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ORIGINAL PAPER

Synchronous fluorescence analysis of phytate in food

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Abstract A novel synchronous fluorescence method is described for determination of phytic acid in food samples. It is based on the formation of a ternary complex between phytate, 1,10-phenanthroline (phen) and Fe^{3+}. The synchronous fluorescence intensity of the solution was accordingly enhanced proportionally to the increased phytate concentration. Synchronous luminescence spectroscopy was adopted in the study, and the $\Delta\lambda$ was set to 40 nm. The calibration graph is linear from 0.33 to 32 mg L^{-1} with a linear equation of I_f=8.770+2.980c (R^2>0.9994). The method was applied to determine phytate in food samples and the found concentrations of phytic acid in food were in the range of 4.62–24.08 mg g^{-1} with recoveries of 92.2%–98.3%. The control experiments were performed using UV-spectrophotometry method, and the results showed the method to be reliable.

Keywords Phytate · Synchronous fluorescence · Complex reaction · 1, 10-Phenanthroline

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Introduction

Phytate, *myo*-inositol hexaphosphoric acid ($InsP_6$), a fully phosphorylated form of inositol [1], is a naturally occurring component and the principal storage source of phosphorus in many plants, such as cereals, soybeans, legumes, oil seeds, pollens, nuts, fruits and vegetables [2–4]. In $InsP_6$ molecule, there are 12 replaceable protons, among which six are strongly (pK_a<3.5) and six are weakly (pK_a=4.6–10) dissociated [5]. $InsP_6$, including its deprotonated species, possesses the high affinity for chelating metal ions such as Fe^{2+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+}, as well as proteins and starch [6, 7]. $InsP_6$ binds with the essential elements in human body, forming complexes with low solubility at physiological pH [8, 9], and thus potentially influences their bioavailability [10]. Hence, $InsP_6$ has long been considered solely as an antinutrient and related studies attracted a great deal of interest in human nutrition [11]. However, a variety of epidemiological and studies on living organisms demonstrated that $InsP_6$ and its lower phosphorylated forms have beneficial functions. For example, it could reduce the risk of cancers [12–14], heart disease, diabetes, and renal calculi [15, 16]. In addition, $InsP_6$ can also bind potentially toxic mineral elements such as Cd^{2+} and Pb^{2+} in human body, and thus influences their toxicity and facilitates their excretion [17].

Subsequently, determination of phytic acid level in dietary products becomes a very important task. Some methods can be used to determine phytic acid concentration in cereal products, biological and urine samples. The analysis of $InsP_6$ in various matrices was usually performed by means of chromatography [18–20] and capillary isotachophoresis [21]. Among them, ion pair chromatography [22] and high-performance ion chromatography [23] are the commonly used methods, in which most are capable of simultaneous

separation and determination of $InsP_6$ and its lower phosphorylated species. Other methods are based on the direct/indirect determination of phosphorus [24] or inositol [25] in $InsP_6$ or its quantitative hydrolytic products by spectrophotometry [26], NMR spectroscopy [27] and inductively coupled plasma atomic emission spectrometry [28]. Moreover, a fluorimetric measurement procedure for $InsP_6$ in human urine and food samples were developed [29, 30]. However, it has no report that determination of $InsP_6$ by synchronous fluorescence spectroscopy method according to our knowledge.

1,10-Phenanthroline (phen), an N-donor ligand with aromatic rings, is known to form the protonated species in the range of pH=2–7, i.e., $H(phen)^+$ and $H(phen)^{2+}$. For $H(phen)^{2+}$, two 1,10-phenanthroline molecules are stacked through their aromatic rings, and it is known that the ligand favorably forms intercalated complexes [31]. Because of their inherent pocket structure, $[M(phen)_3]^{n+}$ complexes have the ability to form outer sphere complexes with anions, neutral molecules, or combination of them [32]. It was found that the synchronous fluorescence of phen will be decrease when Fe^{3+} was added, and the characteristic wavelength with red-movement. Moreover, the synchronous fluorescence increased by addition of $InsP_6$ subsequently, and the characteristic wavelength was also changed. In this procedure, the synchronous fluorescence is constant wavelength synchronous luminescence. By this mean, a novel procedure for the determination of $InsP_6$ in food was developed in this paper. The synchronous fluorescence method has high precision and accuracy, simple, rapid, and it can eliminate the Rayleigh interference effectively. In the proposed procedure, the sensitivity is excellent, but the selectivity can be better. Although this procedure has some disadvantage, we hope it can offer some new thought for the quantification of phytic acid.

Experimental

Apparatus

The fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer equipped with a thermostated compartment using 1.0 cm quartz cuvette. The pH measurements were carried out on a PHS-3C Exact Digital pH meter equipped with Phonix Ag–AgCl reference electrode (Cole-Parmer Instrument Co.), which was calibrated with standard pH buffers. The control experiments were carried out on a SHIMADZU UV-2450 UV–visible spectrophotometer. A CN column (25 cm×4.6 mm i.d.) with 5 μm cyanopropylsilica packing material (Spherisorb) was used in the separation.

Reagents

Sodium $InsP_6$ was purchased from Sigma. 1,10-Phenanthroline (phen) was obtained commercially from Sigma (www.sigmaaldrich.com). A 1,10-Phenanthroline (phen; 0.01 mol L^{-1}) ethanol–water solution was prepared. An aqueous standard solution of sodium $InsP_6$ (0.1000 mol L^{-1}) was prepared and stored in refrigerator prior to use. $FeCl_3$ solution (0.01 mol L^{-1}) was prepared to quench the synchronous fluorescence of phen. Acetate buffer (pH 6.0) as used to maintain the solution pH value throughout the experimental procedure. The cations examined in the disturbance experiments were all chloride salts and the anions were all sodium compounds. $FeCl_3 \cdot 6H_2O$ and 5-sulfosalicylic acid was obtained commercially and used for determination of $InsP_6$ in food by UV-spectrophotometry method. All reagents and solvents were of analytical grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled and deionized water. The AG1-X8 anion-exchange resin (chloride form, 200–400 mesh) was purchased from Sinopharm Chemical Reagent Co., Ltd (www.sinoreagent.com).

Fluorimetric detection

A solution of 3.00 mL 1,10-Phenanthroline (phen) (0.001 mol L^{-1}) containing acetate buffer (pH 6.0, 15 mmol L^{-1}) was transferred to a quartz cell, and then an appropriate aliquot of Fe^{3+} solution (0.01 mol L^{-1}) was added. The resultant mixture was allowed to equilibrate for 3 min at ambient temperature and scanned on the fluorophotometer in the range of 320–420 nm with the $\Delta\lambda$=40.0 nm. For constant wavelength synchronous luminescence was adopted, $\Delta\lambda$ is wavelength space between the excitation wavelength and the emission wavelength. The spectral bandwidths of excitation and emission slits were both 5.0 nm. The synchronous fluorescence of the solution was detected. The variation of synchronous fluorescence of phen against the Fe^{3+} concentration could be observed.

Some 10 mL volumetric flasks were prepared. 1 mL phen solution (0.01 mol L^{-1}) and 1 mL acetate buffer (pH 6.0) was transferred to every volumetric flask, and 0.3 mL $FeCl_3$ solution was added to every flask. Then 5 μL to 400 μL sodium $InsP_6$ standard solution (0.1000 mol L^{-1}) was added to the flasks respectively. After 10 min, the synchronous fluorescence of the solution was recorded and the fluorescent intensity was measured.

Sample treatment

The treatment of food samples was carried out following the literature described procedure with minor modifications as follows [29]. A 0.5–0.25 g amount of the sample was ground

and extracted with 50 ml of 0.5 mol L^{-1} HCl for 3 h at room temperature, according to recommended procedures [33]. The suspension was centrifuged and an aliquot of the solution obtained after centrifugation, and then was purified by means of anionic-exchange treatment. The resin was washed with 50 mL of 0.05 mol L^{-1} HCl at 0.4 mL min^{-1}. The retained phytate was eluted with 5 mL of 2 mol L^{-1} HCl at 0.25 mL min^{-1}. The collected solution was neutralized with 2 mol L^{-1} NaOH and purified via the same process to separate phosphate completely. Then the 2 mol L^{-1} HCl solution containing $InsP_6$ was lyophilized and reconstituted with 5 mL of HCl–NaCl solution (pH 6.6, 10^{-3} mol L^{-1} NaCl). An aliquot of this solution was taken to analyze the content of $InsP_6$.

Results and discussion

Fluorimetric detection

A solution of 1,10-Phenanthroline (phen; 0.001 mol L^{-1}) contain 15 mmol L^{-1} acetate buffer (pH 6.0) show strong synchronous fluorescence with characteristic wavelength at 362.0 nm, when the $\Delta\lambda$ is 40.0 nm. Upon addition of Fe^{3+} (0.01 mol L^{-1}) to the solution, the fluorescence intensity decreased gradually with a little red shift in the characteristic wavelength to 364.0 nm. As the Fig. 1 show, when the concentration of the added Fe^{3+} achieved a certain degree, the fluorescence intensity reached the minimum. Moreover, the peak at 362.0 nm has disappeared, and only a weak peak at 382.0 nm, as shown in the plot of the Fig. 1. It can be calculated the molar ratio of phen to Fe^{3+} is 1:3. So the following experiments all adopted this ratio.

Then, the synchronous fluorescence of the different concentration $InsP_6$ and phen (0.001 mol $L^{-1}$and Fe^{3+} (0.003 mol L^{-1}) mixture solution was tested. We can see from the Fig. 2, at 364.0 nm, a peak was arisen. Furthermore, the fluorescence intensity continually increased with the augment of $InsP_6$ concentration. However, the fluorescence intensity could not recover to the original level, and the characteristic wavelength is 364.0 nm not 362.0 nm. For 1,10-Phenanthroline has the inherent pocket structure, phen metal complexes have the ability to form outer sphere complexes with anions, neutral molecules, or combination of them [34]. Consequently, it could be deduced that $InsP_6$, phen and Fe^{3+} formatted a ternary complex. It made the synchronous fluorescence intensity of the solution increasing.

It was revealed that the formation of $InsP_6$-metal complexes depends on conditions such as pH and the $InsP_6$-to-metal molar ratio [35, 36]. Many results demonstrated that $InsP_6$ can be deprotonated in aqueous solution, giving rise to different anionic species over a wide pH range [37]. And when the pH is about 6.0, it has a good ability of $InsP_6$ binding Fe^{3+}, and the $InsP_6$–Fe^{3+} complex is more onefold [8], so the pH 6.0 was adopted in this study.

Calibration and linearity

As stated in above, the fluorescence of 1,10-Phenanthroline will be quenched with the addition of Fe^{3+}. However, as it shown in Fig. 2, with the concentration of $InsP_6$ increasing, the fluorescence intensity at 364.0 nm was enhanced continuously. And the fluorescence intensity enhancement was proportional with the increasing $InsP_6$ concentration under the stated conditions. Plotting of the fluorescent

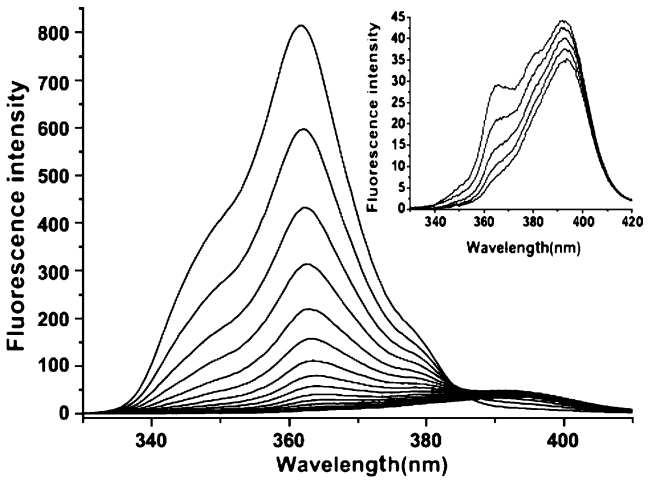

Fig. 1 The synchronous fluorescence of phen was quenched by Fe^{3+}. The *inset* is curves of the last five additions of Fe^{3+}. Phen: 0.001 mol L^{-1}, Fe^{3+}: 0.01 mol L^{-1}, acetate buffer (pH 6.0)

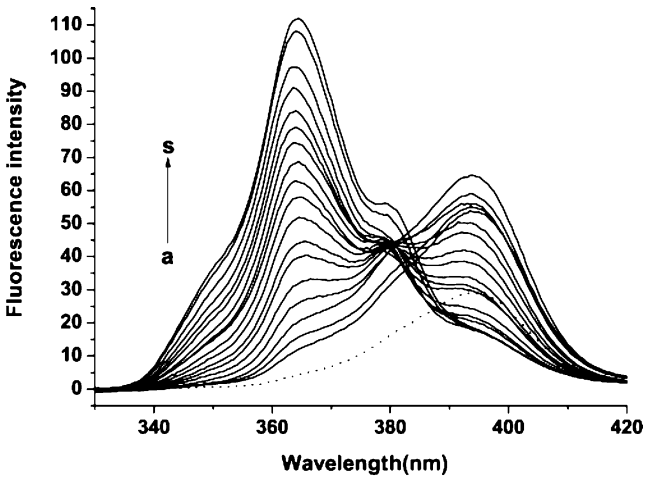

Fig. 2 The variation of the fluorescence intensity of different $InsP_6$ concentration. The *dashed line a* is the sample no $InsP_6$ added, b~s: 0.33 mg ~32.34 mg $InsP_6$. phen: 0.001 mol L^{-1}, Fe^{3+}: 0.003 mol L^{-1}, acetate buffer (pH 6.0)

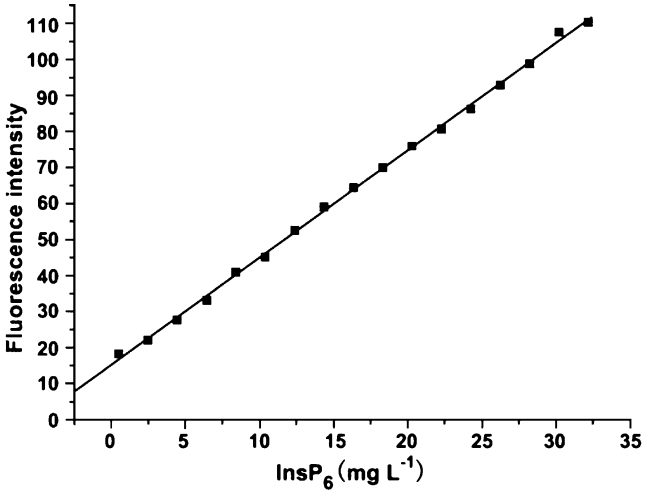

Fig. 3 The calibration curve. The fluorescence intensity is at 364.0 nm. phen: 0.001 mol L^{-1}, Fe^{3+}: 0.003 mol L^{-1}, acetate buffer (pH 6.0)

intensity versus the $InsP_6$ concentration gave rise to a calibration curve that could use to quantitative analysis for the linearity (Fig. 3). The linearity range is 0.33~32.34 mg and with the linear equation of I_f=8.770+2.980c (R^2> 0.9994; I_f represents the fluorescent intensity of the solution at 364.0 nm and c represents the concentration of the $InsP_6$ expressed as mg L^{-1}). Above these concentrations the calibration of $InsP_6$ is not linear, and the probably reason is that $InsP_6$, phen and Fe^{3+} formatted a ternary complex not replacement reaction.

Variables affecting the fluorescence

The variables that affect the synchronous fluorescence of the experiment such as Δλ and foreign ions, were investigated. In this experiment, constant wavelength synchronous luminescence was adopted, the value of Δλ was considered. Generally, the Stokes shift is the best choice. Consequently, the excitation spectrum and emission spectrum were detected, and the Stokes shift is 40 nm. In addition, the synchronous fluorescence was measured with Δλ equal 10.0 nm, 20.0 nm, 30.0 nm, 40.0 nm, 50.0 nm, 60.0 nm, 70.0 nm, 80.0 nm, 90.0 nm respectively, and it was found that 40.0 nm was the most appropriate result. So the Δλ was set 40.0 nm in the study.

The effect of foreign ions and typical chelating agent EDTA on the fluorescence of phen–Fe^{3+}–$InsP_6$ were investigated. The examined foreign ions include K^+, Ba^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Al^{3+}, CO_3^{2-}, PO_4^{3-}, SO_4^{2-}, $P_2O_7^{4-}$, and EDTA. The final concentration of each cation solution was ten times that of the previously added Fe^{3+}, and each anion solution was ten times excess of the added $InsP_6$. As shown in Table 1, among the cation ions, Ba^{2+}, Cu^{2+}, Zn^{2+} can weaken the fluorescence of the phen–Fe^{3+}–$InsP_6$ solution

Table 1 Effect of foreign species on the fluorescence of phen–$Fe3^+$–$InsP_6$

Ion	ΔI^a(%)
K^+	0.04
Na^+	1.05
Ca^{2+}	5.33
Mg^{2+}	16.11
Fe^{2+}	−8.02
Cu^{2+}	−10.93
Zn^{2+}	−7.91
Ba^{2+}	−90.99
Al^{3+}	55.60
CO_3^{2-}	−6.60
SO_4^{2-}	1.71
PO_4^{3-}	30.86
$P_2O_7^-$	11.21
EDTA	−10.52

[a] $\Delta I(\%)=(I(\text{phen–Fe3}^+\text{–InsP}_6+\text{foreign ion})-I(\text{phen–Fe3}^+\text{–InsP}_6))/I(\text{phen–Fe3}^+\text{–InsP}_6)\times 100$, I represents the fluorescent intensity at 362.0 nm.

significant, whereas the Al^{3+} and Mg^{2+} can reversely strengthen the fluorescence. The possible reason is that Ba^{2+}, $Cu^{2+}$and Zn^{2+} have a strong ability combining 1,10-Phenanthroline, and the Al^{3+} and Mg^{2+} had a intense hydrolysis reaction with great influence to the complex reaction. All other cations had little influence on the fluorescence of the system. On the other hand, among the anion ions, only PO_4^{3-} has remarkable influence on the fluorescence of phen–Fe^{3+}–$InsP_6$, the PO_4^{3-}would strengthen the fluorescence intensity. The possible reason is the PO_4^{3-} also can format a complex with phen–Fe^{3+} as $InsP_6$, thus enhanced the fluorescence intensity. However, during the preparation of the food samples, the anion-exchange resin was used and the effect of these foreign ions could be ignored.

Reproducibility of the measurements

Reproducibility is basic for establishing a method for determining an analyte and is one of guarantees for a method precision. The reproducibility of measurements was checked by monitoring the synchronous fluorescent of a

Table 2 Results obtained for the determination of $InsP_6$ in food by the proposed method and by an UV-spectrophotometry method (mean±S. E., n=3)

Sample	$InsP_6$ found/mg g^{-1}			
	Proposed method	UV-spectrophotometry	D (%)[a]	Recovery (%)
Rice	4.62±0.02	4.83±0.03	4.55	92.2
Rapeseed	6.14±0.04	6.41±0.02	4.40	93.3
Peanut	12.55±0.03	12.98±0.04	3.42	95.4
Wheat flour	21.24±0.05	21.85±0.03	2.87	97.7
Soybean	15.32±0.04	15.89±0.04	3.72	95.8
Feed	24.08±0.03	24.82±0.05	3.07	98.3

[a] D (%) is the deviation of the two methods.

phen–Fe^{3+} solution with a certain $InsP_6$ (15 mg L^{-1}) for five replicates every day within a week. RSD was used to present the reproducibility and it was calculated from five (n=5) replicated measurement. The average RSD of the obtained results at 95% confidence interval was found to be less than 2.24%. The result indicated that the reproducibility of the proposed method was excellent.

Determination of $InsP_6$ in food

In order to determine the range of applicability of this method, six different food including rice, peanut, wheat flour, soybean, feed (pig diet), rapeseed, were treated by the aforementioned procedure. The $InsP_6$ level in each sample was measured according to the proposed method. In order to evaluate the recovery of expected $InsP_6$ concentration, an aliquot of 10.0 mg L^{-1} of $InsP_6$ was added into each sample solution and then analyzed. Results are given in Table 2 as the mean of five replicates. Recoveries (%) are in the range of 92.2%–98.3% for $InsP_6$ obtained by subtracting its initial level in samples. The UV-spectrophotometry method [26, 38] was used to compare to the proposed method for determination of $InsP_6$ in the food, and the deviation of the two methods is less than 5%. As can be seen in Table 2, good agreement can be observed between the two methods, thus demonstrating the suitability of the proposed procedure. The results indicated that the proposed method is reliable to be applied for the determination of $InsP_6$ in food. Compared the two method, the synchronous fluorescence method is simple and sensitive, the selectivity is its disadvantage, and should be improved.

Conclusions

A reliable method was developed for determination of phytic acid in food samples based on the synchronous fluorescence measurement. The calibration graph showed good linearity in the $InsP_6$ concentration range of 0.33–32.34 mg L^{-1} with a linear equation of I_f=8.770+2.980c (R^2>0.9994). The reproducibility is satisfied. The method was applied for determination of $InsP_6$ in some food samples, and the suitability of the proposed procedure was also demonstrated.

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