Determination of Isomers of Inositol Mono- to Hexaphosphates in Selected Foods and Intestinal Contents Using High-Performance Ion Chromatography

Erika Skoglund,* Nils-Gunnar Carlsson, and Ann-Sofie Sandberg

Department of Food Science, Chalmers University of Technology, S-402 29 Göteborg, Sweden

A sensitive high-performance ion chromatography (HPIC) method for separation and quantitative determination of inositol mono- to hexaphosphate (IP_1−IP_6) isomers in selected foods and intestinal contents is described. The method includes extraction of samples with HCl, separation of the inositol phosphates from the crude extract by anion-exchange chromatography, separation on high-performance ion-exchange columns with gradient elution in two combined HPIC systems, and detection using either postcolumn reaction and UV detection (system 1) or chemically suppressed conductivity detection (system 2). IP_2−IP_6 in samples were determined on the first system, which also separated isomers of IP_4 and IP_5. Fractions of IP_1−IP_3 from the first system were transferred to the second system after some additional sample preparation and analyzed for isomers. The applicability and sensitivity of the method are illustrated by measuring the content of inositol phosphates in foods, ileal contents, and feces of human balances.

Keywords: *Inositol phosphate isomers; high-performance ion chromatography; HPIC; food; intestinal content*

INTRODUCTION

Efficient analysis of inositol phosphates is difficult, since the compounds do not absorb visible or ultraviolet light, nor can they easily be identified using specific colorimetric reagents. Before HPLC techniques were developed, the quantitative analysis of phytate (inositol hexaphosphate, IP_6) in foods involved either precipitation with ferric chloride, as was first described by Heubner and Stadler (1914), or purification using anion-exchange chromatography (Cosgrove, 1963; Harland and Oberleas, 1986; Smith and Clarke, 1952). One of the disadvantages of these methods is the lack of specificity to distinguish between inositol hexaphosphate and its degradation products (inositol penta- to monophosphates). After the development of ion-pair HPLC procedures, it became possible to study phytate and some of its hydrolysis products (inositol tri-, tetra-, and pentaphosphate) during food processing and digestion in the gut (Sandberg and Ahderinne, 1986). These methods do not differentiate isomeric forms of inositol phosphates, as gradient elution cannot be performed when using refractive index detection. Several isomers of inositol phosphates have shown important physiological functions, such as diabetes complications prevention (Carrington et al., 1993; Ruf et al., 1991), anti-inflammatory effects (Claxon et al., 1990), and secondary messengers in the cell signal transmission (Streb et al., 1983). The position of the phosphate groups on the inositol ring is of great importance for their physiological function. Furthermore, the content of inositol phosphates in certain tissues may respond to nutritional modulation. If physiologically active lower inositol phosphates or precursors to these are absorbed in the alimentary tract of humans, one can expect processed food to have various physiological effects of importance for health. Difficulties in separating the isomers of inositol phosphates have been reported in many analysis approaches. During the past few years a number of isomer specific ion-exchange chromatography methods, with gradient elution for separation of inositol phosphates in biological tissues, have been developed (Mayr, 1988; Phillippy and Bland, 1988; Smith and MacQuarrie, 1988). A remaining problem, however, is to separate isomers from the whole spectrum of inositol phosphates (IP_1−IP_6) in the same run. The variety of methods available for the analysis of inositol phosphates has frequently been reviewed (Crean and Haisman, 1963; Oberleas and Harland, 1986; Reddy et al., 1989; Sandberg, 1995; Xu et al., 1992).

The present investigation describes a method consisting of two combined HPIC systems for the separation and determination of isomers of inositol mono- to hexaphosphates in foods and intestinal contents. The method was applied to the analysis of inositol phosphates in foods, ileal contents, and feces of balance studies on humans.

MATERIALS AND METHODS

Materials. *myo*-Inositol 2-monophosphate, di(cyclohexylammonium) salt, *myo*-inositol 1,4,5-trisphosphate, hexasodiumsalt, *myo*-inositol 1,5,6-trisphosphate, ammonium salt, and *myo*-inositol 1,3,4,6-tetrakisphosphate, ammonium salt, were obtained from Sigma Chemical Co. (St. Louis, MO). Ins(1,2,4)P_3, Ins(1,2,6)P_3, Ins(1,3,4)P_3, Ins(1,2,3,4)P_4, Ins(1,2,5,6)P_4, and Ins(1,2,4,5,6)P_5 were received as a gift from Perstorp Pharma (Perstorp, Sweden). *myo*-Inositol 2,4-diphosphate, tetraammonium salt, was obtained from Calbiochem Corp. (La Jolla, CA), D-*myo*-inositol 1,3,4,5-tetrakisphosphate, ammonium salt, from Boehringer Mannheim GmbH (Mannheim, Germany), and sodium phytate from BDH Chemicals Ltd. (Poole, England). Anion-exchange resin AG 1-X8, 200−400 mesh, was purchased from Bio-Rad (Richmond, CA), and silica-based anion-exchange (SAX) columns (Bond Elut, 500 mg) were purchased from Varian (Harbor City, CA). Type I deionized water for HPIC was purified by a Barnstead NANOpure water

* Address correspondence to this author at the Department of Food Science, Chalmers University of Technology, c/o SIK, Box 5401, S-402 29 Göteborg, Sweden [telephone +46 31 35 13 46; telefax +46 31 83 37 82; e-mail es@sik.se].

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Table 1. Combination of Eluants for the Analysis of Inositol Di- to Hexaphosphates on System 1

time (min)	HCl (%)	2-propanol (%)	water (%)	curve
T(0)	5	2	93	
T(10)	28	2	70	5
T(25)	98	2	0	5

system to a specific resistance of 18 MΩ-cm or greater. Sodium hydroxide 50% solution was purchased from J. T. Baker B.V. (Deventer, Holland). All other reagents used were of analytical grade.

Sample Preparation. The sample preparation was performed according to the method of Sandberg and Ahderinne (1986), with some modifications. Samples of 0.5 g of freeze-dried and ground rye roll, intestinal contents, or feces collections were extracted with 20 mL 0.5 M HCl for 3 h at 20 °C, under agitation. Samples of 0.5 g of pea flour were soaked in 5 mL of buffer, at pH 7 and 45 °C, for 20 h and extracted with 15 mL of 0.67 M HCl for 3 h at 20 °C. The extracts were centrifuged and the supernatant decanted, frozen over night, and centrifuged again. Fifteen milliliters of the supernatant was taken out and evaporated to dryness and dissolved in 15 mL of water. The inositol phosphates were separated from the crude extract by ion-exchange chromatography. Plastic columns with porous polymer filters containing 2.5 mL of resin (AG 1-X8, 200−400 mesh) were used. The samples were washed by 5 and 10 mL of water, and inositol phosphates were removed from the resin with five 4 mL portions of 2 M HCl. The eluants were evaporated to dryness and diluted with 1−2 mL of water.

A reference sample for identification of peaks was prepared by dissolving 1.5 g of natrium phytate in 100 mL of 0.5 M HCl. The solution was reflux boiled for 12 h and evaporated to dryness, and 100 mL of water was added to the hydrolyzed sample.

Sample Analysis. Two systems were combined for the analysis of inositol mono- to hexaphosphate isomers.

System 1. The first system was used to determine IP_2−IP_6 and isomers of IP_4 and IP_5, injected in 50 μL amounts. The chromatograph consisted of a biocompatible HPLC pump (Waters Model 626, Waters Associates, Milford, MA) equipped with a 50 μL injector loop, a HPIC Omni Pac PAX-100 (4 × 250 mm) analytical column, and a PAX-100 (4 × 50 mm) guard column (Dionex Corp., Sunnyvale, CA). Inositol phosphates were detected, after postcolumn reaction, using UV detection (Waters 486, tunable absorbance detector). Absorbance was monitored at 290 nm. IP_2−IP_6 were eluted with a gradient of 5−98% HCl (0.5 M) in conjugation with water and organic solvent (50% 2-propanol). The eluants were combined according to the preprogrammed gradient curve profile 5 and with the proportions listed in Table 1. An equilibration time of 9 min was needed after each run.

The eluants were mixed, in a postcolumn reactor, with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ in a 2% solution of $HClO_4$ according to the method of Phillippy and Bland (1988). The combined flow rate was 1.2 mL/min. A mixing tee (Jour Research, Onsala, Sweden) and a home-made 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.

System 2. The second system was used to determine isomers of IP_1−IP_3, injected in 25 μL amounts. To avoid interference from higher inositol phosphates (IP_4−IP_6), fractions containing IP_1−IP_3 were collected after separation on system 1 as described above. The fractions were evaporated to dryness and dissolved in 0.5 mL of distilled water, before injection on system 2. HPIC was performed on the second system using a gradient pump (Dionex Series 4500 i) equipped with a 25 μL injector loop, a PAX-100 (4 × 50 mm) guard column, and an Omni Pac PAX-100 (4 × 250 mm) analytical column (Dionex Corp.). An anion micromembrane suppressor (AMMS) was applied with conductivity detection. As regenerant for the AMMS, sulfuric acid, 25 mM (15 mL/min), was used. For separation of various isomers of IP_1, IP_2, and IP_3 the gradient elution was generated by mixing 200 mM NaOH, 50% 2-propanol, and water as listed in Table 2. The chromatographic procedure used was outlined by the manufacturer (Dionex Application Note, AN65), except for the linear elution gradient. The column equilibration time was 11 min after a run.

Table 2. Combination of Eluants for the Analysis of Inositol Mono- to Triphosphates on System 2

time (min)	NaOH (%)	2-propanol (%)	water (%)
T(0)	8	6	86
T(0.1)	8	6	86
T(10)	20	6	74
T(30)	35	6	59
T(40)	60	6	34
T(52)	65	6	29

The NaOH eluant was prepared from 50% liquid NaOH (J. T. Baker) to give a low carbonate amount in the solution.

All eluants were sparged with helium in both systems. Detector signals were processed by a laboratory data system (Borwin, Chromatography Software, JMBS Developpments, Grenoble, France).

Analysis of Foods, Intestinal Contents, and Feces Samples. A sour dough fermented rye roll with a low content of IP_5 and IP_6, but a high content of IP_3 and IP_4, was analyzed for inositol phosphate isomers. The rye roll was compared with a dehulled yellow pea flour soaked in 10 mL of buffer, pH 7 at 45 °C, for 20 h. Intestinal content from an ileostomy subject consuming raw wheat bran was analyzed as well for inositol phosphates. More detailed information about the experimental conditions of the ileostomy subject has been given in a previous paper (Sandberg et al., 1982). A feces sample from a subject consuming a white bread supplemented with high amounts of natrium phytate was also studied (Andersson et al., 1983).

RESULTS AND DISCUSSION

The anion-exchange resin AG 1-X8 (200−400 mesh) was chosen, in the preparation of samples containing IPs, as it offers a large loading capacity (1.2 mequiv/mL of resin bed) and was found to have a recovery of 95−98% for inositol mono- to hexaphosphates. Graf and Dintzis (1982) and Sandberg and Ahderinne (1986) obtained similar results for inositol hexaphosphate and inositol tri- to hexaphosphates, respectively. The quality of the anion-exchange resin was verified for phytate during sample preparation, at regular intervals. Comparison was made between AG 1-X8 and silica-based anion-exchange columns (SAX). The SAX column had an inositol mono- to hexaphosphate recovery of 90−92% (for 1 μmol of inositol phosphates on 500 mg SAX columns) and accordingly a lower capacity than AG 1-X8. The more phosphate groups that were attached to the inositol ring, the greater was the tendency for the IPs to be retained on the columns. The size of the SAX column was found to be proportional to the amount of inositol phosphates that disappears on the SAX column. This size dependence can result in complete disappearance of IPs in samples containing low amounts of inositol phosphates.

To avoid problems with aggressive eluants and reagents, the materials in the postcolumn reactor (system 1) were exchanged as far as possible from stainless steel to PEEK (Jour Research, Onsala, Sweden). However, certain leakage of $Fe(NO_3)_3/HClO_3$ solution occurred; therefore, the PEEK materials were concluded to be slightly permeable. The system was regularly regenerated with a mixture of methanol and water (1:1).

The linearity of inositol phosphates on systems 1 and 2, respectively, was demonstrated by plotting peak areas versus the concentrations of different dilutions of selected standard inositol phosphates in Figure 1 (parts

Table 3. IP_1–IP_6 Content (Micromoles per Gram) of Selected Samples

sample[a]	IP_1	IP_2	IP_3	IP_4	IP_5	IP_6
rye roll	0.606 ± 0.036	0.468 ± 0.029	0.808 ± 0.013	0.909 ± 0.014	0.217 ± 0.005	0.171 ± 0.007
ileostomy	0.205 ± 0.012	0.304 ± 0.018	1.755 ± 0.050	3.912 ± 0.109	2.296 ± 0.114	7.629 ± 0.199

[a] Mean ± SD of five replicate extracts. Each sample was prepared and analyzed in duplicate with ion chromatography.

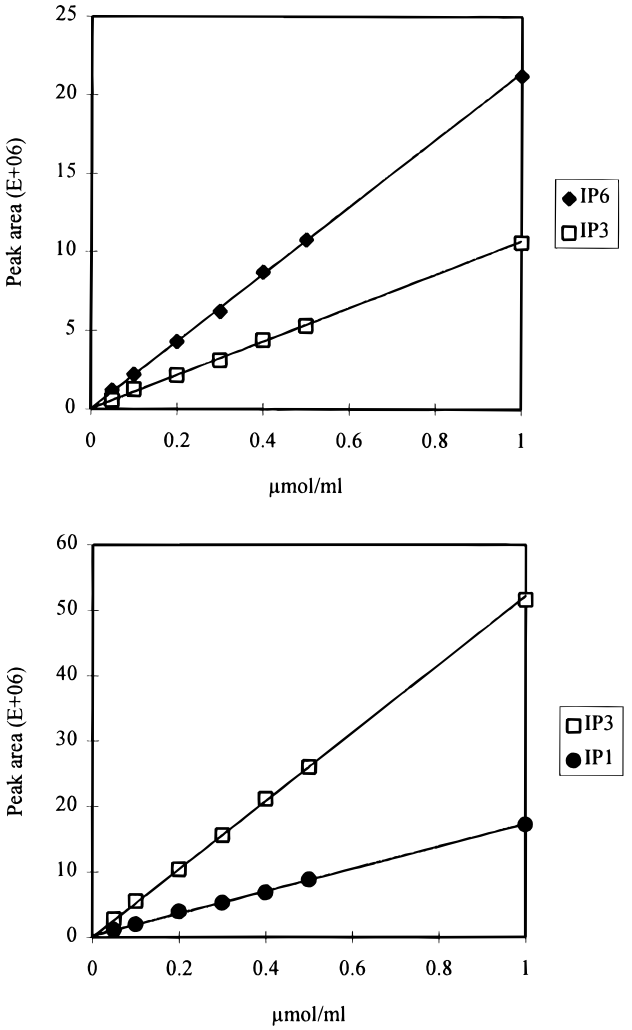

Figure 1. Linearity of (a) system 1 and (b) system 2. The peak areas of selected standards are plotted against the amount of sample. Each point represents the mean of three determinations.

a and b). From these measurements it can be concluded that the peak areas are linearly dependent of the concentrations of inositol phosphates over the entire range (linear regression analysis gave correlation coefficients of 1 for inositol phosphates in both systems). The slope of the linear calibration curve depends on the number of phosphate groups, due to differences in detector responses. Correction factors for IP_2, IP_3, IP_4, IP_5, and IP_6 on system 1 were determined to 3, 2, 3/2, 6/5, and 1, respectively. On system 2 the correction factors were 3, 3/2, and 1 for IP_1, IP_2, and IP_3, respectively. The factors were calculated from standard inositol mono- to hexaphosphates and appeared to increase linearly with decreasing number of phosphate groups connected to the inositol ring. The repeatability of the method is shown in the rye roll and the ileostomy sample for inositol mono- to hexaphosphates (Table 3). When rye roll samples were spiked with inositol mono-, tri-, and hexaphosphate standards, the recovery of added standards was 95–104% (Table 4). The reasons for sloping baselines in chromatograms are due to

Table 4. Recovery (Percent; Mean ± SD) of Standard Additions of Ins(2)P, DL-Ins(1,2,6)P_3, and IP_6 to Rye Roll Samples

µmol of inositol phosphate added	Ins(2)P	DL-Ins(1,2,6)P_3	IP_6
1	98 ± 3.2	96 ± 3.6	95 ± 4.7
2	95 ± 2.2	95 ± 3.7	104 ± 1.7

conductivity changes for conductivity detection and to refractive index (RI) phenomena for UV detection. RI changes can occur owing to the characteristics of solvent flow and composition.

There exist 63 noncyclic and 3 cyclic inositol phosphates. Because the latter compounds are acid-labile, they are difficult to measure and, therefore, their proportion in various cells is unknown (Majerus et al., 1988). About 20 different inositol phosphates of the 66 possible have been identified in animal tissues (Majerus et al., 1988). The elution orders of different isomers of IPs on systems 1 and 2, respectively, were established on the basis of commercially available inositol phosphate isomers [Ins(2)P, Ins(2,4)P_2, Ins(1,5,6)P_3, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, and IP_6] and isomers obtained as a gift from Perstorp Pharma [Ins(1,2,4)P_3, Ins(1,2,6)P_3, Ins(1,3,4)P_3, Ins(1,2,3,4)P_4, Ins(1,2,5,6)P_4, and Ins(1,2,4,5,6)P_5] as well as inositol phosphate isomers prepared by enzymatic hydrolysis of sodium phytate (Türk et al., 1996) [Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5]. The elution orders according to Phillippy and Bland (1988) [Ins(1,2,4,5)P_4, Ins(1,2,4,6)P_4, Ins(2,4,5,6)P_4, Ins(1,4,5,6)P_4, Ins(1,2,3,4,6)P_5, and Ins(1,3,4,5,6)P_5] and Dionex AN65 [Ins(1)P, Ins(4)P, Ins(1,2)P_2, Ins(4,5)P_2, and Ins(2,4,5)P_3] were considered. The order of elution in the first system is in accordance with that suggested by Phillippy and Bland (1988) for all inositol phosphates and with that suggested by Mayr (1988) except for the peak containing DL-Ins(1,2,3,4)P_4 and Ins(1,3,4,6)P_4.

HPIC analyses on systems 1 and 2 of the reference sample show the order in which the inositol phosphate isomers are eluted (Figure 2, parts a and b**)**. A chromatographic run on system 1 takes approximately 20 min and gives adequate separation of IP_4 and IP_5 isomers and phytate down to 5 nmol of IP_6/mL [calculated according to IUPAC (1978), $c_{L(k=3)}$], whereas the separation of IP_2 and IP_3 isomers was better performed on system 2. On the second system, inositol triphosphates are eluted in 45 min. Different isomers of IP_1–IP_3 were separated satisfactory to the lowest range of picomoles, on system 2. Since separation of IP_3–IP_6 on system 2 occurs within 10 min and in mixed order, a fraction from system 1 not containing inositol tetra- to hexaphosphates was needed to study isomers of IP_3 on system 2. As well, the separation of IP_4 isomers can be improved by fractionating on system 1 and separation on system 2.

The analyses of inositol phosphates in foods and intestinal contents on systems 1 and 2 are shown in Figures 3 and 4. One of the dominating isomers of IP_3 in the roll (baked with yeast and sour dough) was DL-Ins(1,2,6)P_3 (Figure 3a), as determined by retention time of pure standard. This isomer is formed when inositol

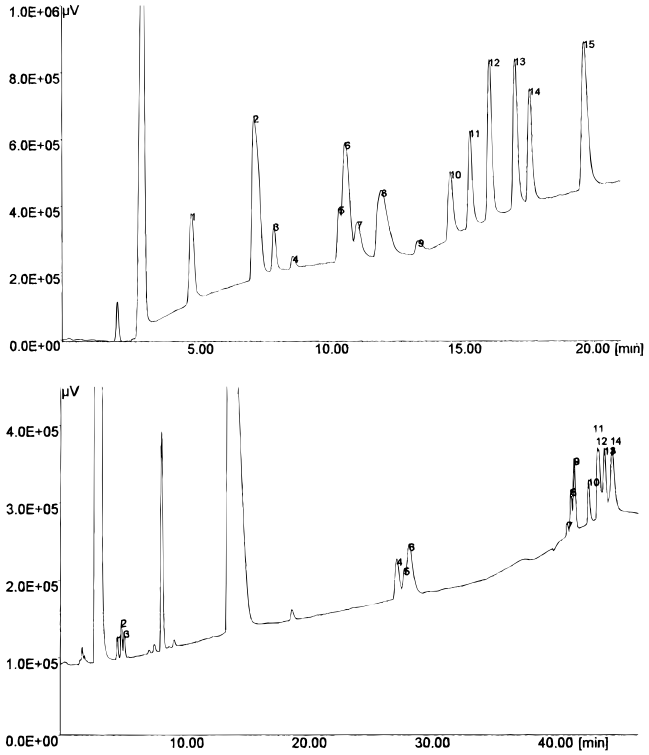

Figure 2. Isomers of inositol phosphates in the reference sample on (a, top) system 1 and (b, bottom) system 2. Unidentified peaks are assigned a star (*). Peaks: (a) (1) $InsP_2$; (2−4) $InsP_3$; (5) DL-Ins(1,2,4,5)P_4; (6) DL-Ins(1,2,3,4)P_4, Ins(1,3,4,6)P_4; (7) DL-Ins(1,2,4,6)P_4; (8) DL-Ins(1,2,5,6)P_4, DL-Ins(1,3,4,5)P_4; (9) Ins(2,4,5,6)P_4; (10) DL-Ins(1,4,5,6)P_4; (11) Ins(1,2,3,4,6)P_5; (12) DL-Ins(1,2,3,4,5)P_5; (13) DL-Ins(1,2,4,5,6)P_5; (14) Ins(1,3,4,5,6)P_5; (15) IP_6. Peaks: (b) (1) Ins(2)P; (2) DL-Ins(1)P; (3) DL-Ins(4)P; (4) DL-Ins(1,4)P_2; (5) DL-Ins(2,4)P_2; (6) DL-Ins(4,5)P_2; (7) *; (8) DL-Ins(1,5,6)P_3; (9) DL-Ins(1,2,6)P_3; (10) *; (11) *; (12) DL-Ins(1,3,4)P_3; (13) DL-Ins(1,4,5)P_3; (14) DL-Ins(2,4,5)P_3, DL-Ins(1,2,4)P_3.

hexaphosphate is hydrolyzed with bakers' yeast. Furthermore, this isomer has been shown to possess several pharmacological properties such as inhibition of platelet thrombus formation (Sirén et al., 1991), prevention of diabetes complications (Carrington et al., 1993; Ruf et al., 1991), and anti-inflammatory effects (Claxon et al., 1990). Among IP_2s the DL-Ins(4,5)P_2 peak was dominating. However, degradation of DL-Ins(1,2,6)P_3 probably results in one or more of DL-Ins(1,2)P_2, DL-Ins(2,6)P_2, and DL-Ins(1,6)P_2. Of these, DL-Ins(1,2)P_2 is most similar to DL-Ins(4,5)P_2 and is therefore expected to have the same retention time. Accordingly, the dominating IP_2 isomer in the rye roll may be DL-Ins(1,2)P_2.

There was a marked difference between the isomers of IP_1−IP_3 in the rye roll (Figure 3a) and in the pea flour (Figure 3b). High amounts of DL-Ins(1,3,4)P_3 and DL-Ins(1,4,5)P_3 were obtained in the dehulled yellow pea flour. The latter isomer, D-*myo*-1,4,5-trisphosphate, has been shown to act as a second messenger in intracellular signal transduction systems (Streb et al., 1983). Among IP_2s in the pea flour, the isomers DL-Ins(1,4)P_2 and DL-Ins(2,4)P_2 dominated. Huang and Liang presented in 1994 (Huang and Liang, 1994) a study indicating Ins(1,4)P_2 as a messenger during cell activation. Their results suggest that Ins(1,4)P_2 controls the organization of actin filaments during cell activation. DL-Ins(1)P was predominant among IP_1s in the pea.

In Figure 4a the ileal content from an ileostomy subject consuming raw wheat bran is shown. As we can see the DL-Ins(1,2,3,4,5)P_5 peak rises above the peaks of the other IP_5s. This indicates hydrolysis by cereal

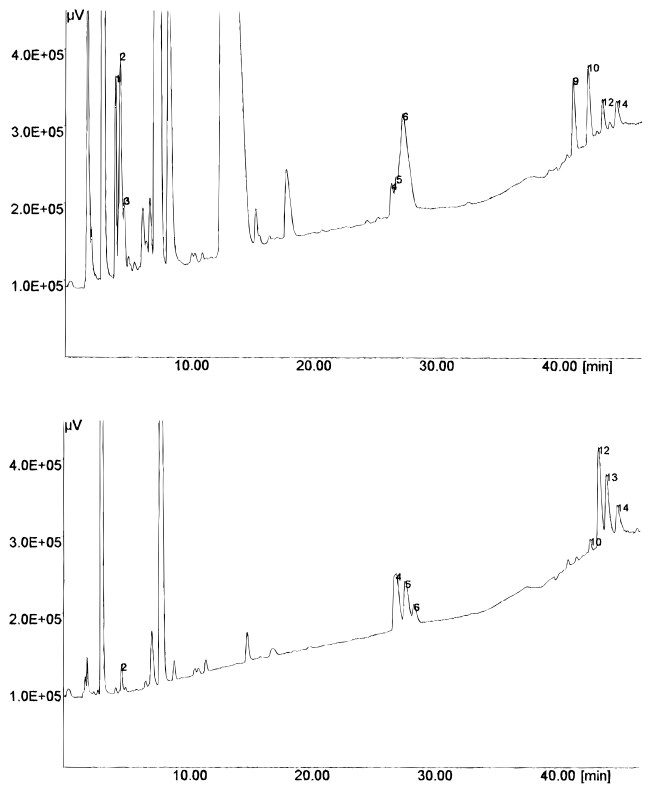

Figure 3. Inositol phosphates on system 2 in (a, top) rye roll and (b, bottom) pea flour. Peaks are numbered according to Figure 2b.

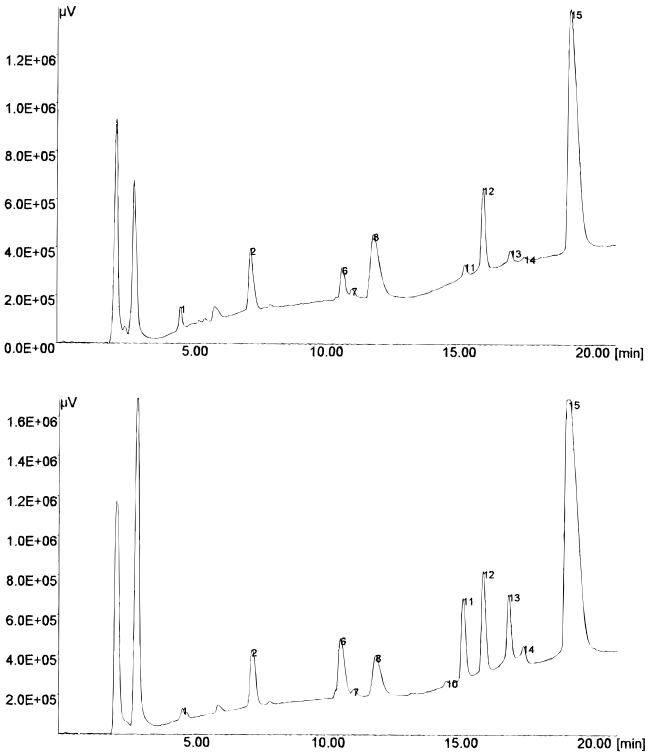

Figure 4. Inositol phosphates on system 1 in (a, top) intestinal content and (b, bottom) feces collection. Peaks are numbered according to Figure 2a.

phytase (EC 3.1.3.26) from the wheat bran, since EC 3.1.3.26 is a 6-phytase, i.e., starting the ester bond hydrolysis at the 6-position. The ileal content was also composed of high IP_4 amounts, with the peak of DL-Ins(1,2,5,6)P_4 and DL-Ins(1,3,4,5)P_4 as predominant. The latter isomer has been proposed to be a second mes-

senger (Batty et al., 1985). However, still in 1995, this hypothesis is controversial (Cullen et al., 1995).

In the feces sample from a subject consuming a white bread supplemented with high amounts of natrium phytate, almost equal amounts of DL-Ins(1,2,3,4,5)P_5 and DL-Ins(1,2,4,5,6)P_5 were found (Figure 4b). Both cereal phytase (EC 3.1.3.26) during bread-making and microbial phytase (EC 3.1.3.8) from the microflora of the colon participate most likely in the degradation of phytate. The phytase usually present in microorganisms, EC 3.1.3.8, is a 3-phytase, first hydrolyzing the 3-position ester bond (Cosgrove, 1970). Moreover, the feces sample contained several different isomers of inositol tetraphosphates.

The choice of samples, to demonstrate the applications for the method, was made on the basis of their origin (food, ileostomy, and feces), their amount of inositol mono- to hexaphosphates, and the differences between various isomers. Samples with inositol phosphate amounts above 1 μmol/mL had to be diluted in order not to overload the systems. The two analysis and detection systems used did distinguish with respect to resolution, separation, elution order, and time. The resolution and separation of IP_4−IP_6 were better performed on system 1, as compared to system 2. To separate IP_1−IP_3, system 2 was superior, as IP_1 was coeluted with the solvent front on system 1 and IP_2−IP_3 isomers were scarcely separated. The sequence of elution on system 2 was difficult to handle since inositol phosphate isomers with different numbers of phosphate groups attached to the inositol ring elute mixed. The first system was superior with regard to elution time.

CONCLUSIONS

The HPIC method described was sensitive and reproducible for inositol phosphate isomers of foods and intestinal contents. Elution orders of 25 different isomers were established. The method will be used for further studies on the effect of food processing and digestion in the gut and on the formation of lower inositol phosphates in cereals and legumes. Additional work is needed to investigate the digestion and absorption of inositol phosphates in humans and animals and their physiological effects.

ABBREVIATIONS USED

AMMS, anion micromembrane suppressor; HPIC, high-performance ion chromatography; IPs, inositol phosphates; IP_1−IP_6, inositol mono−hexaphosphate; Ins, an accepted NC-IUB abbreviation for *myo*-inositol with the numbering of the D configuration unless the prefix L is explicitly added; SAX, silica-based anion exchange.

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