Orthophosphate, Phytate, and Total Phosphorus Determination in Cereals by Flow Injection Analysis

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A flow injection spectrophotometric procedure with enzymatic hydrolysis was developed for determination of orthophosphate, phytate and total phosphorus in cereal samples. Phosphorus species were extracted from cereals with 0.05 mol L^{-1} potassium hydrogen phthalate buffer solution at pH 5.7. Orthophosphate was directly determined in the extracts by molybdenum blue spectrophotometric method. The phytate was hydrolyzed by the enzyme phytase coupled to a solid phase packed into an enzymatic reactor, and the resulting hydrolyzed orthophosphate was also determined by spectrophotometry at 650 nm. After optimization for phosphorus species extraction and enzymatic hydrolysis, a linear calibration graph was obtained up to 196 $\times$ 10^{-6} mol L^{-1} orthophosphate (P conc $= -2.67 + 0.52x$, $r = 0.9998$). Measurements are characterized by relative standard deviation of 1.6% for a standard of 72 $\times$ 10^{-6} mol L^{-1} orthophosphate and no baseline drift was observed during 4 h operation periods. It provides 72 measurements per hour, with 2.4 $\times$ 10^{-6} mol L^{-1} and 7.9 $\times$ 10^{-6} mol L^{-1} as detection and quantification limits, respectively.

KEYWORDS: Enzymatic hydrolysis; immobilization; phytase; phytic acid; and spectophotometry

INTRODUCTION

Phosphorus is an essential nutrient and in plant feedstuffs is found in two separate groups: organically bound phosphorus present as salts of phytic acid (phytate, myo-inositol 1,2,3,4,5,6-hexakisphosphate) and phosphorus present in other forms (nonphytate forms) (*1*). In grains and seeds, phytate is present as 1−5% of the dry matter, which corresponds to 50−90% of total phosphorus (*2*) and is either unavailable or poorly utilized by human and other monogastric animals, due to the very low phytase activity found in the digestive tract (*1*, *3*). Phytases belong to a special group of acid phosphatases that are capable of hydrolyzing phytate (*4*). The individual determination of amounts of P inorganic as phosphate and P as phytate are needed to recommend the optimum dietary intake.

Total phosphorus extraction and determination have been normally done in routine analysis (*1*). A number of methods to determine phytic acid are based on the hydrolysis of phosphoric esters to phosphoric acid and photometric phosphate determination, and phytate purification before determinations is normally necessary (*1*, *6*, *7*). March et al. (*8*) determined phytate in urine based on the reduction of molybdophosphate to molybdenum blue. Extraction with ion pair, sample clean up with activated carbon, and anion exchange chromatography were required before spectrophotometric determination.

The developing of analytical methods that improve the accuracy, minimizing human interference in the analytical steps is the goal of a number of researches. The flow injection approach is include in this task, concerning their advantages such as high sample throughput, low sample and reagent consumption, and the number of chemical manipulations that may be carried out in a contamination-free system with good precision (*9*). To improve flow injection methods, inorganic support materials have been used for enzymatic immobilization, which allows the use of fewer amount of reagents and become possible the on-line enzymatic reaction. Silica is one of the most used support, owing its inert behave to microbial attacks, stability and ease of handling (*10*).

Considering the problems related with the P species determination, the use of an enzymatic reactor coupled to a flow injection system is proposed to determine the inorganic and total phosphorus, as well as the phytate content by difference.

MATERIALS AND METHODS

Materials. A cutting mill (MR 340 Microtec, São Paulo, Brazil) fitted with a 20 mesh screen at the bottom of the cutting chamber was used to ground the samples, previously oven-dried at 60 °C for 48 h in a forced air oven. Total phosphorus decomposition was assisted in a closed vessel microwave oven, ETHOS 1600 (Milestone, Sorisole, Italy). For results and methods comparison, a simultaneous optical emission spectrometer with radial view ICP OES VISTA RL (Varian, Mulgrave, Australia) was used.

Flow Injection System. The flow setup comprised a model ISM 761 peristaltic pump (Ismatec, Switzerland) with Tygon pumping tubes,

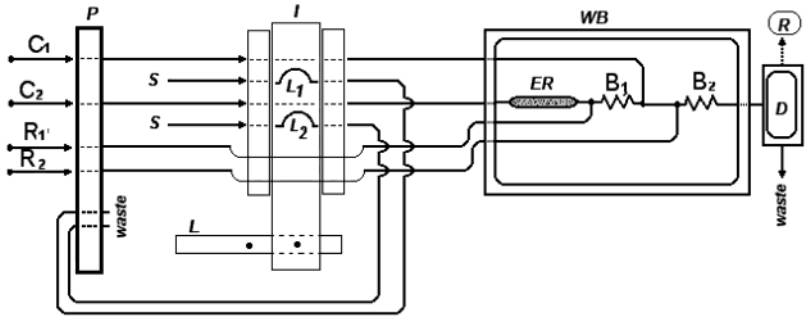

Figure 1. Schematic diagram of the proposed flow injection system. P, peristaltic pump; I, injector–commutator; C_1 and C_2 (water and buffer carrier streams, 1.6 mL min^{-1}); R_1 and R_2 reagents (molybdate and ascorbic acid, 1.6 mL min^{-1}); S, sample solution (3.6 mL min^{-1}); L_1 and L_2, sample loops (72 and 201 μL, respectively); WB, water bath (40 °C); ER, enzymatic reactor; B_1 and B_2, reaction coils (75 and 50 cm, respectively); D, detector (λ = 650 nm) and R, streapchart recorder. More details in the text.

Table 1. Standard Analytical Solutions Used in the Experiments.

standard	concentrations		
	A	B	
	PO_4^{3-}	PO_4^{3-}	$P_{phytate}$
1	0	0	0
2	24	24	27
3	48	48	54
4	72	72	81
5	96	96	107
6	120	120	134
7	144	144	161
8	196	196	215

a laboratory-made injector−commutator (*11*), a model 432 spectrophotometer (FEMTO, São Paulo, Brazil) with a tubular flow cell (inner volume ca. 80 μL, optical path 12 mm) and a model BD111 stripchart recorder (Kipp & Zonen, Delft Holland). The enzymatic unit was built with a glass column with an i.d. of 3 mm and 30-mm length, and the manifold was built up with 0.8-mm i.d. polyethylene tubing of a noncollapsible wall type and Perspex Y-shaped connectors.

Solutions. All solutions were prepared with chemicals of analytical reagent quality. Water: 18 MΩ-cm resistivity, Milli-Q system (Millipore, Bedford, MA) or equivalent. Enzyme: Phytase 2264 U mL^{-1}, derived from *Peniophora lycii* (Roche, São Paulo, Brazil), used for the phytate hydrolysis.

A solution of 0.05 mol L^{-1} potassium hydrogen phthalate ($KC_8H_5O_4$, Vetec, Brazil) buffer solution at pH 5.7 was used for P species extraction. The pH was set with 0.2 mol L^{-1} NaOH. Concentrate nitric acid and 30% H_2O_2 (Mallinckrodt, Mexico) were used for microwave assisted acid digestion of the samples. Solutions of 1.6% w/v ammonium molybdate (Merck, Germany) and 1% w/v ascorbic acid (Synth, Brazil) were used as reagents in the spectrophotometric method.

Controlled pore silica (CPS−60−100 mesh, Aldrich, German) was used as inorganic support. Solutions of 2% v/v (3-aminopropyl)-triethoxisilane and 2.5% v/v glutaraldehyde (Aldrich, German) were used for silanization and activation of the support of CPS, respectively.

Reference analytical solutions with inorganic phosphorus, PO_4^{3-} (dipotassium hydrogen phosphate, K_2HPO_4, Vetec, Brazil), and phytic acid ($P_{phytate}$, inositol hexaphosphoric acid monocalcium, $C_6H_{16}O_{24}P_6Ca$, Sigma, EUA) were prepared as described in the **Table 1**. A solution of 1000 mg L^{-1} P plasma analytical standard (Spex CertiPrep, Metuchen, NJ), diluted to a range from 2.5 to 100 mg L^{-1} was used as reference solution in the total P determination by ICP OES.

Sample Preparation. Samples of corn (*Zea mays* L.), milk cattle ration and soybean (*Glycine max* (L.) Merril) meal were used for experiments evaluation. All samples were first ground in a cutting mill. After they were ground, the samples were dried for 48-hours at 60 °C in a forced air oven.

Sample Extraction. A 250-mg portion of samples were weighed and transferred to glass vessels. A volume of 100 mL of 0.05 mol L^{-1} buffer solution at pH 5.7 was added to each vessel. The solutions were mixed for 1 h in a vortex and then filtered.

Microwave Digestion. For total P determination, microwave assisted acid decomposition was performed at high pressure and temperature, as previously described (*12*).

Enzymatic Immobilization. The immobilization was set in two steps:

(a) Activation of the Support (*10*). Silica beads were silanized with (3-aminopropil)-trietoxisilane−APTS, 3 mL of 2% v/v APTS aqueous solutions were added to 1 g of clean silica. This inorganic support was then coated with the (aminoalkil)silane for 30 min in a vacuum medium. After removing the excess of solution, the inorganic support was heated for 12−15 h at 70−80 °C. The silanized silica was washed with water and dried for 8 h at 100−110 °C in a forced air oven. A mass of 0.5 g of silica plus 1.0 mL of a 2.5% v/v glutaraldehyde solution were mixing inside a vacuum tube especially built to degass the solution with an air flow. A red color formation indicated glutaraldehyde attached to silane. After 30 min reaction time, the particles of the inorganic support were first washed with water to remove the excess of glutaraldehyde and then with 0.05 mol L^{-1} buffer solution (pH 5.7).

(*b*) *Covalent Attachment of the Phytase Enzyme* (*Method of Lam and Malikin* (*13*) *with Modifications*). A 5.0-mL aliquot of phytase (10 mg L^{-1}) in 0.05 mol L^{-1} buffer solution (pH 5.7) was added to 0.5 g of active support. The mixture was degassed with nitrogen and let stand for 4 h at 4 °C. After that, the inorganic support containing immobilized enzyme was washed with 0.5 mol L^{-1} of buffer solution (pH 5.7). The immobilized enzyme in the support was then stored in a 0.05 mol L^{-1} buffer solution at 4 °C in a refrigerator and used in the enzymatic reactor.

Enzymatic Reactor. A mass of 0.3 g of silica with immobilized enzyme was packed in a glass column (3.0-mm i.d. and 30-mm length) containing small pieces of glass wool in the extremities to avoid the leakage of the enzyme. This reactor was coupled to the flow manifold, as presented in **Figure 1**.

Flow Injection Procedure. The proposed flow diagram is shown in **Figure 1**. The volumes of L_1 and L_2 sampling loops through which the sample aliquots were aspirated (4.0 mL min^{-1}) were 72 and 201 μL for *P*-orthophosphate and *P*-(phytate + orthophosphate) determination, respectively. After switching the injector−commutator, the selected sample aliquots were simultaneously introduced into C_1 (water) and C_2 (buffer solution pH 5.7) carrier streams (1.6 mL min^{-1}). The established sample zone formed by the first sample aliquot (C_1) received the R_1 (ammonium molybdate reagent 1.4×10^{-2} mol L^{-1}) and R_2 (ascorbic acid 5.7×10^{-3} mol L^{-1}) reagents (1.6 mL min^{-1}), passed through B_2 coil (50 cm) and reached the detection unit. Passage of the processed sample through the flow cell resulted in a transient signal proportional to the *P*-orthophosphate content in the sample, which was monitored at 650 nm. The established sample zone formed by C_2 passed through the ER, received the R_1 reagent, passed through the B_1 coil (75 cm), received R_2 reagent, passed through the B_2 coil, and reached the detection unit. Passage of L_2 through the enzymatic reactor allows the phytate hydrolysis to phosphate. The ER, B_1, and B_2 were located inside a water bath at 40 °C. The passage of the processed sample

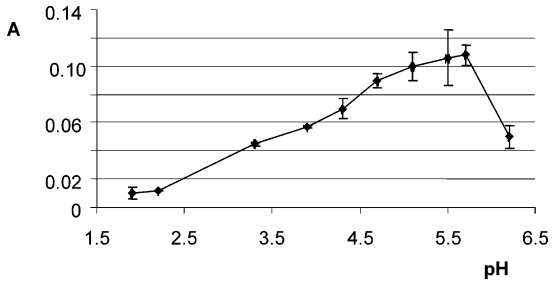

Figure 2. Effect of pH on phytate (as 1.35×10^{-5} mol L^{-1} of phytic acid) hydrolysis by immobilized enzyme. **WB** (**Figure 1**) was set at 25 °C.

through the flow cell resulted in a transient signal proportional to the *P*-(phytate + orthophosphate) content in the sample, which was monitored at 650 nm. After achievement of the analytical signal, the injector−commutator was switched back to position in **Figure 1**, and the next sample aliquots were introduced. The B_1 and B_2 coils were defined to allow similar high signals when phytate was not present in the sample (**Table 1**, column A). Furthermore, the presence of phytate was indicated by an increase in the signal resulting from the second sample aliquot, due to the orthophosphate produced during the enzymatic hydrolysis (**Table 1**, column B).

Study of the Variables Affecting the Analytical System. For system parameters definition, the hydrolysis efficiency, pH of reaction, and temperature dependence were appraised. First, aliquots of 10 mL of different concentrations of analytical solution of phytic acid (**Table 1**) received 5 mL of solutions of phytase in buffer medium with activity equivalent to 0.2, 0.4, and 1.6 U mL^{-1}. The amount of orthophosphate produced was determined by using a flow injection system similar to that described in **Figure 1** without the immobilized column stream, sampling loop L_2, and carrier stream C_2. After that, the system was evaluated with samples of corn grain. A 250-mg sample of corn received 100 mL of buffer extraction solution and was shaken for 1 h before being filtered. A 10-mL aliquot of this solution received 5 mL of solution of phytase in buffer medium with activity equivalent to 1.6 U mL^{-1}. After 15 min, the amount of PO_4^{3-} was determined by using the flow injection system described in the **Figure 1** without the immobilized column stream.

RESULTS AND DISCUSSION

Effect of pH and Temperature. Figure 2 shows the effect of pH variation, from 1.7 to 6.2, on the hydrolysis of phytate. The maximum phytase activity was observed at pH 5.7. At pH 6.0, a significant reduction in substrate hydrolysis was observed. Other authors, employing 3-phytase otherwise derived from *Aspergillus ficuum* to perform the phytate hydrolysis, observed maximum activity at pH 3.0−3.5 (*14*), and 5.5 (*1*). Ullah and Gibson (*15*) reported that purified phytase presented activity at pH 2.2 and 5.0−5.5, with the optimum occurring at pH 5.0.

Temperature dependence of the phytase activity was conducted from 25 to 65 °C using the determination of phosphate in the eluate of the solution at the given temperature (**Figure 3**). Maximum activity of phytase was 55 °C. However, to avoid the formation of bubbles in the flow system, the temperature was set at 40 °C.

Hydrolysis Efficiency. The hydrolysis velocity for corn samples solutions was lower than the hydrolysis velocity obtained with the use of analytical solutions. Corn grain presented approximately 50−70% starch (*16*), which could act as an obstacle to enzyme access to the substrates, limiting the phytate hydrolysis (*17*). To avoid this drawback, 5.0-mL aliquots of a 2.5×10^{-3} mol L^{-1} solution of starch were added to the phytic acid analytical solutions. With this artifice, similar hydrolysis velocity was observed between samples and standard analytical solutions, allowing the determinations with grain

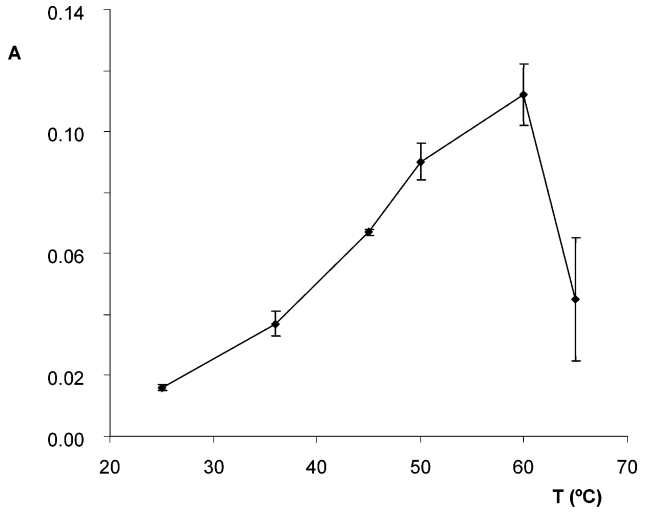

Figure 3. Influence of temperature on phytate (as 1.35×10^{-5} mol L^{-1} of phytic acid) hydrolysis by immobilized enzyme. C_2 buffer carrier (**Figure 1**) was set at pH 5.7.

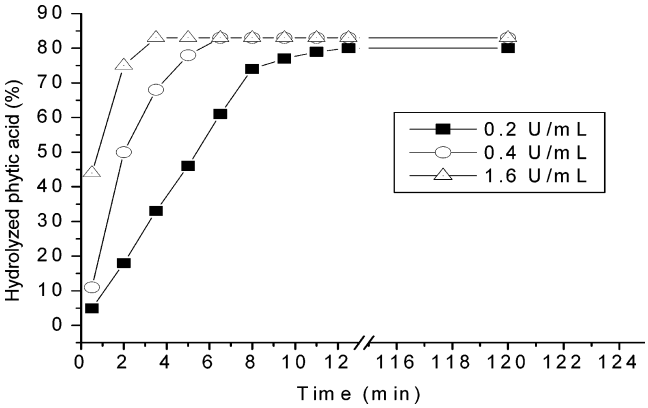

Figure 4. Time of hydrolysis on (%) substrate conversion of phytic acid by immobilized enzyme. Phytic acid 1.35×10^{-5} mol L^{-1}, at 25 °C.

Table 2. Phosphorus Determination in Corn Samples[a]

corn sample	$P_{extracted}$[b]	$P_{phytate}$[c]	$P_{phytate}$[d]
M_1	80.5 ± 1.7	50.5 ± 3.9	51.9 ± 1.7
M_2	76.4 ± 0.9	63.7 ± 5.9	62.3 ± 2.8
M_3	70.9 ± 3.2	63.1 ± 3.2	64.1 ± 0.6
M_4	73.5 ± 4.3	56.8 ± 5.6	57.3 ± 2.3

[a] Values are represented as % of total P present in the samples. [b] Determined by ICP OES. [c] P content in the phytate molecule hydrolyzed in batch solution of phytase. [d] P content in the phytate molecule hydrolyzed in the enzymatic reactor, determined by the proposed flow injection system described in **Figure 1**.

samples without further problems. Five of the six groups present in the phytate molecule, corresponding to approximately 83%, were hydrolyzed (**Figure 4**).

Flow Injection System. A compromise between hydrolysis efficiency and measured frequency had to be done to get analytical performance. Reactors greater than 30 mm significantly reduced the frequency of measures, due to lack of time required to clean the reactor. However, when lower length reactors were connected to the system, a decrease in the signal intensity was observed. The inner diameter used was 3 mm.

Table 2 presents the total extracted phosphorus and a comparison between the phytate hydrolysis obtained in batch and by flow injection coupled to the enzymatic reactor. The data are presented as a percentage of total phosphorus obtained by ICP OES after microwave assisted acid digestion. As

Table 3. Total Phosphorus (P_{total}), Orthophosphate ($P\text{-}PO_4^{3-}$) and Phytate ($P_{phytate}$) Determination after 1 h Extraction in 100 mL of 0.05 mol L^{-1} Buffer Solution (pH 5.7)[a]

sample	P_{total}[a]	$P_{phytate}$[b]	PPO_4^{3-} [d]
M_1	81.0 ± 4.7	52.5 ± 2.7	26.5 ± 1.7
M_2	75.9 ± 1.1	61.2 ± 0.6	13.5 ± 0.4
M_3	71.1 ± 2.0	60.5 ± 1.6	11.9 ± 0.4
M_4	76.6 ± 1.1	59.5 ± 0.7	13.5 ± 0.6
bran soybean	66.2 ± 0.5	50.1 ± 1.1-	20.1 ± 0.7
milk cattle ration	52.8 ± 1.0	41.6 ± 1.8	29.2 ± 0.9

[a] Values are represented as % of total P present in the samples. [b] Determined by ICP OES. [c] $P_{phytate}$ hydrolyzed in enzymatic reactor. [d] Inorganic P content, determined by the proposed flow injection system described in **Figure 1**.

described before, five of the six groups present in the phytate molecule are hydrolyzed by the phytase (**Figure 4**). The values achieved with the flow set up presented lower standard deviation than the batch approach, due to automatic control of flow system. The amount of total phosphorus, phytate, and orthophosphate extracted in samples of corn (M_1, M_2, M_3, and M_4), milk cattle ration, and soybean meal are presented in the **Table 3**. All results were compared with the total phosphorus in the samples. With the used flow injection system (**Figure 1**), the substrate conversion was ca. 23%. It corresponds to hydrolysis about two groups of PO_4^{3-} present in the phytate molecule. The lower conversion percentage, compared with the performance presented in **Figure 4** and previously described, was not a problem in view of high phytate concentration in the samples and the kinetic characteristic of the flow system. The analytical frequency observed was 72 determinations per hour. The detection limit was set as 2.4×10^{-6} mol L^{-1}, and the quantification limit was 7.9×10^{-6} mol L^{-1}.

In conclusion, the flow injection system proved to be suitable for the proposed analytical process, which allowed rapid in-situ phytate hydrolysis and phosphorus species determination in animal food. This method is feasible and seems to be less laborious, presenting low time and reagent consumption if compared with conventional phytate determination procedures.

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