma Chemical Co. (St. Louis, MO). Ins(1,2,4)P_3, Ins(1,2,3)P_3, Ins(1,2,6)P_3, L-Ins(1,3,4)P_3, L-Ins(1,2,3,4)P_4, Ins(1,2,5,6)P_4, and DL-Ins(1,2,4,5,6)P_5 were received as a gift from Perstorp Pharma (Perstorp, Sweden). *myo*-Inositol 2,4-bisphosphate tetraammonium salt was obtained from Calbiochem Corp. (La Jolla, CA). Sodium phytate was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Deionized water was purified by a Millipore water system to a specific resistance of 18 MΩ·cm or greater. All other reagents used were of analytical grade and obtained from commercial sources.

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Table 1. Comparison of Two Methods for the Determination of Phytate Content ($InsP_6$) in Various Foods and Feces[a]

sample	reference method[b]	CV (%)	HPIC method	CV (%)	HPIC method[c]	CV (%)
barley	10.92 ± 0.405	3.7	10.66 ± 0.524	4.9	9.91 ± 0.272	2.7
rolled oats	13.48 ± 0.389	2.9	12.61 ± 0.157	1.2	12.73 ± 0.079	0.6
pig feces	77.29 ± 1.478	1.9	76.09 ± 1.958	2.6		
mixed diet	0.65 ± 0.013	2.0			0.56 ± 0.031	5.5
red grapes	0.49 ± 0.049	10.0			0.63 ± 0.036	5.7
rye bread	3.96 ± 0.140	3.5	3.63 ± 0.060	1.7		
sourdough bread	1.98 ± 0.069	3.5	1.38 ± 0.055	4.0		
rye flour[d]	11.47 ± 0.353	3.1	13.02 ± 0.141	1.1		
long leavened bread	4.38 ± 0.152	3.5	3.98 ± 0.186	4.7		

[a] Values are means ± SD, μmol/g of dm, $n = 10$, for all samples except for breads and rye flour, for which $n = 6$. [b] HPLC method by Sandberg and Ahderinne (*17*) and modified by Sandberg et al. (*18*). [c] The samples were extracted with 5 mL of 0.5 M HCl. [d] Mixture of wheat flour (27%) and rye flour (73%).

Sample Collection. Rolled oats were purchased from a local grocery in Sweden (Axa, Kungsörnen AB, Sweden). Pig feces from a pig consuming a rapeseed diet were obtained from the Danish Institute of Animal Science, Department of Animal Health and Welfare, Tjele, Denmark. More detailed information about the experimental conditions of the pig studied has been given in a previous paper (*19*). The mixed diet was a low-fiber diet obtained from the Department of Clinical Nutrition and Internal Medicine II, University of Göteborg, Sahlgrenska Hospital, Göteborg, Sweden. More detailed information about the diet has been given in a previous paper (*20*). The red grapes (cv. Barlinka, South Africa) were seedless and purchased from a local grocery in Sweden. Rye bread, sourdough bread, rye flour, and long leavened bread in Table 1 were obtained from the Department of Applied Nutrition and Food Chemistry, Lund University, Lund, Sweden. The rye bread was made of 50 g of yeast, 560 g of water, 8 g of salt, 520 g of halved rye kernels, 320 g of wheat flour, and 58 g of flour of halved rye kernels. The rye bread was leavened at 38 °C and baked covered at 120 °C for 6 h. The sourdough bread was made of 50 g of yeast, 400 g of water, 258 g of sourdough, 8 g of salt, 520 g of halved rye kernels, and 230 g of wheat flour. The sourdough bread was leavened at 38 °C and baked covered at 120 °C for 6 h. The rye flour was a mixture of 27% wheat flour and 73% flour of halved rye kernels. The long leavened bread was made of 50 g of yeast, 560 g of water, 8 g of salt, 520 g of halved rye kernels, 230 g of wheat flour, and 50 g of flour of halved rye kernels. The bread was leavened for 2.5 h at room temperature and baked at 120 °C for 2.5 h followed by 30 min at 200 °C. Wheat flour (presented in Table 2) with an extraction rate of 60% (Kungsörnen AB, Sweden) was purchased in a local grocery in Sweden and incubated in 10 volumes of citrate buffer (pH 4.5) for 1 h at 55 °C. Rice and rye bread (presented in Table 2) were obtained from the Research Institute for Human Nutrition, Denmark. Wholemeal gruel, oatmeal gruel, and mild wholemeal gruels were obtained from baby food manufacturers in Sweden. Sourdough breads presented in Table 2 were obtained from the Department of Applied Nutrition and Food Chemistry, Lund University, Lund, Sweden, and contained 60% rye kernels and 40% wheat flour. Peas (*Pisum sativum* var. Jackpot) were harvested in Denmark in the summer of 1998. Barley (cv. Blenheim) was obtained from Skanska Lantmannen, Sweden. The barley samples denoted 1−12 in Table 3 were hydrothermally treated as described earlier (*21*).

Sample Preparation. Duplicate samples [0.5 g of dry matter (dm)] were extracted with 0.5 M HCl (20 or 5 mL) for 3 h at 20 °C under magnetic stirring; 0.5 mL of each sample was placed in an ultracentrifugal filter device (Microcon YM-30, Millipore, Bedford, MA) and centrifuged at 12000*g* for 15 min.

Reference Method. The sample preparation was performed according to the method of Sandberg and Ahderinne (*17*), which was modified by Sandberg et al. (*18*). Duplicate samples (0.5 g of dm) were extracted with 0.5 M HCl (20 mL) for 3 h at 20 °C under magnetic stirring. Extracts were centrifuged and the supernatants decanted, frozen overnight, thawed, and centrifuged again. An aliquot (15 mL) of supernatant was evaporated to dryness and then redissolved in 0.025 M HCl (15 mL). *myo*-Inositol phosphates were separated from the crude extract by ion exchange sample preparation according to the method of Sandberg and Ahderinne (*17*) and Graf and Dintzis (*22*).

Table 2. Comparison of Two Methods for the Determination of Phytate Content ($InsP_6$) in Various Foods and Feces[a]

sample	reference method[b]	HPIC method
barley	11.35 ± 0.375	10.34 ± 0.849
rolled oats	13.24 ± 0.535	12.54 ± 0.233
pig feces	81.25 ± 3.691	77.84 ± 2.312
wheat flour	2.37 ± 0.156	2.09 ± 0.184
rice		
parboiled paddy	7.94 ± 0.000	9.52 ± 0.346
parboiled brown	5.00 ± 0.021	5.05 ± 0.792
parboiled milled	1.98 ± 0.120	1.87 ± 0.007
paddy	9.98 ± 0.445	10.34 ± 0.940
brown	13.72 ± 0.707	15.74 ± 0.233
milled	1.97 ± 0.354	1.97 ± 0.544
gruel		
wholemeal	1.20 ± 0.014	1.46 ± 0.262
oat	1.23 ± 0.057	1.14 ± 0.057
mild wholemeal	3.49 ± 0.042	4.01 ± 0.495
mild wholemeal	1.07 ± 0.014	1.22 ± 0.163
pea	12.27 ± 0.325	13.18 ± 0.014
bread		
rye	0.92 ± 0.064	0.99 ± 0.049
sourdough	2.67 ± 0.354	2.69 ± 0.092
sourdough	4.02 ± 0.233	3.21 ± 0.955

[a] Values are means ± SD, μmol/g of dm, $n = 2$. [b] HPLC method by Sandberg and Ahderinne (*17*) and modified by Sandberg et al. (*18*).

Table 3. Comparison of Two Methods for the Determination of Phytate Content ($InsP_6$)[a]

sample	reference method[b]	HPIC[c] method
rolled oats	13.92 ± 0.0495	13.56 ± 0.191
barley		
1	9.50 ± 0.255	10.43 ± 1.230
2	10.43 ± 0.156	10.61 ± 0.276
3	6.71 ± 0.085	6.74 ± 0.170
4	5.36 ± 0.049	5.25 ± 0.099
5	9.33 ± 0.148	9.22 ± 0.028
6	6.36 ± 0.057	6.56 ± 0.106
7	5.89 ± 0.057	5.87 ± 0.035
8	5.56 ± 0.000	5.74 ± 0.148
9	5.62 ± 0.014	5.63 ± 0.071
10	4.53 ± 0.148	4.69 ± 0.177
11	4.16 ± 0.071	4.30 ± 0.078
12	2.55 ± 0.028	2.76 ± 0.163

[a] Values are means ± SD, μmol/g of dm, $n = 2$. [b] HPLC method by Sandberg and Ahderinne (*17*) and modified by Sandberg et al. (*18*). [c] HPIC with isocratic elution.

Sample Analysis. Samples were injected in 50 μL amounts with an autoinjector (Waters 717, Waters Associates Inc., Milford, MA), and $InsP_2$−$InsP_6$ and isomers of $InsP_3$−$InsP_5$ were determined using HPIC according to the method of Skoglund et al. (*15*). The chromatograph consisted of a

Table 4. Combination of Eluents for the Analysis of Inositol Bis- to Hexakisphosphates with HPIC

time (min)	1 M HCl (%)	water (%)
0	5	95
5	35	65
10	65	35
15−17	95	5
17−25	5	95

Table 5. Combination of Eluents for the Analysis of Phosphate and Free Phosphorus with HPIC

time (min)	0.2 M NaOH (%)	water (%)	50% 2-propanol (%)
0	6	92	2
20	50	48	2
21	6	92	2

biocompatible (PEEK) HPLC pump (Waters model 626) equipped with a PA-100 (50 × 4 mm i.d.) guard column (Dionex Corp., Sunnyvale, CA) and an HPIC CarboPac PA-100 (250 × 4 mm i.d.) analytical column (Dionex Corp.). $InsP_2$−$InsP_6$ were eluted with a gradient of 5−95% HCl (1 M) in conjunction with water according to Table 4. If only a value of the amount of $InsP_6$ was desired for the samples, an isocratic eluent with 80% HCl (1 M) and 20% H_2O was used. The eluants (0.8 mL/min) were mixed with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ (analytical grade, Aldrich Chemical Co. Inc.) in a 2% solution of $HClO_4$ (analytical grade, Aldrich Chemical Co. Inc.) in a postcolumn reactor according to the method of Imanari et al. (*23*) and Phillippy and Bland (*13*). The postcolumn reactor pump used was an HPLC pump, K 500 (Knauer, Berlin, Germany). The combined flow rate was 1.2 mL/min. A mixing tee (Jour Research, Onsala, Sweden) and a homemade reactor coil consisting of a crocheted Teflon tube (i.d. 0.2 mm, 4.5 m), optimized with respect to reaction time and avoidance of peak broadening, were applied to get enough reaction time and a high blending rate.

The inositol phosphates were detected after postcolumn reaction using UV detection (Waters 486, tunable absorbance detector). Absorbance was monitored at 290 nm.

Reference Method. The *myo*-inositol tris- to hexakisphosphates ($InsP_3$−$InsP_6$) were determined by ion-pair C18 reverse-phase HPLC using formic acid/methanol and tetrabutylammonium hydroxide as eluent (*17*, *18*). Injections of 20 μL were made. The HPLC system comprised an HPLC pump (Waters model 510), a C18 Kromasil (5 μm) column (2.1 mm i.d., 150 mm, EKA Nobel, Bohus, Sweden), and a refractive index detector (ERC-7510 RI-detector Erma Optical Works Ltd., Tokyo, Japan). Flow rate was 0.4 mL/min.

External Standards. External standards were prepared from phytic acid, dodecasodium salt hydrate (Aldrich Chemical Co. Inc.). This particular sodium phytate was not certified but was shown to contain less contaminating lower inositol phosphates than other phytate salts tested (phytic acid, dimagensium tetrapotassium salt, and phytic acid, dipotassium salt, Sigma-Aldrich Sweden AB). The purity with regard to lower inositol phosphates was tested by dissolving 10 mg of sodium phytate in 1 mL of water and analyzing the solution using the HPIC system. The sample contained 0.6% $InsP_5$ but no other detectable amounts of lower inositol phosphates. To determine the content of $InsP_6$, six samples (0.15 g) of sodium phytate were hydrolyzed with 5 mL of HCl (6 M) in Teflon-lid sealed Pyrex tubes first at 120 °C for 17 h and then at 140 °C for 2 h. The hydrolysates were diluted to 50 mL, then diluted 1000 times, and analyzed for free phosphate. Phosphate and free phosphorus were analyzed with HPIC on a Dionex system 4500 I (Dionex Corp., Sunnyvale, CA), equipped with a PAX 100 guard column, a PAX 100 analytical column, and a conductivity detector. A linear gradient elution was used with a gradient of 6−50% NaOH (0.2 M) in 20 min in conjunction with water and containing 2% 2-propanol (50%) according to Table 5. Free phosphorus in the unhydrolyzed sample was 0.05%. On the basis of these measurements the $InsP_6$ content was shown to be 827.6 mg/g.

Table 6. Between-Day and Within-Day Variations Estimated on a Sample of Rolled Oats for the HPIC Method with Gradient Elution[a]

	day of extraction and analysis						
run	1	2	3	4	5	6	RSD[b](%)
1	12.7	13.22	12.34	14.74	15.24	13.42	8.16
2	12.82	12	12.27	14.84	14.28	13.69	
3	12.83	11.69					
4	12.41	11.52					
5	12.55	11.8					
6	12.37	11.76					
7	12.69	11.77					
8	12.49	11.18					
9	12.61	11.22					
10	12.63	11.54					
RSD[b] (%)	1.25	4.85					

[a] Values are μmol/g of dm. [b] Relative standard deviation.

External standards were prepared containing between 0.1 and 0.8 mM $InsP_6$ dissolved in water.

Peak Identification. A reference sample for the identification of peaks was prepared by dissolving 5 g of sodium phytate in 100 mL of 0.5 M HCl. The solution was reflux boiled for 22 h and evaporated to dryness, 100 mL of water was added to the hydrolyzed sample, and it was diluted 5−10 times before being injected on the analyze system. The elution order of different isomers of inositol phosphates was established as described by Skoglund et al. (*24*).

Accuracy and Precision. The between-day precision for $InsP_6$ was determined from rolled oats prepared and analyzed in duplicate on six different days (Table 6). The within-day precision for $InsP_6$ was determined from 10 samples of rolled oats analyzed on the same day (Table 6). The precision was stated as the relative standard deviation (RSD).

Statistical Evaluation. The analyses presented in Tables 2, 3, and 7 were carried out in randomized complete block designs. The results were evaluated and fitted to linear models (eq 1) using General Linear Models (GLM, Systat 8.0, 1998, SPSS Inc., Chicago, IL). The estimated model can be described as

$$y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij} \quad (1)$$

where μ is a constant, τ expresses the effect of the methods, and β expresses the effect of the samples. The difference between the estimated model and the experimental data gives the residual, ϵ. Each sample was treated as a block, and only the effect of the methods was evaluated. A Deming method comparison test was run on the results presented in Table 2 using the computer software Analyse-It (*25*). Deming regression has been widely adopted in clinical medicine because, unlike least-squares linear regression that allows for imprecision only in the response variable (y), imprecision can be present in both variables. The Deming method comparison test computed the regression as described by Cornbleet and Gochman (*26*).

RESULTS AND DISCUSSION

The HPIC method described for the analysis of inositol phosphates illustrates the usefulness of centrifugal ultrafiltration for rapid and simple sample preparation.

The methods previously developed at our laboratory (*15*, *17*, *18*) for the analyses of inositol phosphates include a cleanup step with SPE during sample preparation. The present method, however, shows that omitting this step and instead using filtration of 0.5 mL of extract with a centrifuge filter (Microcon YM-30) gives comparable results in the food and feces samples studied so far (Tables 1−3 and 7). The use of centrifugal ultrafiltration in the preparation of the samples is a

Table 7. Comparison of Extracting Samples in 5 mL or 20 mL of 0.5 M HCl before HPIC Determination of Phytate[a]

sample	5 mL	20 mL
barley	10.34 ± 0.849	10.05 ± 0.156
rolled oats	12.54 ± 0.233	12.08 ± 0.799
pea	12.97 ± 0.318	12.46 ± 1.082

[a] Values are means ± SD, μmol/g of dm, $n = 2$.

much simpler, faster, and milder treatment of the samples as compared to the use of evaporation and ion exchange sample preparation. The harsh treatment of evaporation and use of strong acid may also cause redistribution of the phosphate groups on the *myo*-inositol ring and alter the isomer balance. This has been observed in our laboratory in samples treated with various strains of yeast (unpublished results). Samples that do not need to be extracted with an acid (i.e., extracellular media from cells) can be ultracentrifuged directly. We used the centrifugal filter device Microcon YM-30 with a nominal molecular weight limit of 30 kDa to remove enzymes from the samples before injection onto the analysis system. Phytases from cereal and legume sources have molecular masses of ~59−68 kDa (*27*−*30*), and phytases from microbial origin have molecular masses of ~40 kDa (*31*, *32*).

It is possible to replace the anion exchange sample preparation step with centrifugal ultrafiltration when using UV detection because only compounds reacting with $Fe(NO_3)_3$ in the postcolumn and having UV absorbance near 290 nm will be detected (*23*), whereas the refractor detection used in the reference HPLC method detects all compounds that change the refractor index. Hence, the sample injected on the HPLC system of the reference method must be very clean because there is a high possibility that other compounds will disturb the detection of the inositol phosphates, in particular the detection of lower inositol phosphates. Moreover, in the HPIC system, the inositol phosphates adhere firmly to the analytical column while other compounds are quickly eluted. Therefore, the cleaning anion chromatographic step that is used in the sample preparation step in the reference HPLC method is achieved in the analysis step in the HPIC method.

Shown in Table 1 are the phytate contents of samples analyzed in 10 or 6 replicates with the reference HPLC method (*17*, *18*) and the HPIC method with gradient elution further developed in this study. In addition, the phytate contents of samples analyzed using the HPIC method but being extracted with only 5 mL of HCl (0.5 M) are shown (Table 1). The methods gave similar results for $InsP_6$ concentrations, and the CV for all methods was generally low. In Table 2 the phytate contents in 18 various samples analyzed in duplicate with the reference HPLC method and the HPIC method are shown. There was no significant difference between analytical methods (GLM, $P > 0.05$). The agreement between methods was also evaluated by running a Demings method comparison test on the results in Table 2. The Deming regression plot is shown in Figure 1. The two methods are in good agreement over the range of phytate contents that are of interest when various foods are analyzed. The pig feces sample (Table 2) was omitted from the regression because the phytate content of this sample was much higher than the phytate content of the other samples and it would thus have too much of an impact on the regression result, which could, for example, mask curvatures in the lower domain.

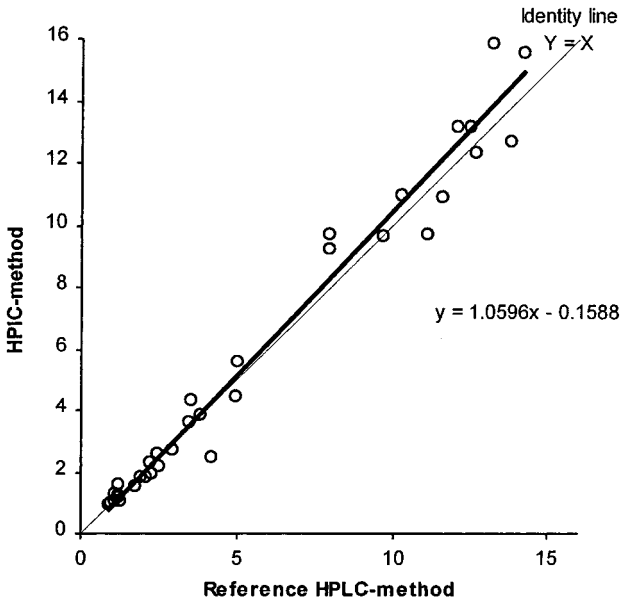

Figure 1. Comparison of the reference HPLC method and the investigated HPIC method with gradient elution for measuring phytate content in various foods: identity line, $y = x$ (—); correlation line (−). Each data point (○) represents the measured value for both methods, presented in Table 1 as mean values.

Extracting samples in 5 or 20 mL of HCl (0.5 M) showed no significant difference (GLM, $P > 0.05$, Table 7). It is valuable to be able to extract samples low in phytate with only 5 mL of HCl (0.5 M) so that it is possible to detect the low amounts of phytate without concentration of the samples by evaporation, which is a harsh treatment for the samples. However, in some cases evaporation of samples has a positive impact on the sample performance during chromatography. Misshapen peaks caused by proteins bound to the inositol phosphates in the samples, for example, can be overcome by evaporating the samples, redissolving them in water, and ultrafiltration. This has especially been seen with certain isomers of $InsP_3$ and $InsP_4$.

Comparing the concentration of $InsP_6$ using the reference HPLC method with the HPIC method with isocratic elution (Table 3) showed that there was no significant difference between methods (GLM, $P > 0.05$). When isocratic elution is used, the chromatographic run takes 5 min and no conditioning is needed, but only a value of $InsP_6$ is attained. Hence, if a lot of samples need to be scanned for phytate, the HPIC method with isocratic elution described in this study is recommended. If the concentration of $InsP_2$−$InsP_6$ and isomers of $InsP_3$−$InsP_5$ is desired, the HPIC method with gradient elution described here can be used. To analyze the isomers of $InsP_2$ the method previously described by us (*24*) can be used, and, finally, to analyze the isomers of $InsP_1$−$InsP_2$, one of the methods described by us in Skoglund et al. (*15* or *16*) can be used.

The most common food samples to be analyzed for phytate content are cereals and legumes. The raw material of such samples normally contains between 11 and 15 μmol/g inositol hexakisphosphate ($InsP_6$). During food processing $InsP_6$ can be degraded to lower inositol phosphates, and in processed samples the content of $InsP_6$ may vary from 0 to 15 μmol/g of dm. With the HPIC method tested in this study it is possible to detect and quantify inositol hexakisphosphate contents in samples as low as 5 μM (*15*), which corresponds to 0.2

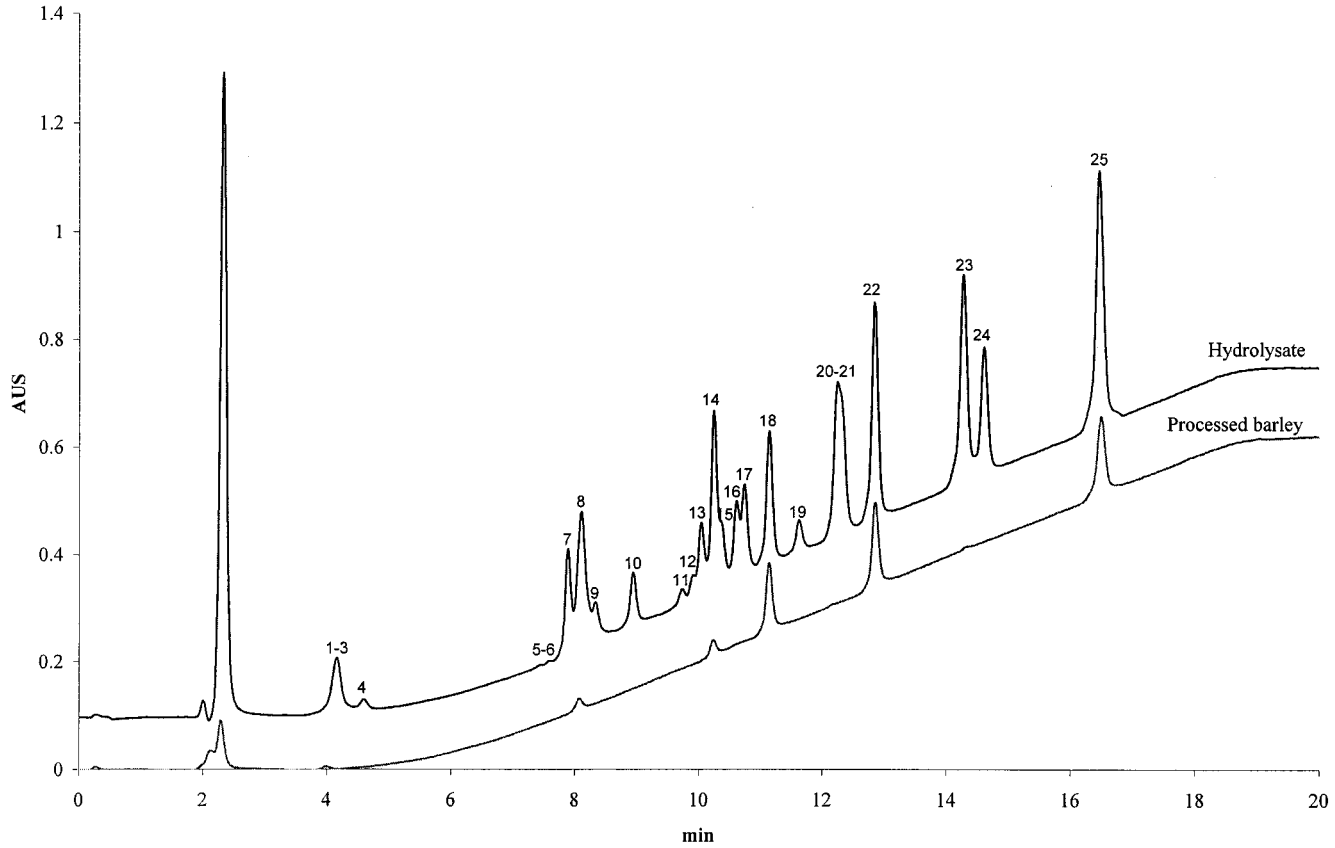

Figure 2. Chromatographic profiles of hydrolyzed sodium phytate and a processed barley sample. The peaks are numbered in accordance with Sandberg et al. (*38*). Peaks: 1−3, $InsP_2$; 4, $InsP_2$; 5 and 6, unidentified; 7, DL-Ins(1,2,4)P_3, DL-Ins(1,3,4)P_3, and Ins(1,2,3)P_3; 8, DL-Ins(1,2,6)P_3 and Ins(1,2,3)P_3; 9, DL-Ins(1,4,5)P_3; 10, DL-Ins(1,5,6)P_3; 11, DL-Ins(4,5,6)P_3; 12, Ins(1,2,3,5)P_4; 13, DL-Ins(1,2,4,6)P_4; 14, DL-Ins(1,2,3,4)P_4; 15, Ins(1,3,4,6)P_4; 16, DL-Ins(1,2,4,5)P_4; 17, DL-Ins(1,3,4,5)P_4; 18, DL-Ins(1,2,5,6)P_4; 19, Ins-(2,4,5,6)P_4; 20, DL-Ins(1,4,5,6)P_4; 21, Ins(1,2,3,4,6)P_5; 22, DL-Ins(1,2,3,4,5)P_5; 23, DL-Ins(1,2,4,5,6)P_5; 24, Ins(1,3,4,5,6)P_5; 25, $InsP_6$.

μmol/g of dm if an extraction volume of 20 mL is used and to 0.05 μmol/g of dm if an extraction volume of 5 mL is used, when 50 μL of sample is injected. Samples that contain <0.05 μmol of $InsP_6$ can be concentrated by evaporation, and a larger injection volume can be used. The linearity of $InsP_6$ was demonstrated by plotting peak areas versus the concentration of different dilutions of standard preparations from 0.1 to 0.8 mM $InsP_6$. Linear regression analysis gave a coefficient of determination (R^2) of 1.0. Parts in the autoinjector used in the HPIC system (717, Waters) not exchanged to PEEK were gradually corroded by the strong acid used in the gradient eluent, and the corrosion was accentuated when the isocratic eluent was used. All stainless steel in the autoinjector needs to be exchanged to PEEK. If this is done, the linearity of the system would probably be extended so that it is possible to have linearity below 0.1 μM on an injection volume of 50 μL.

The HPIC method separates $InsP_3$−$InsP_6$ into specific isomers, and $InsP_2$ are detected in two peeks. This is apparently an advantage if it is desired to know the isomeric composition and balance in a sample, but quantitatively this makes the HPIC method less sensitive for $InsP_3$−$InsP_5$ compared to the HPLC method. However, in most food, feed, and feces samples the $InsP_6$ has been degraded enzymatically, yielding only a few specific isomers of each inositol phosphate (*15*), and the sensitivity is thus not greatly affected.

The methods presented here are highly accurate (Table 6) and selective and have a high throughput of samples. Many studies have been carried out to speed the sample preparation. Lehrfeld (*33*) has previously shown that it is possible to omit the first evaporation step in the sample preparation used in the reference HPLC method by diluting the samples with water before applying them onto the anion exchange sample preparation columns. Talamond et al. (*34*) presented a rapid and sensitive chromatographic method for the determination of $InsP_6$ in food using conductivity detection. In this method the anion exchange sample preparation step was avoided but not the evaporation step. The separation on HPIC was performed by gradient elution using 200 mM NaOH, water, and 2-propanol as solvents. However, when samples containing $InsP_1$−$InsP_6$ were analyzed at our laboratory according to the method described by Talamond et al. (*34*), the $InsP_6$ peak was interfered with by other inositol phosphates, for example, Ins(1,2,6)P_3. When NaOH is used as an eluent solvent at the high levels used by Talamond et al. (*34*), the pH is very high and the hydroxyl groups of the lower inositol phosphates are charged and as firmly adhered to the column particles as $InsP_6$, making them coelute with $InsP_6$. The ion chromatographic methods of Cilliers and van Niekerk (*35*) and Rounds and Nielsen (*36*) use extraction, centrifugation, and filtration to prepare plant, food, or soil samples. In these methods the ion exchange step is also omitted. In the present study we use centrifugal ultrafiltration, which is more efficient than conventional filtration. The method of Rounds and Nielsen (*36*) separates $InsP_2$−$InsP_6$ in a chromatographic run of 30 min, whereas our method separates $InsP_2$−$InsP_6$ and the isomers of $InsP_3$−$InsP_5$ in a chromatographic run of 17 min (Figure 2). The separation between some isomers, for example, DL-Ins(1,2,4,5,6)-

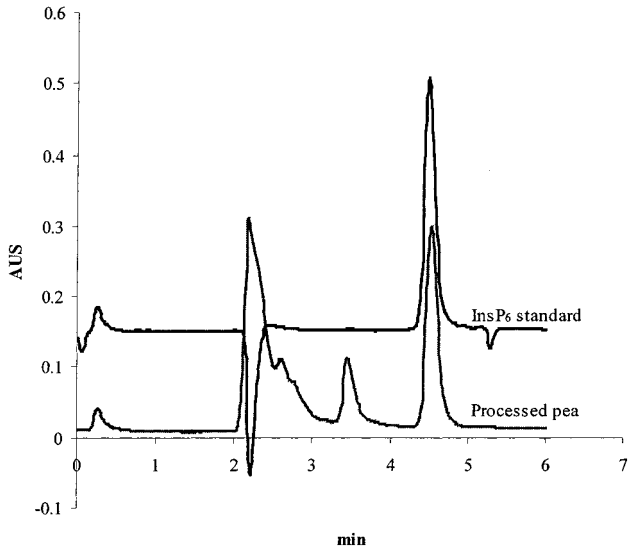

Figure 3. Chromatographic profiles of an $InsP_6$ standard and a processed pea sample analyzed with the HPIC system using isocratic elution.

P_5 and Ins(1,3,4,5,6)P_5 (peaks 23 and 24 in Figure 2), DL-Ins(1,4,5,6)P_4 and Ins(1,2,3,4,6)P_5 (peaks 20 and 21 in Figure 2), DL-Ins(1,2,4,5)P_4 and DL-Ins(1,3,4,5)P_4 (peaks 16 and 17 in Figure 2), and DL-Ins(1,2,3,4)P_4 and Ins(1,3,4,6)P_4 (peaks 14 and 15), is somewhat impaired when the faster gradient presented here is used compared to the gradient used in Skoglund et al. (*15*) when using the PA-100 column that we used here (*37*). However, most samples contain only a few specific isomers of each inositol phosphate, eliminating this problem. We have also shown that an isocratic run of 5 min (Figure 3) gives a reliable value of $InsP_6$.

ABBREVIATIONS USED

cv., cultivar; HPIC, high-performance ion chromatography; HPLC, high-performance liquid chromatography; $InsP_1$−$InsP_6$, inositol mono- to hexakisphosphate; PEEK, polyether ether ketone; RSD, relative standard deviation; SPE, solid phase extraction.

LITERATURE CITED

(1) Nävert, B.; Sandström, B.; Cederblad, Å. Reduction of the phytate content of bran by leavening in bread and its effect on absorption of zinc in man. *Br. J. Nutr.* **1985**, *53*, 47−53.

(2) Hallberg, L.; Rossander, L.; Skånberg, A.-B. Phytates and the inhibitory effect of bran on iron absorption in man. *Am. J. Clin. Nutr.* **1987**, *45*, 988−996.

(3) Kivistö, B.; Cederblad, Å.; Davidsson, L.; Sandberg, A.-S.; Sandström, B. Effect of meal composition and phytate content on zinc absorption in humans from an extruded bran product. *J. Cereal Sci.* **1989**, *10*, 189−197.

(4) Brune, M.; Rossander-Hulthén, L.; Hallberg, L.; Gleerup, A.; Sandberg, A.-S. Iron absorption from bread in humans: Inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J. Nutr.* **1992**, *122*, 442−449.

(5) Weaver, C. M.; Heaney, R. P.; Proulx, W. R.; Hinders, S. M.; Packard, P. T. Absorbability of calcium from common beans. *J. Food Sci.* **1993**, *58*, 1401−1403.

(6) Cosgrove, D. J. 11. Intermediates in the dephosphorylation of P6-inositols by phytase enzymes. In *Inositol Phosphates: Their Chemistry, Biochemistry, and Physiology*; Elsevier Scientific Publishing: Amsterdam, The Netherlands, 1980; pp 99−117.

(7) Oberleas, D.; Harland, B. F. Analytical methods for phytate. In *Phytic Acid: Chemistry and Applications*; Graf, E., Ed.; Pilatus Press: Minneapolis, MN, 1986.

(8) Reddy, N. R.; Pierson, M. D.; Sathe, S. K.; Salunkhe, D. K. Methods for analysis of phytate. In *Phytate in Cereals and Legumes*; CRC Press: Boca Raton, FL, 1989.

(9) Xu, P.; Price, J.; Aggett, P. J. Recent advances in methodology for analysis of phytate and inositol phosphates in foods. *Prog. Food Nutr. Sci.* **1992**, *16*, 245−262.

(10) Sandberg, A.-S. *Determination of Pytic Acid. COST 92 Metabolic and Physiological Aspects of Dietary Fibre in Food. Recent Progress in the Analysis of Dietary Fibre*; European Commission: Copenhagen, Denmark, 1995.

(11) Phillippy, B. Q.; Johnston, M. R. Determination of phytic acid in foods by ion chromatography with post-column derivatization. *J. Food Sci.* **1985**, *50*, 541−542.

(12) Mayr, G. W. A novel metal-dye detection system permits picomolar-range HPLC analysis of inositol phosphates from non-radioactively labelled cell or tissue specimens. *Biochem. J.* **1988**, *254*, 585−591.

(13) Phillippy, B. Q.; Bland, J. M. Gradient ion chromatography of inositol phosphates. *Anal. Biochem.* **1988**, *175*, 162−166.

(14) Franz, H.; Maier, H. G. Inositolphosphate in Kaffee und Kaffeemitteln. I. Identifizierung und Bestimmungsmethode. *Dtsch. Lebensm.-Rundsch.* **1993**, *89* (9), 276−282.

(15) Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. *J. Agric. Food Chem.* **1997**, *45*, 431−436.

(16) Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. Analysis of inositol mono- and diphosphate isomers using high-performance ion chromatography and pulsed amperometric detection. *J. Agric. Food Chem.* **1997**, *45*, 4668−4673.

(17) Sandberg, A.-S.; Ahderinne, R. HPLC method for determination of inositol tri-, tetra-, penta-, and hexaphosphates in foods and intestinal contents. *J. Food Sci.* **1986**, *51* (3), 547−550.

(18) Sandberg, A.-S.; Carlsson, N.-G.; Svanberg, U. Effects of inositol tri-, tetra-, penta-, and hexaphosphates on *in vitro* estimation of iron availability. *J. Food Sci.* **1989**, *54* (1), 159−161, 186.

(19) Sandberg, A.-S.; Larsen, T.; Sandström, B. High dietary calcium level decreases colonic phytate degradation in pigs fed a rapeseed diet. *Nutr. Req. Interact.* **1993**, *123*, 559−566.

(20) Sandberg, A.-S.; Ahderinne, R.; Andersson, H.; Hallgren, B.; Hulthén, L. The effect of citrus pectin on the absorption of nutrients in the small intestine. *Human Nutr.: Clin. Nutr.* **1983**, *37C*, 171−183.

(21) Bergman, E.-L.; Fredlund, K.; Reinikainen, P.; Sandberg, A.-S. Hydrothermal processing of barley (cv. Blenheim): Optimisation of phytate degradation and increase of free *myo*-inositol. *J. Cereal Sci.* **1999**, *29*, 261−272.

(22) Graf, E.; Dintzis, F. R. Determination of phytic acid in foods by high-performance liquid chromatography. *J. Agric. Food Chem.* **1982**, *30*, 1094−1097.

(23) Imanari, T.; Tanabe, S.; Toida, T.; Kawanishi, T. High-performance liquid chromatography of inorganic anions using Fe^{3+} as a detection reagent. *J. Chromatogr.* **1982**, *250*, 55−61.

(24) Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. High-performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns. *J. Agric. Food Chem.* **1998**, *46*, 1877−1882.

(25) Analyse-It Software Ltd. *Analyse-It for Microsoft Excel*; Leeds, U.K., 2000.
(26) Cornbleet, P. J.; Gochman, N. Incorrect least-squares regression coefficients in method comparison analysis. *Clin. Chem*. **1979**, *25* (3), 432−438.
(27) Gibson, D. M.; Ullah, A. H. J. Purification and characterization of phytases from cotelydons of germinating soybean seeds. *Arch. Biochem. Biophys.* **1988**, *260* (2), 503−513.
(28) Konietzny, U.; Greiner, R.; Jany, K.-D. Purification and characterization of a phytase from spelt. *J. Food Biochem*. **1995**, *18*, 165−183.
(29) Greiner, R.; Konietzny, U.; Jany, K. D. Purification and properties of a phytase from rye. *J. Food Biochem*. **1998**, *22*, 143−161.
(30) Greiner, R.; Jany, K.-D.; Alminger, M. Identification and properties of *myo*-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). *J. Cereal Sci.* **2000**, *31*, 127−139.
(31) Greiner, R.; Konietzny, U.; Jany, K. D. Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys.* **1993**, *303* (1), 107−113.
(32) Greiner, R.; Haller, E.; Konietzny, U.; Jany, K.-D. Purification and characterization of a phytase from *Klebsiella terrigena*. *Arch. Biochem. Biophys.* **1997**, *341* (2), 201−206.
(33) Lehrfeld, J. High-performance liquid chromatography analysis of phytic acid on a pH-stable, macroporous polymer column. *Cereal Chem*. **1989**, *66* (6), 510−515.
(34) Talamond, P.; Gallon, G.; Treche, S. Rapid and sensitive liquid chromatographic method using a conductivity detector for the determination of phytic acid in food. *J. Chromatogr. A* **1998**, *805* (1/2), 143−147.
(35) Cilliers, J. J. L.; van Niekerk, P. J. LC determination of phytic acid in food by postcolumn colorimetric detection. *J. Agric. Food Chem*. **1986**, *34*, 680−683.
(36) Rounds, M. A.; Nielsen, S. S. Anion-exchange high-performance liquid chromatography with post-column detection for the analysis of phytic acid and other inositol phosphates. *J. Chromatogr. A* **1993**, *653*, 148−152.
(37) Skoglund, E. Inositol phosphates. Ion chromatography methods, intestinal degradation and influence on iron bioavailability. Ph.D. thesis, Department of Food Science, Chalmers University of Technology, Göteborg, Sweden, 1998; pp 38−40.
(38) Sandberg, A.-S.; Brune, M.; Carlsson, N.-G.; Hallberg, L.; Skoglund, E.; Rossander-Hulthén, L. Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 240−246.

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