Potentiometric Determination of Phytic Acid and Investigations of Phytate Interactions with Some Metal Ions

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Dedicated to the memory of Prof. Dr. Jurij Brenčič

Abstract

Determination of correct amount (concentration) of phytic acid is of vital importance when dealing with protonation and/or metal complexation equilibria. A novel approach for precise and reliable assay of phytic acid, based on the difference between end points by potentiometric titration, has been presented. Twelve phytic acid protons are classified into three groups of acidity, which enables detection of 2 to 3 distinct equivalent points (EPs) depending on experimental conditions, *e.g.* counter-ion concentration. Using the differences between individual EPs enables correct phytate determination as well as identification of potential contamination and/or determination of initial protonation degree. Impact of uncertainty of phytate amount on the calculation of protonation constants has been evaluated using computer simulation program (Hyperquad2013). With the analysis of titration curves different binding sites on phytate ligand have been proposed for complexation of Ca^{2+} and Fe^{3+} ions.

Keywords: Phytic acid; potentiometric standardization; calcium(II) and iron(III) phytates

1. Introduction

Myo-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate), known as phytic acid (H_{12}Phy), exhibits six acidic phosphate groups around a cyclohexane ring (Fig. 1). From a chemical point of view it is an unique molecule containing 12 reversibly exchangeable protons and as multidentate ligand shows high ability to form stable coordination compounds with numerous metallic ions.[1] Inositol phosphates (IP_x, $1 \leq x \leq 6$) are naturally occurring compounds synthetized by plants and widely found in natural environment. Many papers deal with antioxidative[2–4] and anticorrosion[5–7] properties of phytates and their role in the environment,[8] biological functions,[9] human nutrition,[10–11] and interactions with protons[12–14] and metallic ions[1,15–17] in aqueous solutions.

The magnitude of the interaction between metal ions and chelating agents is defined by the corresponding equilibrium constants, and nowadays most of them are determined experimentally by spectrophotometry, potentiometry and other techniques (NMR) in combination with computational analysis of data.[18] Protonation of phytates and/or determination of stability constants with metallic ions are the subject of numerous publications.[1,12,19] With help of computerized programs determination of such data becomes easier and published constants are nowadays of high precision, which was not the case by data published in the classical monographs on stability constants,[20–21] where data frequently scatter between different methods and sources for more orders of magnitude.

The main aim of this work was to check parameters influencing uncertainty of the determination of phytate protonation constants by potentiometric measurements and computerized fitting of titration data. To improve accuracy and reliability of the results involving phytates, a new approach for the alkalimetric standardization of phytic acid was elaborated. It was demonstrated that uncertainty of the ligand concentration participating in the equilibria between phytate and protons/metal ions is one of the most important source of errors by investigation of metal to ligand interactions.
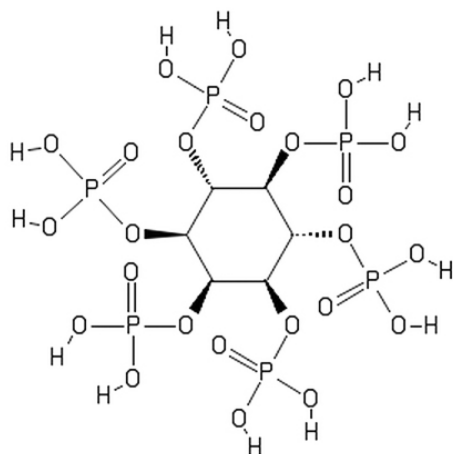
Figure 1. Structure of phytic acid

2. Experimental

2. 1. Materials and Methods

Stock solution of phytic acid was prepared by weighing the dipotassium salt ($K_2H_{10}Phy$, Sigma-Aldrich, min. 95%) and dissolving in ultrapure water with resistivity > 18.2 MΩ/cm (Millipore/MilliQ system). Protonated form of phytate was obtained by passing the solution over a strong cationic exchange resin (Dowex 50WX8). Potassium, sodium, calcium and magnesium concentrations in the eluate were analyzed by atomic absorption spectrometer, (Varian AA240), and were all below 3.0×10^{-6} M after single ion exchange procedure. NaCl, $CaCl_2$ (both Carlo Erba, p. a. grade) and $Fe(NO_3)_3 \cdot 9H_2O$ (Merck, p. a. grade) were used without further purification. Iron(III) salt was dissolved in 0.100 M HNO_3 to avoid metal hydrolysis and the titration data were corrected for the contribution of HNO_3 added into titration cell.

2. 2. Potentiometric Titrations

Potentiometric titrations were performed at 25 ± 1 °C using automatic titrator Metrohm 799 GPT Titrino, equipped with 20 mL burette (accuracy of the increment ±0.5 μL), carbon dioxide trap, magnetic stirrer (Metrohm) and combined glass electrode (Metrohm 6.0234.100, pH 0–14), which was calibrated at least with two buffer solutions (pH 4.00 and 7.00, Merck) if not stated otherwise. Due to calibration of glass electrode with standard buffers, pH is given in term of activity (Eq. 2). Titrations were carried out by TiNet 2.4 software (Metrohm). For each titration curve 120–140 data points were collected, and pH range between 1.5 and 11.0 was used for calculations. Both titrants carbonate-free 0.10 M NaOH (prepared from concentrated NaOH, Carlo Erba, p. a. grade) and 0.070 M Bu_4NOH (prepared from 40% solution, Fluka) were dissolved in ultrapure water deaerated with argon and standardized weekly by potassium hydrogen phthalate pri-

mary standard (Merck). For titrations at 1.0 M ionic strength analyte and NaOH titrant solutions were prepared in 1.0 M NaCl if not stated otherwise. Initial volume of titration solution was set to 50.0 mL.

2. 3. Calculations

Equilibrium constants were derived from potentiometric titration data using the Hyperquad2013 software,[22] and simulated titration curves were produced using the HySS2009 software.[23]

3. Results and Discussion

The general protonation equilibrium of phytate (Phy^{12-}) can be given by the reaction

$$H^+ + H_{i-1}Phy^{(12-i+1)-} \xrightarrow{K_i^H} H_iPhy^{(12-i)-}, \qquad (1)$$
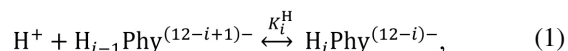
where Phy^{12-} represents completely deprotonated form of phytic acid $H_{12}Phy$ and index i is the number of protonation step, $0 \leq i \leq 12$. At constant ion strength I and temperature T one can define apparent protonation constant K_i^H, which is given according to the equilibrium (1)

$$K_i^H = \frac{[H_iPhy^{(12-i)-}]}{[H^+][H_{i-1}Phy^{(12-i+1)-}]}, \qquad (2)$$

where $[H^+]$ is given as activity, while other species are given as molar concentrations, therefore constants calculated in this work are mixed (apparent) macroscopic constants. Overall equilibrium constant β_i^H is defined by equation (3)

$$iH^+ + Phy^{12-} \xrightarrow{\beta_i^H} H_iPhy^{(12-i)-}, \quad \beta_i^H = \Pi K_i^H \qquad (3)$$
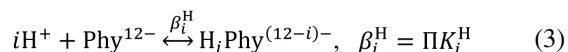
Titration curve of phytic acid (Fig. 2) shows rather complicated characteristics due to the high number of
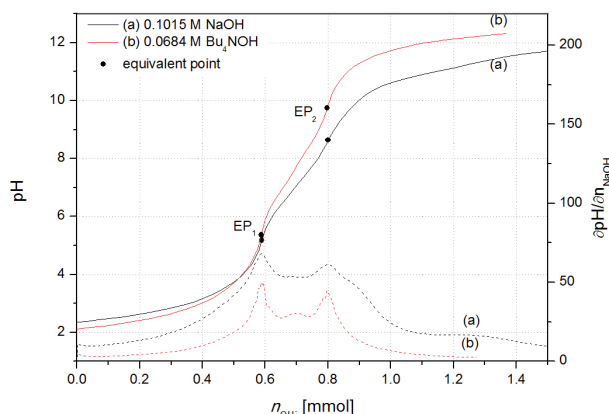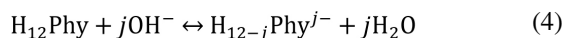

Figure 2. Titration of 0.1061 mmol $H_{12}Phy$ with (a) 0.1015 M NaOH and (b) 0.0684 M Bu_4NOH. Dashed lines represent the corresponding derivatives $\partial pH/\partial n_{OH^-}$.

(de)protonation steps and relatively small differences between values of protonation constants, K_i^H, collected in a review.[1] The titration of phytic acid proceeds according to the reaction:

$$H_{12}Phy + jOH^- \leftrightarrow H_{12-j}Phy^{j-} + jH_2O \qquad (4)$$

where index j represents the molar ratio between titrant and phytate: $j = n_{OH}/n_{Phy}$. From the titration curve it can be seen, that only certain (de)protonation steps are accompanied with a remarkable pH change during the titration and only two equivalent points (EPs) can be determined successfully from the corresponding derivatives, $\partial pH/\partial n_{NaOH}$ of the titration curves. As evident from Fig. 2 titration of phytic acid $H_{12}Phy$ with NaOH (curve a) differs from that when Bu_4NOH titrant was used (curve b). This effect is known well and is due to the complexation of Na^+ ions with phytate as phytic acid obtains an enhanced acidic character in the presence of alkali ions due to exchange of protons with Na^+ ions.[14,24–26] However, in both cases only two EPs (EP$_1$ at pH 5.3 and EP$_2$ at pH 8.5–9.5) can be determined unambiguously. Further from the course of the titration curves is evident that phytic acid contains three categories of protons. In the region before first EP$_1$ a group of strong acidic protons exists with an average dissociation constants pK_a around 2.6 ± 0.1, the second group appears between EP$_1$ and EP$_2$ with an average pK_a of 7.2 ± 0.2, and a third group of protons can be predicted at an average pK_a of 11.5 ± 0.5. Interestingly, these values are very close to that of ortophosphoric acid,[14,20] but the titration curve of H_3PO_4 shown in Fig. 3 (curve b), reveals due to a low number of protons a much sharper pH transition in the range around both equivalence points, and its content can be determined very accurately by an alkalimetric titration. In the past, assays of phytic acid were based on the precipitation of phytate with iron(III), however, Thompson and Erdman[27] reported that iron-phosphorus ratio was subject to variation and that assays based on determination of iron from ferric phytate precipitate are not recommended. So titrimetry stays the only promising direct analytical technique, for fast and accurate determination of substances with expressed acidic properties.

From the analytical point of view there appears the question, how to determine an unknown amount of phytic acid on the basis of alkalimetric titration data shown in Fig. 2. We will try to explain this problem on the basis of the above mentioned similarity between phytic and phosphoric acid shown in Figure 3. Such similarity inside of phosphate chemistry is quite common as described a long time ago in a series of papers by Van Wazer *et al.*[28–29] It is known that titration of H_3PO_4 up to the first EP$_1$ (Fig. 3, curve b), corresponds to the neutralization of first most acidic proton, and therefore by analogy EP$_1$ of phytic acid should correspond to neutralization of the first group of most acidic protons (pK_a 2.6 ± 0.1, Fig. 2). On the basis of fundamental knowledge of phosphate chemistry,[28] it can be presumed that phytic acid also contains only one strongly ionized hydrogen for each phosphorous atom, and that six protons are neutralized at EP$_1$. However, due to existence of different isomeric forms of IP$_6$ (*e.g. myo, scyllo, neo,...*)[8,30] and pH dependent conformation change between equatorial (1a5e) and axial (5a1e) orientation of phosphate groups of *myo*-IP6,[12,31] deprotonation of $H_{12}Phy$ is quite more complex process. Brigando *et al.*[12] proposed on the basis of ^{31}P NMR titrations that highly acidic protons belong to P[2], P[5] and P[1,3] phosphate group of $H_{12}Phy$. Recently Veiga and coworkers[31] with the aid of molecular modelling and NMR spectroscopy explain pH dependent conformational transition and protonation sequence of phytate. These findings may be very helpful for the explanation of intramolecular processes and microequilibria, as well as for the elucidation of the overall deprotonation process of $H_{12}Phy$.

While the exact number of protons consumed up to EP$_1$ cannot be determined by alkalimetric titration of an unknown amount of phytic acid, some additional data was required to answer this question.

For a comparison we simulated alkalimetric titration of an equal amounts of phosphate(V) in the presence of an excess and deficit of protons as shown in Figure 3. Curve (c) shows titration of a mixture of ortophosphoric acid and a strong acid (*e.g.* HCl), and curve (a) represents an equimolar mixture of H_3PO_4 and $H_2PO_4^-$. In this case the consumption of titrant up to the first EP$_1$ on the curve (c) correspond to the sum of the amounts of HCl (1 mmol) and H_3PO_4 (1 mmol), or in the case of phosphate buffer (curve a), only to the amount of H_3PO_4 (0.5 mmol). Therefore, if H_3PO_4 or analogously phytic acid is not pure, its amount cannot be determined from the first EP$_1$ only, and for determination of the composition of the mixture, the titration at least to the second EP$_2$ should be performed. For example, by titration of $H_3PO_4/H_2PO_4^-$ buffer (Fig. 3, cur-
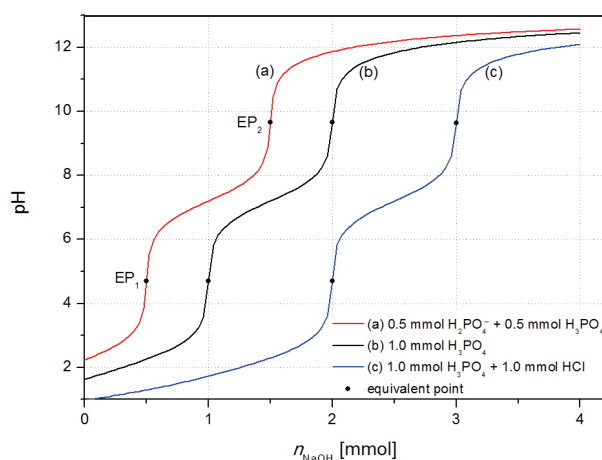

Figure 3. Simulated titration of 10 mL of a) 0.05 mmol H_3PO_4 + 0.05 mmol $H_2PO_4^-$, b) 1 mmol H_3PO_4, and c) 1 mmol H_3PO_4 + 1 mmol HCl with 0.20 M NaOH.
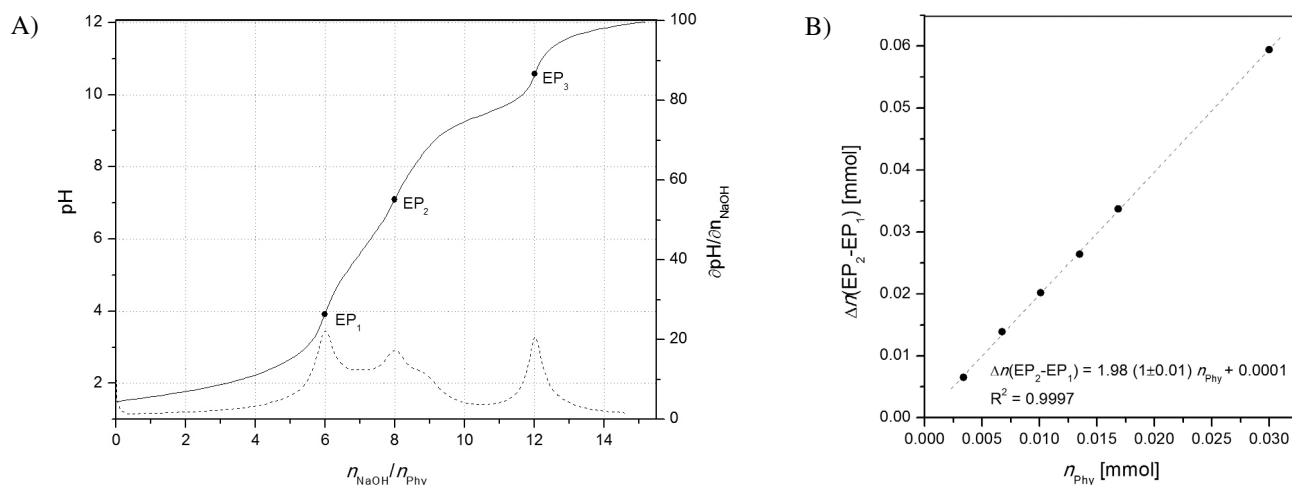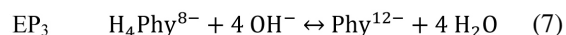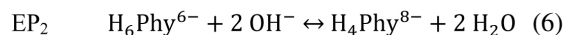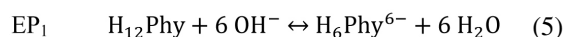
Figure 4. (A) Titration of 0.3081 mmol $H_{12}Phy$ in 1.0 M NaCl with 0.0996 M NaOH. Equivalents of NaOH per mole of phytate are given on x-axis. Dashed line represents the corresponding derivative $\partial pH/\partial n_{NaOH}$. (B) Linear dependence of $\Delta n(EP_2–EP_1)$ versus n_{Phy} added into titration cell.

ve a), the difference between EP_2 and EP_1 gives the total amount of phosphates as by the titration of pure H_3PO_4 (curve b), and EP_1 then equals to the concentration of H_3PO_4 originally present in the buffer. Also a mixture of H_3PO_4 and HCl (curve c) can be analyzed on this basis since difference $EP_2–EP_1$ equals to the amount of phosphoric acid and EP_1 represent the sum of the amounts of HCl and H_3PO_4.

By analogy, difference between EP_2 and EP_1 by alkalimetric titration of phytates should give the unknown amount of the analyte. However, again the equivalent amount of protons consumed between EP_2 and EP_1 remains unknown. The solution of this complex task was found in the interaction between phytate and metallic ions and formation of a relatively strong complexes of the alkaline cations with phytates.[14,24–26] As mentioned above sodium ions significantly increase acidity of phytate due to complexation (Fig. 2, curve a), but at this low concentration of titrant (low ionic strength, *I*), complete deprotonation to its final 12th step does not occur. This step can be detected just by very low maximum on the derivative curve at approx. 1.25 mmols of added NaOH. This is in accordance with relatively high K_i^H values of phytate at low ionic strength for $i \leq 3$.[32] To stimulate deprotonation of phytic acid therefore the exchange with sodium or other alkaline ions should be exploited.[14,24]

In Fig. 4A alkalimetric titration of phytic acid in the presence of 1 M NaCl is shown. From the curve is evident, that high concentration of Na^+ ions enhanced substantially the acidity of the last group of protons still bonded to the phytate ligand at pH above 8. As a consequence of the formation of a stable Na-Phy complexes (log $K_1^H = 8.69$)[25], a third well shaped equivalence point EP_3 on the titration curve appears at pH 10.6 indicating the final (12th) deprotonation step of phytate. Appearance of this third EP_3 finally clarifies the pathway of the titration route of phytic

acid by standardized solution of NaOH. Using the known amount of phytate (n_{Phy}) and adjustment of moles of NaOH to equivalents per mole of phytate *j* (n_{OH}/n_{Phy}), one can deduce that the EP_1, EP_2 and EP_3 correspond to 6th, 8th and 12th deprotonation step, respectively according to the reactions:

$$EP_1 \qquad H_{12}Phy + 6\,OH^- \leftrightarrow H_6Phy^{6-} + 6\,H_2O \qquad (5)$$

$$EP_2 \qquad H_6Phy^{6-} + 2\,OH^- \leftrightarrow H_4Phy^{8-} + 2\,H_2O \qquad (6)$$

$$EP_3 \qquad H_4Phy^{8-} + 4\,OH^- \leftrightarrow Phy^{12-} + 4\,H_2O \qquad (7)$$

Due to a strong affinity of Phy to sodium ions, the above mentioned species at high *I* and excess of Na^+ ions do not exist in its written forms in reality. For example H_4Phy^{8-} and Phy^{12-} most probably exists in forms of $Na_3H_4Phy^{5-}$ and Na_6Phy^{6-} species, respectively.[14,19]

To our knowledge only Bieth and Spiess[13] reported standardization of the amount (concentration) of phytate with the differential technique. They used the difference between two inflection points corresponding to the neutralization of 6 protons of phytic acid at molar concentration of $NaClO_4$. Although not explained into the details, they probably took into account the final deprotonation step (EP_3 in Fig. 4), which required an addition of an excess of alkali metal (≥ 1.0 M) into titration solution. Whilst other of numerous research groups dealing with the protonation and/or metal complexation equilibria of phytates[15,24–25,33–34] reported on an "alkalimetric/acidimetric" standardization of the ligand concentrations, or by elemental analysis[35] or simply used commercialy supplied chemicals without any proof of its purity.

Since the accuracy of a titrimetric standardization procedure is of vital importance for interaction and complexation studies of phytates with metallic ions, any un-

certainties in the determination of EPs should be minimized and every systematic error in the evaluation procedures of the analyte determination should be avoided. On the basis of titration shown in Fig. 4A, reactions (5) to (7), and discussion and explanations stated above, the differential approach should be used for an accurate and reliable determination of an unknown amount of phytate. Although first and third EPs shows much better transition than EP_2 (Fig. 4A), we found that the difference between EP_2 and EP_1 remains constant also in the presence of alkaline metallic ions, and by variation of phytate concentrations. Linear dependence between the amount of OH^- consumed between EP_1 and EP_2 ($\Delta n(EP_2 - EP_1)$) and n_{Phy} is shown in Figure 4B with the slope value of 1.98 ± 0.02 ($R^2 = 0.9997$). On the basis of this data one can again confirm, that 2 moles of OH^- per 1 mole of phytate are consumed between EP_1 and EP_2 according to the relationship:

$$\Delta n(EP_2 - EP_1) = 2\,n_{Phy}. \tag{8}$$

This relation enables a precise ($s_{rel.} < 1\%$, $N = 5$, n_{Phy} = 7.18 μmol) and reliable determination of low amounts of phytate, particularly in cases with difficulties with final EP_3 detection. For instance, titration in the electrolyte with low ionic strength (Fig 2, curve a) and/or titration with a bulky counter cation titrants, *e.g.* Bu_4NOH (Fig. 2, curve b), or the presence of other multivalent cations (see below).

On the basis of equation (8) an excess of a strong acid (*e.g.* HNO_3) added to the phytic acid solution is easily detected by an equivalent shift of EP_1 to higher values, but difference between EP_2 and EP_1 remains constant and is twice the amount of phytic acid in a mixture. Also a mixture of an equivalent amounts of $H_{12}Phy$ and $K_2H_{10}Phy$ can be analyzed, where first EP_1 reflects the sum of the amounts of six and four of the most acidic protons of $H_{12}Phy$ and $K_2H_{10}Phy$ according to the reaction (5), respectively, and the difference $EP_2 - EP_1$ then corresponds according to the reaction (6) to the transition of H_6Phy to H_4Phy of both phytate salts and is thus proportional to the total amount of phytates present in the mixture.

It is important to note that any significant deviation from above mentioned course of the titration (*e.g.* shift of EPs) indicate that analyzed phytic acid $H_{12}Phy$ is not pure, and may contain moisture, other phytate salts or impurities, and/or multivalent metallic cations. Declared purity of commercially available phytic acid and its salts ($K_2H_{10}Phy$, Na_6H_6Phy, $Na_{12}Phy$) is less than 95%. Due to natural origin of IP_6 and its hydrolysis it may contain lower inositol phosphates (IP_2–IP_5), which can affect the shape of the titration curve. As shown by Persson *et al.*[36] alkalimetric titration of IP_3, IP_4, IP_5, and IP_6 results in an appropriate shift of the first EP. Therefore if sample contains lower IPs a separation before the titration is required. Such impurities can be identified using HPLC[37] or ion chromatography.[38]
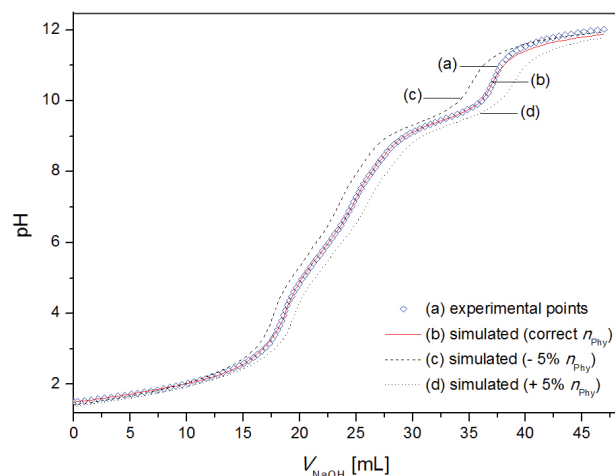

Figure 5. Titration of 0.3081 mmol $H_{12}Phy$ with 0.0996 M NaOH in 1.0 M NaCl. (a) Squares represent observed experimental points and lines represent simulated curves using (b) correct amount of phytate, (c) –5% and (d) +5% deviation in amount of total phytate. Protonation constants used for simulations (c) and (d) were same as calculated for the correct amount of phytate (curve b). Initial molar ratio between protons and phytate was set to 12.0 in all cases.

The importance of an accurate standardization of phytate ligands used in the complexation studies can be seen from the Figure 5 and Table 1, where an experimental titration curve was fitted by a computer. As shown, a relatively small error in phytate amount generates large differences in calculated protonation constants. Figure 5 curve (a), shows experimental points (labeled as squares) obtained by potentiometric titration of pure phytic acid $H_{12}Phy$ with NaOH in the presence of 1.0 M NaCl. Apparent macroscopic protonation constants of phytate were then calculated (Table 1) by fitting the potentiometric data (curve b) using the Hyperquad2013 program. If the titration curve were simulated by the same calculated constants and with 5% lower (curve c) and 5% higher amounts of phytate (curve d), a significant deviation from the experimental titration curve is observed (Fig. 5). Results listed in Tabele 1 reveal considerable differences between K_i^H values calculated when a small deviations from correct n_{Phy} are applied to simulation program. For instance, at fourth protonation step ($i = 4$) values of 7.25, 7.95 and 8.60 were obtained for log K_i^H at –5%, 0% and +5% deviation of n_{Phy} from the experimental true value, respectively.

Differences between logarithm values can be converted to factors of 5.04 and 4.47 on the linear scale, while already a 35-fold higher K_4^H was calculated when +10% error in n_{Phy} was applied to the refinement process. Evidently, a brief attention should be given to accurate phytate determination, particularly when dealing with protonation and/or complexation equilibria as stated also by Brigando *et al.*[12] Moreover, precision of the reported protonation and stability constants in the literature without a care-

Table 1. Apparent protonation constants with calculated standard deviations of phytate in 1.0 M NaCl at $T = 298$ K, calculated from experimental data shown in Fig. 5 (a) using different deviations from the actual amount of phytate present in titration cell and comparison with literature.

Error of n_{Phy}	−5%	0%[b]	+5%	+10%	Ref[32]
log K_1^{Ha}	9 ± 1	9.6 ± 0.2	11.0 ± 0.7	15 ± 1	8.69
log K_2^H	9.7 ± 0.9	9.3 ± 0.2	8.6 ± 0.7	10 ± 1	8.95
log K_3^H	8.6 ± 0.1	9.4 ± 0.1	10.2 ± 0.1	9.9 ± 0.3	8.56
log K_4^H	7.25 ± 0.07	7.95 ± 0.03	8.60 ± 0.08	9.5 ± 0.1	7.21
log K_5^H	5.79 ± 0.09	6.23 ± 0.03	6.89 ± 0.05	7.50 ± 0.09	5.65
log K_6^H	4.35 ± 0.07	4.96 ± 0.03	5.44 ± 0.05	5.82 ± 0.8	4.42
log K_7^H	1.92 ± 0.06	2.73 ± 0.07	3.48 ± 0.06	4.06 ± 0.08	2.22
log K_8^H	1.93 ± 0.09	1.92 ± 0.06	1.75 ± 0.08	2.1 ± 0.1	
log K_9^H	*< 1.5*[c]	*< 1.5*	*< 1.5*	*< 1.5*	
log K_{10}^H	*< 1.5*	*< 1.5*	*< 1.5*	*< 1.5*	
log K_{11}^H	*< 1.5*	*< 1.5*	*< 1.5*	*< 1.5*	
log K_{12}^H	*< 1.5*	*< 1.5*	*< 1.5*	*< 1.5*	

[a] K_i^H refers to the equilibrium reaction (1). [b] Values calculated from titration curve (a). [c] Estimated values are given in italics.

ful standardization of the ligand, should be addressed with precaution despite being determined with highly powerful computation programs and measured at well-established experimental conditions, *e.g.* temperature, ionic strengths, calibration of burettes and electrodes etc.

From the protonation constants given in Table 1 we can also see, that phytic acid actually contains three different categories of protons as presumed above. A first group of six the most acidic protons have log protonation constants less than 2.7 (log K_{12}^H to log K_7^H) two protons of an intermediate acidity with protonation constants log K_6^H = 4.96 and log K_5^H = 6.23, and the last group of four most strongly bonded protons have the log protonation constants between 7.95 (log K_4^H) and 9.6 (log K_1^H). Such differences in the acidity is a consequence of strong intramolecular hydrogen bonding between phosphate groups and pH dependent stabilization of two conformations.[12]

As we mentioned above, presence of the multivalent cations affect the shape of the titration curve of phytate with NaOH as shown on Fig. 6. It can be seen that addition of Ca^{2+} to phytic acid due to complexation and consequent substitution of protons increased its acidity in region between EP_1 and EP_2. Three EPs were detected from titration curve of phytic acid in the presence of various amounts of calcium (Fig. 6A). If molar ratio between M^{z+} (Ca^{2+} in this case) and phytate $m = n_M/n_{Phy}$ is increased from 0:1 (curve a) to 1:1 (curve b) and 2:1 (curve c), a significant shift of EP_2 is observed, while EP_1 and EP_3 remain constant. Also the course of the titration curve before the first and after the last equivalent point stays the same as in the absence of the metal. This indicate that equation (8) will not give the correct amount of phytate when solution contains Ca^{2+} (or other alkaline earth cations) as an impurities. In this case for titrimetric determination of phytic acid the difference between EP_3 and EP_1 should be used according to reactions (5) to (7):

$$\Delta n(EP_3 - EP_1) = 6 \, n_{Phy}. \qquad (9)$$

Figure 6B shows dependence of NaOH equivalents (j) consumed at different EPs on the equivalents of calcium (m) added into solution. A linear relation with a slope value of +0.59 is obtained for EP_2, indicating that over a half of mole of protons was released from phytate per one mole of added calcium around pH 7.5. Calcium ions like alkaline ions evidently compete for binding sites on phosphate group(s) with H^+ ions of low acidity. The latter is, however, not the case for the group of six most acidic protons, which are not subjected to the influence of calcium, as the titration curve remains unchanged up to the EP_1, where the 6th equivalent of H^+ is neutralized, as also reported by Martin and Evans[16] and Veiga *et al.*[31] After the final deprotonation step (EP_3) both titration curves recorded in the presence of Ca^{2+} (curves b, c) follow the same shape as the one obtained in the absence of Ca^{2+} and the amount of NaOH consumed to neutralize the 12th proton remains practically unchanged (slope value of EP_3 < −0.03, Fig. 6B). Similarly as in the case of Na^+ (Fig. 4A) an evident increase of derivative peak $\partial pH/\partial n_{OH^-}$ is observed at EP_3 when Ca^{2+} is present in solution, indicating a decrease of apparent protonation constants of phytate at low protonation degree ($i \le 4$) due to formation of stable coordination compounds.[17] Since EP_1 and EP_3 retain their respective positions upon addition of Ca^{2+}, the above described shift of EP_2 could be used as direct indication of contamination of phytic acid with divalent cation, *e.g.* Ca^{2+} or Mg^{2+}, which is often the case for naturally occurring phytate salts.[39]

Phytates interact strongly also with iron(III) ions and play an important role in biologically-relevant processes as described in numerous papers.[8,35,40–44] Complexation of iron(III) ions with phytate show different behavior than alkaline or alkaline earth cations. Three distinct EPs are detected on titration curves of phytic acid in the presence of various amounts of Fe^{3+} ions (Fig. 7A) and a shift of all three EPs is observed upon addition of Fe^{3+}. Presence of Fe^{3+} causes two additional complications as iron(III)
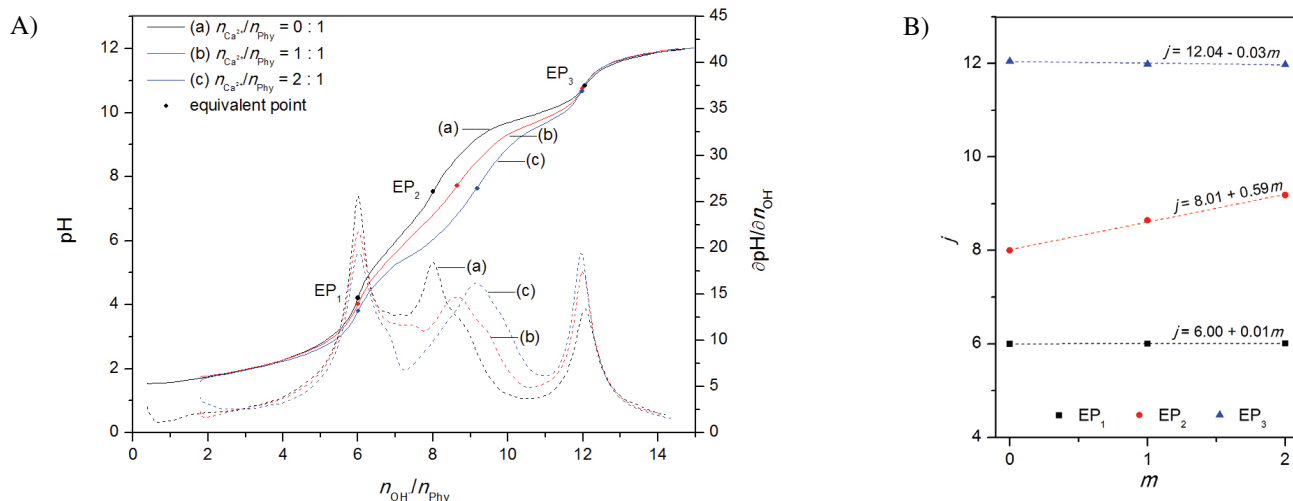
Figure 6. (A) Titration of 0.3104 mmol $H_{12}Phy$ (in the presence of 0.25 M NaCl) with 0.1032 M NaOH at various molar ratios $n_{Ca^{2+}}/n_{Phy}$: (a) 0:1, (b) 1:1, and (c) 2:1, accompanied with corresponding derivatives $\partial pH/\partial n_{OH^-}$. (B) Equivalents of OH^- per amount of phytate consumed at equivalent points from part A.

undergoes hydrolysis[35,45] and at pH above 9.5 precipitation of brown iron(III) hydroxides despite the presence of strong phytate ligand.[35,44–45] As reported by Šala *et. al.*[43] and Mali *et. al.*[46] a white-colored iron(III)-phytate precipitate is formed in acidic solution at molar ratios *m* (n_{Fe}/n_{Phy}) above 0.25. Due to the latter, EPs recorded for *m* > 0.25 (shown as grey marks in Figure 7B) were excluded from further calculations, hereinafter. Linear dependence of NaOH equivalents (*j*) consumed at each EPs on the molar ratio *m* (Fig. 7B) gives slope values of 1.67, 2.26, and 1.94 for EP_1, EP_2 and EP_3, respectively. Shift of EP_1 indicates that over 1.5 mole of H^+ is released per 1 mole of Fe^{3+} bound to phytate, which is close to results reported in the literature.[43] Fe^{3+} apparently competes with H^+ ions for binding sites on phytate and enhanced acidity of protons with intermediate character. The released protons behave

as a strong acid and are neutralized earlier, i.e. before EP_1 and the solution show properties of a mixture of strong and weak acids like in Fig. 3c. This is different than in the presence of Ca^{2+} (Fig. 6B), where only EP_2 was shifted upon addition of the metal, and is a clear indication of different binding sites for Fe^{3+} and Ca^{2+} on phytate ligand.

Greater shift of EP_2 (slope value of 2.26, Fig. 7B) can be again explained with pronounced acidity of certain protons and/or with more intensive Fe^{3+} hydrolysis side reaction, which requires additional consumption of NaOH. The final equivalent point (EP_3) corresponds to the neutralization of 12 protons and the consumption of NaOH at the EP_3 is increased for +1.94 mole per 1 mole of Fe^{3+}. This is a clear evidence of parallel metal hydrolysis, also visibly observed as brownish precipitate formed during the titration at pH above 9.5. Taking into account
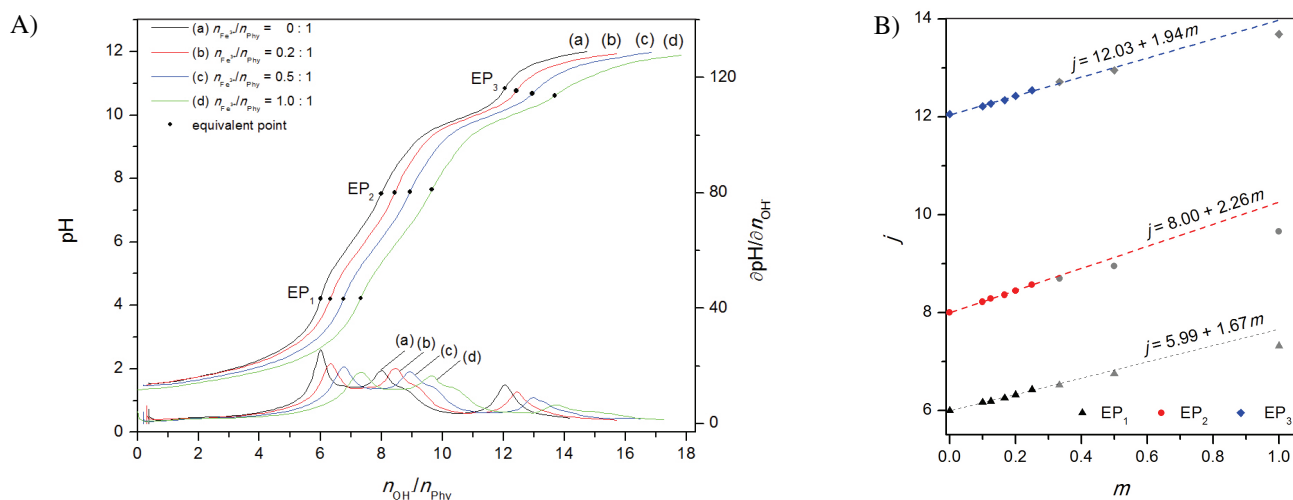

Figure 7. (A) Titration of 0.3104 mmol $H_{12}Phy$ (in the presence of 0.25 M NaCl) with 0.1008 M NaOH at various molar ratios $n_{Fe^{3+}}/n_{Phy}$ (a) 0:1, (b) 0.2:1, (c) 0.5:1, and (d) 1:1, accompanied with corresponding derivatives $\partial pH/\partial n_{OH^-}$. (B) Equivalents of OH^- per amount of phytate (*j*) versus molar ratios *m* ($m = n_{Fe}/n_{Phy}$) consumed at different EPs from part A.

theoretical (3 moles) and actual (1.94 mole) consumption of NaOH per 1 mole of added Fe^{3+} one can calculate that around 35% of Fe^{3+} is still present as soluble phytate complex at EP_3 despite relatively high pH (> 10.6). This is in accordance with high stability constants of iron(III)-phytate complexes reported in the literature.[35]

From the analytical point of view, contamination of phytic acid with Fe^{3+} can be distinguished from the one with Ca^{2+}, as the consumption of NaOH at each equivalent point, namely EP_1, EP_2 and EP_3 differs from theoretical 6, 8 and 12 equivalents, respectively. However, due to accompanying competitive reactions (metal hydrolysis, complexation, and complex precipitation), titrimetric standardization of phytic acid in the presence of Fe^{3+} is a challenging task, as neither of equations (8) and (9) can be applied. Therefore Fe^{3+} removal prior to performing titrimetric analysis is required.

4. Conclusions

The main aim of the present work was to evaluate phytic acid/phytate standardization with potentiometric titrations. Correct determination of phytate is of vital importance, as relatively small errors in determination of total phytate amount generate large differences in protonation constants calculated using computer simulation programs. A novel differential titration method was elaborated on the basis of equivalent point difference (EP_2–EP_1 and/or EP_3–EP_1) calculation. Presented method enables precise and reliable determination of low amounts of phytic acid down to μmol levels, particularly in the case of electrolytes with low ionic strength and/or when bulky counter cation titrants (*e.g.* Bu_4NOH) are used for potentiometric titrations. Moreover, contamination of phytic acid with multivalent cations, such as Ca^{2+} and Fe^{3+}, can be detected distinguishably, and in the case of alkaline earth ions correct determination of phytate is available as well. Different binding sites on phytate ligand were predicted for Ca^{2+} and Fe^{3+} complexation using the analysis of titration curves of phytic acid recorded in the absence and presence of both compared metals.

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6. References

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Povzetek

Pri določevanju ravnotežnih konstant protonacije in/ali konstant stabilnosti kovinskih kompleksov s fitati je ključnega pomena standardizacija, oziroma pravilna določitev množine (koncentracije) fitinske kisline. V tem delu je predstavljen nov pristop za precizno in zanesljivo določevanje fitinske kisline s potenciometrično titracijo, ki temelji na izračunu razlik med ekvivalentnimi točkami. Glede na kislost je dvanajst protonov fitinske kisline razvrščenih v tri glavne skupine, kar nam v odvisnosti od eksperimentalnih pogojev, tj. tipa in koncentracije proti-ionov, omogoča detekcijo dveh do treh izrazito razvidnih ekvivalentnih točk. Poleg pravilne določitve množine fitinske kisline je s pomočjo primerjave razlik med posameznimi ekvivalentnimi točkami možna tudi identifikacija morebitne kontaminacije in/ali napoved prvotne stopnje protonacije fitata. Vpliv negotovosti v poznavanju množine fitinske kisline na izračun konstant protonacije smo ovrednotili s pomočjo simulacijskega računalniškega programa Hyperquad2013. S podrobnejšo analizo titracijskih krivulj, posnetih v prisotnosti Ca^{2+} in Fe^{3+} ionov, smo napovedali, da se Ca^{2+} veže na drugo vezavno mesto na fitatnem ligandu kot Fe^{3+}.