Journal of Chromatography B, 878 (2010) 347–354

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High-performance ion chromatography method for separation and quantification of inositol phosphates in diets and digesta

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ARTICLE INFO

Article history:
Received 8 July 2009
Accepted 30 November 2009
Available online 4 December 2009

Keywords:
Inositol phosphates
Phytate
Phytic acid
HPIC
Methanesulfonic acid
Correction factor

ABSTRACT

A gradient high-performance ion chromatographic method for separation and quantification of inositol phosphates ($InsP_2$–$InsP_6$) in feedstuffs, diets, gastric and ileal digesta from pigs was developed and validated. The $InsP_2$–$InsP_6$ were separated on a Dionex CarboPac™ PA1 column using a gradient with 1.5 mol L^{-1} methanesulfonic acid and water. The exchange of the commonly used HCl with methanesulfonic acid has two advantages: (i) the obtained baseline during the separation is almost horizontal and (ii) it is not necessary to use an inert HPIC equipment as the methanesulfonic acid is not as aggressive as HCl. Twenty-three of the 27 separated inositol phosphate isomers were isolated. ICP-MS was used for quantification of phosphorus in the isolated isomers and used for calculation of correction factors for each isomer allowing $InsP_6$ to be used as calibration standard. The detection limits for $InsP_2$–$InsP_6$ were in the range of 0.9–4.4 mg phosphorus L^{-1}. The recovery of the major part of the inositol phosphates was 80–100%, and the CV for repeatability and reproducibility were 1–17% and 1–14%, respectively.

1. Introduction

Phytate, the salt of *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate ($InsP_6$), represents the main storage form of phosphorus (P) in cereals, legumes and oilseeds [1,2]. It consists of a *myo*-inositol esterified with symmetrically distributed phosphate groups (Fig. 1) [3]. $InsP_6$ is remarkably stable and is often considered as an antinutrient because it forms complexes with divalent metal ions such as Zn^{2+}, Fe^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+}. This lowers the nutritional availability of these minerals [4,5]. However, it has also been recognised that $InsP_6$ and some of its hydrolysis products may have potential health benefits such as antioxidant properties, lowering serum cholesterol and triglycerides and preventing heart disease, colon cancer, renal stone formation [6]. Furthermore, $InsP_3$ functions as intracellular second messenger regulating the release of Ca^{2+} [7]. Perturbation of the $InsP_3$/Ca^{2+} signal transduction pathway implicates a variety of disorders including bipolar affective disorder, Alzheimer's disease, Parkinson's disease and malignant hyperthermia [7].

For the last two decades, there has been focus on $InsP_6$ because of environmental concerns. $InsP_6$ bound P is poorly available to monogastric animals like pigs causing excessive P excretion, which results in environmental pollution in areas with high densities of animals [8,9]. Naturally occurring plant phytase in the feed [10–13] or added commercial microbial phytase [14–16] catalyses the hydrolysis of $InsP_6$, producing *myo*-inositol pentakis-, tetrakis-, tris-, bis-, monophosphates ($InsP_5$–$InsP_1$) and finally *myo*-inositol [17]. The extent to which $InsP_6$ and its degradation products are hydrolysed before and during digestion is of significance for the availability of P. Therefore, it is of great interest to investigate the $InsP_6$ degradation pathways and products.

High-performance ion chromatography (HPIC) with gradient elution is the most commonly used method for separation of inositol phosphates and their isomers in foods, feeds and intestinal contents [18–21]. However, a persistent problem is that the baseline of the chromatographic systems is increasing during the separation as the content of HCl of the mobile phase increases. Consequently, it is difficult to integrate the peak areas and thereby quantify the amounts of inositol phosphates. Another disadvantage of using HCl in the eluent is that it is an aggressive acid, which means that the HPIC system must consist of PEEK or other inert material as a normal stainless steel system rapidly corrodes. The objective of this study was to develop an analytical method to separate inositol phosphates present in feedstuffs, diets, gastric and ileal digesta using an eluent which creates a horizontal baseline during the chromatographic separation and which can be used in a non-PEEK HPIC system.

2. Materials and methods

2.1. Chemicals

Hydrochloric acid, methanesulfonic acid, iron (III) nitrate nonahydrate minimum 98%, perchloric acid 70–72%, formic acid,

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doi:10.1016/j.jchromb.2009.11.046

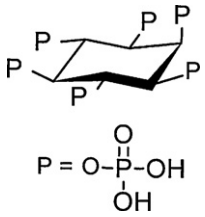

Fig. 1. Structure of phytic acid, the free form of phytate ($InsP_6$).

sulphuric acid, nitric acid, acetic acid and phosphorus pentaoxide (P_2O_5) (Sicapent with indicator) (Merck, Darmstadt, Germany). Phytic acid dodecasodium salt hydrate, from rice, minimum 90% (P-0109) (Sigma–Aldrich Inc., Sct. Louis, MO, USA). All water was treated in a Milli Q Plus (Millipore, Bedford, MA, USA).

The purity of the phytic acid dodecasodium salt was verified by determination of (i) water content by drying until constant weight at 103 °C or drying in vacuum over P_2O_5 for 5 days, (ii) sodium content by flame photometry, (iii) total P content and (iv) free P content by 2009/152/EF with and without preashing and (v) lower inositols by the developed HPIC method.

A reference sample for identification of peaks was prepared by partial hydrolysis of phytic acid dodecasodium salt. Hydrolysis was performed by boiling 1.5 g phytic acid with 100 mL of 0.5 mol L^{-1} HCl with reflux for 16 h. The solution was evaporated to dryness in a vacuum centrifuge at ambient temperature. The residue were dissolved in 100 mL water and stored at −20 °C [18]. The reference sample was analysed in between every 15 and 20 samples to verify the retention times. Furthermore, the amounts of the different inositol phosphates in the reference sample were quantified after fractionation of the individual isomers (see Section 2.4) and quantification of the P content in the pure fractions using ICP-MS (see Section 2.5). The reference sample was used for spiking experiments.

2.2. High-performance ion chromatography system

The HPIC system was a Dionex BioLC system made of PEEK (Dionex, Sunnyvale, CA, USA) consisting of a Model AS50 autosampler, a thermostated column compartment, a Model GP50 gradient pump, a Model IS25 isocratic pump and a Model Dionex AD25 absorbance detector. The system was equipped with a Dionex CarboPac™ PA1 guard column (50 mm × 4 mm i.d., 10 μm) and a CarboPac™ PA1 analytical column (250 mm × 4 mm i.d., 10 μm). Chromeleon software (Dionex) was used for control of the HPIC and collection of data.

Two mobile phases were used: (A) 1.5 mol L^{-1} methanesulfonic acid and (B) water. The separation of $InsP_2$–$InsP_6$ was performed by gradient elution (Table 1) at a column temperature of 30 °C. If only the amount of $InsP_6$ was desired an isocratic elution with 85% A and 15% B was used at a column temperature of 30 °C. The eluate were monitored at 290 nm after post-column reaction with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ and 2% $HClO_4$ in water (w/v) forming InsP–Fe complexes [22]. The flow rates of eluents and post-column reaction solution were 1.0 and 0.65 mL min^{-1}, respectively. The injection volume was 50 μL, however, the volume was reduced if the peak height exceeded 1 absorbance unit. A Dionex mixing tee and a knitted Dionex reaction coil (750 μL, 0.019 mm i.d. × 0.050 mm o.d.) was used. Total time for separation of $InsP_6$ and $InsP_2$–$InsP_6$ was 11 and 28.1 min, respectively with an equilibration time of 0 and 4 min.

Table 1
Gradient elution with methanesulfonic acid and water was used for analysis of $InsP_2$–$InsP_6$ by HPIC on a CarboPac™ PA1 column. Column temperature was 30 °C, flow rates were 1.0 and 0.65 mL min^{-1} for eluent and post-column colour reagent, respectively. The equilibration time was 4 min. The change in composition was linear.

Time (min)	1.5 mol L^{-1} CH_3SO_3H (%)	Water (%)
0.0	5	95
20.0	28	72
21.0	85	15
28.0	85	15
28.1	5	95

2.3. Samples and sample preparation

Samples of wheat (non-heat-treated), soybean meal (solvent extraction) and rapeseed cake (mechanical extraction) ground by a roller mill were used. Furthermore, samples of a heat-treated compound diet (37.8% barley, 37.8% wheat, 22.1% soybean meal, 1.4% calcium carbonate, 0.4% amino acids, 0.3% sodium chloride and 0.2% vitamin/mineral premix) were used. The heat-treatment involved steam injection at about 70 °C in a cascade mixer before pelleting at 90 °C through 3 mm dies followed by cooling. Additionally, gastric and ileal digesta samples collected from pigs fitted with a cannula in the stomach (fundic region) and in the terminal ileum (15 cm anterior to the ileal–caecal junction), respectively, were used. Both the gastric and ileal cannulated pigs were fed the compound diet described above. Gastric digesta was collected from the pigs 3 h after feeding, while the ileal digesta was collected from 4 to 6 h after feeding. The digesta samples were frozen after collection in order to stop further degradation of the inositol phosphates. Detailed information about the experimental conditions of these studies is given by [23,24].

Preparation of samples for analysis of $InsP_6$ and lower inositol phosphates ($InsP_5$–$InsP_2$) were performed according to [19] with modifications. Freeze-dried samples (0.5 g) ground by a hammer mill (1 mm screen) were extracted with 10 mL of 0.5 mol L^{-1} HCl for 1 h at 20 °C by ultrasonication. The extracts were then centrifuged for 10 min (2200 × *g*), and 5.0 mL of the supernatant was evaporated to dryness in a vacuum centrifuge at ambient temperature. The samples were re-dissolved in 1 mL water by ultrasonication for 1 h at 20 °C and centrifuged for 15 min (18,000 × *g*). The supernatants were filtered through a 13 mm syringe filter with a 0.45 μm GHP membrane (Bulk GHP Acrodisc®, Pall Corporation, Ann Arbor, MI, USA) and placed in a Microcon® membrane filter device with a cut of on 30 kDa (Ultracel YM-30, Millipore Corporation, Bedford, MA, USA) containing cation exchanger (H^+) (Dowex®, Merck) and finally centrifuged for 30 min (approximately 9000 × *g*). The samples were then analysed for $InsP_2$–$InsP_6$. If only determination of $InsP_6$ was desired the evaporation and re-dissolving steps were omitted.

2.4. Identification of inositol phosphates

The commercial availability of the different inositol phosphate isomers is limited. Therefore, to isolate and identify the different isomers the system with a CarboPac™ PA100 column was used in a semi-preparative mode. The post-column system was dismounted, and the outlet from the column was connected directly to a Gilson 203 B Fraction Collector (Gilson Inc., Middleton, WI, USA). Fractions were isolated in intervals of 0.2 min from 2 to 16 min and from 18 to 20 min. In each separation 50 μL of reference sample was injected. Fractions from 40 consecutive separations were pooled and evaporated to dryness in a vacuum centrifuge at ambient temperature and re-dissolved in 1 mL water. This was repeated three times. The pooled isolated fractions were analysed on the CarboPac™ PA100 column. The gradient elution was modified according to Carlsson et al. [19] and Chen and Li [20] and was effected with a mixture of HCl (1.0 mol L^{-1}) and water: 0–13 min 5–26% HCl (linear curve); 13–21 min 26–80% HCl (weak concave curve); 21–25 min 80% HCl;

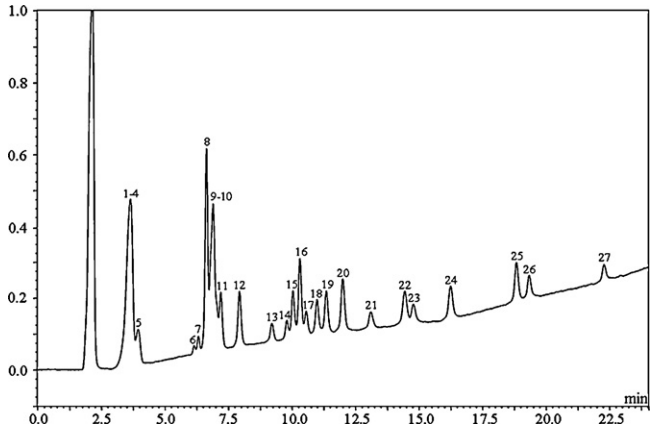

Fig. 2. Chromatogram of reference sample separated on a CarboPac™ PA100 using a gradient with HCl and water. (1–5) $InsP_2$, (6) $Ins(1,3,5)P_3$, (7) $Ins(2,4,6)P_3$, (8–11) $InsP_3$, (12) DL-$Ins(1,5,6)P_3$, (13) DL-$Ins(4,5,6)P_3$, (14) $Ins(1,2,3,5)P_4$, (15) DL-$Ins(1,2,4,6)P_4$, (16) DL-$Ins(1,2,3,4)P_4$, (17) $Ins(1,3,4,6)P_4$, (18) DL-$Ins(1,2,4,5)P_4$, (19) DL-$Ins(1,3,4,5)P_4$, (20) DL-$Ins(1,2,5,6)P_4$, (21) $Ins(2,4,5,6)P_4$, (22) DL-$Ins(1,4,5,6)P_4$, (23) $Ins(1,2,3,4,6)P_5$, (24) DL-$Ins(1,2,3,4,5)P_5$, (25) DL-$Ins(1,2,4,5,6)P_5$, (26) $Ins(1,3,4,5,6)P_5$, (27) $InsP_6$. Peaks are identified according to Carlsson et al. [19] and Chen and Li [20].

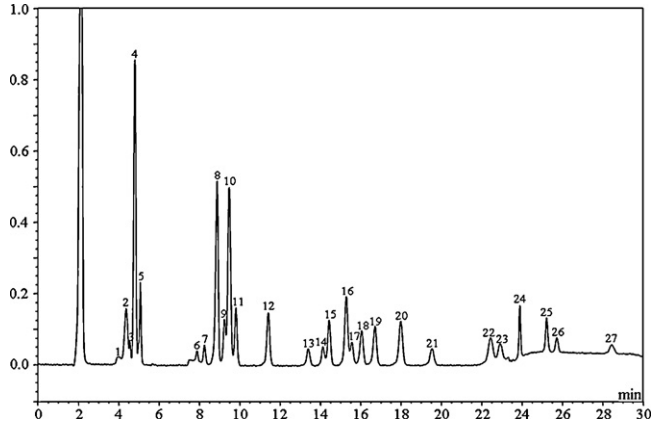

Fig. 3. Chromatogram of reference sample separated on a CarboPac™ PA1 using a gradient with methanesulfonic acid and water. (1–5) $InsP_2$, (6) $Ins(1,3,5)P_3$, (7) $Ins(2,4,6)P_3$, (8–11) $InsP_3$, (12) DL-$Ins(1,5,6)P_3$, (13) DL-$Ins(4,5,6)P_3$, (14) $Ins(1,2,3,5)P_4$, (15) DL-$Ins(1,2,4,6)P_4$, (16) DL-$Ins(1,2,3,4)P_4$, (17) $Ins(1,3,4,6)P_4$, (18) DL-$Ins(1,2,4,5)P_4$, (19) DL-$Ins(1,3,4,5)P_4$, (20) DL-$Ins(1,2,5,6)P_4$, (21) $Ins(2,4,5,6)P_4$, (22) DL-$Ins(1,4,5,6)P_4$, (23) $Ins(1,2,3,4,6)P_5$, (24) DL-$Ins(1,2,3,4,5)P_5$, (25) DL-$Ins(1,2,4,5,6)P_5$, (26) $Ins(1,3,4,5,6)P_5$, (27) $InsP_6$.

25–25.1 min 80–5% HCl (linear curve). The flow rates of eluents and post-column colour reagent were 1.0 and 0.65 mL min^{-1}, respectively. The column temperature was maintained at 10 °C and the equilibration time was 6 min. Peaks were assigned according to a modified version of the methods by Carlsson et al. [19] and Chen and Li [20] (Fig. 2). Fractions containing single peaks were further analysed on the CarboPac™ PA1 column (Fig. 3) to verify which peaks are identical in the two systems (Fig. 4).

For further identification of the inositol phosphates present in the isolated fractions, a mass spectrum was collected using positive electrospray on a Quattro LC tandem mass spectrometer (Micromass, Manchester, UK). A carrier solvent consisting of 50% methanol and 0.25% acetic acid at a speed of 0.4 mL min^{-1} was used. The fractions were teed in the carrier solvent at a speed of 2 μL min^{-1} using a Harward 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). The settings for the electrospray were: desolvation gas (N_2) 550 L h^{-1} at a temperature of 350 °C, cone gas (N_2) 30 L h^{-1} capillary voltage 3.0 kV, and source temperature 120 °C. The cone voltage was set individually for each of the inositol phosphates.

2.5. Quantification of inositol phosphates

The lack of lower inositol phosphates makes it difficult to prepare individual standard curves. Therefore, the phytic acid dodecasodium salt hydrate was used as standard both for $InsP_6$ and the lower inositol phosphates. Correction factors for difference in detector response for $InsP_5$, $InsP_4$, $InsP_3$ and $InsP_2$ isomers compared to $InsP_6$ were calculated. The ratio between peak area and phosphorus amount (area/mmol L^{-1}) of the inositol phosphates in the isolated fractions was calculated and set in relation to $InsP_6$ to estimate factors which correct for difference in detector response of the different inositol phosphate isomers.

The amount of P in each of the isolated fractions was determined by means of ICP-MS on an X Series^{II} ICP-MS equipped with a conventional Meinhard nebulizer and a Peltier cooled quartz impact bead spray chamber operated at 3 °C (Thermo Electron Corporation, Bremen, Germany). Data were collected using PlasmaLab version 2.5.9.30 (Thermo Electron). The instrument settings were: forward power 1444 W, plasma gas (Ar) 14 L min^{-1}, nebulizer gas (Ar) 0.87 L min^{-1}, auxiliary gas (Ar) 0.7 L min^{-1}. Sample uptake was approximately 0.4 mL min^{-1} and ^6Li and ^{45}Sc were used as internal standards with interpolation. The fractions were diluted 50–1000 times with 2% nitric acid depending on the expected content of P.

2.6. Validation

The linearity of the standard curves was investigated in the interval from 2 to 2000 mg P L^{-1} by serial dilution of the reference sample and $InsP_6$ stock solution.

The recovery of $InsP_6$–$InsP_2$ was determined by spiking the five different matrices wheat, soybean meal, rapeseed cake, gastric and ileal digesta from pigs with known amounts of reference sample at a high and a low level before extraction and compared with the corresponding non-spiked samples.

For within-day and between-day variations the same five matrices were used. To determine the between-day variation the samples were prepared and analysed in duplicate on five dif-

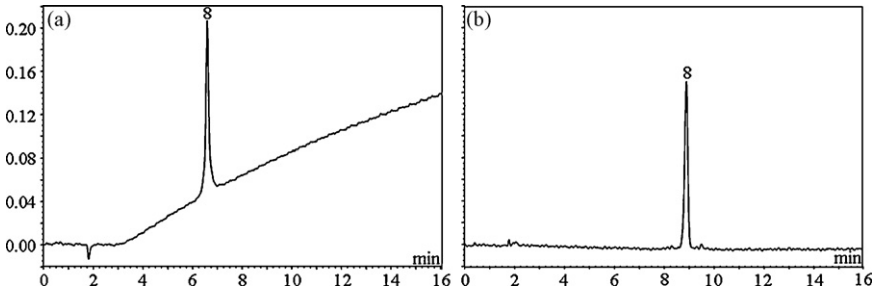

Fig. 4. Example of a chromatogram of an isolated fraction containing $InsP_3$ separated on a (a) CarboPac™ PA100 using a gradient with HCl according to Carlsson et al. [19] and Chen and Li [20] and (b) CarboPac™ PA1 column using a gradient with methanesulfonic acid.

ferent days. Furthermore, on one of these days seven replicates were prepared and analysed to determine the within-day variation.

3. Results and discussion

3.1. Purity of $InsP_6$

The content of water of $InsP_6$ (phytic acid dodecasodium salt) was 6.5% and 12.9%, respectively, when using drying in vacuum at ambient temperature and 103 °C. The water content is declared to be 12.6% after drying 2 h at 110 °C. The free content of phosphate was less than 0.02%, while the content of lower inositol phosphates was below the limit of detection. The content of sodium and total phosphorus was 29.3% and 18.3%. The theoretical content is 29.9% and 20.1% which gives a purity of 98% and 91%, respectively. The purity of 91% (total P) was used for construction of standard curves.

3.2. Test of different columns, eluents and column temperatures

Three different anion exchange columns were tested: CarboPac™ PA200 (3 mm × 250 mm i.d., 5.5 μm), CarboPac™ PA100 (4 mm × 250 mm i.d., 8.5 μm) and CarboPac™ PA1 (4 mm × 250 mm i.d., 10 μm). The small particle size of the PA200 column resulted in a high back pressure when identical flow rates were used. Alternatively, it should be operated at lower flow rates with longer separation times. However, the number of isomers separated in the reference sample was lower than the number found using the PA1 column which made the PA200 column less usable. The PA1 had a better separation of the isomers than the PA100. Several PA100 columns with different lot numbers were tested. Some of these lots separated the isomers considerably poorer than the lot used during the initial optimization of the gradient. The same variability in the separation was not observed with different lot numbers of the PA1 column. Thus, the PA1 column was considered more robust and was selected for further method validation.

A common disadvantage of the methods published [18–21] is the increasing baselines which makes it difficult to quantify compounds which is located at the increasing baseline. The increasing baseline has been explained as side effects of the HCl gradient used due to a refractive index phenomena caused by the changing HCl concentration [18]. In the search for alternative acids to be used in the gradient instead of HCl, formic acid, sulphuric acid, nitric acid and methanesulfonic acid were tested. Methanesulfonic acid appeared to be capable of separating the highest number of inositol phosphates. By substituting 1 mol L^{-1} HCl (pH 0.16) with 1.5 mol L^{-1} methanesulfonic acid (pH 0.26) the elutrophic strength of the acids was comparable when the start percent of the acid is 5% and the final percent is 80–85%, it may therefore be concluded that the methanesulfonic acid is a weaker eluent than HCl. However, methanesulfonic acid resulted in an almost horizontal baseline (Fig. 3). This makes the integration and thus quantification of the amounts of inositol phosphates much more precise. Secondly, samples with a high concentration of $InsP_6$ in relation to $InsP_5$–$InsP_2$ can to a greater extent be analysed for $InsP_6$ and $InsP_5$–$InsP_2$ during the same separation, because the absorbance range is not narrowed by an increasing baseline. Furthermore, methanesulfonic acid is less aggressive than HCl, which allows the use of a non-PEEK HPIC system.

Increasing the column temperature from 30 to 40 °C using the PA1 column resulted in a better resolution between peaks 1 to 11 ($InsP_2$ and $InsP_3$ isomers), whereas the resolution between peaks 14 to 17 ($InsP_4$ isomers) was deteriorated. In contrast, Chen and Li [20] found that by increasing the column temperature from 20 to 45 °C (PA100 column, gradient elution: HCl and water) the resolution between $InsP_2$ and $InsP_3$ isomers decreased, whereas the resolution between $InsP_4$ isomers improved. Furthermore, by decreasing the column temperature from 30 to 10 °C in the present study the reso-

Table 2
Linearity of the standard curves[a] of inositol phosphates and limit of detection (LOD) (S/N ratio = 3). The LOD was determined in the isolated fractions. The analyses were performed on the PA1 column. Three determinations were conducted.

No.	Inositol phosphates	Linearity (mg P L^{-1})	R^2	Slope	Intercept	LOD[b] (mg P L^{-1})
1[c]	$InsP_2$					
2	$InsP_2$	5–40	0.998	0.0548	−0.0229	1.9
3[c]	$InsP_2$	5–40	0.307	−0.0009	0.0070	
4[c]	$InsP_2$	5–40	0.953	0.0507	0.0453	
5	$InsP_2$	4–85	0.989	0.0189	0.0019	1.6
6	Ins(1,3,5)P_3	11–220	0.848	0.0027	0.0016	4.2
7	Ins(2,4,6)P_3	12–235	0.984	0.0036	0.0025	4.4
8	$InsP_3$	6–120	0.997	0.0711	0.0083	2.1
9[c]	$InsP_3$		0.952	0.0110	0.0034	
10	$InsP_3$	5–110	0.997	0.0770	0.0087	1.9
11	$InsP_3$	5–90	0.997	0.0176	0.0031	1.7
12	DL-Ins(1,5,6)P_3	5–90	0.997	0.0247	0.0028	1.7
13	DL-Ins(4,5,6)P_3	3–70	0.997	0.0083	0.0009	1.3
14	Ins(1,2,3,5)P_4	8–160	0.996	0.0083	0.0006	2.9
15	DL-Ins(1,2,4,6)P_4	7–140	0.997	0.0192	0.0022	2.6
16	DL-Ins(1,2,3,4)P_4	6–130	0.997	0.0327	0.0034	1.7
17	Ins(1,3,4,6)P_4	6–130	0.997	0.0110	0.0012	2.9
18	DL-Ins(1,2,4,5)P_4	7–290	0.996	0.0167	0.0019	2.7
19	DL-Ins(1,3,4,5)P_4	6–120	0.996	0.0211	0.0020	2.4
20	DL-Ins(1,2,5,6)P_4	6–120	0.997	0.0237	0.0024	2.3
21	Ins(2,4,5,6)P_4	2–290	0.998	0.0092	0.0006	2.7
22	DL-Ins(1,4,5,6)P_4	9–350	0.992	0.0219	−0.0009	3.3
23	Ins(1,2,3,4,6)P_5	5–200	0.995	0.0107	0.0004	1.9
24	DL-Ins(1,2,3,4,5)P_5	3–140	0.993	0.0130	0.0004	1.3
25	DL-Ins(1,2,4,5,6)P_5	4–170	0.993	0.0115	0.0004	1.6
26	Ins(1,3,4,5,6)P_5	2–192	0.992	0.0074	−0.0012	0.9
27	$InsP_6$	11–920	0.992	0.0082	−0.0029	4.3

[a] Serial dilution of the reference sample.
[b] Injection volume 50 μL.
[c] Values are missing for the inositol phosphates of which it was not possible to isolate a fraction containing only the desired inositol phosphate.

Table 3
Identification of inositol phosphates in isolated fractions by means of LC-MS[a] ($M+1 = M/z$)[b]. The numbers in parentheses refer to the relative intensity (%) of the fragments compared to the base peak (100%). One determination was conducted.

No.	Inositol phosphates	M/z			
		$M+1$			
1–4	$InsP_2$	340.9	109.1 (100)	242.9 (35)	224.9 (33)
5	$InsP_2$	340.9	81.3 (100)	242.8 (97)	144.8 (38)
6	Ins(1,3,5)P_3	420.8	403.0 (100)	305.2 (40)	304.6 (60)
7	Ins(2,4,6)P_3	420.8	304.8 (100)	403.0 (58)	224.9 (60)
8	$InsP_3$	420.8	402.8 (100)	304.8 (95)	225.0 (68)
9–10	$InsP_3$	420.8	323.0 (100)	402.9 (63)	305.0 (63)
11	$InsP_3$	420.8	109.1 (100)	403.0 (36)	323.0 (37)
12	DL-Ins(1,5,6)P_3	420.8	109.1 (100)	323.0 (45)	305.0 (60)
13	DL-Ins(4,5,6)P_3	420.8	99.1 (100)	323.0 (45)	197.0 (31)
14	Ins(1,2,3,5)P_4	500.8	384.8 (100)	482.9 (66)	402.9 (42)
15	DL-Ins(1,2,4,6)P_4	500.7	384.9 (100)	482.9 (54)	402.8 (30)
16	DL-Ins(1,2,3,4)P_4	500.8	384.9 (100)	482.9 (57)	402.8 (57)
17	Ins(1,3,4,6)P_4	500.8	482.8 (100)	483.0 (59)	482.5 (37)
18	DL-Ins(1,2,4,5)P_4	500.9	384.8 (100)	482.8 (25)	482.5 (15)
19	DL-Ins(1,3,4,5)P_4	500.8	384.8 (100)	482.9 (74)	402.8 (16)
20	DL-Ins(1,2,5,6)P_4	500.8	384.8 (100)	482.8 (79)	403.0 (26)
21	Ins(2,4,5,6)P_4	500.8	384.9 (100)	482.9 (55)	402.9 (17)
22	DL-Ins(1,4,5,6)P_4	500.8	384.9 (100)	482.9 (54)	402.9 (24)
23	Ins(1,2,3,4,6)P_5	580.7	562.7 (100)	483.0 (46)	464.9 (85)
24	DL-Ins(1,2,3,4,5)P_5	580.6	562.8 (100)	483.1 (18)	464.9 (37)
25	DL-Ins(1,2,4,5,6)P_5	580.6	385.0 (100)	562.5 (49)	465.0 (61)
26	Ins(1,3,4,5,6)P_5	580.7	562.9 (100)	464.8 (80)	366.8 (71)
27[c]	$InsP_6$	660.9	446.9 (100)	465.0 (55)	367.0 (34)

[a] LC-MS: liquid chromatography mass spectrometry.
[b] M + 1: molecular ion + hydrogen, M/z: mass to charge ratio.
[c] Spectrum obtained from a standard solution of phytic acid dodecasodium salt.

lution between peaks 14 to 17 ($InsP_4$ isomers) improved along with a decreased resolution between 1 to 11 ($InsP_2$ and $InsP_3$ isomers) and 22 to 23 ($InsP_4$ and $InsP_5$ isomers). No considerable change for separation of peaks 18 to 21 ($InsP_4$ isomers) and 24 to 26 ($InsP_5$ isomers) appeared when changing the column temperature. A column temperature of 30 °C was chosen as the best compromise.

3.3. Separation of inositol phosphates on the chromatographic system

Inspecting the chromatogram of the reference sample (Fig. 3), a total of 27 peaks can be counted. In comparison, Carlsson et al. [19] separated 22 peaks within 17 min, while Chen and Li [20] separated

Table 4
Correction factors for use of the standard curve for $InsP_6$ to quantify the phosphorus content in the other inositol phosphates separated on the PA1 column (mean ± sd; four determinations were conducted). The calculation is based on the determination of phosphorus content in the isolated fractions.

No.	Inositol phosphates	Factor	CV (%)
1[a]	$InsP_2$		
2	$InsP_2$	2.2 ± 0.03	1.5
3[a]	$InsP_2$		
4[a]	$InsP_2$		
5	$InsP_2$	2.1 ± 0.11	5.3
6	Ins(1,3,5)P_3	3.7 ± 0.03	8.2
7	Ins(2,4,6)P_3	2.5 ± 0.24	9.4
8	$InsP_3$	1.6 ± 0.15	9.7
9[a]	$InsP_3$		
10	$InsP_3$	1.2 ± 0.03	3.1
11[b]	$InsP_3$	1.3	
12	DL-Ins(1,5,6)P_3	1.2 ± 0.06	4.8
13	DL-Ins(4,5,6)P_3	1.1 ± 0.14	12.9
14	Ins(1,2,3,5)P_4	1.5 ± 0.24	16.2
15	DL-Ins(1,2,4,6)P_4	1.3 ± 0.07	5.6
16	DL-Ins(1,2,3,4)P_4	1.0 ± 0.09	8.8
17	Ins(1,3,4,6)P_4	1.2 ± 0.07	6.3
18	DL-Ins(1,2,4,5)P_4	1.3 ± 0.05	4.1
19	DL-Ins(1,3,4,5)P_4	1.1 ± 0.13	11.3
20	DL-Ins(1,2,5,6)P_4	1.1 ± 0.10	8.8
21	Ins(2,4,5,6)P_4	1.2 ± 0.07	6.0
22	DL-Ins(1,4,5,6)P_4	1.4 ± 0.19	13.3
23	Ins(1,2,3,4,6)P_5	1.3 ± 0.15	11.9
24	DL-Ins(1,2,3,4,5)P_5	1.7 ± 0.03	1.9
25	DL-Ins(1,2,4,5,6)P_5	1.9 ± 0.21	11.1
26	Ins(1,3,4,5,6)P_5	1.8 ± 0.14	7.9
27	$InsP_6$	1.0	

[a] Values are missing for the inositol phosphates of which it was not possible to isolate a fraction containing only the desired inositol phosphate.
[b] Only one fraction contained the desired inositol phosphate ($n = 1$).

Table 5
Recovery (%) of inositol phosphates in six different matrices separated on the PA1 column. Samples were spiked with a low or a high level of reference sample as well as stock solution of $InsP_6$ (mean ± sd; three determinations were conducted).

No.	Inositol phosphates	High level[a]							Low level[b]						
		mg P added	Wheat	Soybean meal	Rapeseed cake	Diet	Gastric digesta	Ileal digesta	mg P added	Wheat	Soybean meal	Rapeseed cake	Diet	Gastric digesta	Ileal digesta
8	$InsP_3$	3.40	91 ± 1	86 ± 4	110 ± 1	107 ± 9	102 ± 9	83 ± 1	0.85	96 ± 2	79 ± 4	98 ± 3	85 ± 0	86 ± 2	54 ± 2
9	$InsP_3$	0.40	60 ± 2	91 ± 5	130 ± 2	109 ± 4	103 ± 9	70 ± 4	0.10	94 ± 1	59 ± 9	83 ± 6	69 ± 6	53 ± 7	99 ± 4
10	$InsP_3$	5.00	100 ± 2	85 ± 3	95 ± 1	103 ± 9	103 ± 9	90 ± 2	1.25	103 ± 2	89 ± 4	98 ± 1	94 ± 2	98 ± 2	85 ± 2
11	$InsP_3$	1.00	78 ± 2	79 ± 6	113 ± 1	88 ± 6	83 ± 8	68 ± 2	0.25	93 ± 3	79 ± 5	79 ± 6	86 ± 7	81 ± 10	107 ± 30
12	DL-Ins(1,5,6)P_3	1.40	101 ± 2	100 ± 2	101 ± 4	123 ± 11	120 ± 9	100 ± 1	0.35	100 ± 2	110 ± 6	100 ± 7	99 ± 3	106 ± 2	98 ± 7
13	DL-Ins(4,5,6)P_3	0.40	97 ± 3	99 ± 10	86 ± 6	117 ± 16	149 ± 11	105 ± 4	0.10	98 ± 1	121 ± 9	99 ± 6	103 ± 1		96 ± 4
14	Ins(1,2,3,5)P_4	0.40	89 ± 0	93 ± 3	102 ± 5	99 ± 8	107 ± 8	89 ± 4	0.10	102 ± 3	118 ± 2	117 ± 17	103 ± 15	103 ± 9	103 ± 5
15	DL-Ins(1,2,4,6)P_4	1.20	86 ± 1	83 ± 2	89 ± 1	94 ± 7	93 ± 8	87 ± 1	0.30	98 ± 2	88 ± 3	94 ± 13	81 ± 8	90 ± 4	104 ± 0
16	DL-Ins(1,2,3,4)P_4	3.00	91 ± 2	84 ± 2	89 ± 2	105 ± 11	117 ± 11	110 ± 1	0.75	107 ± 3	100 ± 11	106 ± 5	84 ± 9	111 ± 7	109 ± 17
17	Ins(1,3,4,6)P_4	0.60	101 ± 1	91 ± 6	94 ± 1	108 ± 14	105 ± 11	74 ± 4	0.15	97 ± 3	94 ± 7	94 ± 7	82 ± 16	80 ± 8	102 ± 37
18	DL-Ins(1,2,4,5)P_4	1.00	86 ± 2	85 ± 3	84 ± 4	99 ± 10	98 ± 8	88 ± 5	0.25	90 ± 1	107 ± 8	97 ± 8	77 ± 8	88 ± 5	95 ± 22
19	DL-Ins(1,3,4,5)P_4	1.47	99 ± 1	96 ± 2	95 ± 2	110 ± 14	111 ± 13	90 ± 2	0.37	93 ± 1	95 ± 4	102 ± 9	83 ± 8	93 ± 11	73 ± 14
20	DL-Ins(1,2,5,6)P_4	2.00	85 ± 2	91 ± 3	87 ± 3	89 ± 5	99 ± 8	81 ± 1	0.50	96 ± 2	94 ± 11	83 ± 2	78 ± 3	100 ± 3	118 ± 12
21	Ins(2,4,5,6)P_4	0.47	76 ± 3	82 ± 4	69 ± 2	78 ± 6	82 ± 8	48 ± 4	0.12	82 ± 10	95 ± 9	84 ± 5	74 ± 7	70 ± 3	
22	DL-Ins(1,4,5,6)P_4	1.00	94 ± 2	96 ± 3	98 ± 3	93 ± 6	98 ± 10	70 ± 2	0.25	98 ± 6	109 ± 18	109 ± 3	79 ± 9	98 ± 2	107 ± 6
23	Ins(1,2,3,4,6)P_5	0.80	86 ± 7	98 ± 6	94 ± 2	95 ± 9	111 ± 22	85 ± 4	0.20	113 ± 3	111 ± 26	146 ± 12	91 ± 14	85 ± 20	109 ± 18
24	DL-Ins(1,2,3,4,5)P_5	0.80	95 ± 3	93 ± 3	86 ± 3	126 ± 7	122 ± 21	105 ± 8	0.20	107 ± 11	81 ± 12	114 ± 5	73 ± 12	69 ± 11	98 ± 10
25	DL-Ins(1,2,4,5,6)P_5	0.70	97 ± 0	85 ± 7	87 ± 3	123 ± 18	111 ± 14	113 ± 1	0.18	117 ± 3	120 ± 5	121 ± 8	82 ± 5	94 ± 4	109 ± 7
26	Ins(1,3,4,5,6)P_5	0.20	106 ± 2	99 ± 7	80 ± 7	140 ± 12	128 ± 16	113 ± 4	0.05	103 ± 5	94 ± 20	69 ± 13	64 ± 11	81 ± 6	96 ± 15
27	$InsP_6$	48.0	95 ± 3	96 ± 1	75 ± 3	99 ± 3	95 ± 4	75 ± 1	19.0	93 ± 3	70 ± 1	43 ± 7	97 ± 31	124 ± 13	113 ± 8

[a]Injection volume 15 μL. [b]The injection volume was 50 μL for wheat and 25 μL for soybean meal, rapeseed cake, diet, gastric digesta and ileal digesta.

Table 6
Within-day ($n = 7$) and between-day ($n = 5$) variations of the inositol phosphates present in wheat, soybean meal, rapeseed cake, diet, gastric and ileal digesta when separated on the PA1 column. Not all inositol phosphates were present in the different matrices. Concentrations in $\mu g\,P\,kg^{-1}$ dry matter (mean ± sd).

No.	Wheat				Soybean meal[a]				Rapeseed cake			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)
1									7.4 ± 0.4	5.4	6.8 ± 0.4	6.4
4					64.1 ± 4.4	6.9	64.4 ± 3.4	5.2	13.2 ± 0.4	3.0	13.1 ± 0.3	2.5
5	36.9 ± 0.9	2.5	36.2 ± 0.6	1.6								
6					1.9 ± 0.1	4.8	1.9 ± 0.2	7.9				
8					4.0 ± 0.2	4.9	4.1 ± 0.1	3.2	8.6 ± 1.5	17.6	7.7 ± 1.1	14.3
13					6.4 ± 0.6	9.0	6.1 ± 0.4	6.2				
23	4.2 ± 0.1	2.8	4.1 ± 0.1	1.3	6.4 ± 0.3	4.8	6.9 ± 0.5	7.1	11.6 ± 0.4	3.6	12.4 ± 0.4	3.1
24	4.1 ± 0.1	2.9	4.2 ± 0.1	1.8	7.7 ± 0.3	3.5	8.2 ± 0.5	6.6	11.6 ± 0.5	4.4	12.3 ± 0.4	3.5
26					4.5 ± 0.2	3.6	4.8 ± 0.2	4.2	5.0 ± 0.3	6.4	5.4 ± 0.2	3.2
27[b]	1.7 ± 0.1	4.8	1.7 ± 0.03	2.1	2.8 ± 0.2	7.2	3.1 ± 0.2	5.3	5.6 ± 0.1	1.4	6.3 ± 0.1	1.8

No.	Diet				Gastric digesta				Ileal digesta			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)	Mean ± sd	CV (%)
1									4.1 ± 0.5	11.9	4.1 ± 0.3	7.4
3									7.1 ± 0.6	8.7	6.4 ± 0.3	4.0
4	38.0 ± 2.4	6.4	36.7 ± 0.9	2.4	33.4 ± 0.7	2.0	33.4 ± 0.5	1.6				
5									84 ± 1.2	1.4	87 ± 0.9	1.1
6					1.8 ± 0.0	1.6	1.9 ± 0.1	5.7				
9					5.9 ± 0.0	0.7	6.1 ± 0.1	1.9	11.0 ± 0.7	6.2	11.1 ± 0.2	1.8
10									7.9 ± 0.5	6.8	8.2 ± 0.3	4.2
13					8.1 ± 1.2	14.3	7.8 ± 1.1	13.6				
16					10.5 ± 0.2	2.3	11.1 ± 0.4	4.0	22.6 ± 1.2	5.1	22.8 ± 0.6	2.6
17									11.8 ± 0.6	5.1	11.4 ± 0.3	3.0
19	6.6 ± 0.2	3.2	7.1 ± 0.1	1.8	6.3 ± 0.1	1.4	6.3 ± 0.2	2.6	12.4 ± 0.4	2.9	12.4 ± 0.3	2.7
20					7.2 ± 0.1	1.7	7.7 ± 0.3	3.6	15.9 ± 1.4	8.5	16.5 ± 0.6	3.9
23					7.2 ± 0.2	2.5	7.9 ± 0.3	4.1	18.6 ± 0.5	2.5	17.8 ± 0.5	2.8
24	4.0 ± 0.3	6.6	4.2 ± 0.3	6.9	8.2 ± 0.3	3.7	8.7 ± 0.2	2.0	22.8 ± 0.5	2.1	22.3 ± 0.4	1.9
26					3.0 ± 0.1	4.3	3.1 ± 0.1	2.6				
27[b]	2.3 ± 0.1	4.5	2.4 ± 0.1	1.8	1.7 ± 0.1	3.6	1.8 ± 0.03	1.7	6.1 ± 0.1	2.2	6.1 ± 0.1	1.2

[a] Within-day ($n = 6$) and between-day ($n = 5$).
[b] Concentrations in $g\,P\,kg^{-1}$ dry matter.

27 peaks within 50 min, which is 20 min longer than in the present methods (30 min). The inositol phosphates eluted in the order of increasing numbers of phosphate groups on the inositol ring, which is in accordance with Carlsson et al. [19] and Chen and Li [20]. The present method separated $InsP_2$ and $InsP_3$ superiorly compared to the method by Carlsson et al. [19], but not as good as the method by Chen and Li [20]. On the other hand, the separation of $InsP_4$ and $InsP_5$ by the present method was superior to the method by Chen and Li [20] while it was equal to the method by Carlsson et al. [19].

3.4. Linearity and detection limits

Serial dilution of the reference sample was used to study the linearity of the different inositol phosphates on PA1 by plotting peak areas versus the concentrations of different dilutions. The correlation coefficient (R^2), slope and intercept of the plots are given in Table 2. The peak areas were linearly dependent on the concentrations of inositol phosphates within the range of 2–12 to 40–920 $mg\,P\,L^{-1}$ for the different isomers. The span of linearity was the highest for $InsP_6$ with a range from 11 to 920 $mg\,P\,L^{-1}$. The linear regression analysis gave correlation coefficients of approximately 1 for most of the inositol phosphates except for nos. 3 and 6 which may be due to the small peak area of these components.

The limit of detection (S/N = 3) for the different inositol phosphates was determined in the isolated fractions (Table 2). The detection limits for $InsP_2$–$InsP_6$ were in the range 0.9–4.4 $mg\,P\,L^{-1}$. Using LC-MS/MS Liu et al. [25] reported a LOD for $InsP_6$ of 60 pmol which is a 20 times increase in the sensitivity compared with the present HPIC method (LOD $InsP_6$ of 4.4 $mg\,L^{-1}$ corresponding to 1.2 nmol injected). However, the LC-MS/MS method by Liu et al. [25] was only capable of separating 6 isomers compared with 27 in the present method. Furthermore, the LOD for $InsP_6$ in the present study was approximately 10 times higher than the LOD obtained by the HPIC method described by Chen [21]. However, the linearity of the present method was better for $InsP_6$ and $InsP_5$ up to 4.9–1.3 $mmol\,L^{-1}$ compared with 1.2–0.5 $mmol\,L^{-1}$ found in the study by Chen [21].

3.5. Identification and correction factors for quantification of inositol phosphates

With the exception of the $InsP_2$ and $InsP_3$ components (no. 8–11 in Fig. 3), the order of elution of the inositol phosphates on the PA1 corresponded with the order on the PA100 found by Carlsson et al. [19] and Chen and Li [20]. Compared with the chromatogram of Carlsson et al. [19] and Chen and Li [20] it seems that one or more peaks in the group of peaks 8–11 shifts position.

The mol weight of the different inositol phosphates in the isolated fractions were determined using electrospray ionization mass spectrometry for further identification of the inositol phosphates (Table 3). The assignment of the peaks within the groups of $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$ corresponds precisely with the determined mol weight which confirm the identification of the compounds. Additionally, the mol weight of these compounds matches the mol weight of different inositol phosphates measured by Cooper et al. [26] and Liu et al. [25].

Correction factors for each of the inositol phosphate isomers were calculated (Table 4). Sandberg and Ahderinne [27] calculated

that the correction factors for $InsP_3$, $InsP_4$ and $InsP_5$ were 2.4, 1.5 and 1.1 using a reverse phase HPLC method with a gradient between formic acid/methanol and tetrabutylammonium hydroxide and refractive index detection. Skoglund et al. [18] showed, using a HPIC method with post-column reaction with $Fe(NO_3)_3$, that the calculated correction factors for $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$ were 3, 2, 1.5, 1.2 and 1, respectively, when using a OmniPac PAX100 column and a HCL gradient. These factors differ from the factors calculated in the present study possibly because a different column is used and most likely because methanesulfonic acid, which is a completely different acid compared to HCl, is used in the eluent. As such, it is important to use correction factors which are established in the matching system.

3.6. Validation

It was not possible to find any matrices for validation that did not contain any inositol phosphates. However, all the samples selected had a low content of all the compounds except for $InsP_6$. Table 5 presents the recoveries of $InsP_3$–$InsP_6$. At both spiking levels, the recovery of $InsP_2$ (nos. 1–5), Ins(1,3,5)P_3 (no. 6) and Ins(2,4,6)P_3 (no. 7) was poor (data not shown) probably because most of the peaks were relatively small and eluted very close to each other which makes accurate peak integration difficult. The recovery of the remaining inositol phosphates was in general good (80–100%). In a study with rye roll spiked with 1 or 2 μmol of InsP(2)P, DL-Ins(1,2,6)P_3 and $InsP_6$, the recovery of the added standards was 95–104% [18]. Liu et al. [25] conducted a similar recovery study on oat as the present study. The recoveries were in the same range with recoveries close to 100% at the high level and higher variations at the lower spiking level [25]. In the present study, the recovery of $InsP_6$ was in general better at the high compared with the low spiking level of $InsP_6$. At the high spiking level, the recovery of $InsP_6$ was 95–99% for wheat, soybean meal, diet and gastric digesta, whereas it was 75% for rapeseed cake and ileal digesta. The poor recovery of $InsP_6$ in rapeseed cake and ileal digesta may be due to the higher content of $InsP_6$ in rapeseed cake and ileal digesta compared with the rest of the samples. Therefore, an even higher $InsP_6$ spiking level is required to obtain a better recovery of $InsP_6$ in this type of matrices. This is confirmed by the present results obtained by the "short $InsP_6$ method" (isocratic elution), where the amount of $InsP_6$ in the sample was less concentrated as the samples were not evaporated. As such, the amount of spiked $InsP_6$ and the amount of $InsP_6$ in the sample was smaller compared with the evaporated samples ("long method"), resulting in good recovery (90–111%) of $InsP_6$ for all samples at the high $InsP_6$ spiking level.

The within-day ($n = 7$) and between-day ($n = 5$) coefficient of variations for $InsP_2$–$InsP_6$ are shown in Table 6. In general, the CV was low. For most of the compounds the CV values were highest for the soybean meal compared with the other matrices except for peak no. 13, which was highest for rapeseed cake and gastric digesta. The occurrence of more isomer forms of inositol phosphates in soybean meal and rapeseed cake compared with wheat corresponds with results by Kasim and Edwards [28] and Pontoppidan et al. [29] and is most likely caused by the extreme processing of the oilseeds. The gastric and ileal digesta contained considerably more different isomer forms of inositol phosphates than the diet fed to the pigs. This is due to the hydrolysis of $InsP_6$ to the lower inositol phosphates along the gastrointestinal tract of the pig.

4. Conclusion

A sensitive and reproducible HPIC method for separation of 27 different inositol phosphates in feedstuffs, diets, gastric and ileal digesta from pigs was developed using methanesulfonic acid in the eluent instead of HCl that is normally used. This exchange to methanesulfonic acid resulted in an almost horizontal baseline during the chromatographic separation whereby the isomers could be integrated more accurately. The standard curve for $InsP_6$ was used for quantification of all the isomers. Due to the new column and eluent it was necessary to establish new correction factors for quantification of the different inositol phosphates in the tested matrixes using $InsP_6$ as calibration standard. The obtained correction factors were different compared to former published correction factors using other columns and eluents emphasizing that it seems to be important to use correction factors established by use of matching systems. Additionally, the new method based on methanesulfonic acid is more flexible and non-corrosive whereby the method can be established on a non-PEEK HPIC system.

Acknowledgement

The project was granted by The Danish Food Industry Agency, The Ministry of Food, Agriculture and Fisheries.

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