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Application of a Modified Haug and Lantzsch Method for the Rapid and Accurate Photometrical Phytate Determination in Soybean, Wheat, and Maize Meals

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A modified version of the Haug and Lantzsch method for rapid photometrical phytate determination was applied for the analysis of phytate in soybean, wheat, and maize meals. In comparison to the original protocol, the amount of the toxic reagent thioglycolic acid is reduced substantially to minimize potential health risks for laboratory personnel. Different extraction conditions for soybean meal were tested, and boiling for at least 30 min was found to be necessary to remove an interfering compound in soybean meal extracts. Phytate contents determined according to the modified Haug and Lantzsch method did not differ from those obtained by measuring total precipitated phosphorus or by sensitive and specific high-performance ion chromatography. Applicability and accuracy of the modified method for phytate analysis in major feed substrates, including soy-based textured vegetable protein, were demonstrated.

KEYWORDS: Maize; photometrical determination; phytic acid; phytate; soybean meal; wheat

INTRODUCTION

Phytate (*myo*-inositol[1,2,3,4,5,6]hexakisphosphate) is the major phosphorus (P) storage compound in plant seeds, accounting for 60–90% of total seed phosphorus. As a highly negatively charged anion, phytate is a potent chelator of mineral cations as well as protein and is therefore considered to be an antinutritional factor (*1*). Monogastric animals such as pigs and poultry have only a very limited capability to degrade phytate due to insufficient levels of phytase in their gastrointestinal tracts. Traditionally, the limited bioavailability of plant phosphate was compensated by adding inorganic feed phosphates in animal rations. This praxis has led to increasing phosphate pollution in regions with intense livestock production. In recent years, the increasing use of microbial phytase as a feed additive has been shown to be a more cost-attractive and environmentally sustainable route to improve phosphate digestibility (*2*).

From this perspective, the analytical determination of phytate levels in feed raw materials is of interest for agro-economy and ecology. Most methods for photometrical determination of phytate are based on indirect measurements. Initially, acidic extraction is carried out with HCl, H_2SO_4, or trichloroacetic acid followed by precipitation with Fe^{3+} (*3*). Unprecipitated ferric ions are determined by spectrophotometry. The difference between initial and remaining ferric ion concentration is then used to calculate phytate concentration. Acid extraction is preferable, because raw materials such as wheat might contain high levels of endogenous phytase, which could degrade phytate if allowed.

A well-known photometrical phytate method is the protocol developed by Haug and Lantzsch that uses 2,2′-bipyridine as a complexing chromogenic agent to quantify ferric ions (*4*). Although the rapid and sensitive character of the method is appealing, the published method has been evaluated for maize only. Following the authors' recommendation to investigate carefully the applicability of their protocol for the analysis of phytate from other plant substrates, we tested the modified method first in soybean meal (SBM) and then in SBM/maize, wheat, and maize meal. The rationale for proceeding in this order was that analytical interference was most likely to arise from SBM, which has a high phytate content [approximately 4 mg of phytate-bound phosphorus per gram of diet (*5*)] as well as protein [up to 40–55% (*6*)]. Thus, complex formation between phytate and soybean protein or peptides was expected to be a potential source of interference (*7*). We used high-performance ion chromatography (HPIC) as an advanced and specific method to quantify inositol phosphates (*8*) to evaluate the accuracy of the modified method.

Furthermore, we examined whether the concentration of thioglycolic acid (TGA) could be reduced from the relatively high level of 1% (v/v) prescribed in the original protocol. TGA is toxic and corrosive according to European Union directives (*9*) and has a highly unpleasant smell. In the present study we have attempted to determine to what extent the amount of TGA can be reduced without affecting measurement results.

MATERIALS AND METHODS

Materials. Maize, SBM (44% protein), wheat meal, and a 30:70 maize/SBM mixed diet were used. All diets were ground to pass a 1 mm sieve before extraction.

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Table 1. PAP in a SBM Sample Determined Using Different TGA Concentrations[a]

TGA (v/v)	*n*	g of PAP/kg of DM	SD	CV%
1.00%	7	3.9 a	±0.06	1.6
0.50%	6	4.0 a	±0.11	2.7
0.13%	6	4.0 a	±0.09	2.4

[a] DM, dry matter; SD, standard deviation; CV%, coefficient of variation in percent. Entries followed by the same letter are not significantly different (Tukey test, all pairwise, 95%).

Haug and Lantzsch's Photometric Determination of Phytic Acid Phosphorus. Phytic acid dodecasodium salt (Sigma-Aldrich, P-8810, 10.06% water) was used without further purification. A stock solution containing 200 μg of phytate accessible phosphorus (PAP)/mL was prepared in 0.2 M HCl (made from 1 M HCl, p.a., Bie & Berntsen, LAB00433.1000); this solution is stable for at least a year. Reference solutions were prepared by diluting the stock solution with 0.2 M HCl, yielding working solutions with 6.25–50 μg of PAP/mL. Ferric solution was prepared by dissolving 0.05 g of $FeCl_3$ (Merck, >98% pure, 803945) in 500 mL of 0.2 M HCl. Complexing reagent comprised 2.5 g of 2,2′-bipyridine (VWR International, p.a., 23576.128) and 325 μL of TGA (Merck, p.a., 1.00700.0100) dissolved in 250 mL of 0.2 M HCl. This reagent was stable for at least a week. This method is very sensitive to iron; hence, all solutions should be prepared using glass magnetic stir bars.

Samples (0.05 g) were placed in 1.5 mL tubes, and 1 mL of 1 M HCl was added. The tubes were incubated on a thermomixer at 100 °C for 45 min under vigorous mixing at 1000 rpm (alternatively, a water bath and a vortex mixer can be used). After extraction, samples were centrifuged for 5 min at 13000*g*. Five hundred microliter supernatant aliquots were transferred to new tubes, and 2 mL of deionized water was added. Eight hundred microliters of ferric solution was added to 400 μL of diluted extract or standards, and tubes were incubated on a thermomixer at 100 °C and 300 rpm for 45 min. Samples were cooled on ice for 15 min to allow iron phytate precipitate to form and then centrifuged at 13000*g* for 10 min at 0 °C. Six hundred microliter supernatant aliquots were transferred to microcuvettes, 800 μL of complexing reagent was added to each cuvette, and absorbances were measured at 540 nm.

HPIC Method, Direct Quantification. The method used was the same as described by Pontoppidan et al. (*8*). Briefly, samples were extracted in 0.5 M HCl and centrifuged through 30 kDa cutoff filter tubes (Microcon YM-30, Millipore) to remove particles that would potentially disturb the sensitive Dionex PAC-100 HPIC column used (250 × 4 mm). Inositol phosphates were eluted with a H_2O/HCl gradient (1 mL/min), and postcolumn detection was achieved using a solution of 0.1% $FeNO_3$ and 2% $HClO_4$ dissolved in deionized water (0.4 mL/min). Standards of 0.05−1 μg of PAP/mL as well as purified meal extracts were analyzed, and the content of inositol phosphates was calculated using linear regression.

Measurement of Total P. The analysis of total P in iron phytate precipitates was carried out at Eurofins, DK-Kolding, according to AOAC official method 985.01. In short, the sample is dry-ashed, treated with HNO_3, and dissolved in HCl, and total P is determined by ICP emission spectroscopy at 214.9 nm using $NH_4H_2PO_4$ as standard.

RESULTS AND DISCUSSION

Reducing the Amount of TGA Reagent. We hoped to reduce substantially the amount of TGA levels used in the assay without affecting the results to minimize potential health risks and improve handling convenience. TGA is used as an antioxidant in the complexing reagent containing 2,2′-bipyridine. TGA levels were reduced from 1.0 to 0.13% (v/v). Reducing the TGA concentration resulted in somewhat slower color development upon addition of the complexing reagent compared with the original method. However, this did not affect the measurement, as shown in **Table 1**. The results at the three different TGA levels tested were not statistically different from each other.

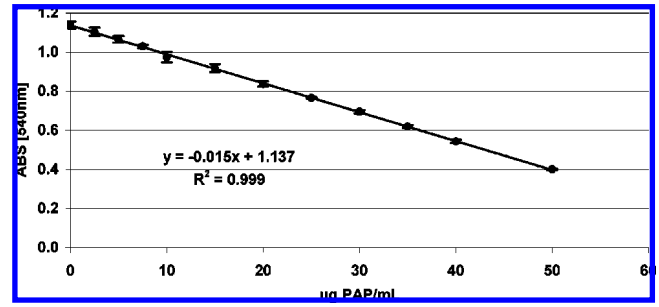

Figure 1. Testing linearity of the optimized method. Error bars represent standard deviations based on four measurements.

Linearity and Sensitivity. The test of linearity was based on the assumption that standard deviations were constant across all observations. Standard deviations obtained for phytate standards were very low, resulting in very low limits of detection and quantification (**Figure 1**).

To be sure that limits are set according to standard deviations for samples, operational limits of detection and quantification were introduced instead, representing 3 and 10 times the observed average standard deviation when samples were measured (data not shown). The operational limit of detection (LOD) was found to be 2 μg/mL, whereas the operational limit of quantification (LOQ) was 7 μg/mL. It should be noted that the Cl^- ions present might influence the precipitation of iron phytate, and hence the concentration of HCl was 0.2 M in all diluted samples and standards.

Varying Extraction Conditions during of Analysis SBM. Haug and Lantzsch evaluated the accuracy and sensitivity of their method by conducting measurements on phytate extracted from maize. Consequently, the authors recommended careful testing and evaluation when other plant materials were used. SBM is an important feed raw material because of its high content of vegetable protein. For the evaluation of assay applicability with substrates besides maize, we chose a standard SBM preparation used for animal nutrition purposes with 44% crude protein. The duration of the initial extraction period, performed at 100 °C, was varied from 5 to 60 min (**Table 2**). Somewhat surprisingly, significantly higher measurement values were obtained with a short extraction of 1 min. Even higher values were measured if extractions were performed at 37 °C. Hence, both extraction temperature and extraction time seemed to have an impact on the measured phytate content. Because the assay is based on indirect determination of precipitated iron phytate by measuring unprecipitated ferric ions, these results suggested that another compound(s) besides phytate formed precipitate(s) with ferric ions. The hypothesis of an interfering compound was further strengthened by determining phytate via analysis of total P in the iron precipitates (AOAC official method 985.01, see Materials and Methods). Precipitates of 5, 15, and 60 min extracts, obtained at 100 °C, were washed to remove soluble unprecipitated phosphate compounds and analyzed subsequently (**Table 2**). In the precipitate of the 5 min extract total P was measured to be only 1.3 g/kg, indicating that most of the precipitated material was not phytate. In the case of the 15 min extract, the discrepancy between the photometrically obtained value and the measurement of total precipitated P was much less pronounced, suggesting that most of the iron-precipitated material following the longer extraction was indeed phytate. This evidence was further corroborated when PAP values for the 60 min extracts using both methods were

Table 2. Influence of Extraction Conditions on Measured PAP in SBM Using the Modified Haug and Lantzsch and Official AOAC Methods (*10*)[a]

		photometric method				AOAC official method 985.01	
		extraction at 100 °C		extraction at 37 °C		extraction at 100 °C	
extraction period (min)	*n*	PAP (g/kg of DM)	SD	PAP (g/kg of DM)	SD	P (g/kg of DM)	SD
1	4	4.4 a	±0.07				
5	4	4.1 b	±0.06			1.3	±0.02
15	3	4.1 b	±0.09			3.9	±0.05
30	3	4.0 b	±0.07	4.3	±0.2		
45	3	3.9 b	±0.2				
60	3	4.0 b	±0.2	4.5	±0.08	4.0	±0.05

[a] DM, dry matter; SD, standard deviation. Entries followed by the same letter are not significantly different (Tukey test, all pairwise, 95%).

Table 3. PAP in SBM, Maize Meal, SBM/Maize Meal, and Wheat Meal As Measured with the Modified Haug and Lantzsch and HPIC Methods

	photometrical method			HPIC[a]			
substrate	*n*	PAP (g/kg of DM)	SD	*n*	PAP (g/kg of DM)	SD	difference (%)
SBM	6	3.9	±0.2	3	3.8	±0.07	3.8
maize meal	6	2.3	±0.1	3	2.3	±0.06	−1.3
SBM/maize diet	6	2.9	±0.2	3	2.9	±0.03	**1.3**
wheat	3	2.7	±0.1	3	2.6	±0.07	3.3

[a] A different extraction procedure is used when samples are analyzed on the Dionex HPIC (see text).

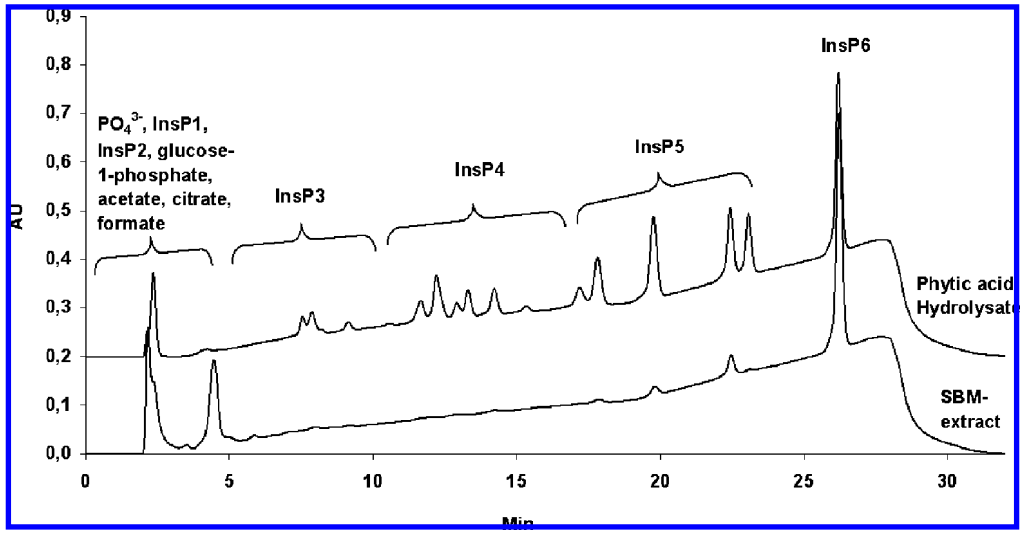

Figure 2. HPIC analysis of HCl-extracted SBM. A phytate hydrolysate served as a reference. Order of elution of various inositol phosphates was established as described in refs *8, 11*, and *12*.

compared. These values were nearly identical, suggesting total elimination of the interfering compound. Given the high content of vegetable protein in SBM, it was speculated that the interfering compound was proteinacous. To try to identify possible contamination, iron extract precipitates as obtained from the procedure were analyzed by measuring nitrogen (LECO, FP-528 nitrogen analyzer). However, the values obtained were in the range of 1–3% w/w content (data not shown) and, hence, too small to support this theory.

Increasing the extraction temperature from 37 to 100 °C resulted in a decrease of the amount of phytate measured using the photometric method. Furthermore, the results suggest that all phytate present in the SBM sample was extracted within 5 min. This does not seem likely, and hence analysis of total P was conducted on the iron phytate precipitates (see Materials and Methods). The levels of total P in the samples verified the assumption that all phytate was in fact not extracted after 5 min, and an interfering compound(s) seemed to substantially elevate the measured amount of PAP. Furthermore, the results showed that extraction at 100 °C did not degrade phytate but removed the interfering compound, giving a very close correlation between total P measurement and the photometric method. To be sure that the interfering compound was removed and that the risk of thermal degradation of phytate was minimized, 45 min and 100 °C were chosen as standard extraction parameters of the modified method.

Applying the Modified Method on SBM/Maize, Maize, and Wheat Meal. On the basis of the evaluation of the modified method, maize meal, wheat meal, and mixed diets (based on SBM and maize) were analyzed. The results obtained verified the reliability of the developed procedure. Note that all trials were carried out separately, and hence standard deviations vary slightly between days in **Tables 1**−**3**. Direct quantification of PAP in the same raw materials using HPIC (*8*) confirmed the levels obtained by the optimized photometric method (**Table 3**).

The HPIC method was applied as described previously (*8, 11, 12*). Diets were extracted with hydrochloric acid, and supernatants were ultrafiltered. Briefly, inositol phosphates were separated and quantified using HPIC with gradient elution (H_2O/HCl) on an anion exchange column. Following postcolumn derivatization with Fe^{3+}, iron−inositol phosphate complexes

were measured at 294 nm. PAP determination by HPIC was free of interference, because interfering proteins were removed by ultrafiltration (30 kDa cutoff membrane) and interfering anions eluted from the column much earlier than inositol phosphates, including phytate (**Figure 2**).

The present work demonstrates the applicability of the modified Haug and Lantzsch protocol for photometrical determination of phytate from maize, wheat, SBM, and SBM/maize meals. We optimized the extraction time (45 min, 100 °C) and reduced the amount of toxic TGA used in the complexing reagent (from 1 to 0.13% v/v). Corroborating the photometric data by means of HPIC was important because it established correlation between an indirect method that can be influenced by interfering compounds and the interference-free HPIC method.

However, the time and expense associated with the HPIC equipment are very high, often exceeding the budgets of agro-analytical laboratories located in developing countries. Thus, even though more accurate chromatographic separation methods are available, there is still a need to develop further the more traditional and simple photometric methods. In this respect, using SBM to develop an assay for phytate determination makes sense, because SBM is a widely used feedstuff and contains compounds that might interfere with phytate determination assays based on iron (*13*).

ABBREVIATIONS USED

HPIC, high-performance ion chromatography; LOD, limit of detection; LOQ, limit of quantification; PAP, phytate accessible phosphorus; SBM, soybean meal; TGA, thioglycolic acid.

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