Determination of Phytic Acid in Cereal Grains, Legumes, and Feeds by Capillary Isotachophoresis

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A method is described for the determination of phytic acid in various plant materials and feeds based on isotachophoresis. The electrophoretic migration properties of phytic acid, the lower inositol phosphates, and phosphate were investigated in various buffering systems. It was found that the electrolyte system consisting of 0.01 mol/L hydrochloric acid, 0.0056 mol/L bis-tris-propane (pH 6.1) as leading electrolyte, and 0.005 mol/L 2-morpholinoethanesulfonic acid as terminating electrolyte is most appropriate for the analysis of phytic acid in real samples using a volume-coupling isotachophoretic instrument. Calibration was carried out in the concentration range between 10^{-5} and 1.2×10^{-4} mol/L. The detection limit was less than 10^{-6} mol/L, which corresponds to a phytic acid content of the samples of less than 0.08%. The precision of the determinations including sample pretreatment, expressed by the relative standard deviation, was 3.8%.

Keywords: *Phytic acid; isotachophoresis; determination; cereals; legumes; feeds*

INTRODUCTION

Phytic acid (inositol hexaphosphoric acid, IP6) is a common component in grains. For the plants it is a source of phosphate as well as inositol. In human and animal nourishment IP6 is, on the other hand, considered to be an antinutrient because it decreases the bioavailability of trace elements and minerals. Due to this activity its control in food is an important task.

Most methods for the determination of IP6 are based on the insolubility of its ferric salt. The IP6 concentration is not measured directly, but indirectly from the iron or phosphate content of the precipitate (Sandberg et al., 1989; Doherty et al., 1982). Thompson and Erdman (1982a) determined IP6 indirectly, too, by the measurement of residual iron remaining in solution after the precipitation of the Fe–IP6 complex. In the above-mentioned methods the concentration of IP6 is derived from the assumed iron-to-phosphorus ratio in the precipitate. Significant variations of this ratio occur, however, as discussed by the same authors (Thompson and Erdman, 1982b). Consequently, they do not recommend the use of such determinations. One method is based on the enzymatic hydrolysis of IP6 by phytase (Uppström and Svenson, 1980). Released phosphate is then determined spectrophotometrically. Frølich et al. (1986) analyzed IP6 by ^{31}P-NMR spectroscopy. Liquid chromatography was also used for the phytic acid determination. Most techniques use rather complicated sample pretreatment prior to HPLC analysis. Lehrfeld and Morris (1982) presented a method in which IP6 and other inositol phosphates were separated in a reversed-phase system with ion-pair chromatography. Cosgrove (1980) determined the phosphorus content of suitable aliquots of chromatographic fractions obtained from an anion exchange resin column. A rapid HPLC determination of phytic acid was published by Knuckles et al. (1982).

Capillary isotachophoresis (ITP) was also used for the analysis of IP6 (Kikunaga et al., 1985; Spano, 1992). Prior to the determination by ITP the plant extract was purified by precipitation of IP6 with Fe(III), in the same way as mentioned above. For electrophoresis the precipitate was converted into sodium phytate by the addition of sodium hydroxide.

It is seen that all approaches are based on partially complex preparations of the sample, mostly followed by indirect determinations of IP6, rather than by directly measuring the IP6 content. Therefore, it was the aim of this investigation to work out a most simple method for the analysis of IP6. It is based on the capillary isotachophoretic determination of IP6 directly in the plant extract. As the separation according to the pK of the separands is hardly possible (it can be expected that all solutes have too similar values), an approach based on the different degrees of complexation with the counterion of the buffering electrolyte was applied. The migration behavior of IP6, other inositol phosphates (inositol monophosphate to inositol pentaphosphate, IP1–IP5), and phosphate (P) was thus investigated in various electrolyte systems to enable the selection of favorable separation conditions, and its applicability was evaluated for a number of different plant matrices.

MATERIALS AND METHODS

Apparatus. Two different instruments were used. The volume-coupling instrument (Ionosep 900.1, Recman-Laboratorní technika, Ostrava, Czech Republic) was equipped with Teflon capillaries (preseparation part 50 × 1 mm, separation part 150 × 0.45 mm, detection part 70 × 0.3 mm). Detection was carried out with a contactless high-frequency conductivity cell.

The column-coupling instrument (Trace 1, United Research, Vienna, Austria) was equipped with capillaries (preseparation capillary 160 × 0.8 mm, analytical capillary 90 × 0.3 mm) made of a fluorinated ethylene–propylene copolymer. Detection was carried out with two conductivity cells consisting of platinum wires placed directly into the preseparation and the analytical capillaries, respectively.

The following constant current was applied: volume-coupling isotachophoretic instrument, initially 70 μA (15 μA during detection); column-coupling isotachophoretic instrument, preseparation capillary 250 μA, analytical capillary initially 60 μA (30 μA during the detection).

Reagents. The following chemicals were used: phytic acid (as dodecasodium salt), 1,3-bis[tris(hydroxy-

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0021-8561/95/1443-0129$09.00/0

Table 1. Electrolyte Systems Investigated for the Determination of Phytic Acid

system	leading electrolyte[a]	pH	terminating electrolyte
1	0.01 mol/L HCl + EACA[b]	4.5	0.005 mol/L caproic acid
2	0.01 mol/L HCl + 0.001 mol/L BTP + EACA[b]	4.5	0.005 mol/L caproic acid
3	0.01 mol/L HCl + 0.002 mol/L BTP + EACA[b]	4.5	0.005 mol/L caproic acid
4	0.01 mol/L HCl + 0.003 mol/L BTP + EACA[b]	4.5	0.005 mol/L caproic acid
5	0.01 mol/L HCl + 0.004 mol/L BTP + EACA[b]	4.5	0.005 mol/L caproic acid
6	0.01 mol/L HCl + His[b]	5.0	0.005 mol/L caproic acid
7	0.01 mol/L HCl + BTP[b]	6.1	0.005 mol/L MES
8	0.01 mol/L HCl + BTP[b]	6.3	0.005 mol/L MES
9	0.01 mol/L HCl + BTP[b]	6.5	0.005 mol/L MES
10	0.01 mol/L HCl + BTP[b]	6.9	0.005 mol/L MES

[a] In all systems HEC was added to the leading electrolyte at a concentration of 0.1%. [b] Substance used to adjust the given pH.

Table 2. Relative Step Heights (RSHs) of Inositol Phosphates and Phosphate in Different Electrolyte Systems[a]

	RSH (%)				
system	IP6	IP5	IP4–IP2	IP1	phosphate
1	6.2	6.2	7.5–23.5	64.5	33.1
2	19.2	15.7	14.3–28.7	73.2	37.7
3	24.3	19.1	18.0–28.7	69.0	36.1
4	41.9	25.1	25.5–31.0	74.4	38.3
5	57.9	43.8	31.2–37.2	85.1	41.7
6	17.5	17.5	*b*	*b*	55.8
7	71.5	63.6	39.7–54.7	53.6	44.5
8	75.1	67.4	*b*	*b*	41.9
9	79.6	73.0	44.2–57.8	52.0	39.1
10	84.1	78.1	40.0–63.0	52.9	37.2

[a] The RSH is related to the step heights of the terminating ion. Number of systems as in Table 1. Values are average from three separate replications; the relative standard deviation of RSHs (IP6, IP5, IP1, and phosphate) varied between 2 and 4%. Due to difficulties with evaluation of step heights of IP2–IP4 (mixed zones), the relative standard deviations are not calculated. [b] Not measured.

methyl)methylamino]propane (bis-tris-propane, BTP 99+%), and (hydroxyethyl)cellulose (HEC) (all Aldrich, Steinheim, Germany); phytase (crude from wheat, activity approximately 0.015 unit/mg of solid) from Sigma (Deisenhofen, Germany). For the preparation of the buffers water deionized on a mixed-bed exchanger was used.

Procedure. Two to five grams of the ground sample is weighed into a 100 mL volumetric flask, and 50 mL of HCl of different concentrations (0–5%) is added. IP6 is extracted from the sample by shaking for 1 h at room temperature. After this period, the mixture is filtered, diluted, and directly analyzed by isotachophoresis.

RESULTS AND DISCUSSION

Migration Properties of the Solutes in Different Buffering Electrolyte Systems. The buffering electrolyte system must enable the separation of IP6 from its degradation products, namely the other inositol phosphates (IP1–IP5) and phosphate. It should be pointed out that the separation of the lower inositol phosphates from each other is not of interest for the goal of this contribution and has therefore no influence on the selection of the appropriate systems. To find such a separation system, a number of different buffers were investigated, which are listed in Table 1. Reference compounds for the inositol phosphates were taken from the reaction mixture prepared by hydrolysis with phytase for different lengths of time as described elsewhere (Blatny et al., 1994).

It should be mentioned that two possibilities exist to describe the composition of the electrolyte system in capillary isotachophoresis. The one consists in the description of the concentration of the leading electrolyte (and its co-counterion, if present) and the denotation of the pH value, which is adjusted by the use of the buffering counterion. The other is to define the concentrations of all electrolytes involved without adjustment to a given pH value. It is an advantage of the latter description that it will not include systematic errors, e.g. due to a false calibration of the glass electrode used. In addition, this description allows faster electrolyte preparation and easier handling. It is an advantage of the former that the effective mobilities of the separands at the pH of the buffer can be estimated easily, given that their pK values and actual mobilities are known.

The relative step heights of the separands in the buffer systems investigated are given in Table 2. In Figure 1 isotachopherograms of a mixture of IP6, IP5, and phosphate in systems 6 and 7 are shown for comparison. It was found that system 6 [with His as counterion as described by Kikunaga et al. (1985) for the determination of IP6] is not able to separate IP6 and IP5. This is also the case for system 1 that consists of EACA as buffering counterion of the leading electrolyte, although separation from phosphate can be carried out in both systems.

It is likely that the separation based on the pK values of the analytes is not possible. The selectivity of the

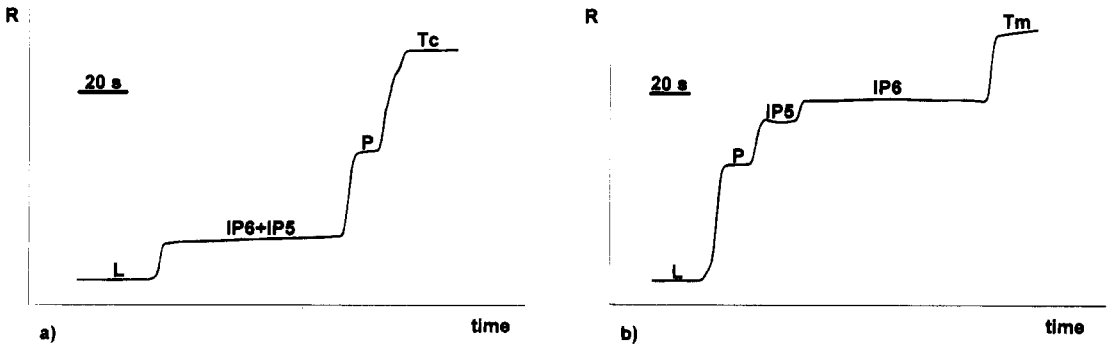

Figure 1. Isotachopherograms of a model mixture: (a) measured in the electrolyte system presented by Kikunaga (Table 1, system 6); (b) measured in our developed electrolyte system (Table 1, system 7). The sample consists of a reaction mixture after 2.5 min of enzymatic hydrolysis of phytic acid (IP6). Numbering of the electrolyte systems is as in Table 1. L, leading electrolyte (chloride); Tc, terminating electrolyte (caproic acid); Tm, terminating electrolyte (MES); IP5, inositol pentaphosphate; P, phosphate; R, resistance (detector response). The volume-coupling instrument was used (see Materials and Methods).

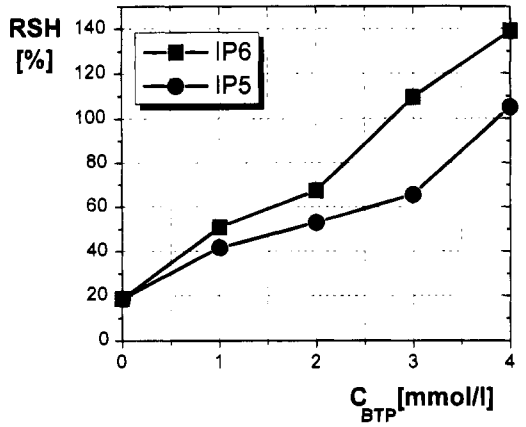

Figure 2. Migration behavior of phytic acid (IP6) and inositol pentaphosphate (IP5) in the electrolyte systems at pH 4.5 (Table 1, systems 1–5), showing dependence on the concentration of bis-tris-propane (BTP), the co-counterion of the leading electrolyte. The relative step height, RSH, is related to phosphate (P).

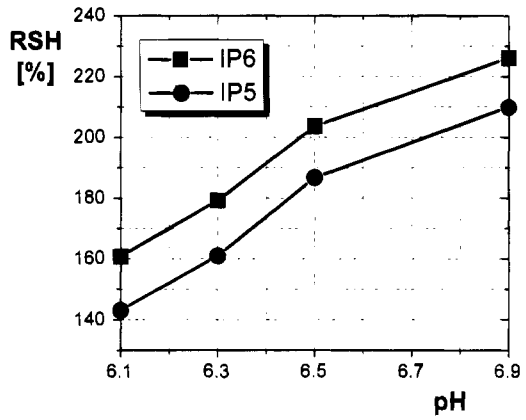

Figure 3. Migration behavior of IP6 and IP5 as a function of the pH in the selected electrolyte systems (Table 1, systems 7–10). The relative step height, RSH, is related to phosphate.

system can be enhanced, however, by complexation of the anionic analytes with higher charged cations. It is well-known that metal ions such as Ca, Mg, and Fe (see Introduction) form salts with very low water solubility and can therefore not be taken for this purpose. Thus, a doubly charged amine, BTP, was applied as co-counterion in systems 2–5 or as counterion with complex-forming ability in systems 7–10.

The presence of BTP as co-counterion indeed improves the separation of IP6 from IP5, as can be seen from Figure 2. The increase of the concentrations of BTP at a constant pH, e.g. 4.5, decreases the mobility of IP6 and IP5 relative to phosphate (indicated by the increase of the relative step heights), leading even to a reversal of the migration sequence at a BTP concentration of 4 mmol/L. The variation of the concentration of BTP, the component used for the adjustment of the pH of the 0.01 mol/L HCl solution for systems 7–10 (consequently leading to a change of the pH), also increases the selectivity of IP6 and IP5, respectively, in relation to phosphate, as can be seen from Figure 3. The step height difference between IP6 and IP5 remains, however, constant in these separation systems with increasing pH.

It can be seen from the step heights from Table 2 that systems 2, 3, and 7–10, all consisting of BTP, are indeed superior to those without BTP. On the basis of these

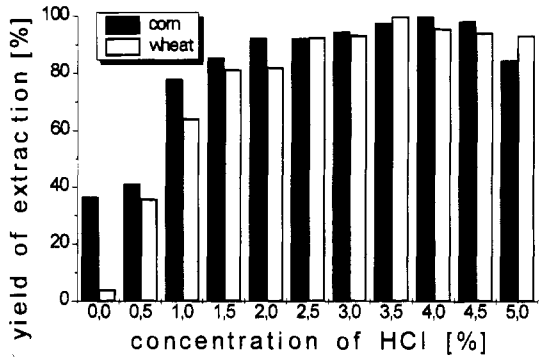

Figure 4. Optimization study of the extraction yield of phytic acid from corn and wheat: percentage IP6 (w/w) determined as a function of the concentration of HCl used as the extraction medium. Phytic acid content found by extraction with 3.5% HCl was taken as 100% yield of extraction. The IP6 content was measured isotachophoretically in the plant extracts with the volume-coupling device.

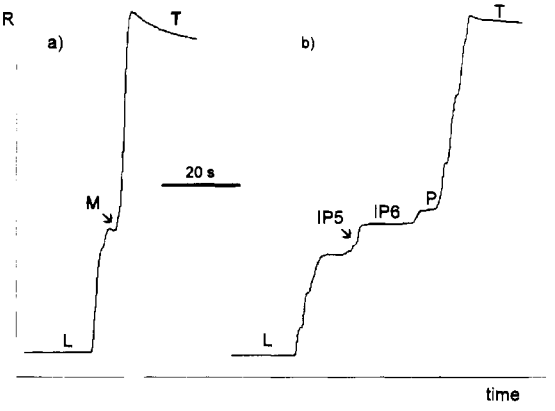

Figure 5. Isotachopherograms of a real sample (corn) obtained with the column-coupling device using electrolyte system 3 (Table 1). The extract from 10 g of plant material was directly injected after 40-fold dilution. Isotachopherograms were obtained from the preseparation (a) and the analytical capillaries (b). L, leading anion (chloride); T, terminating anion (caproic acid); M, mixed zone of IP5, IP6, and P; R, resistance (detector response). For details see text.

results it can be concluded that system 7 will be most favorable for the determination of IP6 in real samples. This is *per se* not valid for the separation of the lower *myo*-inositol phosphates, where system 2 would be more appropriate.

Quantitation. On the basis of the results discussed above, buffering system 7 was chosen for the calibration analysis. An external standard calibration method was used with dodecasodium phytate as standard. Seven calibration points were measured with the volume-coupling instrument in the range between 0.01 and 0.12 mmol/L (6.5–78 μg/mL). Standard solutions were injected by a sample valve with a fixed volume of 20 μL. As a result, the calibration line can be expressed by $y = 17780x + 112$, where y is the zone length of IP6 (given by the number of counts collected at a sampling rate of 20 Hz) and x is its concentration in mmol/L. The correlation coefficient, r, was 0.99948. It should be pointed out that in system 7, together with the quantitation of IP6, that of IP5 and phosphate can be carried out simultaneously.

The limit of detection using system 7 was assumed to be 10^{-6} mol/L IP6. This corresponds to less than 0.08% of IP6 in a sample, when an aliquot of 5 g is extracted as described under Materials and Methods, and the extract is diluted 50-fold.

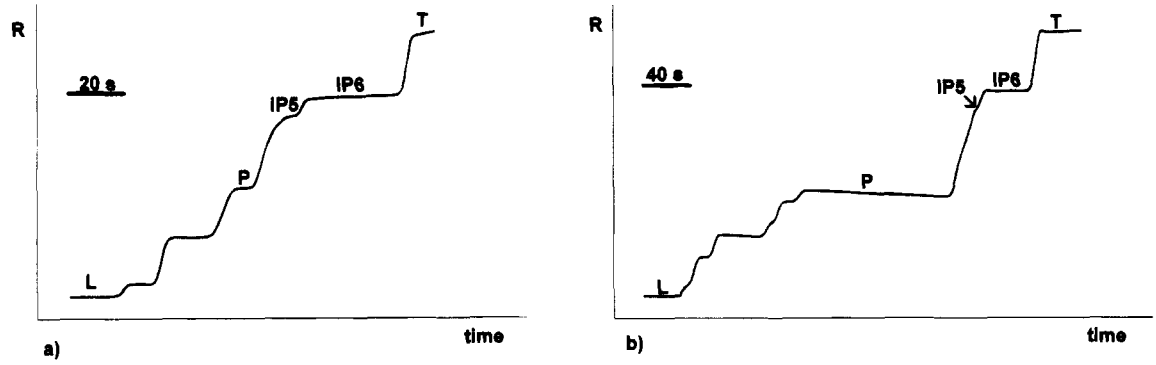

Figure 6. Isotachopherograms of real samples obtained with the volume-coupling device using electrolyte system 7 (Table 1): (a) extract of pea (50-fold diluted); (b) extract of feed sample (50-fold diluted). In both cases 5 g of sample was weighted for the extraction. L, leading ion (chloride); T, terminating anion (MES); R, resistance (detector response).

Optimization of the Extraction Procedure for Real Samples. IP6 is present in plants as so-called "phytate", which is its Ca and Mg salt. This material is, however, insoluble in water but can be dissolved in acidic media. Therefore, HCl at different concentrations between 0 and 5% was used as solvent to investigate the conditions for maximum yield of extraction of IP6 from the plant matrix. The content of IP6 was quantified by isotachophoresis. Two cereals were investigated, corn and wheat. The results of the extractions at 11 different HCl concentrations are depicted in Figure 4. The yield is very low at low concentrations, increases as the HCl content increases, approaches a plateau, and decreases slightly at higher concentrations. The concentration of 3.5% gives the best results for both types of samples; this was therefore taken for further quantitations.

IP6 Content of Real Samples. The applicability of systems 2, 3, and 7–10 was examined for the analysis of real samples. Analysis were carried out with the volume-coupling isotachophoretic instrument. It was found that in system 2 IP6 forms a mixed zone with an unknown compound present in the samples. Although in system 3 the separation of IP6, IP5, and phosphate can be achieved, it was found that its relatively low separation capacity allows the analysis of very diluted samples only; otherwise, mixed zones remain. Such a high dilution could result, on the other hand, in a poor detectability of the zones. To obtain zones with sufficient lengths in this system, the column-coupling isotachophoretic instrument had to be used. This instrumentation allows a higher sample load at the preseparation capillary (about a factor of 5 higher compared to that of the volume-coupling instrument) and sufficient separation capacity after the zones of interest are switched onto the analytical capillary. The resulting isotachopherograms are shown for one real sample in Figure 5. It can be seen that the constituents forming a mixed zone in the preseparation capillary are clearly separated after switching into the analytical capillary.

By the use of systems 7–10 the samples can be analyzed directly after dilution with the volume-coupling instrument, most favorably with system 7, which was therefore used for all further determinations. Due to reasons discussed above, the composition of this electrolyte system is given as follows: leading electrolyte, 0.01 mol/L HCl; 0.0056 mol/L BTP; 0.1% HEC; terminating electrolyte, 0.005 mol/L MES.

Isotachopherograms of real samples obtained with this electrolyte system are shown in Figure 6. The presence of IP6, IP5, and phosphate can clearly be seen.

Table 3. Results of Determinations of Phytic Acid in Real Samples

sample	phytic acid concn[a] (%)
corn	1.29 ± 0.04
barley	1.15 ± 0.02
poultry feed	1.05 ± 0.04
animal feed for pigs	1.97 ± 0.07
soy	2.74 ± 0.09
pea	2.20 ± 0.12
wheat fraction 1 (>0.71 mm)	10.53 ± 0.59
wheat fraction 2 (between 0.25 and 0.71 mm)	5.75 ± 0.08
wheat fraction 3 (<0.25 mm)	0.99 ± 0.03

[a] Values are average from two separate determinations ± standard deviation.

In Table 3 the result of quantitation is given for different materials. The total concentration of phytic acid varies from 1 to 3% (w/w), which is in accordance with the values known from the literature (Frølich et al., 1988; Knuckles et al., 1982).

Three fractions of ground wheat with different particle sizes were investigated, too. They were obtained by sieving the ground material through sieves with mesh sizes of 0.71 and 0.25 mm, respectively. The results are also shown in Table 3. It can be seen that the bran fraction (with particles larger than 0.71 mm) has the highest IP6 content, namely more than 10%. This is double the content of the medium fraction (between 0.25 and 0.71 mm). The finest fraction, corresponding to white flour, has the lowest concentration, less than 1%: phytic acid is in fact enriched in the bran of wheat grain.

The precision of the ITP determination of IP6, given by the relative standard deviation from 10 repetitive injections of a reference sample, is 2.2%. Including the sample pretreatment, a relative standard deviation of 3.8% was found from nine independent extractions.

The recovery of IP6 when added to real samples was between 94.3 and 102.2% for all cases; the amount of IP6 added did not exceed about half of the native content. It can be thus concluded that the isotachophoretic procedure introduced in this paper is a simple method for the precise and accurate determination of IP6 in grains and feeds.

ABBREVIATIONS USED

BTP, bis-tris-propane; EACA ϵ-aminocaproic acid; HEC, (hydroxyethyl)cellulose; His, histidine; IP6, phytic acid; IP5, IP4, IP3, IP2, and IP1 inositol penta-, tetra-, tri-, di-, and monophosphate; ITP, isotachophoresis;

MES, morpholinoethanesulfonic acid; P, phosphate; RSH (relative step height), ratio of step height of analyzed compound and the one of a selected standard or terminating ion.

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Received for review April 20, 1994. Revised manuscript received October 6, 1994. Accepted November 4, 1994.[⊗]

JF9402082

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1994.