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Flow Injection Potentiometric System for the Simultaneous Determination of Inositol Phosphates and Phosphate: Phosphorus Nutritional Evaluation on Seeds and Grains

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A simple flow injection potentiometric (FIP) system, which uses a tubular cobalt electrode, has been developed for phosphorus nutritional evaluation of seeds and grains. Inorganic phosphorus, P_i, is determined using a 1×10^{-2} mol·L^{-1} potassium phthalate buffer solution adjusted at pH 4. A sensitivity of 47 mV/decade and an operating range from 10 to 1000 mg·L^{-1} (1×10^{-4}−1×10^{-2} M) of dihydrogen phosphate are obtained. The inositol phosphates amount, which is referred to the organic phosphorus, P_{org}, is directly determined from extracts using a 1×10^{-2} mol·L^{-1} Tris-HCl buffer solution adjusted at pH 8. A sensitivity of 127 mV/decade and an operating range of 10−1000 mg·L^{-1} (2.5×10^{-4}−5×10^{-3} M) of P_{org} (expressed as inositol hexakisphosphoric acid monocalcium) are achieved. Some samples of seed and grain are analyzed by an ICP-OES and a spectrophotometric method to compare results to the developed flow system; no significant differences at the 95% confidence level are observed using a paired *t* test. Other samples such as animal nursing feed, soybean meal, and corn are also analyzed with the proposed FIP system, showing a good correlation to the ICP-OES values.

KEYWORDS: Phosphate; inositol phosphates; cobalt electrode; flow injection potentiometry

INTRODUCTION

myo-Inositol phosphates are phosphate esters of a cyclic alcohol derived from glucose. The best known component of this family is phytic acid, which was identified for the first time in 1855 and which is characterized to include 12 replaceable protons in its structure. Therefore, it can be found in many different forms, which are called phytates. Moreover, phytates are the major phosphorus-containing constituents in cereals and legumes, which normally hold up to the 85% of the total phosphorus amount. These compounds have a significant influence on the functional and nutritional properties of food and represent the primary storage of phosphorus. They play an essential role in the germination process by contributing to the viability and vigor of the seeds (*1*−*4* and refs therein).

Although inositol phosphates, especially phytate, serve as the major source of energy and phosphorus, the latter by itself is poorly available to simple-stomached animals. Thus, inorganic phosphorus, a nonrenewable and expensive mineral, is supplemented in diets for swine, poultry, and fish to complement their nutrient requirements. Meanwhile, the unutilized organic phosphorus phosphoric phytate, contained in the animal excrements, is becoming an important environmental pollutant in areas of intensive animal agriculture. The excessive phosphorus content in soil lixiviates to lakes and the sea, leading to eutrophication and stimulating the growth of aquatic organisms capable of producing injurious neurotoxins to humans (*5*).

Nevertheless, the manipulation of seeds and grain crops to produce lower phytate contents could have unacceptable effects on agricultural production, especially in geographical areas where soils have a low phosphorus concentration and/or poor micronutrient richness (*3*). Moreover, phytate is known as an antinutrient compound due to its chelating ability toward several metallic cations such as Ca^{2+}, Na^{+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and, in particular, Zn^{2+}. It modifies the nutritional bioavailability of these dietary metal ions (*2*).

The analysis of inositol phosphates could be considered a difficult task because of its poor spectral characteristics and the lack of specific reagents. To solve this problem, both direct and indirect methods based on complex analytical techniques have been proposed. Direct methods include CE-MS (*6*), high-performance liquid chromatography (*7*, *8*), mid-infrared spectroscopy (*9*), ^{31}P nuclear magnetic resonance spectroscopy (*10*),

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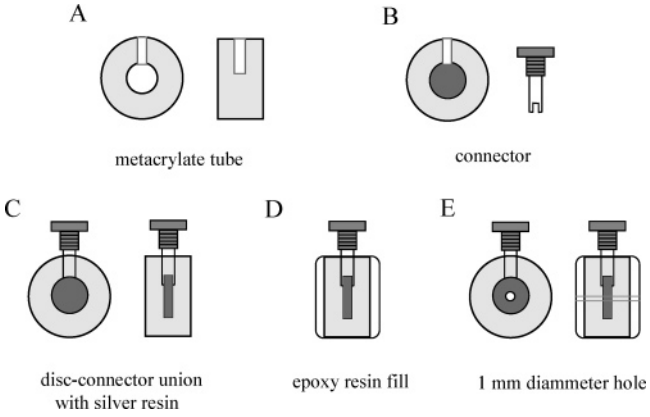

Figure 1. Construction of the cobalt electrode with a tubular configuration.

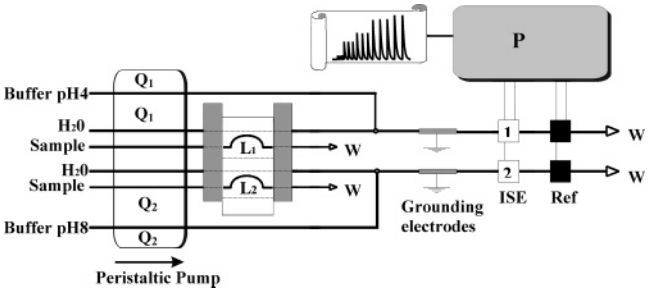

Figure 2. Developed flow injection system: P, potentiometer; ISE (1,2), cobalt electrodes; Ref., reference electrodes; L_1, sample loop for "P_i" determination (500 μL); L_2, sample loop for "P_{org}" determination (75 μL); buffer pH 4, potassium hydrogenphthalate; buffer pH 8, Tris-HCl; flow rate, Q_1, 1.4 mL min^{-1}, Q_2, 0.7 mL min^{-1}; W, waste.

or inductively coupled plasma mass spectrometry (*11*). Indirect methods are mainly based on the enzymatic hydrolysis of inositol phosphates to *myo*-inositol and phosphate with subsequent determination of these ions by gas chromatography (*12*) and UV−visible spectrophotometry (*13*), respectively. However, simple, reliable, low-cost, and less time-consuming methods for the routine determination of inositol phosphates are still necessary in a wide range of applications.

This paper reports a new direct method for a simple, rapid, and accurate determination of inositol phosphates, especially phytate, in agricultural and food samples. This methodology is based on a flow injection potentiometric system that uses a cobalt electrode as a phytate and a phosphate sensor. It allows evaluation of the phosphorus nutritional content, including both organic (P_{org}) and inorganic (P_i) phosphorus, in aqueous extracts of seeds and grains.

EXPERIMENTAL PROCEDURES

Reagents. Analytical grade reagents and Milli-Q water (Millipore, Bedford, MA) were, when possible, always employed. Standard solutions of P_i were prepared from NaH_2PO_4 and Na_2HPO_4. Standard solutions of P_{org} were made using inositol hexaphosphoric acid monocalcium, $C_6H_{16}O_{24}P_6Ca$ (90% purity). Buffered standard solutions of P_i were prepared in a 1×10^{-2} mol·L^{-1} potassium hydrogenphthalate solution at pH 4. Buffered standard solutions of Na_2HPO_4 and phytic acid were obtained using 1×10^{-2} mol·L^{-1} Tris-HCl, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS)−KOH, and H_3BO_3−KOH buffers at pH 8. They were adjusted to the desired pH value by the addition of concentrated hydrochloric acid, potassium hydroxide, and potassium chloride solutions. pH measurements were performed using a combined glass electrode (Ingold model 10/402/3092, Crison Instruments, Barcelona, Spain). All chemicals were purchased from Sigma-Aldrich, Europe.

Cobalt Electrode Construction and Conditioning. The tubular flow-through cobalt electrode construction was carried out using a modification of a well-established methodology, which was proposed in the research group (*14*, *15*) and which is illustrated in **Figure 1**. This technique consists of the following steps: a methacrylate cylindrical tube (8 mm length and 5 mm internal diameter) is built so that a metallic cobalt disk (99.99% purity) is perfectly inserted in the opening area (**Figure 1A**). After that, a tongue-like form connector is introduced in the tube (**Figure 1B**) and is joined to the cobalt disk with a silver-epoxy resin that guarantees the electrical connection (**Figure 1C**). Then, both faces of the electrode are isolated from the external medium with an epoxy resin (**Figure 1D**), which is cured at 40 °C during 24 h. Finally, the center of the device is longitudinally perforated with a 1 mm diameter drill (**Figure 1E**).

Sensors were conditioned in phosphate solutions prior to analysis. For the determination of P_{org}, the cobalt electrode was soaked in a 1×10^{-1} mol·L^{-1} phytic acid solution during 24 h, whereas for the determination of P_i, a 1×10^{-1} mol·L^{-1} NaH_2PO_4 solution was employed.

FIP Instrumentation. The potentiometric response of cobalt electrodes was measured in the flow analysis system depicted in **Figure 2**. This system comprised a peristaltic pump (Ismatec, 78001-12, Glattbrug, Switzerland), a potentiometer (Mettler-Toledo, 355 ion analyzer, Leicester, U.K.), a potentiometric recorder (Kipp and Zonen, model BD112, Delft, The Netherlands), and a double-junction Ag/AgCl reference electrode (Orion, 90-02-00). A 10% KNO_3 solution was used as the external reference solution. To minimize the possible non-Faradaic current coming from the electrical supply and the static (harmonic) effects generated by the friction between the propulsion tubes and the peristaltic pump roulettes, a steel tube connected to a ground was additionally coupled to the main channel prior to the detector.

Sample Pretreatment and Experimental Procedures. The evaluation methodology of phosphorus involves a previous sample preparation followed by an extraction procedure. For the validation of the proposed FIA system, samples of corn (*Zea mays* L.), animal nursing feed, and soybean meal (*Glycine max* L.) were used. First, a cutting mill (MR 340 Microtec, Campo Belo, Brazil) fitted to a 20 mesh screen at the bottom of the cutting chamber was used to grind samples. They were previously dried at 60 °C during 48 h in a forced-air oven. For the sample extraction stage, a 1.0 g portion of each sample was weighed out and transferred to a glass vessel. Twenty-five milliliters of boiling Milli-Q water at 100 °C was added to each vessel. The resulting suspensions were mixed in a vortex for 30 min and were centrifuged at 2000 rpm for 10 min. Then, the phosphorus determination was performed.

For the potentiometric determination, samples were injected in a water carrier stream of two parallel flow systems, and then they were mixed through a confluence point with their respective buffer solution. The resulting solutions, which define the baseline signal, were simultaneously registered. For the P_i determination a 1×10^{-2} mol·L^{-1} potassium phthalate solution at pH 4 was used as the buffer solution, whereas for the P_{org} determination the chosen buffer was a 1×10^{-2} mol·L^{-1} Tris-HCl solution at pH 8. To obtain the calibration curves, sodium dihydrogen phosphate was used as the inorganic phosphorus standard solution and phytic acid was employed to describe the organic phosphorus content. Both solutions were prepared from 10 to 1000 mg·L^{-1} P.

To optimize the flow injection system, the peak heights of two standard solutions were evaluated. A triplicate analysis was carried out using 50 and 500 mg·L^{-1} solutions, respectively corresponding to the lower and a medium sample concentration of the linear working range. The evaluation criterion to select the experimental conditions was based on the best relationship between the highest peak height with the best sample throughput. For comparison purposes two methodologies have been used. A solution of 1500 mg·L^{-1} P plasma analytical standard (Spex, CertiPrep, Metuchen, NJ) was used as the reference solution in the total P determination by ICP-OES (ICP-OES VISTA RL, Varian, Mulgrave, Australia). A flow injection spectrophotometric procedure based on an enzymatic hydrolysis was employed. P_i was directly

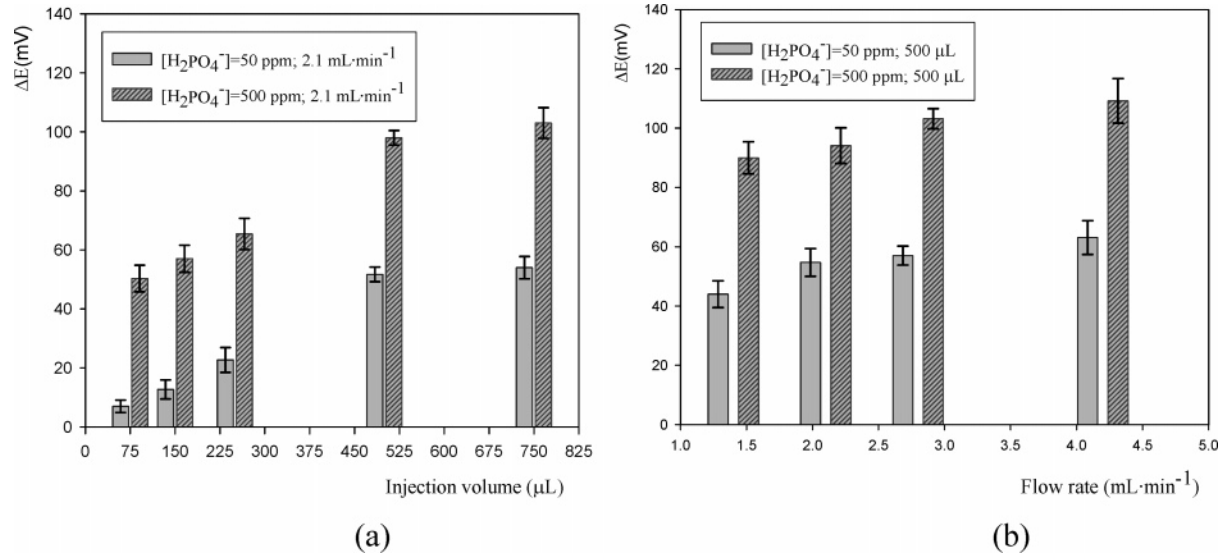

Figure 3. Optimization of the flow injection system for the P_i determination: (**a**) influence of sample volume on peak height (conditions: standard solutions of 50 and 500 mg·L^{-1}; 1×10^{-2} mol·L^{-1} potassium phthalate buffer at pH 4, and a global flow rate of 2.1 mL·min^{-1}); (**b**) influence of global flow rate on peak height (conditions: standard solutions of 50 and 500 mg L^{-1}; 1×10^{-2} mol·L^{-1} potassium phthalate buffer at pH 4, and sample volume of 500 μL).

determined by the molybdenum blue spectrophotometric method. Phytate was hydrolyzed by the phytase enzyme meanwhile packed into an enzymatic reactor, and the resulting hydrolyzed P_i was determined by spectrophotometry at 650 nm (*13*).

RESULTS AND DISCUSSION

P_i Determination. To establish the chemical and hydrodynamic variables of the continuous flow system and optimize the response to determine inorganic phosphorus, some parameters such as the composition and pH of the solution used as the buffer, the flow rate, and the sample volume were tested. According to the previously described works related to the phosphate determination using a cobalt electrode (*16*−*20*), a potassium phthalate buffer adjusted at pH 4 was chosen. Thus, the flow system optimization was first focused on the evaluation of the flow rate and the sample volume influence on the system response. All of the analytical signals presented in this work are relative potential values obtained from the difference between the potential measured at the maximum peak height and the potential baseline signal.

Panels **a** and **b** of **Figure 3**, respectively, show the influence of the sample volume and flow rate on the peak height. A sample volume of 500 μL and a global flow rate of 2.8 mL·min^{-1} were chosen to be the optimum values as a compromise between the peak height and the sample throughput.

To determine the analytical features obtained under these experimental flow conditions, a calibration curve was done. A linear correlation between ΔE and log [$H_2PO_4^-$] (in molar scale, M) was obtained, which shows a sensitivity of 47 mV/decade in a working range from 10 to 1000 mg·L^{-1} of dihydrogen phosphate. These calibration parameters are in good agreement with those described in the literature (*16, 20*) and, moreover, the obtained linear range using the cobalt sensor conveniently covers the commonly observed concentration levels of the evaluated samples.

P_{org} Determination. In the same way, to determine organic phosphorus using the cobalt electrode, the optimization of the flow system was done. The phytate molecule holds an inositol nucleus, which contains 6 phosphate groups with 12 acidic functionalities, which are capable of releasing protons. Six of these functionalities have very weak pK_a values (1.1−2.1) and are completely dissociated in solution; another three have nearly neutral character, with pK_a values of 5.7, 6.9, and 7.6, and the remaining ones show pK_a values of >10.0 and are difficult to dissociate (*21*). Therefore, it can be concluded that there are three differentiated pH regions that would lead to a response using the cobalt electrode, which are related to the predominant different species of phytic acid.

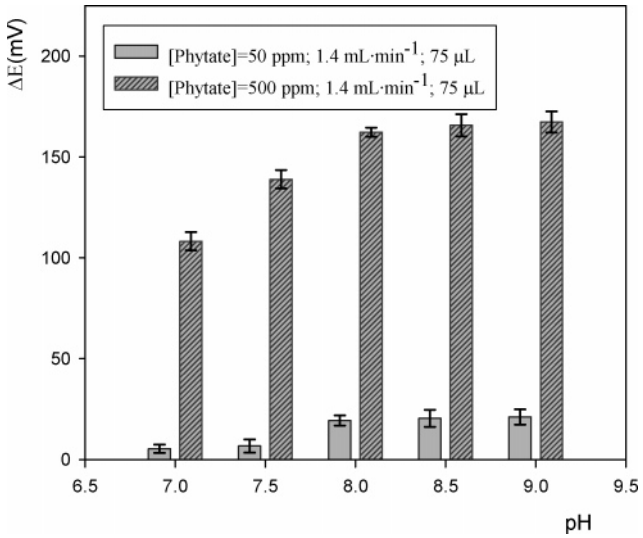

Figure 4. Optimization of the flow injection system for P_{org} determination. Effect of the buffer pH on the peak height. Standard solutions, 50 and 500 mg·L^{-1}. Conditions: 75 μL sample volume; 1×10^{-2} mol·L^{-1} TRIS–HCl buffer solution; global flow rate, 1.4 mL·min^{-1}.

Initially, the potentiometric response to phytic acid was tested under the same conditions, which were established for the inorganic phosphorus determination (potassium hydrogenphthalate buffer at pH 4). In those conditions, the electrode response was not affected by the phytic acid concentration and, therefore, it assured an accurate analysis of the inorganic phosphorus. As a result, we could conclude that the cobalt electrode was not able to detect the phytate species with a charge lower than or equal to −6, which is predominant at this pH. The electrode response was then evaluated at the pH region

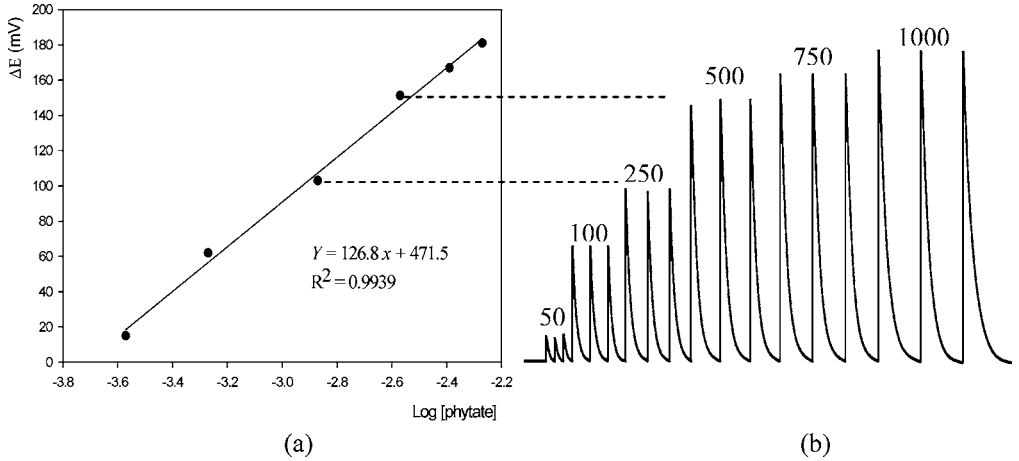

Figure 5. (a) Calibration curve obtained with the optimized P_{org} flow injection system and linear correlation obtained between ΔE vs log [phytate] (in molar scale, M) (b) FIA peaks recorded during the phytate calibration run. Phytate concentrations are in mg·L^{-1}.

Table 1. Optimization of the Phytate Flow Injection System[a]

buffer	detection limit[b,c] (mg·L^{-1})	working range (mg·L^{-1})	sensitivity[c] (mV·decade^{-1})
Tris-HCl	10 ± 4	50–1000	126.8 ± 2.5
EPPS–KOH	230 ± 24	250–1000	101.1 ± 7.7
H_3BO_3–KOH	80 ± 9	100–1000	43.6 ± 0.4

[a] Effect of the buffer composition on the analytical characteristics of the FIA system. Conditions: pH 8.0, global flow rate of 1.4 mL·min^{-1}, and sample volume of 75 μL. [b] Detection limit obtained using IUPAC recommendations. [c] Errors obtained by linear regression (95% confidence level).

between 7 and 9 values. **Figure 4** shows the obtained results using a 1×10^{-2} mol·L^{-1} Tris-HCl buffer solution. As it can be seen, the cobalt electrode response continuously rises by following the appearance of phytate (−9) species versus pH. The maximum signal was obtained at pH 8, at which this species is predominant. A further increase in the pH solution value does not show any signal variation, whereas it worsens reproducibility. This fact was referred to the possible cobalt electrode surface modification, which can occur at higher pH values (*22*, *23*). Therefore, a buffer solution at pH 8 was chosen to achieve the best relationship between the peak height and the precision of the measurement. Additionally, the influence of the monohydrogen phosphate anion on the electrode response was evaluated. Its response was negligible at this pH value.

Due to the chelating ability of phytate, the influence of the chemical composition of the buffer solution on the electrode response must be also evaluated. Three different 1×10^{-2} mol·L^{-1} concentration buffer solutions (Tris-HCl, EPPS−KOH, and H_3BO_3−KOH) were tested at pH 8. As shown in **Table 1**, the electrode sensitivity, measured as the slope of the calibration curve, dramatically varies according to the buffer used. These results seem to be related to the effective net charge of the phytate species, which is modulated by its interaction with the buffer substances. According to the previously described results (*24*), the highest slope observed using the Tris-HCl buffer solution could be explained by the formation of strong phytate−amine complexes that reduce the net charge of the phytate ion, which is favored by the amine concentration in the buffer composition. EPPS−KOH and H_3BO_3−KOH buffer solutions, which are not able to form this kind of complex, give lower analytical sensitivities and a narrower working range. Nevertheless, phytate ions can also form weak complexes with potassium ion (*25*), which is present at different concentration levels in the last mentioned buffer solutions. This can explain the

Table 2. Determination of the Phosphorus Content of Different Animal Feeding Samples[a]

	P_i (mg·L^{-1})		P_{org} (mg·L^{-1})		P_{total} (mg·L^{-1})	
sample	spectro	pot.	spectro	pot.	pot.	ICP-OES
1	51 ± 1	56 ± 2	258 ± 15	278 ± 20	334 ± 20	321 ± 12
2	70 ± 2	79 ± 4	402 ± 18	391 ± 23	470 ± 23	479 ± 19
3	125 ± 3	130 ± 5	785 ± 24	732 ± 29	862 ± 29	877 ± 21
4	148 ± 4	146 ± 6	957 ± 28	930 ± 37	1076 ± 37	1061 ± 25
5	165 ± 8	170 ± 10	964 ± 29	1001 ± 41	1171 ± 42	1154 ± 31
paired *t* test (95%)	$t_{cal} = \|2.475\|$, $t_{tab} = 2.776$; no significant differences		$t_{cal} = 0.421$, $t_{tab} = 2.776$ no significant differences			

[a] Errors obtained by linear regression from triplicates for each sample (95% confidence level).

Table 3. Nutritional Evaluation of Grain and Seed Samples Using the Flow Injection System Proposed[a]

sample	P_i (mg·L^{-1}) pot.	P_{org} (mg·L^{-1}) pot.	P_{total} (mg·L^{-1}) pot.	P_{total} (mg·L^{-1}) ICP-OES
animal fodder	78 ± 4	276 ± 19	354 ± 23	361 ± 14
soy flour	69 ± 3	471 ± 23	540 ± 25	551 ± 21
corn without fertilization	25 ± 1	166± 11	191 ± 14	201 ± 7
corn with fertilization	51 ± 2	166± 10	217 ± 17	221 ± 7

[a] Errors obtained by linear regression from triplicates for each sample (95% confidence level).

observed differences between them regarding the electrode sensitivity.

After this stage, the influence of the sample volume and flow rate on the electrode response was examined. Using the same criteria described above, a sample volume of 75 μL and a global flow rate of 1.4 mL·min^{-1} were chosen. To optimize the response of the cobalt electrode to the phytate concentration, a calibration experiment was performed at the conditions established before (**Figure 5**). The electrode shows an average sensitivity of 127 mV/decade. According to the Nernst law, phytate seems to have less than half of the effective charge, most likely due to the interaction maintained with the buffer solution. A linear working range from 10 to 1000 mg·L^{-1} of phytate was obtained under these experimental conditions. Notice that this range is adequate for the evaluated samples. A global sampling rate of 25 samples per hour was achieved, which included P_i and P_{org} analysis.

Phosphorus Speciation in Seeds and Grains. The developed flow injection system was applied for the evaluation of the nutritional properties of seeds and grains (based on the phosphorus content), which are commonly used in animal feed. The obtained results for both P_i and P_{org} were compared to those provided by the method previously proposed by the research group (*13*). The obtained values of the analysis of the samples by both methods are summarized in **Table 2**. No significant differences between them were observed using a paired *t* test at a 95% confidence level. The total phosphorus content was also determined by ICP-OES.

To demonstrate the applicability of the proposed system to other types of samples, animal nursing fodders, soybean flours, and corn were also analyzed. In this case, P_{org} and P_i were also determined but compared to total phosphorus content, which was determined by ICP-OES. Results are summarized in **Table 3**. The phytate content found in animal fodder was ~80% of P_{total} in accordance with the values obtained from the literature. The proposed system can be also applied to follow the effect of the inorganic phosphorus fertilization in the corn crop. As can be noted from the two last samples, the fertilization process leads to an increase in the proportion of the inorganic phosphorus, which is quickly available to animals, without decreasing the phytate supply of the grain.

Values of P_{total}, obtained as the sum of P_{org} and P_i, for all samples ($n = 9$), are in good agreement with the results supplied by ICP-OES showing no significant differences by a paired *t* test at a 95% confidence level ($n = 9$; $t_{cal} = |0.292|$, $t_{tab} = 2.306$).

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