Fluorimetric determination of phytic acid based on the activation of the oxidation of 2,2′-dipyridyl ketone hydrazone catalysed by Cu(II)

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Phytic acid exerts an activation effect on the oxidation of 2,2′-dipyridyl ketone hydrazone catalysed by Cu(II) ion and the oxidation product is highly fluorescent. A fixed time method for the fluorimetric determination of phytic acid based on this effect is described. The calibration graph is linear over the range 0.05–0.6 mg l^{-1} phytic acid, resulting in a limit of detection of 0.03 mg l^{-1} phytic acid. The relative standard deviation is in the range 1.4–1.8%, depending on the sample analysed. The method was successfully applied to the determination of phytic acid in human urine (20 samples) and food samples (nine different products). The results obtained for urine samples ranged from 0.31 to 3.6 mg l^{-1} phytic acid and for food samples from 3.8 to 22 mg g^{-1} phytic acid. This is the first procedure to be reported for the determination of phytic acid based on fluorimetric measurements.

Introduction

Phytic acid (inositolhexaphosphoric acid) is a natural plant constituent that is found in legumes, cereals, oil seeds, pollens and nuts.[1] In recent years, a number of benefits to human health have been attributed to this compound, *e.g.*, its preventive and therapeutic action as an anticancer agent.[2] Moreover, *in vitro* and *in vivo* studies demonstrated that phytate at mg l^{-1} concentrations is a powerful inhibitor of calcium oxalate and calcium phosphate crystallization.[3–5] Further, phytic acid has been proved to act as a preventive agent against renal calculi formation in the human urinary tract.[6]

Other studies attribute an anti-nutritional role to phytic acid because it combines with metal ions (Ca, Mg, Fe, Zn, Cu, Co) in the intestinal tract, inhibiting their absorption.[7] Some experiments have indicated that such an anti-nutritional role in humans is not significant in ordinary diets. At present, the predominant scientific opinion tends to recommend the presence of phytic acid in human diet. In fact, various dietary complements containing phytic acid have been commercialized. Hence there is interest in analysing clinical and food samples for phytic acid.

The procedures for phytic acid determination are basically limited to refractive index HPLC procedures[8] owing to its poor spectrophotometric properties. Other methods are based on the quantitative hydrolysis of phytic acid and the determination of inositol[9] or phosphate.[10] A new alternative for the determination of phytic acid, based on the use of a replacement reaction in which a metal ion complex is dissociated proportionally to the amount of phytic acid, has recently been proposed, using zinc chloranilate.[11] The sensitivity of such procedures is limited by the stoichiometric relation between the metal ion and phytic acid.

The use of replacement reactions has also been applied to the determination of lower phosphate esters of inositol, *e.g.*, a fluorimetric detection system for inositol 1,2,6-triphosphate based on the dissociation of the fluorescent Fe(III)–methylcalcein blue complex has been published.[12]

The reported methods have permitted the study of the phytic acid content in food samples. Nevertheless, in general, better analytical characteristics (mainly sensitivity) are required for the analysis of biological samples.

The aim of this work was to develop a new analytical approach for phytic acid determination based on its action on a metal ion catalysed reaction. The reaction selected to carry out the study was the oxidative transformation of 2,2′-dipyridyl ketone hydrazone to a fluorescent product, catalysed by Cu(II) ion.[13] This is the first proposed procedure for the determination of phytic acid based on fluorimetric measurements.

Experimental

Apparatus

The fluorescence measurements were performed with a Perkin-Elmer (Norwalk, CT, USA) Model LS-5 spectrofluorimeter with a 10 mm quartz cell. Lyophilization was performed using a Telstar lyophilizer.

Chemicals and solutions

All chemicals were of analytical-reagent grade. Granular activated carbon (100 mesh) was purchased from Panreac (Barcelona, Spain), the anion exchange resin was AG 1-X8 (200–400 mesh) from Bio-Rad, Laboratories (Richmond, CA, USA) and myo-inositolhexaphosphoric acid dodecasodium salt from corn from Sigma (St. Louis, MO, USA). 2,2′-Dipyridyl ketone hydrazone (DPKH) was synthesized by the reaction of di-2-pyridyl ketone with hydrazine monohydrate as described in the literature.[14] A stock standard solution containing 10^{-3} M KCl and of pH 6.6, obtained by addition of HCl, was prepared. This solution was used to prepare the other solutions used in this study, namely 3.9×10^{-5} M $Cu(NO_3)_2$ and 1.5×10^{-4} M DPKH.

Determination of phytic acid

A 0.4 ml volume of 3.9×10^{-5} M Cu(II) solution and 0.4 ml of 1.5×10^{-4} M DPKH solution were added to a 3.2 ml aliquot containing from 0.2 to 2.4 μg of phytic acid in 10^{-3} M KCl (pH 6.6). The solution was left for 60 min at room temperature

(22–25 °C), then 0.5 ml of 3.6 M HCl was added to stop the reaction. In order to avoid quenching effects, 0.5 ml of this solution was diluted with 4.5 ml of 0.4 M HCl. The fluorescense intensity was measured (λ_{ex} = 349 nm, λ_{em} = 435 nm, spectral bandwidth = 1 nm). A graph of the increment of fluorescence due to the presence of phytic acid *versus* the logarithm of the phytic acid concentration was used as a calibration graph.

Determination of phytic acid in urine

After clean-up of the sample by passing it through a 20 mm id syringe containing 2 g of activated carbon, 1 ml of the eluted sample was then passed through an 8 mm id syringe containing 1.5 g of the anion-exchange resin, previously equilibrated with 0.05 M hydrochloric acid. The resin was washed with 50 ml of 0.05 M HCl at 0.4 ml min^{-1}. The retained phytate was eluted with 5 ml of 2 M HCl at 0.25 ml min^{-1}. This solution was then neutralized with NaOH and a new purification process with the anion-exchange resin, as described, was carried out in order to ensure complete separation from phosphate. Then the 2 M HCl solution containing phytic acid was lyophilized, and reconstituted with 5 ml of HCl–KCl stock standard solution (pH 6.6, 10^{-3} M KCl). The pH was checked (when it differed from 6.6, it was adjusted with potassium hydroxide). An aliquot of this solution was taken to carry out the analysis for phytic acid.

Determination of phytic acid in food samples

A 0.5–0.25 g amount of the sample was ground and extracted with 50 ml of 0.5 M HCl for 3 h at room temperature, according to recommended procedures.[15] The suspension was centrifuged and an aliquot of the solution obtained after centrifugation was adjusted to pH 3–4 with NaOH, then was purified by means of anionic-exchange treatment as described for urine analysis (owing to the low content of phosphate in such samples, just one purification process was necessary). The 2 M HCl eluate containing phytic acid was then lyophilized and treated as described.

Results and discussion

The oxidative reaction of DPKH with oxygen to form a fluorescent product (λ_{ex} = 349 nm, λ_{em} = 435 nm) is catalysed by the presence of trace amounts of Cu(II). Such an oxidative reaction is stopped in acidic media. Moreover, it was found that phytic acid exerts an activation effect on such a catalysed reaction. On the basis that a stable, soluble Cu(II)–phytate complex has been reported,[16] a Cu(II)–phytate complex, with a more active catalytic action, is assumed to be the cause of this effect. Hence, the increment in the reaction rate, measured by the fluorescence intensity increment, is related to the phytic acid content. Experimental studies of the variables affecting the rate of the fluorescent product formation are described below.

Study of the variables affecting the reaction rate

The effects of the pH and ionic strength on the reaction rate are shown in Fig. 1. For better reproducibility and good sensitivity a pH of 6.6 was selected as optimum. In basic media, probably owing to the formation of other complex bonds of Cu(II) with the hydroxy group, the fluorescence emission corresponding to the blank increased and the effect of phytic acid became less noticeable. The effect of the ionic strength was studied using KCl. As can be seen, on increasing the KCl concentration the analytical signal decreases. For this reason, to analyse purified real samples obtained in 2 M HCl (see procedure), the excess of HCl was eliminated by lyophilization instead of neutralization. It was demonstrated that such a lyophylization process permitted a reproducible ionic strength of samples to be obtained and, as mentioned, higher sensitivity (Table 1). Other species (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, BO_2^-, HBO_2, NH_3, NH_4^+) were studied (Table 1). As can be seen, the presence of the studied species decreased the analytical signal considerably, and therefore the use of pH buffers would lead to lower sensitivity and hence is not recommended.

The effects of Cu(II) ion and DPKH concentrations on the reaction rate are shown in Fig. 2. A maximum fluorescence emission increment was obtained at a 5.5 × 10^{-6} M Cu(II) concentration. Nevertheless, as the fluorescence corresponding to the blank increases with increase in Cu(II) concentration, better reproducibility was obtained at lower Cu(II) concentrations and 3.9 × 10^{-6} M Cu(II) was adopted. With regard to DPKH concentration, maximum sensitivity was obtained at 1.5 × 10^{-4} M and this DPKH concentration was adopted. The

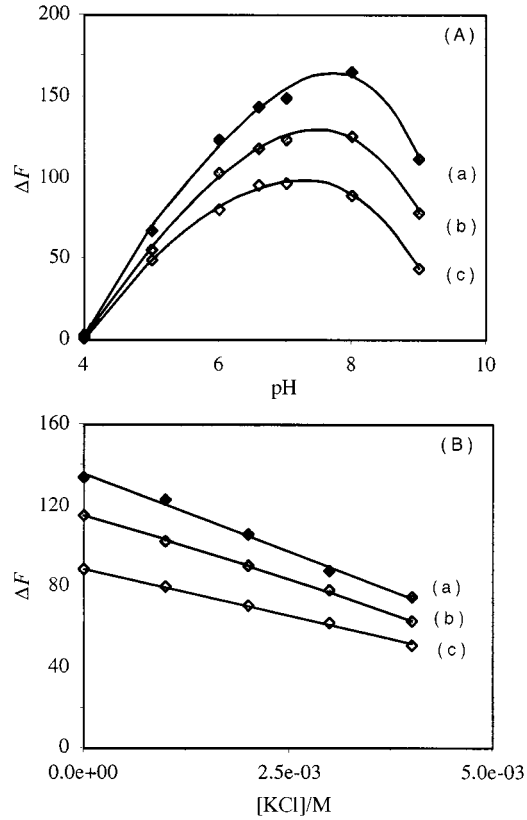

Fig. 1 Influence of (A) pH and (B) KCl concentration on the increment of fluorescence intensity for three phytic acid concentrations: (a) 2.0, (b) 1.0 and (c) 0.5 mg l^{-1}. Common conditions: [Cu(II)] = 1.6 × 10^{-7} M, [DPKH] = 3.8 × 10^{-5} M, time = 60 min, [HCl] to stop the reaction = 0.4 M. Dilution factor of the reaction mixture prior to fluorescence measurements: 1:46. Instrumental conditions: λ_{ex} = 349 nm, λ_{em} = 415 nm. (A) [KCl] = 1.0 × 10^{-3} M; (B) pH 6.0

Table 1 Effect of different species on the fluorescence increment, corresponding to 4 mg l^{-1} phytic acid

Species	Concentration/M	pH	ΔF
Na_2HPO_4–Na_3PO_4	1 × 10^{-2}	11.0	5.0
NaH_2PO_4–Na_2HPO_4	1 × 10^{-2}	6.0	4.2
NaH_2PO_4–Na_2HPO_4	1 × 10^{-3}	6.0	7.8
HBO_2–$NaBO_2$	1 × 10^{-2}	9.0	11.3
NaOH	1 × 10^{-2}	12.0	14.8
NH_4Cl–NH_3	1 × 10^{-2}	9.5	3.7
KCl	1 × 10^{-3}	6.6	80.1[a]

[a] 0.5 mg l^{-1} phytic acid.

decreases observed at higher concentrations were due to quenching effects.

The sensitivity of the fixed time determination proposed in this paper is directly related to the time of reaction owing to the slowness of the oxidative reaction. Depending on the concentration of the sample to be analysed, a convenient reaction time could be chosen. A 60 min reaction time was selected to carry out this study. When the determination was performed with a 30 min reaction time, the slope of the calibration graph diminished to 61% of that obtained at 60 min (see analytical characteristics). A 100 min reaction time led to a slope increase of 10% (referred to 60 min). Reaction times longer than 100 min led to a slight increment of the slope of the calibration graph.

The analytical signal diminished slightly (fluorescence increment due to 0.5 mg l^{-1} phytic acid from 91.2 to 76.9) on increasing the temperature (from 12 to 37 °C). Room temperature (22–25 °C) was adopted.

Fig. 3 shows the evolution of the analytical signal with phytic acid concentration. The shape of the curve agrees with the assumption that the formation of a stoichiometric Cu(II)-phytate complex is responsible for the fluorescence increment. In order to obtain a calibration graph with a higher linear range, ΔF *versus* log (phytic acid concentration) is recommended for analytical purposes.

Analytical characteristics

With the recommended procedure, a linear function (ΔF = 122.0log[phytic acid] + 188.0, r^2 = 0.994) was obtained over the phytic acid concentration range 0.05–0.6 mg l^{-1}. The limit of detection was 0.03 mg l^{-1}. Such concentrations are referred to the volume where the analytical reaction took place. The relative standard deviation (RSD) for a phytic acid concentration of 0.5 mg l^{-1} was 1.4% (five replicates).

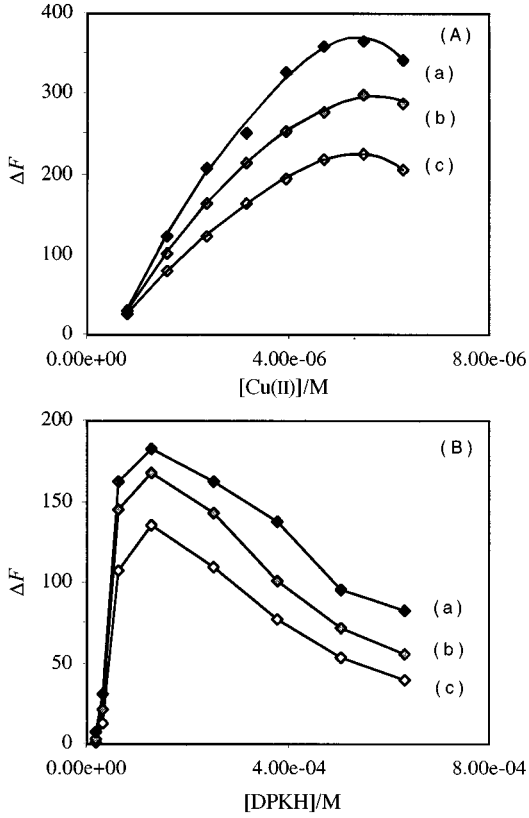

Fig. 2 Influence of (A) Cu(II) concentration and (B) DPKH concentration on the increment of fluorescence intensity for three phytic acid concentrations: (a) 2.0, (b) 1.0 and (c) 0.5 mg l^{-1}. Common conditions: [KCl] = 1.0 × 10^{-3} M, pH 6.0, time = 60 min, [HCl] to stop the reaction = 0.4 M. Dilution factor of the reaction mixture previous to fluorescence measurements: 1 : 46. Instrumental conditions: λ_{ex} = 349 nm, λ_{em} = 415 nm. (A) [DPKH] = 3.8 × 10^{-5} M; (B) [Cu(II)] = 1.6 × 10^{-7} M.

Determination of phytic acid in urine

Twenty urine samples were analysed using the proposed procedure. An indirect method based on the quantitative hydrolysis of phytic acid to phosphate and its photometric determination after liquid–liquid extraction[10] was also used to compare results (Table 2). A regression of results obtained with the fluorimetric procedure *versus* results obtained with the extraction–photometric method led to a linear graph comparable to the theoretical line with slope = 1 and intercept = 0 at the 95% confidence level.

In order to provide more evidence on the applicability of the proposed procedure to determine phytic acid in urine samples, five spiked samples were analysed. The natural phytic acid concentration in such samples ranged from 1.0 to 3.5 mg l^{-1}. The increment in phytic acid concentration due to the addition of a standard was of 0.3 mg l^{-1}. A graph of phytic acid found in natural samples *versus* phytic acid found in spiked samples minus added phytic acid was linear (slope = 1.03, intercept = −0.07, r^2 = 0.998). Such a function is comparable to the

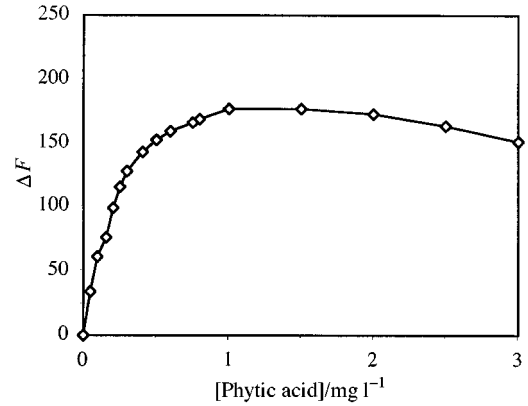

Fig. 3 Influence of phytic acid concentration on the analytical signal (ΔF). The recommended procedure was followed.

Table 2 Results obtained for the determination of phytic acid in urine by the proposed fluorimetric method and by an extraction–photometric method.[10] The results are averages of three determinations ± standard deviation for $n - 1$ degrees of freedom

	Phytic acid found/mg l^{-1}	
Sample No.	Fluorimetric method	Photometric method
1	1.87 ± 0.04	1.98 ± 0.03
2	2.20 ± 0.05	2.31 ± 0.02
3	3.15 ± 0.07	3.29 ± 0.04
4	3.42 ± 0.05	3.56 ± 0.04
5	1.10 ± 0.03	1.23 ± 0.02
6	1.41 ± 0.02	1.57 ± 0.03
7	1.84 ± 0.05	2.05 ± 0.03
8	0.68 ± 0.02	0.66 ± 0.02
9	0.27 ± 0.02	0.31 ± 0.03
10	2.80 ± 0.08	2.82 ± 0.05
11	0.14 ± 0.03	0.13 ± 0.03
12	3.20 ± 0.09	3.15 ± 0.06
13	2.70 ± 0.05	2.72 ± 0.03
14	2.43 ± 0.04	2.31 ± 0.03
15	1.78 ± 0.05	1.92 ± 0.03
16	1.53 ± 0.04	1.56 ± 0.03
17	0.84 ± 0.03	0.94 ± 0.02
18	2.21 ± 0.06	2.27 ± 0.03
19	3.10 ± 0.10	3.17 ± 0.04
20	2.34 ± 0.06	2.36 ± 0.03

theoretical one (slope = 1, intercept = 0), implying a 100% recovery at the 95% confidence level. The reproducibility of the determination of phytic acid in urine, including purification and analysis, was calculated from eight replicates of a sample containing, according to the proposed procedure, 2.09 mg l^{-1}. An RSD of 1.75% was obtained.

Determination of phytic acid in food samples

Table 3 gives the results for the determination of phytic acid in food samples. They were also analysed by using the indirect photometric method mentioned earlier. The application of the same statistical test as indicated for urine demonstrated that both methods are comparable at the 95% confidence level. The reproducibility of the determination (extraction, purification and analysis) in food samples was evaluated from results corresponding to one sample. The RSD for five replicates was 1.6%.

Table 3 Results obtained for the determination of phytic acid in food by the proposed fluorimetric method and by an extraction–photometric method.[10] The results are averages of three determinations ± standard deviation for $n - 1$ degrees of freedom

	Phytic acid found/mg l^{-1}	
Sample	Fluorimetric method	Photometric method
Whole grain oatmeal	4.8 ± 0.2	4.7 ± 0.1
Wheat bran	3.8 ± 0.1	3.6 ± 0.l
Carob seed germ	20.2 ± 0.5	19.6 ± 0.3
Carob seed germ, purified	9.6 ± 0.3	9.7 ± 0.1
Whole grain wheat bran	6.6 ± 0.2	6.7 ± 0.1
Wheat germ flour	22.3 ± 0.5	22.5 ± 0.4
Rat chow	8.1 ± 0.2	8.2 ± 0.1
Grape	2.1 ± 0.1	2.1 ± 0.1
Almond	21.1 ± 0.4	21.2 ± 0.3

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