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An overview of the analytical methods for food phytates

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Abstract
Phytate, an antinutrtional factor impairing mineral absorption and protein digestion even when present in small amount has been a subject of investigation from nutritional and chemical viewpoints. Phytic acid which is the inositol combined with six phosphates group is a common constituent in most of the plants as an important source of phosphorus therefore accurate methods for its determination is needed. As there is no specific reagent for the determination of phyate; many methods involve the determination where inositol or phosphates are estimated or by stoichiometric ratios between phytates and specific cations. Direct methods are the most popular method for determining phytates in food and feed stuffs. Extraction being the preliminary stage in determination of phytates, dilute Hydrochloric acid (HCl), Trichloroacetic acid (TCA) are used in common. Quantitative methods for phytates are presented under colourimetric determination, ion exchange chromatography, HPLC, NMR.

Keywords: analytical methods, food phytates, antinutrtional factor

Introduction
Phytic acid is the potential source of inositol and storage phosphorus in plant seeds contributing approximately 70% of total phosphorus (Greiner *et al*., 2006) [1]. According to Lolas and Markakis, (1975) [2] phytate accounts for 80% of the total phosphorus in legumes. Moreover, phytates are naturally formed compounds during the maturation phase of plant seeds and grains. Many plant derived foods are also contributed by a range of phytates for their phytochemical profiles. (Greiner *et al*. 2006) [1]. Highest phytate content is found in milled fractions of cereals and protein products. In dicotyledons seeds such as legumes, nuts and oilseeds, phytates are found closely associated with proteins and is often isolated or concentrated with protein fraction of these foods, which are the stores for phosphate and mineral nutrients that are important for plant nutrition and especially vulnerable during germination (Bora, 2014) [3]. In addition, phytate is a polydentate ligand which can chelate metal ions at physiological pH reducing the bioavailability of essential dietary nutrients such as minerals (Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe2+/3+), proteins and amino acids (García-Estepa *et al*., 1999; Dahiya, 2006) [4, 5]. Phytic acid is enzymatically hydrolyzed by phytases, or chemically to lower inositol phosphates such as inositol pentaphosphate (IP5), inositol tetraphosphate (IP4), inositol triphosphate (IP3) during storage, fermentation, germination, food processing and digestion in the human gut. Only IP6 and IP5 have a negative effect on bioavailability of minerals (Shashi Kiran Misra, 2012) [6]. Phytates are known for exhibiting antioxidant properties as forms an iron chelate which inhibits iron-mediated oxidative reactions and limits site-specific DNA damage and can prevent tumor growth by suppressing the formation of the highly reactive $OH^{\bullet}$ and other ROS (Watson & Zibadi, 2014) [7].
Analysis of phytates is relied upon indirect quantification, as there is no specific reagent available for determination of phytates. Determination of phytates rely upon quantification of Inisitol or Phosphate is used as indirect quantification tool or the stoichiometric relationship between phytates with some cation that is easy to measure. Qualitative methods are based on the separation and identification of inositol phosphate esters namely Paper chromatography, paper electrophoresis, thin layer chromatography, ion exchange chromatography (N.R. Reddy *et al*, 1989) [9]. This review focuses on the novel approaches to determine phyates quantitatively by popular methods such as HPLC, Fourier Transform Nuclear Mangnetic Resonance for the quantitation.

Quantitative methods

Extraction is the initial step before the determination of phytic content. Trichloroacetic acid is beneficial over Hydrochloric acid because it denatures and coagulates proteins in the extract thus preventing possible interferences. Later it was reported 3% sulphuric acid is effective than TCA or HCl. The samples containing more than 15% of fat, fat should be extracted with petroleum ether prior to the extraction of phytates, to prevent possible interference. Also the fat extraction can be replaced with solid-phase extraction cartridge to remove hydrophobic compounds (N.R. Reddy *et al*, 1989) [9].

Precipitation methods

Phytates form an insoluble complex (precipitate) with Ferric ions in dilute acid solutions, which is the basis of many analytical methods. Determination of phytates is based on the analysis of phosphorus or Iron in the isolated ferric phyate or based on the determination on residual iron in the solution after the precipitation of Ferric phytates from a known concentration of ferric salt in acidic solution. Heubner and Stadler, 1914 [10] in the beginning phytate extract was titrated with standardized Ferric Chloride with ammonium thiocyanate indicator, there were difficulties in determining the end point of the titration due to the formation of the precipitate. Later the above was modified by associating a step for the removal of phytate by filtration.

Earlier phytic acid was determined by complexometric titration of excess Iron (III) where the phytate was precipitated as insoluble Ferric phytate in acidic medium. The above was proposed by Heubner and Stadler initially, shortcomings of the method involved the varying rations of the Iron (III) to phytate molecule as only the 8 protons of a phytate molecule can be dissociated at the working pH so that each phytate would require 2.8 Iron (III) ions, which is not constant. Soon a modified method was put forward where the phytates in a solution were saturated with Iron of a known quantity and then back titration of the excess Iron with a ligand most preferably Sulphosalicylic acid which is stable, reacting only with the free Iron in the solution (not with the Iron that is bound to Phytates) forming an intermediate complex and gives a visible end point (Garcia-Villanova *et al*., 1982) [8]. It has been reported that the assay requires steps to remove the precipitate of Iron-Phytate before determining the excess Iron in the solution which is one of the drawbacks led to low accuracy.

Makower, 1970 [11] analysed the phytate contents in Pinto beans of different stages of maturity in different ways: precipitation of Ferric phytate with Ferric Chloride, after centrifugation the supernatant was used for the determination of phytic acid based on the residual Iron. The precipitate of Ferric hydroxide obtained (done in quadruplicate) by adding 0.6N NaOH to the supernatant, was dissolved by 0.5N HCl. The iron was determined colourimetrically with *o*–phenanthroline after reducing Fe^{3+} to Fe^{2+} with hydroxylamine and measured at 515mμ. Other samples of Ferric phytates were dry ashed with H_2O_2 and perchloric acid for determination of phosphorus. Wheeler and Ferrel, 1971 [12] determined the phytic acid contents in wheat and wheat fractions colourimetrically from the known ratio of Fe:P.

Colourimetric determination

Later a colourimetric method which was developed based on the reaction between Fe^{3+} with Sulphosalicylic acid (Wade reagent) forming a complex that can be monitored spectrophotometrically at 500nm. Before the colourimetric dosing, the sample is eluted by an anion exchange resin to separate the phytates – phosphorus from the inorganic Phosphorus in the sample. Thereafter from the Phytate standard curve, the value obtained through correlation should be multiplied by 0.282 (molar ratio of phytate - phosphorus in a molecule of phytate) to express the respective phytate content in the sample (Naves *et al*., 2014) [13]. This method can be said to be time consuming due to the anion exchange chromatographic separation of phytates. A modified colourimetric method proposed by Maroof *et al*. (2007) [14] to overcome the poor reproducibility and poor precision, where the extraction time was extended to 16 hours, centrifugation temperature was lowered to 10ºC and a matrix cleaning step to improve the phytate recovery was included. At the same time it has been found that colourimetric method overestimated the phytate content in samples with lower percentage of phytates.

Mohomed *et al*., 1986 [15] introduced a colourimetric method to analyze phytic acid without acid digestion to inorganic phosphate. The principle of the method involves conversion of Ferric phytate to Sodium phytate and thereafter reacting phytate with chromogenic reagent to form a blue molybdenum complex, which has maximum absorption at 830nm.

Due to the nature of the phytate molecule no UV or Visible spectra are available. (Spiller, 2001) [16]. The differential refractive index detector was the first detector to be used successfully in the HPLC analysis, yet it has some drawbacks such as being the least sensitive of the detectors, it could detect only the changes in the refractive index and it must be used only in the isocratic system. Detection of inositol phosphates by HPLC needs series of independent phases as follows, effective extraction, extract purification, separation of individual compounds, detection and quantification. (Burbano *et al*., 1995) [17].

Chromatographic techniques

Ion exchange chromatography, which is also known as adsorption chromatography, is a useful and popular method due to its; high capacity, high resolving power, mild separation conditions, versatility and wide speared applicability, tendency to concentrate the sample, relatively low cost. Harland and Oberlease in 1977 [18] introduced an ion exchange chromatographic method with stepwise gradient elution for quantification of inositol-6-phosphate. A glass column about 0.7mm x 30mm with a valve filled with anion exchange resin AG 1- X 4 Chloride form, 100-200 mesh is used. Initially the recovery of the column is tested using standard Sodium phytate solution. Phytates in the sample is extracted using dilute HCl, the exract is mixed with Na_2EDTA-NaOH solution and placed on an ion exchange column. The fraction containing phytate eluted with 0.7M NaCl is wet digested with a mixture of concentrated acids H_2SO_4 and HNO_3 to release inorganic Phosphorus which is measured colourimetrically at 640nm using UV-VIS spectrophotometer. Standard curve plotted using Standard phosphate solution (Primary standard KH_2PO_4, 80μg/ml) is used in determining the content of phytates (Herath *et al*.2017) [19]. But it is rather lengthy and fails to determine the lower inositol esters. With the identified draw backs and later on with modifications, this method has been adopted as an official AOAC method (AOAC, 2012) [20].

The previous applications of HPLC involved the usage of reverse phase C-18 Silica column and refractive index

detectors. Problems associated with overlapping of quantitating peaks of the solvent front and that of phytic acid made quatitation difficult. As stated by Reddy, 1982 [21], for the first time phytate in rice bran was analyzed by HPLC method, where without any preparation filtered sample extract of 3% TCA (Trichloroacetic acid) was injected to the Bondapak C-18 (30cm X 4mm) reverse phase column and eluted phytate with 5mM Sodium acetate at the flow rate of 0.5 to 2.0ml/min. Later the above method was modified by Graf and Dintzis, (1982) [22] as follows: plastic columns with glass filter and containing 0.65 mL resin (AG l-X8, 200 - 400 mesh) were used. The filtrate was evaporated to dryness at reduced pressure and a water bath temperature not exceeding 40ºC, dissolved 0.025M HCl and passed through the column (0.4 mL/min.) followed by 0.025M HCl. Inositol phosphates were removed from the resin with 2M HCl. The eluent was diluted with water after evaporation. The dried sample was re-solubilized in 5mM Sodium acetate prior to the HPLC injection. Mobile used was 5mM Sodium acetate. This procedure eliminates the error occurring from solvent components and it is highly sensitive.

Knuckles *et al*., 1982 [23] extracted samples with 3% TCA, later the sample was diluted and pH adjusted to 6.0 by the addition of 0.5M KH_2PO_4. The diluted sample extract was filtered through a 0.2µm membrane filter and injected to the HPLC column. A 25mM KH_2PO_4 (pH 6.0) was used as the mobile phase. Phytates eluted at retention time of 1.1 to 1.2 min.

Sandberg, (1986) [24] used the method put forward by Graf and Dintzis (1982) [22] where an ion exchange method was used to concentrate the phytates into the sample. The mobile phase consisted of 0.05M formic acid: methanol, TBA-OH (Tetrabutylammonium hydroxide) (Fluka, 40% in water). The pH was adjusted to 4.3 by addition of 9M sulfuric acid. The mobile phase was filtered under vacuum and degassed by sonication. A C-18 reverse column of 5 micron particle size was used. Inositol phosphates were measured by refractive index. The pH of the mobile phase set to 4.3 led to the separation of tri to hexa inositol phosphates identified by different retention times of the reference compounds. The same time it was proved that iron precipitation methods lead to over estimation of inositol phosphates as it can cause co precipitation of lower inositol phosphates.

An HPLC method is quicker and thus the lower phosphates esters of myoinositol can be identified. Lehrfeld, (1989) [25] reported a HPLC analysis for phytic acid using PRP-1 5-µm (150 X 4.1 mm) reversed-phase analvtical column which can function in a wide range of pH from 1 -14; and it is stable in wide range of solvents. The supernatant sample was prepared by pouring the sample (supernatant - which has been centrifuged and the residue has been discarded) thereafter eluting the Silica based anion exchange (SAX) commercial column with 2M HCl. A sample added with 0.5M HCI and stirred to ensure removal of air pockets. After sonication, the suspension was centrifuged. An aliquot of supernatant was diluted with H_20 and poured onto an Analytichem silica-based anion-exchange (SAX) column (quaternary amine Bond Elute column, 500 mg), that was connected to a vacuum manifold set at 50-75 mmHg. The loaded SAX column was washed with 0.05M HCI, and the inositol polyphosphates then were eluted with 2M HCI and dried by vortex evaporation. The sample was eluted from Hamilton PRP-I column with a 0.015M formic acid solution containing 60% methanol and 0.4% tetrabutvlammonium hydoxide. The pH of the eluent was adjusted to 4.3 with Sulfuric acid. The column temperature was 28ºC. and the elution rate was 0.5 ml/ min. RI or UV at wavelength 190. From the results obtained for the grain samples that were analyzed IP6 content was substantially higher than the other lower inositols (IP6>IP5>IP4).

Burbano *et al*.(1995) [17] compared the concentration of phytic acid by AG l-X8 column (Dowex) and a silica based quaternary amine column (SAX) and found that slightly higher results were obtained by Dowex column. SAX column was considered to be less time consuming and more reproducibility and thus used for the analysis of phytates in legumes. Sodium phytate was used as the external standard. Initially due to the poor retention of inositols in the stationary phase some modifications were made where 0.012M formic acid, and 0.8% tetrabutylammonium hydroxide in 51.5% methanol (pH 4.3) stationary phase – Spherisorb column thermostated at 45ºC. Inositol phosphates were detected and quantified using refractive index. The flow rate was 1.2ml/min.

Spiller, 2001 [16] proposed Ferric Iron-to-Sulfosalicylic acid HPLC phytate analysis method, where the principle of the method was to separate various inorganic phosphates and the inositol phosphates of a sample by eluting them from the column at different gradients of NaCl or NaNO3, when eluted they form a stable complex with Ferric iron than does Sulfosalicylic acid. Ferric phyate is white and it reduces the colour of the complex Ferric sulfosalicylate. The decreased colour is a measure of decreased absorbance and it is proportional to the phytates or inositol phosphates in the eluted sample. Wade's reagent, ferric chloride and Sulfosalicylic acid initially should have a pH of 2.5 – 3.0 which is reddish purple in colour. It has a maximum absorbance of 500nm. And it provides pH range at 2 – 2.2. A similar method was explained by Bos *et al*., 1991 [26] where for the separation and the quantification an anion exchange column was used thereafter phytic acid was detected at 300nm following a in line post column reaction with Ferric Chloride.

Samples were extracted by stirring vigorously with either 10% TCA or 0.5mol/L HCl or 0.66mol/L HCl. The samples must be extracted by gently agitation over a large surface area. A polyethylene or a Polypropylene centrifuge tube taped to an orbital shaker or a mild shaking device for 3 hours or more is satisfactory. The extract is removed from the, matrix by centrifugation twice (later in a micro centrifugal tube) and the supernatant is injected to the vial. Ferric phytate is insoluble and forms a precipitate in the system this must be removed by inland filter frit if not the pressures increase rapidly. The frits can be cleaned by pumping 0.2mol/L NaOH, then flushing thoroughly with water or replaced with clean filter frits or sonicated with 30% HNO_3.

The column type is PL-SAX or any strong anion exchange column (50 X 4.6mm, particle size 8µm, 100nm pore type). A UV/Vis detector capable of monitoring specific wavelengths at 500nm. Also a secondary pump to pump Wade's reagent, for the post column reactions with phytates and inositol phosphates. Total run time is 31 to 36 minutes. Flow rate is 1 ml/min for the baseline buffer and gradient buffer salt solutions. 0.5ml/min wade's reagent. Pressures is 200 – 400 pounds per square inch (psi).

Kwanyuen & Burton, (2005) [27] proposed a simple HPLC method for determination of phytates in soyabeans with minimal sample preparation where the sample was ground to 0.5mm particle size, then it was extracted for phytates in 0.5N HCl for 1hour in a ratio of 1:20. The crude extract was

centrifuged and the supernatant was filtered with a tuberculin syringe and then with a 0.45µm syringe filter. Sample analysis was done by the post column reaction of wade's reagent with phytate but the procedure had certain modifications. Elution of phytate was achieved with a 30-min linear gradient of 0.01M 1-methylpiperazine, pH 4.0, to 0.5 M $NaNO_3$ in 0.01M 1-methylpiperazine, pH 4.0, at a flow rate of 1 mL/min. The post column reaction was allowed to take place in a 0.05 × 210 cm polyetheretherketone tubing at the combined flow rate of 2 mL/min.

Phescatcha, 2012 [28] uses the hydroxide-selective Dionex IonPac™ AS11 column to separate phytate from other anions (including the remaining chloride from the sample preparation) with an isocratic method. This column is paired with an eluent generator that produces the eluent automatically by just adding deionized water to the system, eliminating the time required and possible error associated with manual eluent preparation. Phytate is detected by suppressed conductivity detection. Altogether, the system is a Reagent-Free™ IC (RFIC™) system. Therefore, the analyst just prepares the samples, puts them in the autosampler, adds DI water to the eluent bottle, and programs the system with the Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software to determine phytate in samples. The IC method requires less than 10 min per injection.

NMR

PFT_NMR is specific for inositol hexaphosphates and discriminates lower inositol phosphates and inorganic phosphates. O'Neill *et al*. (1980) [29] reported the phytate extraction procedure where 3% Trichloroacetic acid containing Sodium cyclotetrametaphosphate. After centrifuging the supernatant's pH was adjusted to 4.5 ± 0.5 by adding tetrasodium EDTA. The viscosity was increased by addition of sucrose and the extract was filtered with a 0.45µm Millipore. The extract has been said to be stable for several weeks. Aras *et al*. (1990) [30] who determined the phytate content in Turkish diet, modified the method of sample extraction by filtering the sample extract and thereafter adding 0.1N NaOH to adjust pH to 4.5 ± 0.5. Excess quantities of EDTA was added to sufficiently chelate interfering paramagnetic ions for a well resolved phosphorus NMR spectrum. Also the use of sucrose has been eliminated for improved accuracy, sensitivity and specific for phytates.

Maroof *et al*., 2007 [14] used ^{31}P Nuclear magnetic resonance analysis. The solution ^{31}P NMR spectra were acquired on a Varian Unity 400 MHz spectrometer Walnut Creek, CA) with an automated ^{31}P probe robust operating at 161.9 MHz. A 70° pulse was used with 0.819 s acquisition time, 3 s delay, and broadband proton decoupling. Phytic acid was quantified by its C2–P chemical shift (5.95 ppm), which was isolated from the rest of monoester peaks.

Blatny *et al*. 1995 [31] reported a method called Capillary Isotachophoresis, electrophoretic migration properties of phytic acid, the lower inositol phosphates, and phosphate can be determined at different buffer systems. It was found that the electrolyte system consisting of 0.01 mol & hydrochloric acid, 0.0056 mol & 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 x 10^{-4} mol. 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%. 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-IPB), and phosphate (PI 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.

Conclusion

Phytate is considered as an undesirable component in many instances due to its chelating property of ions that are essential. And with the marked evidence showing the significance of phytates in reduction of colorectal cancer and tumor formation it is highly essential to determine the quantity of phytates in the diet we consume. And thus techniques like ion exchange chromatography, high performance liquid chromatography can uniquely identify and quantify the IP6 (phytic acid) and hence they can be used for the analysis of phytates in food products.

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