Problems Associated with Measuring Phytate in Infant Cereals†

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The inositol hexaphosphate (IP6) content of commercially available dried infant cereals was measured by ion pair high-pressure liquid chromatography (ion pair HPLC) and ion exchange high-pressure liquid chromatography (ion exchange HPLC). Large differences between methods were apparent: ion pair HPLC gave values 14 to 190-fold lower than the values from ion exchange HPLC. Poor recoveries of added IP6 (25 to 60%) by ion pair HPLC suggested that some component of the infant cereal was responsible for the difference. Further experimentation suggested that an excess of minerals (approximately 11 mg/g calcium and 0.3 mg/g iron) in these samples sequestered the endogenously low phytate content. This problem may be unique to samples with low IP6 and high mineral content as wheat bran was not problematic. These results suggest that ion exchange HPLC is the method of choice for measuring inositol phosphates in infant cereals.

Keywords: *Phytate; infant cereals; HPLC; minerals*

INTRODUCTION

Phytic acid (IP6) is a hexaphosphate-substituted inositol ring compound that, in the deprotonated form, has a high affinity for divalent minerals such as calcium, magnesium, zinc, iron, and cobalt (*1* and *2*). The Ca/Mg salt of IP6 is a naturally occurring phosphate storage compound found in plants. Phytic acid binds minerals in the gastrointestinal tract, potentially making them biologically unavailable. As evidence of this, IP6 compromises the zinc status and growth rate of many species (*3* and *4*). The importance of IP6 in altering mineral status has led to guidelines for ensuring adequate zinc bioavailability (*5*−*8*).

The potentially detrimental effect of IP6 on mineral status and growth has led to the development of several methods to measure its content in foods. These methods range from colorimetric and chromatographic procedures to NMR spectroscopy and capillary isotachophoresis (*9*−*18*). Some of the early spectrophotometric methods could measure only total inositol phosphates and, therefore, overestimated the IP6 content of foods (*10* and *19*). In these methods, inositol hexaphosphate was measured along with inositol penta-, tetra-, tri-, di-, and mono-phosphates. Modern methods use high-pressure liquid chromatography (HPLC) to fractionate the inositol phosphates into their components, allowing accurate determination of most (or all) inositol phosphates (*12*, *15*, *16*). Although the HPLC methods produce identical results with wheat bran and purified inositol phosphates, no direct comparison between methods has been undertaken with a specific food group.

The present study compared two common HPLC methods with respect to their ability to measure concentrations of inositol phosphates in commercially available dry infant food cereals. Ion pair HPLC makes use of reversed-phase chromatography in the presence of counterions to alter the partition coefficient of the compound of interest. Ion exchange chromatography uses a column with fixed cationic charges to bind the highly anionic IP6 and its lower phosphate forms. A salt gradient is used to elute these components separately from the column. We focused on infant cereals for the following reasons. First, the potential for zinc and iron deficiencies exists with high IP6 diets. This is of special concern to infants because zinc deficiency has been associated with low growth rates (*3* and *4*), and iron deficiency can affect many biochemical and physiological processes (*20*). Second, IP6 is often associated with whole grain products because it is localized in the aleurone cell layer. It should be noted, however, that whole grain products do not always contain high amounts of IP6 because processing has been shown to reduce IP6 content either through milling to remove the aleurone cell layer (*10*) or heat treatment in the presence of active phytases (*10*, *21*, and *22*). Third, there has been a recent trend toward recommending increased fiber intake in infants (*23*) to levels similar to those in some European countries (*24*). And finally, the widespread use of soy-based cereals is of concern because phytate is typically high in soy products (*25*).

MATERIALS AND METHODS

Dried infant cereals were purchased from local food and drug stores in communities around Ottawa, Ontario, during February and March 1998. The cereals were stored at room temperature until analyzed. Sodium IP6 (dodecasodium salt hydrate from corn) and cation-exchange resin AG 50W0 × 4 50−100 mesh H^+ were purchased from Sigma Chemical Co. (St. Louis, MO). The purity of the sodium IP6 was assessed by NMR spectroscopy and the IP6 concentration was verified by total phosphorus analysis after complete ashing of the sample (*26*). Silica-based anion exchange columns (3 cm^3 SAX columns) were purchased from Varian (Harbor City, CA). Classic Sep-Pak columns (360 mg of sorbent) and Classic Accel Plus QMA columns (360 mg of sorbent) were obtained from Waters Limited (Mississauga, ON). 8-Hydroxyquinoline was from Aldrich Chemicals (Mississauga, ON). Water was purified by a Barnstead NANOpure apparatus. Nitric acid and HCl

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Ion Exchange HPLC. The method is based on that described by Rounds and Nielsen (*15*). Approximately 0.4 g of infant cereal was accurately weighed into a 40-mL, round-bottom Teflon centrifuge tube. Fat interferes with the chromatography so the samples were extracted first with 35 mL of 10% ethyl ether in hexane by mixing in a rocking-type shaker (Reliable Scientific, obtained from Diamed Lab Supplies, Inc., Mississauga, ON) for 20 h in a 37 °C incubator. Hexane and sample were separated by centrifugation at 3000*g* for 10 min. The majority of the hexane was aspirated and the sample was washed with 35 mL of hexane and recentrifuged. The majority of the hexane was again aspirated, and the remaining solvent was removed by drying under a steam of nitrogen gas at room temperature. The dried sample was resuspended in 4 mL of 0.61 M tricholoracetic acid (10% w/v TCA) and sonicated for 1.5 min at 200 W. A 2-mL aliquot was removed and centrifuged at 17 000*g* in a refrigerated microfuge. The supernatant was filtered through a 0.45-μm poly(tetrafluoroethylene) syringe filter (Chromatographic Specialities, Brockville, ON) and injected directly into the HPLC system.

HPLC analysis was performed using Waters HPLC pumps (models 510 and 590), a Waters 717+ autosampler connected to a PL-SAX 50 × 2.1 mm, 8 μm column (Bodman Industries, Aston, PA) and a Waters 490 multiwavelength detector as described by Rounds and Nielsen (*15*). Where possible, PEEK plastic tubing was used to help reduce problems of IP6 adsorption to stainless steel (*16*). Data were collected and analyzed using Waters Millennium software (version 2.10). Chromatography was performed at ambient temperature using a linear gradient mobile phase of 100% Buffer A (0.1 M methyl piperazine adjusted to pH 4.0 with 1 M HNO_3) to 100% Buffer B (0.5 M $NaNO_3$ in 0.1 M piperazine adjusted to pH 4.0 with 1 M HNO_3). Gradients were run for 28 min at a flow rate of 1.0 mL/min. The column was re-stabilized post chromatography by washing with Buffer B (2 min) and Buffer A (3 min). Inositol phosphates were monitored by adding Wade's reagent (0.74 mM ferric chloride and 6.87 mM 5-sulfosalicylic acid, adjusted to pH 1.8 with 1 M HNO_3) to the column eluant at a constant rate of 0.5 mL/min and mixed by passing through a PEEK 13 × $^1/_{16}$ OD × 0.02 ID in. spiral link tube (Upchurch Scientific, Oak Harbor, WA; *15*). Baseline noise was reduced by adding a stainless steel pulse damper (Scientific Systems Inc., State College, PA). Decreases in absorbance were measured at 500 nm. Injection volumes were 50 μL.

Linear standard curves ($r^2 \geq 0.99$) were generated by injecting 16.7 to 50 μg of commercially available IP6 in 50 μL of 10% TCA. The system was also calibrated for the relative (to IP6) response of inositol penta- (IP5), tetra- (IP4), and tri- (IP3) phosphates by preparing solutions containing different concentrations of IP6, IP5, IP4, and IP3 (*16*) and analyzing the chromatograms by multiple regression. Specifically, 1.5 g of IP6 was dissolved in 7.5 mL of water and placed onto a 20-mL column of cation-exchange resin 50W-X8 H^+ to produce the acid form of IP6. Phytic acid was then eluted with 20 mL of water and boiled or autoclaved for various times to produce mixtures of partially hydrolyzed products. The concentrations of IP6, IP5, IP4, and IP3 varied with length of boiling or autoclaving. Final analysis gave relative (to IP6) response factors ($n = 20$ samples; regression parameter ± standard error) of 0.70 ± 0.07 (IP5), 0.68 ± 0.16 (IP4), and 1.39 ± 0.20 (IP3).

Ion pair HPLC. Unless otherwise stated, sample preparation and chromatography were performed essentially as described by Lehrfeld (*16*). Specifically, 0.4 g of infant cereal was accurately weighed into a 40-mL, round-bottom Teflon centrifuge tube. To reduce the fat content, the samples were extracted with 30 mL of hexane by heating in a 40 °C shaking water bath for 15 min. Hexane and sample were separated by centrifugation at 3000*g* for 5 min. The majority of the hexane was aspirated, and the remaining solvent was removed by drying under a steam of nitrogen gas at room temperature. The dried sample was resuspended in 4 mL of 0.5 N HCl and

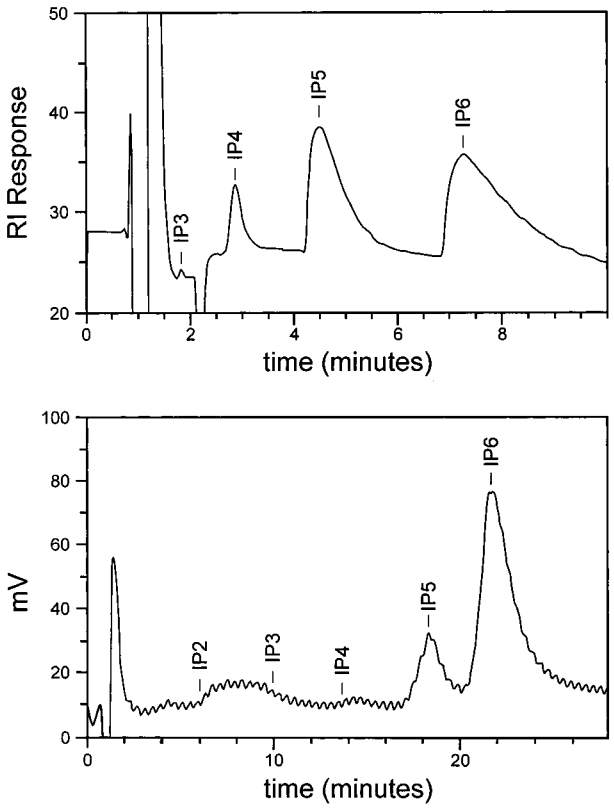

Figure 1. Ion pair HPLC (top) and ion exchange HPLC (bottom) of oatmeal infant cereal (Brand A). Chromatographic procedures were as described in the Materials and Methods section. Peak locations were assigned using partially hydrolyzed phytic acid samples. Top figure shows refractometer response as a function of time. The peaks represent approximately 16 μg IP6, 20 μg IP5, 10 μg IP4, and 2 μg IP3. Bottom figure shows mV output from the absorbance detector as a function of time. The peaks represent approximately 40 μg IP6 and 13 μg IP5. The reported amounts have been corrected for the relative refractometer or spectrophotometer response.

sonicated for 1.5 min at 200 W. The sample was then diluted to 40 mL with water and centrifuged at 17 000*g* for 30 min. The supernatant was decanted directly onto a silica-based anion exchange column pre-washed 3 times with 2 mL of water. The column was washed twice with 2 mL of H_2O and the inositol phosphates were eluted with 2 mL of 2 N HCl. The IP6-containing HCl eluant was collected in a glass test tube and dried under nitrogen gas at 40 °C. The residue was reconstituted by adding 2 mL of water and placing the test tube into a sonicating bath for 6 min. The sample was then centrifuged at 17 000*g* for 10 min to remove particulates and injected directly into the HPLC system.

High-pressure liquid chromatographic analysis was performed using a Waters pump model 510, a Waters 717+ autosampler connected to a macroporous polymer HPLC column PRP-1, 5 μm (150 × 4.1 mm; Hamilton Co., Reno, NV), and a Waters 410 differential refractometer. Data were collected and analyzed using Waters Millennium software (version 2.10). The mobile phase was prepared by first mixing 440 mL of water and 560 mL of acetonitrile. To this mixture was added 0.855 mL of 88% formic acid, 10 mL of tetrabutylammonium hydroxide, and 0.2 mL of 5 mg/mL phytic acid (prepared by cation exchange chromatography; *16*). The pH was adjusted to 4.3 with 72% sulfuric acid. Injection volumes were 100 μL, the flow rate was 1.5 mL/min, and the column and refractometer temperatures were 45 °C. Phytic acid eluted after 5 to 7 min; the exact elution time varied with the total amount (mg) injected. The conditions were selected to separate the negative peak associated with injection from the IP4 peak (see Figure 1, top). Linear standard curves ($r^2 \geq 0.99$) were

Table 1. Inositol Phosphate Content of Selected Commercial Infant Cereals as Measured by Two HPLC Methods

description	method	mg/100 g dry weight[a] IP3	IP4	IP5	IP6
barley (A)[b]	ion pair HPLC	52 ± 35	39 ± 18	50 ± 18	46 ± 27
	ion exchange HPLC	N. D.[c]	N. D.	232 ± 32	627 ± 71
oatmeal (A)	ion pair HPLC	58 ± 43	54 ± 23	51 ± 29	40 ± 33
	ion exchange HPLC	N. D.	N. D.	272 ± 23	799 ± 183
mixed cereal with fruit (B)	ion pair HPLC	76 ± 41	50 ± 11	50 ± 27	39 ± 36
	ion exchange HPLC	N. D.	N. D.	243 ± 43	617 ± 33
mixed cereal (B)	ion pair HPLC	108 ± 97	52 ± 26	35 ± 29	37 ± 24
	ion exchange HPLC	N. D.	N. D.	245 ± 31	641 ± 39
rice cereal, whole grain (C)[d]	ion pair HPLC	N. D.	6	38	16
	ion exchange HPLC	N. D.	N. D.	232	850
rice cereal (A)	ion pair HPLC	52 ± 50	2 ± 4	N. D.	N. D.
	ion exchange HPLC	N. D.	N. D.	N. D.	366 ± 28
mixed cereal (A)	ion pair HPLC	71 ± 45	13 ± 7	N. D.	N. D.
	ion exchange HPLC	N. D.	N. D.	227 ± 43	470 ± 25
soya based cereal (D)	ion pair HPLC	55 ± 32	13 ± 12	7 ± 10	N. D.
	ion exchange HPLC	N. D.	N. D.	247 ± 43	890 ± 57

[a] Corrected for water content. [b] Letters denote different brands. [c] N. D., below detection limit for the method. [d] Values represent means ± SD for $N = 4$ different lot numbers (2 determinations), except for rice cereal, whole grain ($N = 1$).

generated by injecting 6−23.5 μg of commercially available IP6 in 20 μL of water. The correction factors for the relative (to IP6) responses of IP5, IP4, and IP3 were 1.1, 1.5, and 2.4, respectively (*13*).

RESULTS

Comparison Between Ion Exchange HPLC and Ion Pair HPLC. The IP6 content of American Association of Cereal Chemists (AACC) hard red spring wheat bran was identical when measured by ion pair chromatography (4.2% w/w) or by ion exchange chromatography (4.2% w/w). These values compared favorably with the value of 3.95% obtained by Lehrfeld (*27*) and the value of 3.8% reported by Graf and Dintizis (*28*).

Table 1 presents the IP3, IP4, IP5, and IP6 content of commercially available infant cereals as measured by ion pair HPLC and by ion exchange HPLC. Large differences in IP3, IP4, and IP5 content were apparent when the two methods were compared. The ion exchange HPLC method gave IP5 values that were 5−33-fold higher than those observed by the ion pair HPLC method, and the IP6 values were 14−190-fold higher when measured by the ion exchange HPLC method. IP3 and IP4 peaks were not detected by the ion exchange HPLC method (Figure 1, bottom) but were clearly visible in the ion pair HPLC chromatograms (Figure 1, top).

The total inositol phosphate content of the infant cereals was also compared against published literature values (*25*) to provide an external check of the method performance. Because the AOAC spectrophotometric method (*25*) measures total inositol phosphates as IP6, some overestimation of IP6 occurs (*10* and *20*). This was apparent when ion pair HPLC and ion exchange HPLC determined IP6 values were compared with spectrophotometrically determined IP6 values for similar cereals (Table 2). However, the total inositol phosphate content as measured by ion exchange HPLC was similar to the spectrophotometrically determined IP6 values, whereas the total inositol phosphate content measured by ion pair HPLC was 5−18-fold lower.

Investigation of Ion Pair HPLC Methodology as Applied to Infant Foods. *IP6 Recovery*. The initial analyses relied exclusively on ion pair HPLC, but a rigorous validation process was conducted because the procedure gave values that were consistently lower than those found in the literature (see Table 2). IP6 recoveries were tested by adding pure IP6 or a mixture of inositol

Table 2. Total Inositol Phosphates in Selected Infant Cereals: Comparison with Literature Values

cereal description	total mg inositol phosphates/100 g dry weight[a] ion exchange HPLC	ion pair HPLC	Harland and Oberleas, 1987
barley (A)[b]	858	187	1000
oatmeal (A)	1071	204	1000
mixed cereal with fruit (B)	860	214	N. A.[c]
mixed cereal (B)	886	232	809
rice cereal, whole grain (C)[d]	1082	61	N. A.
rice cereal (A)	366	55	980
mixed cereal (A)	698	93	809
soya based cereal (D)	1138	80	N. A.

[a] Corrected for water content. [b] Letters denote different brands. [c] N. A., values not available. [d] Values represent means ± SD for $N = 4$ different lot numbers (2 determinations), except for rice cereal, whole grain ($N = 1$).

phosphates (produced by hydrolysis; *16*) to barley, oatmeal, rice, and mixed cereals (Table 3). This procedure does not test the efficacy of the extraction process but rather the recovery of inositol phosphates in the subsequent steps. The recoveries of IP6 (25 to 60%), IP5 (120 to 350%), IP4 (100 to 200%), and IP3 (84 to 106%) demonstrated no consistent pattern, showing that a complex interaction existed between all inositol phosphates and the food matrix. Problems with the food matrix were suspected because running pure IP6 through the ion pair procedure gave essentially 100% recoveries. For comparison purposes, spiked samples were also run with the ion exchange HPLC method (Table 3). Recoveries of 97 to 105% were observed when pure IP6 was added to barley, oatmeal, rice, and mixed cereals. In addition, 104% of the IP3, 109% of the IP4, and 97% of the IP5 was recovered when oatmeal cereal was spiked with a partially hydrolyzed IP6 sample.

Tests of Individual Ion Pair HPLC Steps. The ion pair HPLC procedure was further investigated in an attempt to identify the step(s) responsible for the inositol phosphate losses. Partially hydrolyzed IP6 was used to test for sample loss at the fat extraction step. Full recovery of all inositol phosphates was observed with and without the fat extraction step included, suggesting that this was not the problem (Table 3). The drying/heating step (required to concentrate the strong anion exchange eluate and remove HCl) was tested by heating a

Table 3. Summary of Experiments Investigating Ion Pair HPLC Methodology

procedure	sample	method	result
spike samples	cereal + pure IP6	ion pair HPLC	25−60% recovery of IP6; 120−350% recovery of IP5; 100−200% recovery of IP4, 84−106% recovery of IP3
spike samples	ceral + pure IP6	ion exchange HPLC	97−105% recovery of IP6; 97% of IP5; 109% of the IP4; 104% of IP3
include or exclude the fat extraction step	hydrolyzed IP6	ion pair HPLC	No difference in recovery
exchange ion pair HPLC column for ion exchange HPLC column	mixed cereal	ion pair HPLC (sample prep) − ion exchange HPLC column	No visible inositol phosphate peaks
heat and dry for various times	hydrolyzed IP6	ion pair HPLC	Complete recovery of all inositol phosphates
add 10x minerals	mixed cereal ± pure IP6	ion pair and ion exchange HPLC	No visible inositol phosphate peaks
40 mM EDTA + 5 mM ascorbic acid	rice cereal + pure IP6	ion pair HPLC	Increased recoveries of IP6 spike to 53%.
8-hydroxyquinoline (fat-soluble metal chelator)	rice cereal + pure IP6	ion pair HPLC	No difference in recoveries
chromatograph on Dowex 50W-X4 prior to anion exchange − elute with HCl or water	rice cereal or pure IP6	ion pair HPLC	No quantifiable peaks
exchange SAX column for QMA or C-18 reverse-phase column	rice cereal + pure IP6	ion pair HPLC (modified SAX)	No difference in recoveries
pretreat column by washing with 5 mg/20 mL pure IP6	rice cereal + pure IP6	ion pair HPLC	Increased recoveries to 60% (maximum) but inconsistent recoveries.
precipitate IP6 by sonicating in 0.5 N HCl plus 2 mg of calcium, iron and zinc. Incubate for 24 h at room temperature. Extract by incubating in 0.2 N NaOH for 24 h	rice cereal + pure IP6	ion pair HPLC	40% yield of added IP6.

partially hydrolyzed IP6 sample in 0.05 N HCl for 20 min (approximately the time required to completely dry the sample), or for 1, 2, and 4 h. All samples gave 100% recovery. Commercial mixed cereal was used to test the strong anion exchange step that is an essential part of the ion pair HPLC method. This was done by substituting the macroporous polymer HPLC column PRP-1 (of the ion pair HPLC method) for the PL-SAX column of the ion exchange HPLC method. Thus, the test involved injecting the resuspended dried eluant from the strong anion exchange column directly onto the PL-SAX column of the ion exchange HPLC method. No inositol phosphate peaks were visible although mixed cereal contains appreciable IP5 and IP6 (Table 1). This suggested that the strong anion exchange step of the ion pair HPLC method was problematic.

Mineral Interference. Different explanations can account for the poor performance of the ion pair strong anion exchange step. It is possible that the high mineral content of the infant foods is responsible for the poor recoveries. The nutrition labels reported mineral contents of approximately 11 mg/g calcium, 0.3 mg/g iron, and 0.03 mg/g zinc. IP6 has a strong affinity for minerals as well as a high binding capacity for minerals (*2* and *29*). Iron can readily bind at low pH values (*29*), although calcium and zinc apparently do not (*2* and *30*). The effect of minerals was tested directly by adding approximately 10 times the mineral content of calcium, iron, and zinc to commercial mixed cereal. Some of these samples were also spiked with purified IP6. The final samples contained a white precipitate (which was probably a mineral−IP6 complex) that could be removed by centrifugation. Chromatography by ion pair HPLC or by ion exchange HPLC showed that no IP6 was present in the final supernatant. This demonstrates that minerals can affect yields.

Methodological Variations. Several different methodological variations were employed in an attempt to counteract the potential negative effect of the high mineral content on the ion pair HPLC method. Adding 40 mM EDTA plus 5 mM ascorbic acid (to prevent possible oxidation by iron−EDTA complexes) increased the measured IP6 content of rice cereal to 19.8 mg/g dry weight (from a value that was below the detection limit of the method), but only 53% of a IP6 spike was recovered (Table 3). Pretreating a rice cereal sample that had been sonicated in HCl with Dowex 50W-X4 to remove the minerals gave a value below the detection limit of the method. This was due to irreversible IP6 binding to the Dowex column as shown by an experiment with purified IP6 dissolved in 0.5 N HCl. Exchanging the HCl for water did not solve the problem, probably because water is not a good medium for extracting IP6 from foods. Strong anion-exchange columns obtained from different suppliers (SAX versus QMA) performed equally poorly. Exchanging the strong anion-exchange column for a reversed-phase C-18 column did not improve recoveries. Addition of 8-hydroxyquinoline as a chelator to the fat extraction step failed to remove the minerals. Potential adsorption of IP6 to

the strong anion-exchange column was tested by preloading the strong anion-exchange column with 5 mg of purified IP6 dissolved in 20 mL of water. This improved yields in some cereal samples to 60% but failed to give consistent results.

A final methodological variation involved quantitatively precipitating all the IP6 from the food samples by sonicating in 0.5 N HCl plus 2 mg each of calcium, iron, and zinc (Table 3). In the procedure that gave the highest yield, the sample was incubated for 24 h at room temperature and centrifuged. The addition of excess minerals completely precipitated all the inositol phosphates in the cereal samples as shown by the absence of any detectable inositol phosphates in the supernatant as measured by ion pair HPLC. The precipitate was extracted by incubating in 0.2 N NaOH for 24 h to redissolve the inositol phosphates and precipitate the minerals. Although the procedure improved the final yield, analyzing the redissolved pellet was problematic. The samples were extremely viscous and yielded very little liquid after centrifugation or after filtration. In addition, the chromatographic peaks were broad with considerable tailing and the column pressure increased dramatically. Maximal recoveries were also low (40% at best) and required overnight incubations at room temperature.

DISCUSSION

The poor recovery of IP6 and IP5 from the ion pair HPLC procedure was probably due to a combination of factors including a low total inositol phosphate content and a high mineral content. The results show that a high mineral content and adsorption to the column matrix during strong anion-exchange (SAX) chromatography step combined to make ion pair HPLC unsuitable for measuring IP6 in infant cereals. It is surprising that the SAX column was identified as a problem area because both HPLC methods use this technique and it is part of the official AOAC procedure (*31*). The difference may be due to the 10-fold dilution required to load the samples onto the relatively large (3 mL) column in the ion pair HPLC method. This can lead to two different problems. First, the relatively small IP6 content of the infant cereals may adsorb nonspecifically to the column matrix and become difficult to remove with a salt gradient. Nonspecific absorption can be a problem as evidenced by the necessity to add IP6 to the buffers of the ion pair HPLC method, although this is thought to be due to IP6 binding to stainless steel (*16*). This problem might not occur with samples containing higher amounts of IP6 where this loss may be negligible. Nonspecific absorption was suggested by an increase in IP6 recovery after pre-loading the SAX columns with IP6 (Table 3). Second, the large calcium and iron content of the samples may be problematic. Although binding of IP6 to minerals is weak at low pH values (*1* and *2*), the 10-fold lower HCl concentration during loading of the SAX column may promote mineral−IP6 binding. In addition, the high mineral content of the samples will help drive the equilibrium toward formation of IP6−mineral complexes that will pass through the SAX column. These problems are avoided in the ion exchange HPLC method and the original AOAC method because in both cases a small amount of concentrated sample is loaded onto the SAX columns at a low pH, and the inositol phosphates are eluted with nitric acid or sodium chloride gradients and measured directly.

It was possible that the high mineral content also interfered with the initial IP6 extraction from the samples. This was tested by varying the length of time during extraction: 1.5 min with sonication, 4 h with shaking, or overnight with shaking. Identical results in all three cases ruled out any problems. Time is an important consideration because precipitation is a slow process and occurs over several hours. Although the extraction conditions appeared adequate for the cereal samples, when cereal samples were spiked with 10 times the original calcium, iron, and zinc concentrations, no IP6 was recovered by either ion pair HPLC or ion exchange HPLC. The molar mineral/IP6 ratios in unspiked cereal were approximately 30 (calcium), 0.6 (iron), and 0.06 (zinc), indicating that there was enough mineral to completely precipitate the IP6 in the original extraction. This suggests that the minerals are sequestered somehow in the foods through binding to other components to allow IP6 recovery.

The IP3 and IP4 peaks observed by ion pair HPLC were not apparent in the ion exchange HPLC runs. A difference in the relative sensitivity of the ion exchange HPLC method (compared to IP6) does not explain this observation nor does a lower recovery of IP3, IP4, and IP5 by ion exchange HPLC. These two potential explanations were ruled out by recovery studies with spiked samples. In addition to the absence of the IP3 and IP4 peaks in the ion exchange HPLC method, a difference in the IP6/IP5 ratio was noted for samples measured by the two methods (Table 1). One possible explanation is IP6 degradation during the ion pair HPLC procedure. This would explain both the lower IP6 yield as well as the relatively higher IP3, IP4, and IP5 content measured by the ion pair HPLC method. In addition, it would explain an increase in the recoveries of IP3 and IP4 that was observed after spiking cereal samples with purified IP6. Experiments with purified IP6 and mixtures of inositol phosphates apparently rule out this explanation because complete recovery was observed. However, it is possible that the food matrix of the cereal samples may catalyze IP6 degradation through an unknown mechanism. Other explanations for these differences are possible. The strong anion-exchange column of the ion pair HPLC method could have adsorbed IP6 and IP5 preferentially. As was the case with potential IP6 degradation, this could also explain the increased IP3 and IP4 recoveries that were measured after spiking the cereals with IP6. In this scenario, IP6 would displace adsorbed IP3 and IP4 from the strong anion-exchange column giving higher yields. If IP6 and IP5 were preferentially adsorbed to the strong anion-exchange column, and the amounts of IP3 and IP4 measured by ion pair HPLC were reasonably accurate, one can calculate that approximately 2.5 μg and 4 μg of IP4 and IP3, respectively, should have been present in the ion exchange HPLC chromatograms. These amounts are too small to be visualized under our conditions (50 μL/injection). Even if there were twice as much IP3 and IP4 in the cereals (as suggested by increased IP3 and IP4 yields when the cereals were spiked with purified IP6), then the amounts would still fall below the detection limit of ion exchange HPLC. As indicated above, IP6 adsorption was tested by pretreating the strong anion-exchange column with purified IP6. The increased yields show that IP6 adsorption to the column was part of the problem but not the complete answer.

Last, the IP6 concentrations measured by the ion exchange HPLC method were lower than previously reported IP6 concentrations measured by the spectrophotometric procedure (*25*). These results were expected because previous studies had demonstrated discrepancies between chromatographically measured IP6 and spectrophotometrically measured IP6 (*10* and *19*). These differences were due to an overestimation of IP6 by the spectrophotometric method that does not distinguish between IP6 and other inositol phosphates.

In conclusion, ion exchange HPLC appears to be the method of choice for measuring inositol phosphates in infant cereal samples that contain high amounts of added calcium and iron. This is suggested by excellent recoveries of added IP6, IP5, IP4, and IP3. In addition, the close agreement between total inositol phosphates as measured by the ion exchange HPLC method and the AOAC method (as reported in the literature) suggests the complete recovery of inositol phosphates from the cereal samples.

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