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Short communication

Anion-exchange high-performance liquid chromatography with conductivity detection for the analysis of phytic acid in food

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Abstract

A sensitive method for the accurate determination of phytic acid in food samples is described. The proposed procedure involves the anion-exchange liquid chromatography with conductivity detection. Initially, two methods of determination of phytic acid were compared: absorptiometry and high-performance ion chromatography (HPIC) with chemically suppressed conductivity detector. Unlike most conventional methods involving precipitation by FeCl_3 , the simpler and more reliable HPIC assay avoids the numerous assumptions inherent in the iron precipitation and the accuracy is independent of the phytate content. The protocol was also applied to a survey of phytic acid concentration in some cereal, oil and legume seeds. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The number of recognized roles of phytic acid in numerous biochemical pathways and physiological processes has been increasing in accordance with its apparent ubiquity. Long regarded as an antinutrient in seeds due to its ability to chelate minerals and reduce their solubility and bioavailability, the role of phytic acid in foods to prevent and possibly reverse carcinogenesis is now recognized [1,2]. Phytic acid is an antioxidant [3], an anti-inflammatory selectin inhibitor [4], an energy store [5] and a regulator of vesicular binding to various proteins.

The growing interest in the phytic acid and the inherent problems with its detection (it does not

absorb visible or ultraviolet light) are reflected in the diverse analytical approaches.

The conventional methods for determination of phytic acid derive from the absorptiometric method of Heubner and Stadler [6]. In these methods, after extraction of samples with an acid, phytic acid is precipitated by ferric chloride [7,8]. Different assumptions, especially the ratio of iron to phytate phosphorus make these methods unreliable and the precipitation of lower polyphosphorylated compounds in lower sensitivity [10]. Because of these disadvantages, HPLC methods were developed to improve the determination of phytic acid. Many liquid chromatographic systems have been described for the analysis of phytates in food. The first systems incorporated refractive index detection coupled with reversed-phase separation [9–13]. The relatively poor sensitivity associated with refractometry has

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stimulated the search for alternative methods. Then, inositol phosphates were detected by derivatizing in another method of detection [14–16] and separated by a variety of chromatographic techniques. These techniques are all limited either in their ability to separate structural isomers, the need for pre- or post-column derivatization, or by time required for analysis. In spite of improvements, a prepurification is still needed.

The principal aim of this paper was to develop a technique without a prepurification step making easier the routine determination of phytic acid in food and to give a few values for common foods.

2. Materials and methods

2.1. Materials

2.1.1. Analytical instruments

Beckman (Fullerton, USA) DU 70 spectrophotometer was used for absorptiometric determination. High-performance ion chromatography (HPIC) analyses were performed with a 4500i Dionex (Sunnyvale, CA, USA) liquid chromatograph equipped with an eluent delivery pump, an auto-injector and using chemically suppressed conductivity. A 50- μ l constant volume injection loop was used throughout. A centrifugal evaporator Jouan (France) RC10.10 fitted with a refrigerated trap cooled at -60°C was used for sample preparation.

2.1.2. Reagents and solutions

All chemicals used were of analytical grade and deionized water was used for preparing the reagent solutions. Deionized water was purified by Millipore ultra pure system to a specific resistance of 18 $\text{m}\Omega$ cm or greater. Sodium phytate (Sigma Ref. 3168) was used for the preparation of standard phytic acid solutions.

Ferric solution ($50 \mu\text{g ml}^{-1}$) for the absorptiometric method: 625 μl of concentrated HNO_3 was added to 25 ml of a commercial standard iron solution (Fe^{3+} , 1 g l^{-1} , Titrisol, Merck, Ref. 9972) and completed to 500 ml deionized water to give the final solution ($50 \mu\text{g ml}^{-1}$).

HPIC analysis: the mobile phase was a mixture of three solutions A, B and C. Solution A was prepared

by adding 10.4 ml NaOH (commercial solution at 50% (w/v) in water, Baker) in water (final volume: 1 l). Solution B was deionized water–isopropanol (50:50, v/v). Solution C was deionized water. Regenerated solution of anion suppressor was 25 mmol l^{-1} sulphuric acid solution.

2.2. Methods

2.2.1. Absorptiometric method

Triplicate samples (0.5 g) of the freeze-dried, finely-ground products were extracted with 20 ml $0.5 \text{ mol l}^{-1} \text{HNO}_3$ for 3–4 h with continuous shaking. After filtering, phytate analysis was performed on the filtrate by a modification of Holt's method (see [8]).

The modified Holt procedure [7] adopted routinely in our laboratories for phytate analysis was as follows: 0.2–0.5 ml of the filtrate or standard sodium phytate solution (0.2 mmol l^{-1}) was diluted with distilled water to a final volume of 1.4 ml. Then, 1.0 ml of a solution of ferric solution containing $50 \mu\text{g ml}^{-1} \text{Fe}^{3+}$ was added. After mixing, the test tubes were capped, placed in a boiling waterbath for 20 min and cooled to room temperature. A 5-ml volume of amyl alcohol was added to each test tube followed by 0.1 ml of a solution of ammonium thiocyanate (100 g l^{-1}).

The contents of the test tubes were immediately mixed and centrifuged at 4000 g for 5 min. The intensity of the colour in the amyl layer was determined at 465 nm using a spectrophotometer against an amyl alcohol blank, exactly 15 min after the addition of CHN_5S . Under these conditions an inverse relationship was found over a range of phytate concentrations from 0.0286 to 0.114 mmol l^{-1} .

2.2.2. HPIC analysis

2.2.2.1. Fat extraction

When the fat content exceeds 15 g/100 g dry matter, fat extraction is carried out with 10 ml light petroleum ether on a 0.2-g flour sample. The lipids mixed with water, prevented the evaporation to dryness. Other compounds like proteins, starch, pigments and carbohydrates, did not interfere with the assay. The mixture was stirred by vortex and then centrifuged at 4000 g for 10 min. The precipi-

Table 1
Gradient elution program for the separation of phytic acid

Elution time (min)	Flow-rate (ml/min)	A (%)	B (%)	C (%)
0.0	1	35	2	63
2.0	1	65	2	33
9.5	1	65	2	33
10.5	1	35	2	63
15	1	35	2	63

tate was recovered and 10 ml of 0.5 mol l⁻¹ HCl was added in order to begin the phytate extraction.

2.2.2.2. Phytate extraction

A 0.2-g sample of cereal flour, oil or legume seeds was introduced to a Pyrex vial fitted with a PTFE screw-cap. A 10-ml volume of 0.5 mol l⁻¹ HCl was added and the vial was capped. The mixture was heated under stirring for 5 min by immersing the vial in boiling water. It was then centrifuged at 4000 *g* for 10 min. The supernatant was recovered and 1.5 ml of 12 mol l⁻¹ HCl were added to obtain a 2 mol l⁻¹ HCl concentration in order to ensure the decomplexation of phytates. This procedure was found to give the best extraction conditions. The resulting solution was then shaken and evaporated to dryness in a centrifugal evaporator. The vial was stored at 8°C. The residue was resuspended in 2 ml of deionized water 10 min before the injection, and passed through a 0.2 µm disposable filter (Acrodisc) tip-syringe assembly. The filtrate was then diluted in deionized water (1:25) and injected into the liquid chromatograph.

2.2.2.3. HPIC procedure

Separation of phytic acid was achieved using an Omnipac Pax-100 anion-exchange column (250×4 mm I.D., Dionex) equipped with an Omnipac Pax-100 (8 µm) precolumn and an anion suppressor (ASRS-I 4 mm). The Omnipac column requires a minimum of 1% organic solvent at all times. The gradient elution using three eluents (Table 1) was selected with a total chromatography run time of 15 min.

3. Results and discussion

3.1. Precision and accuracy of the HPIC method

The separation of phytic acid was achieved by an anion-exchange column with chemically suppressed conductivity detector with concentrations ranging from 0.01 to 0.16 mmol l⁻¹. The retention time of phytate was 6.3±0.3 min with no day-to-day variation over a 24-month period. The variation in retention time was due to temperature differences during the measurements. In order to avoid this effect both the column and the solvent were thermostated. The area under the conductivity peak is proportional to the phytic acid concentration. In Fig. 1 the chromatograms of separation of phytic acid from standard and food sample are shown.

To investigate the method repeatability, six cowpea samples were analysed and the relative standard deviation (RSD) of repeatability was <5%. The procedure was repeated daily for a 4-day period (*n*=24) for reproducibility. Over 3 years, the precision was assessed under different conditions: assistance operator, analytical instrument, new column, another laboratory. Therefore, the method was found to be robust in regard to the precision which ranged from 2.7 to 8.3% (RSD of reproducibility), shown in Table 2. Recovery was 99% with a (95%) confidence range. The HPIC method allows the quantitation of phytic acid down to 0.1 µmol l⁻¹. The signal-to-noise ratio was higher than 10: the limit of detection was therefore less than 0.0001 µmol l⁻¹.

3.2. Comparison of the two methods

The HPIC method and the classical absorptiometric method were compared because in agreement Rounds and Nielsen [14], we observed with the reversed-phase HPLC method two main disadvantages: poor separation and low sensitivity in the refractometric detection system (unpublished results). The absence of chromophoric functional groups within inositol has led to development of methods based on post-column derivatization and spectrophotometric detection. Despite a high sensitivity of detection, the additional derivatization reaction constitutes a potential source of error and is also time-consuming. For these reasons, we decided to com-

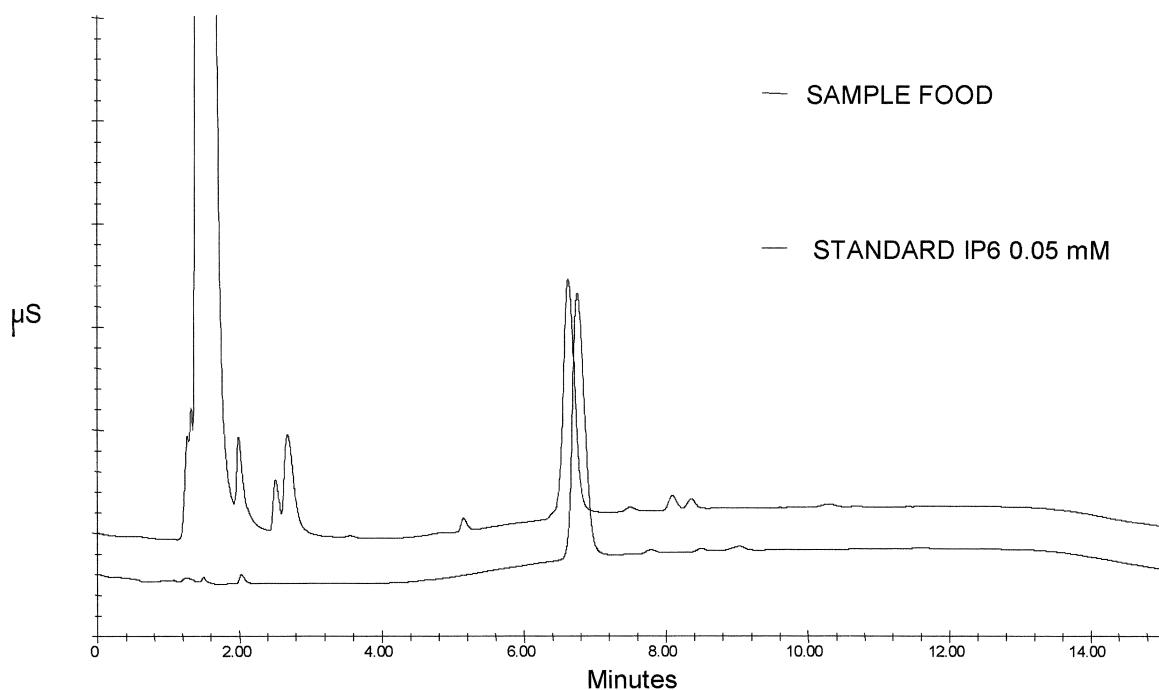


Fig. 1. Elution profile of phytic acid standard and of the cowpea sample (legume seed) on an Omnipac Pax-100 column: eluents: 200 mM NaOH, water–isopropanol (1:1, v/v) and water (18 mΩ) detection: chemically suppressed conductivity using an ASRS-I 4 mm.

Table 2
Determination of RSD of reproducibility over 3 years

Years	1997	1998	1999
Number of replicates	24	6	6
Average value (mmol l ⁻¹)	1.70	1.75	1.73
RSD (%)	7.3	2.7	8.3

pare the HPIC method with the classic absorptiometric method in use in our laboratory [7]. The study with phytate standard solutions showed that the HPIC method was more precise than the absorptiometric method and could be directly applied to samples without purification. Millet and cowpea

were extracted and phytate was quantified by both methods. Whatever flour was used the data obtained by the absorptiometric method were systematically higher by about 27% than those of the HPIC method (Table 3). These foods generally contain the lower inositol phosphates (penta-, tetra- and triphosphates) in addition to phytic acid and these substances are included in the calculation of phytic acid as determined by the absorptiometric method. This result is in agreement with those of other authors [9,10,12,13,16] who obtained higher values with the absorptiometric method than the reversed-phase method. The ferric precipitation method cannot be

Table 3
Comparison of absorptiometric and HPIC methods for determination phytic acid in food samples

Sample	Phytic acid (g/100 g dry matter) ^a		
	Absorptiometric method (A)	HPIC method (B)	Overestimation ^b (%)
Millet souna	0.807±0.06	0.587±0.06	27.3
Cowpea	1.32±0.03	0.97±0.02	26.5

^a Mean±SD of three replicate samples.

^b Percentage difference between the absorptiometric and HPIC methods.

used for the determination of phytic acid in all foods because the presence of interfering substances, such as reducing compounds, leads to high results.

3.3. Phytate levels in food

The phytic acid content of some raw cereal, oil and legume seeds was determined (Table 4). Soybean had the highest phytate content (1.25 g phytic acid/100 g dry matter). This value is close to those given by Sudarmadji [17] and Erdman [19] for raw soybean (1.4–1.6 g/100 g dry matter). For other seeds, our values were slightly lower than those previously reported by other authors, generally obtained using absorptiometric method [17–22]. This difference is not surprising in view of the fact that treatment of the extracts with a ferric solution precipitates small amounts of inorganic phosphates and phosphates from other phosphorylated compounds. Furthermore, this technique does not allow a distinction to be made between different forms of inositol mono- to hexaphosphate (IP1–IP6) [23].

The HPLC method was sensitive, rapid and precise for phytic acid determination. The method will be

used for further studies on the effect of food processing. It is a useful method for the determination of phytic acid in all foods or other biological samples because the presence of interfering substances does not disturb the assay.

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Table 4
Phytic acid content of cereals, oil and legume seeds

Sample	Origin	Phytic acid (g/100 g dry matter) ^a
Cereals		
Pearl millet (<i>Pennisetum nigritarum</i>)	Burkina Faso	0.74
Maize (<i>Zea Mays</i>)	Cote Ivoire	0.50
Sorghum (<i>Sorghum candatum</i>)	Benin	0.76
Oilseeds		
Peanut (<i>Arachys hypogea</i>)	Burkina Faso	0.68
Soybean (<i>Glycine max</i>) white variety	Cote Ivoire	1.28
Soybean (<i>Glycine max</i>) black variety	Burkina Faso	1.20
Legume seeds		
Kidney bean (<i>Phaseolus vulgaris</i>)		
Bico de Ouro variety	Madagascar	0.49
Goiano variety	Madagascar	0.96
G13671 variety	Madagascar	0.56
Kinumba variety	Madagascar	0.94
Xan 76 variety	Madagascar	0.96
Lingot blanc variety	Madagascar	1.06
Rice bean (<i>Vigna umbellata</i>) green variety	Madagascar	0.27
Rice bean (<i>Vigna umbellata</i>) red variety	Madagascar	0.29
Cowpea (<i>Vigna unguiculata</i>)	Senegal	0.82

^a Mean of three replicate samples.

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