Received: 13 January 2023 | Revised: 23 February 2023 | Accepted: 13 March 2023
DOI: 10.1002/jsf2.109

RESEARCH ARTICLE

Enzyme-assisted HPTLC method for the simultaneous analysis of inositol phosphates and phosphate

Corinna Henninger[1] | Bernd Spangenberg[1] | Mario Schmidt[2] | Katrin Ochsenreither[3] | Thomas Eisele[1]

[1]Hochschule Offenburg, Fakultät Maschinenbau und Verfahrenstechnik, Badstraße 24, 77652 Offenburg, Germany

[2]Kaesler Research Institute, Kaesler Nutrition GmbH, Fischkai 1, 27572 Bremerhaven, Germany

[3]Karlsruhe Institute of Technology (KIT), Institute of Process Engineering in Life Sciences 2—Technical Biology, Fritz-Haber-Weg 4, 76131 Karlsruhe, Germany

Correspondence
Thomas Eisele, Hochschule Offenburg, Badstraße 24, 77652 Offenburg, Germany.
Email: thomas.eisele@hs-offenburg.de

Abstract

Background: The analysis of myo-inositol phosphates ($InsP_x$) released by phytases during phytic acid degradation is challenging and time-consuming, particularly in terms of sample preparation, isomer separation, and detection. However, a fast and robust analysis method is crucial when screening for phytases during protein engineering approaches, which result in a large number of samples, to ensure reliable identification of promising novel enzymes or target variants with improved characteristics, for example, pH range, thermal stability, and phosphate release kinetics.

Results: The simultaneous analysis of several $InsP_x$ ($InsP_1$-$InsP_4$ and $InsP_{5+6}$) as well as free phosphate was established on cellulose HPTLC plates using a buffered mobile phase. Inositol phosphates were subsequently stained using a novel enzyme-assisted staining procedure. Immobilized $InsP_x$ were hydrolyzed by a phytase solution of Quantum® $Blue_{liquid}$ 5G followed by a molybdate reagent derivatization. Resulting blue zones were captured by DAD scan. The method shows good repeatability (intra-day and intra-lab) with maximum deviations of the Rf value of 0.01. The HPTLC method was applied to three commercially available phytases at two pH levels relevant to the gastrointestinal tract of poultry (pH 5.5 and pH 3.6) to observe their phytate degradation pattern and thus visualize their $InsP_x$ fingerprint.

Conclusion: This HPTLC method presents a semi-high-throughput analysis for the simultaneous analysis of phytic acid and the resulting lower inositol phosphates after its enzymatic hydrolysis and is also an effective tool to visualize the $InsP_x$ fingerprints and possible accumulations of inositol phosphates.

KEYWORDS
animal feed, HPTLC, inositol phosphates, phosphate release, phytase, phytate dephosphorylation

INTRODUCTION

Phosphate is the limiting factor for the growth of plants and animals on Earth, as it is a key component for many essential molecules such as DNA, phospho-lipids and ATP.[1] In plant seeds and grains, the major storage form for phosphorous (P) is phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or $InsP_6$) with a content of 1%–3% (w/w), which typically accounts for 60%–90% of the total P content depending on the plant species.[2] However, phytic acid shows poor digestibility in nonruminant animals such as swine, poultry, and fish due to the lack or low activity of $InsP_6$-hydrolyzing enzymes in their gastrointestinal tract.[3,4] Phytases (EC: 3.1.3.26) are an enzyme

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *JSFA Reports* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

class that enable the sequential dephosphorylation of $InsP_6$ to lower inositol phosphate esters such as $InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$, and eventually $InsP_1$. The use of phytases as feed additives in animal nutrition is already an established method[5,6] to provide additional phosphate in the diet, thus eliminating the need for external phosphate sources. The advantages of phytase addition become more and more apparent considering the limitation of the resource and the need for minimizing the environmental impact of P extraction.[1] For a phytase to be used as a feed additive, it must meet specific requirements including high specific activity, broad thermal (60°C–90°C) and pH profile (pH 2 to 7.5), and protease resistance to withstand gastric environment.[6,7] For ecological and economic reasons, there is a need for novel phytases with an enhanced ability to release phosphate as in vivo studies suggest that 30%–50% of phytate passes the gastrointestinal tract unused.[8] Inositol phosphates ($InsP_x$) play an important role in various biological systems;[1] therefore, the demand for suitable analytical methods to analyze $InsP_x$ is high.

Also, for screening of novel and engineered phytase variants, it is necessary to characterize and constantly monitor changes in $InsP_6$ degradation and to detect possible accumulations of lower inositol phosphates. This requires a fast and robust screening method with a high sample turnover, since a large number of samples have to be processed.

Common challenges with inositol phosphate analysis include sample preparation, isomer separation and detection.[9] Traditionally, phytic acid is analyzed via precipitation with Fe^{3+} ions. However, this method is strongly pH dependent, influenced by other ions and not very selective, since lower inositol phosphates also form a precipitate leading to an overestimation of the $InsP_6$ content.[9] Over the past decades, a wide variety of separation techniques have been devised for the analysis of single or multiple inositol phosphates. These include various chromatographic systems such as ion pair chromatography, thin-layer chromatography, gas chromatography, ion-exchange chromatography, and electrophoresis. Since inositol phosphates are not optically active, a derivatization is necessary for the detection by absorbance measurement. For this purpose, a post-column derivatization using the Wade reagent is often applied.[10] Other detection methods are radiolabeling, mass spectrometry (MS) and tandem mass spectrometry (MS/MS), nuclear magnetic resonance spectroscopy (NMR), or inductively coupled plasma (ICP). In more recent years, enzymatic sensors were developed to detect $InsP_6$ online.[9] Despite the wide range of analytical systems available, only a few of them are suitable for the simultaneous analysis of phytic acid and its degradation products. For example, it is only possible to separate either higher or lower inositol phosphates simultaneously by HPLC.[11,12] In addition, mainly pools of $InsP_x$ isomers are separated from each other. Separation of isomers is possible with ion-exchange chromatography.[10] However, the analysis is very time-consuming and only allows for a limited number of samples per day.

The aim of this study is to develop an analytical method for phosphate, phytic acid, and lower inositol phosphates to monitor the enzymatic degradation of $InsP_6$ and possible accumulation of $InsP_x$ pools. For this purpose, high-performance thin-layer chromatography (HPTLC) is a robust method that allows a high daily sample throughput.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were of analytical grade and were obtained from either Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany), unless stated otherwise. Commercial phytase products used in this study were Quantum® Blue (AB Vista, Woodstock, UK), Axtra® PHY (Danisco Animal Nutrition, Wilmington, Delaware, USA), and Enzy Phostar (Kaesler Nutrition, Cuxhaven, Germany), in solid formulation, which were provided by Kaesler Nutrition GmbH (Cuxhaven, Germany). A liquid formulation Quantum® Blue 5G (AB Vista, Woodstock, UK) was obtained from AB Vista. The phosphate standard for IC (TraceCERT®, 1000 mg/L ± 4 mg/L) was ordered from Sigma-Aldrich (Darmstadt, Germany). Inositol phosphate standards Ins(3)P_1 (sodium salt), Ins(2,4)P_2 (sodium salt), Ins(1,4,5)P_3 (sodium salt), Ins(2,3,5,6)P_4 (sodium salt), Ins(1,3,4,5,6)P_5 (sodium salt) were sourced from Cayman Chemical (Ann Arbor, Michigan, USA), Ins (1,2,3,4,5,6)P_6 (sodium salt) from Merck (Darmstadt, Germany), and Ins(1)P_1 (potassium salt) from ChemCruz (SCBT, Dallas, Texas, USA).

Material and instrumentation

High-performance thin-layer chromatography (HPTLC) was performed on HPTLC Cellulose F 20 × 10 Glass plates (1.15036.0001, Lot: HX69191936) from Merck (Darmstadt, Germany) in a 20 × 10 Twin Trough Chamber with glass lid from CAMAG (Muttenz, Switzerland). Samples were applied by the Automatic TLC Sampler 4 by CAMAG (Muttenz, Switzerland), derivatized in the CAMAG® Derivatizer (CAMAG, Muttenz, Switzerland), and visualized in a CabUVIS from DESAGA (Wiesloch, Germany) equipped with a Canon EOS 600D camera or by a Visualizer 3 from CAMAG (Muttenz, Switzerland). For direct DAD-spectrophotometry of UV spectra from HPTLC plates, a TIDAS TLC S700 system from J&M (Aalen, Germany) was used. A ThermoMixer® C manufactured by Eppendorf (Hamburg, Germany) was used to incubate all reaction tubes. Spectrophotometric measurements in microtiter plates were performed in an Epoch 2 manufactured by Biotek (Winooski, Vermont, USA). An ICP-OES spectrometer iCAP 7000 series from Thermo Fisher Scientific (Dreireich, Germany) was utilized.

Statistical method and evaluation

Spectral measurement from plate

A TIDAS TLC S700 system from J&M (Aalen, Germany) with a reflection attachment consisting of two rows of optical fibers was used for

the spectral measurements of the plate, which has a wavelength resolution of 0.8 nm and a spatial resolution on the plate of 100 μm. The measurement time for a single spectrum in the wavelength range from 190 to 1000 nm was 25 ms. The raw data of the measurement were evaluated using expression (1) derived from the extended Kubelka–Munk equation.[13]

$$KM(p,q) = \frac{(J_0 - J)(pJ_0 - qJ)}{JJ_0} = \frac{a}{1-a} \quad (1)$$

$$p + q = 1 \quad (2)$$

where p is backscattering factor ($0 \le p \le 1$); a is absorption coefficient; J_0 is reflected light intensity measured from a neat plate part; J is reflected light intensity measured from a track.

The factors p and q adapt Equation (1) to special measurement conditions.[13] In trace analysis, for example, almost all analyte molecules are located near the surface of the layer. In the deeper parts of the layer, no light is needed to measure the sample molecules and thus no backscattering is needed to illuminate all analyte molecules. This is considered by setting the backscatter factor p in Equation (1) to $p = 1$, which leads to Equation (3).[13]

$$KM(p=1) = \frac{J_0}{J} - 1 = \frac{a}{1-a} \quad (3)$$

In the case of fluorescence, backscattering need not be considered, so the corresponding equation from Equation (1) is reproduced for $p = 0$. If the analyte is present in the stationary phase at such a high concentration, that parts of the stationary phase are not illuminated by the incident light, which is corrected by a backscatter factor of $p = ½$. For this value, Equation (1) gives the equation known as the Kubelka–Munk equation.

Limit of detection (LOD) and limit of quantification (LOQ)

The external standard method was used for statistical verification of inorganic phosphate (P_i) content. For this purpose, 16 calibration points resulting from six different dilutions of a phosphate standard solution were sprayed (2–9 μL) onto a plate in duplicate, resulting in a range between 5 and 150 ng of phosphate. The TraceCERT® phosphate standard for IC (Sigma-Aldrich, Darmstadt, Germany) used is a certified reference material for ion chromatography measurement. For the inositol phosphates Ins(1)P_1, Ins(2,4)P_2, Ins(1,4,5)P_3, Ins(2,3,5,6)P_4, and Ins(1,3,4,5,6)P_5, the LODs and LOQs were determined using an external standard. For each $InsP_x$, a range between 8 and 500 ng based on their free acid base was applied to an HPTLC plate. For evaluation, the backscattering factor was chosen to maximize the correlation coefficient for linear regression. LOD was calculated as 3 σ and LOQ as 9 σ, with the standard deviation σ of the calibration function calculated at a significance level of $\alpha = 0.002$.[14]

Phosphorous content in $InsP_x$ standard solutions

As a control, the $InsP_x$ standard solutions and phosphate standard solution were checked for their phosphorus content by ICP-OES measurement. For this purpose, the solutions were diluted to a theoretical P content of 100 μg/L and measured in triplicate. Calibration was performed using an external standard between 20 and 300 μg/L P. Argon was used as a carrier gas with an auxiliary stream of 0.5 L/min and an RF power of 1150 W. The sample was nebulized in a glass concentric type diffuser and P was measured on the spectral line 177.495 nm.

Reproducibility and repeatability

Reproducibility and repeatability (intra-day and intra-lab) of the method were determined based on Rf values. For this purpose, three plates were evaluated within one day and three plates on three consecutive days. $InsP_1$–$InsP_6$ and free phosphate were applied on separate lanes on each plate. To qualify the chromatographic system (quality and reproducibility) on each plate, a system suitability test (SST) was established based on five inositol phosphate standards (300 ng Ins(3)P_1 (sodium salt), 200 ng Ins(2,4)P_2 (sodium salt), 200 ng Ins(1,4,5)P_3 (sodium salt), 100 ng Ins(2,3,5,6)P_4 (sodium salt), and 100 ng Ins(1,3,4,5,6)P_5 (sodium salt)).

Protocols

HPTLC (high-performance thin-layer chromatography)

The Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland) sprayed 6-mm wide bands onto cellulose F glass plates (200 × 100 mm) (Merck, Darmstadt, Germany), 10 mm from the lower edge and 15 mm from the left edge at a rate of 40 nL/s. The plate was developed in a twin trough chamber (200 × 100 mm with a glass lid), which was previously saturated with mobile phase consisting of an aqueous salt solution (20 mM NaOAc, 10 mM NH_4Cl)/2-propanol/1,4-dioxane/acetic acid 5/5.2/2/0.06 (v/v/v/v) for 30 min at ambient temperature (~20°C). The development was performed over a migration distance of 75 mm from the lower edge for approximately 2.5 h. Consequently, the plate was dried at 105°C in a drying oven over night (minimum 12 h) to remove all solvent residues.

Derivatization of *myo*-inositol phosphates

With the Derivatizer (CAMAG, Muttenz, Switzerland) 1 mL of enzyme solution (250-fold diluted Quantum® $Blue_{liquid}$ 5G in 50 mM NaOAc, pH 4.5) was sprayed onto the still warm plate (nozzle red, level 4, precleaned with ddH_2O). The plate was pre-incubated for 5 min at room temperature and then incubated on a plate heater for 15 min at 55°C. Subsequently the plate was dried in the drying oven at 105°C for 30 min. The hydrolyzed free phosphate was then stained with a

modified molybdate reagent according to Spangenberg et al.[14] Using the Derivatizer, 0.5 mL of molybdate reagent (5 mL of a 10 g/L ammonium-molybdate heptahydrate aq. solution mixed with 200 μL of concentrated sulfuric acid freshly every day) were sprayed onto the plate, which had cooled down to ambient temperature (nozzle red, level 4). The plate was then treated with UV light (254 nm)[15] for 15 min and scanned directly afterwards using a DAD scanner (J&M, Aalen, Germany) in a wavelength range of 200–900 nm. The resulting blue zones were evaluated at the absorption maximum of 774 nm. It is necessary that the plate is dry and cooled down to ambient temperature before the color reagent is applied to avoid background staining during derivatization.

Enzyme preparation

Solid phytase formulations were mortared to a fine powder and made up to solutions of 5 g/L in 5 mM NaOAc buffer at 4.5 pH. The solutions were vortexed for 1 min and stored on ice until use. Enzyme activity was measured at pH 5.5 at 37°C and was subsequently adjusted to 100 U/L.

Enzymatic activity

Phytase activity was determined in triplicates according to a slightly modified method of Greiner et al.[16] Exactly 200 μL of a phytic acid solution (50 mM NaOAc and 1.66 g/L sodium phytate buffer, set to pH 5.5) was pre-incubated for 5 min at 37°C. Hydrolysis was started by adding 10 μL of an appropriately diluted enzyme solution and stopped after 5 min with 100 μL of an acidic molybdate-vanadate reagent according to DIN EN ISO 30024.[17] Any precipitate formed was centrifuged (17,000xg for 2 min) and the supernatant was measured photometrically at 405 nm. The stop reagent consists of one-part solution A (10-% (w/w) ammonium-molybdate in 0.25% (*v*/*v*) ammonia solution), one-part solution B (aqueous 0.24-% (w/w) ammonium vanadate solution) and two parts of 21.7% of nitric acid. Stock solution A and B can be stored refrigerated and dark for up to two months, the stop reagent must be prepared fresh daily.[17]

$InsP_6$ degradation assay

In a 50 mL falcon tube, 9 mL of phytic acid solution (50 mM NaOAc and 1.66 g/L sodium phytate buffer, set to pH 5.5 and 3.6 respectively) was pre-incubated at 37°C for 10 min on an Eppendorf ThermoMixer® C at 800 RPM. Hydrolysis was started by adding 1 mL of enzyme solution to a resulting final activity of 10 U/L. Samples of 500 μL were taken periodically (after 5, 30, 60, 120, 180, 240, 300 min and 24 h) and stopped by heat, diluting the sample twofold in 95°C H_2O_{dd} and inactivating it for 1 h. For the blank, an appropriately diluted enzyme solution was inactivated for 1 h at 95°C before adding it to the phytic acid solution and leaving at 95°C for 1 h.

Complete $InsP_6$ hydrolysis

As a comparative measure, phytic acid was completely hydrolyzed by acid hydrolysis to determine the maximum phosphate release. For this purpose, a phytic acid solution of 1.66 g/L in 0.25 M HCl was incubated for five days at 95°C in a drying oven. The solution was then diluted in the same manner as the degradation assay and free phosphate content was determined via HPTLC.

RESULTS AND DISCUSSION

Separation and detection of inositol phosphates

Phosphorous (P) is a pivotal element for the growth of microorganisms, animals, and humans as it is a component in bones, cell membranes, and DNA.[1] In animal feed, P may be added as inorganic phosphate (P_i) salts or as an alternative, and commercial phytases may be added to hydrolyze the natural phosphate storage phytic acid to liberate phosphate.[5,6] For the separation of the enzymatically generated degradation products, such as inositol phosphates ($InsP_x$), a stationary phase consisting of cellulose was selected in combination with an acidic buffer system NaOAc/Ac as a mobile phase (aq. salt solution (20 mM NaOAc, 10 mM NH_4Cl)/2-propanol/1,4-dioxane/acetic acid 5/5.2/2/0.06 (*v*/*v*/*v*/*v*) over a distance of 75 mm for 2.3 h). The moderate acidity of the applied mobile phase gives optimal protonation levels of the analytes (pH 4.3),[18] which was also utilized by Sandberg et al.[12] for a HPLC method. Hatzack et al.[19] previously reported the use of cellulose as a stationary phase, however with an alkaline mobile phase (1-propanol/25% aqueous ammonia solution/H_2O 5/4/1 (*v*/*v*/*v*)) over a distance of 100 mm for 5 h). The modern HPTLC plates applied in this work were not compatible with the mobile phase of Hatzack et al.

The HPTLC method outlined in Figure 1 is suitable for the separation of $InsP_x$ pools as well as P_i. The isomers Ins(3)P_1, Ins(2,4)P_2, Ins(1,4,5)P_3, and free phosphate are baseline separated. Ins(2,3,5,6)P_4 and Ins(1,3,4,5,6)P_5 may be quantified by the peak splitting method. $InsP_6$ shows two bands in a concentration-dependent manner. Presumably, the part that is present as an undissolved salt remains on the application line, while the free base migrates to an Rf value of 0.06 and thus co-migrating with Ins(1,3,4,5,6)P_5 (Rf = 0.07).

Compared with other chromatographic techniques, in HPTLC, the analytes are immobilized on the dried plate after separation. The present HPTLC method uses a novel approach in which the separated inositol phosphates are treated with a commercial phytase (Quantum Blue 5G) using a "micro droplet" spraying technology, in order to release free phosphate (P_i). The phytase solution (>10,000 U/L) is utilized at an optimal pH of Quantum Blue (pH 4.5) with a maximum volume of 1 mL to provide moisture and soak through the stationary phase (cellulose) without exceeding the maximum moisture

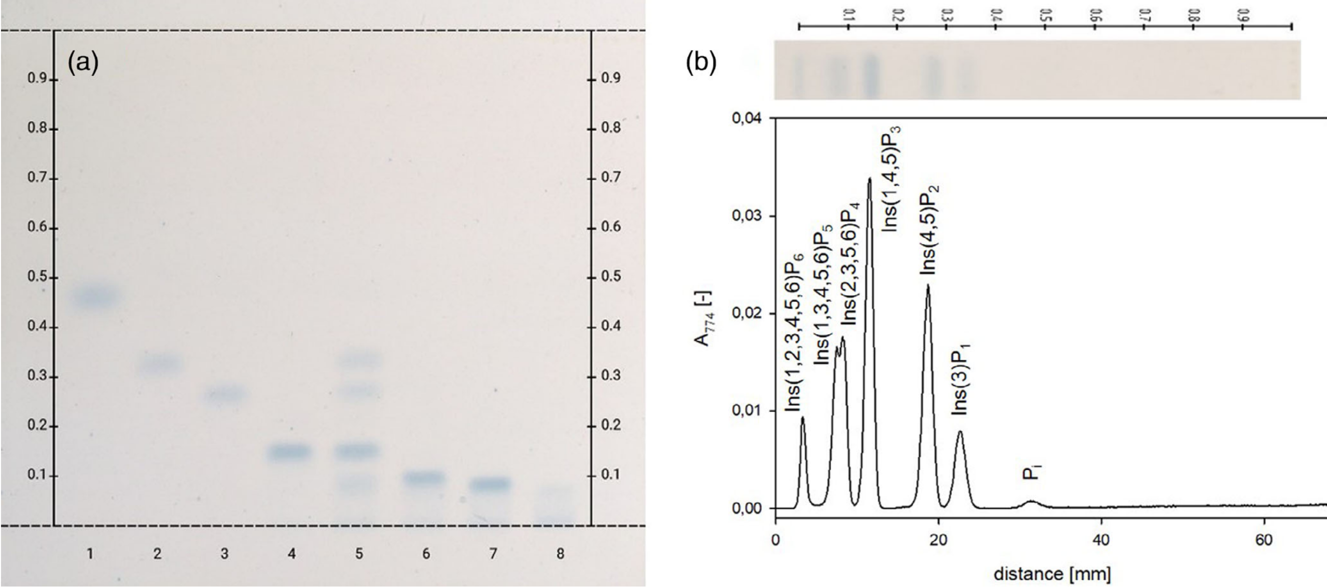

FIGURE 1 (a) Separation of myo-inositol phosphate standards on cellulose: 1: 100 ng P_i, 2: 300 ng Ins(3)P_1 (sodium salt), 3: 200 ng Ins(2,4)P_2 (sodium salt), 4: 200 ng Ins(1,4,5)P_3 (sodium salt), 5: System suitability test ($InsP_1$-$InsP_5$), 6: 200 ng Ins(2,3,5,6)P_4 (sodium salt), 7: 100 ng Ins (1,3,4,5,6)P_5 (sodium salt), 8: 100 ng $InsP_6$ (sodium salt) captured with Visualizer 2 (VC v3.1, RemissionVis, Exposure 0.045 s, contrast 2.0). (b) Chromatogram from DAD scan of separated $InsP_x$ standards $InsP_1$—$InsP_6$ and P_i on one lane.

TABLE 1 HPTLC method parameters for separation of myo-inositol phosphate standards with evaluation parameters.

	Rf [−][a]	LOD [ng]	LOQ [ng]	Smallest detected quantity [pmol]	Backscattering factor p [−]
P_i	0.46	6	7	32	0.75
Ins(1)P_1	0.32	30	42	68	0.65
Ins(2,4)P_2	0.26	29	39	42	0.55
Ins(1,4,5)P_3	0.14	27	45	35	0.6
Ins(2,3,5,6)P_4	0.09	23	34	13	0.6
Ins(1,3,4,5,6)P_5	0.07	29	41	23	0.6
Ins(1,2,3,4,5,6) P_6	0.00 // 0.06				

[a]Reproducibility (intra-day, intra-lab) with maximum deviation of Rf value of 0.01.

absorbance of the material. The enzymatically liberated P_i is derivatized in a following step using a molybdate reagent resulting in blue zones on the HPTLC plate. The reagent was simplified compared with recipes found in the literature[14,19] by only using two ingredients (ammonium-molybdate and sulfuric acid). The optimal mixture ratio in terms of contrast to the background and signal intensity of the zones was achieved by applying 10 g/L of ammonium-molybdate at a pH <1. This reagent reacts specifically with free phosphate and does not stain inositol phosphates.

The HPTLC method shows good repeatability (intra-day and intra-lab) with deviations of the Rf values of max. 0.01 (Table 1) under constant conditions. Acidic conditions or salt containing samples may affect Rf values, however not the overall separation of inositol phosphates. Other $InsP_x$ isomers may migrate slightly differently (Table S1). The quantification of the $InsP_x$ isomers can be performed by external standards and linear regression. The limit of detections (LODs) and limit of quantifications (LOQs) were determined for P_i as well as selected $InsP_x$ standards. The LODs and LOQs for each inositol phosphate are shown in Table 1. Considering the light intensity scattered by the plate background as described in the materials and methods section, the calibration lines were each evaluated with an individual backscattering factor p. For free phosphate, two linear ranges were found between 5–15 ng (with $p = 0.75$) and 20–150 ng (with $p = 0.5$) with correlation coefficients of 0.99. Free phosphate was detected with an LOD and LOQ of 5.7 and 6.9 ng (63.2 and 73.7 pmol), respectively. For inositol phosphates (free acid base), LODs and LOQs were found to be in the range of 23–30 ng (39–89 pmol) and 34–45 ng (58–125 pmol), respectively. Determination of LOD and LOQ for $InsP_6$ was not determined as the analyte is only partially migrating from the application line.

Quantification of inositol phosphates via the calibration curve of free phosphate is not applicable due to the broadening of the bands on the thin-layer plate with increasing solvent height (compare Figure 2a) This phenomenon is common in thin-layer chromatography.[20,21] Both relative and quantitative evaluation of inositol phosphates is possible, for example, via the external standards or relative to each other. In the present work, the inositol phosphates were evaluated relative to each other. Exemplarily, the $InsP_3$ calibration curve was evaluated via a phosphate calibration curve to confirm the linear relationship between the applied $InsP_x$ amount and the released phosphate. To the best of our knowledge, existing methods are not suitable for a rapid detection of $InsP_4$-accumulating phytases as well as the baseline separation of $InsP_3$, $InsP_2$, and particularly $InsP_1$.

In recent decades, many approaches to analyze inositol phosphates have been pursued, and Marolt and Kolar et al. have written a comprehensive review about the subject.[9] Only a few analytical methods are suitable for the simultaneous analysis of $InsP_6$ and its degradation products, mainly thin-layer chromatography (present work $InsP_6$-$InsP_1$ and P_i), HPLC ($InsP_6$-$InsP_3$),[9] and ion-exchange chromatography ($InsP_6$-$InsP_2$ and P_i).[9] All of these methods are either costly in terms of equipment, time intensive, or only capable of separating a limited amount of $InsP_x$ pools.

There are only few reports on the use of thin-layer chromatography for the analysis of inositol phosphates apart from Hatzack et al.[19] The HPTLC method described by Hatzack et al.[19] reports LODs in the range of 100–200 pmol for Ins(1)P_1, Ins(1,4,5)P_3, and $InsP_6$. Sandberg et al.[12] published an ion pair C_{18} reversed-phase HPLC separating $InsP_x$ pools of $InsP_6$ to $InsP_3$ at 4.3 pH. The $InsP_x$ were detected by refractive index detector. No detection limits were reported for the HPLC method; however, the lower end of the $InsP_6$ calibration was 2 μmol/mL (20 μL injection volume), corresponding to 40 nmol on an HPTLC plate in this work. Oates et al.[10] presented an ion-exchange chromatography (IC) method separating 25 inositol phosphate isomers ($InsP_2$–$InsP_6$) on a Dionex CarboPac PA100 column using an HCl gradient followed by post-column derivatization with ferric nitrate and UV detection at 290 nm. LODs and LOQs of 1 and 3.2 mg/L (100 μL injection volume) were reported for individually tested isomers. This translates to an amount of 100 and 320 ng on a HPTLC plate. However, with IC, only a small number of samples can be processed in one day as one analysis takes approximately 45 min.[10] Considering sample throughput and number of separated isomeric pools, HPTLC is most suitable as a fast screening method. However, for a more in-depth analysis of enzymatic $InsP_x$ degradation or regioisomers, a different analysis method may be chosen.

$InsP_x$ fingerprint and substrate specificity

The necessity for a fast and simple phytic acid degradation assay to screen for protein engineered phytase variants with undesired accumulations of lower inositol phosphates is apparent. The degradation of phytic acid in the digestive tract of poultry was simulated at pH 3.6 and 5.5 to mimic the gizzard and crop, respectively. Different in vitro assays for the evaluation of phytases in application-relevant matrices can be found in the literature.[22] For example, Sommerfeld et al.[5] described a continuous three-step in vitro assay to simulate the gastrointestinal tract (GIT) of chicken. Here, water content, temperature, residence time, pH, and proteolytic enzymes were considered and IC was used to analyze the degradation pattern of phytic acid. For the initial evaluation of dozens of phytase variants simultaneously, a static assay at two pH values relevant to the GIT is sufficient.

In the present work, a phytase activity of 10 U/L (at pH 5.5, 37°C) was applied to a standardized phytic acid solution (1.66 g/L) at pH 3.6 and 5.5 at 37°C. Samples were taken periodically until 5 hours of hydrolysis time and a final sample after 24 h (5, 30, 60, 120, 180, 240, 300 min and 24 h) to observe the differences in the $InsP_x$ finger print (supplement data Figure S1). The time point, at which the highest concentration for each particular $InsP_x$ pool was reached, was set to 100%. Thus, $InsP_x$ and P_i were evaluated as relative amounts and all peaks were evaluated using the Kubelka–Munk equation ($p = 0.5$). P_i, $InsP_1$-$InsP_3$ were base line separated and $InsP_4$ was quantified by the peak splitting method. $InsP_5$ and parts of $InsP_6$ were co-migrating and thus evaluated as a sum parameter (Figure 1). During the hydrolysis of phytic acid by Quantum® Blue (Figure 2) at 3.6 and 5.5 pH values, the $InsP_{5+6}$ concentration decreased rapidly within the first 30 min and was almost completely degraded after 60 min. The $InsP_4$ pool concentration resulting from the hydrolysis of $InsP_{5+6}$ increased almost linearly during hydrolysis at pH 3.6 and pH 5.5, respectively, until the hydrolysis time of 30 min. The degradation of the $InsP_4$ pool at pH 3.6 was completed after 120 min. In contrast, the degradation of the $InsP_4$ pool at pH 5.5 required 180 min to be completed. For the pool of $InsP_3$, there were even more significant differences for both, the formation and degradation kinetics at the two pH values (pH 3.6 and 5.5). The highest concentration of $InsP_3$ was determined at pH 3.6 after 60 min, whereas for hydrolysis at pH 5.5, the highest concentration was detected after 120 min. The degradation of $InsP_3$ at pH 3.6 was completed within 60 min, whereas the complete degradation of $InsP_3$ at pH 5.5 required 120 min. The pool of $InsP_2$ reached its maximum after 120 min for the hydrolysis at pH 3.6, whereas the maximum for the hydrolysis at pH 5.5 was reached after 180 min. After 180 min, the previously formed $InsP_2$ was completely degraded during hydrolysis at pH 3.6. On the other hand, the hydrolysis at pH 5.5 showed an accumulation of $InsP_2$, which was only slowly degraded over the entire hydrolysis time of up to 24 h. During hydrolysis at pH 3.6, the formation of $InsP_1$ occurs from the degradation of $InsP_2$. This reaches its maximum (180 min) once the degradation of $InsP_2$ is finalized. At pH 3.6, an accumulation of $InsP_1$ can be observed and only ~35% of the $InsP_1$ is degraded in the remaining hours. At pH 5.5, no significant formation of $InsP_1$ occurs due to the accumulation of $InsP_2$.

Moreover, the phytic acid degradation by Axtra® PHY (Figure S2) and Enzy Phostar (Figure S3) was evaluated in this work. Both phytases showed a high substrate specificity for $InsP_5$ and $InsP_6$ as the degradation was almost completed within 60 min. Enzy Phostar showed similar substrate specificity as Quantum® Blue. $InsP_2$ was degraded at both pH values over a period of several hours, however not completely. $InsP_1$

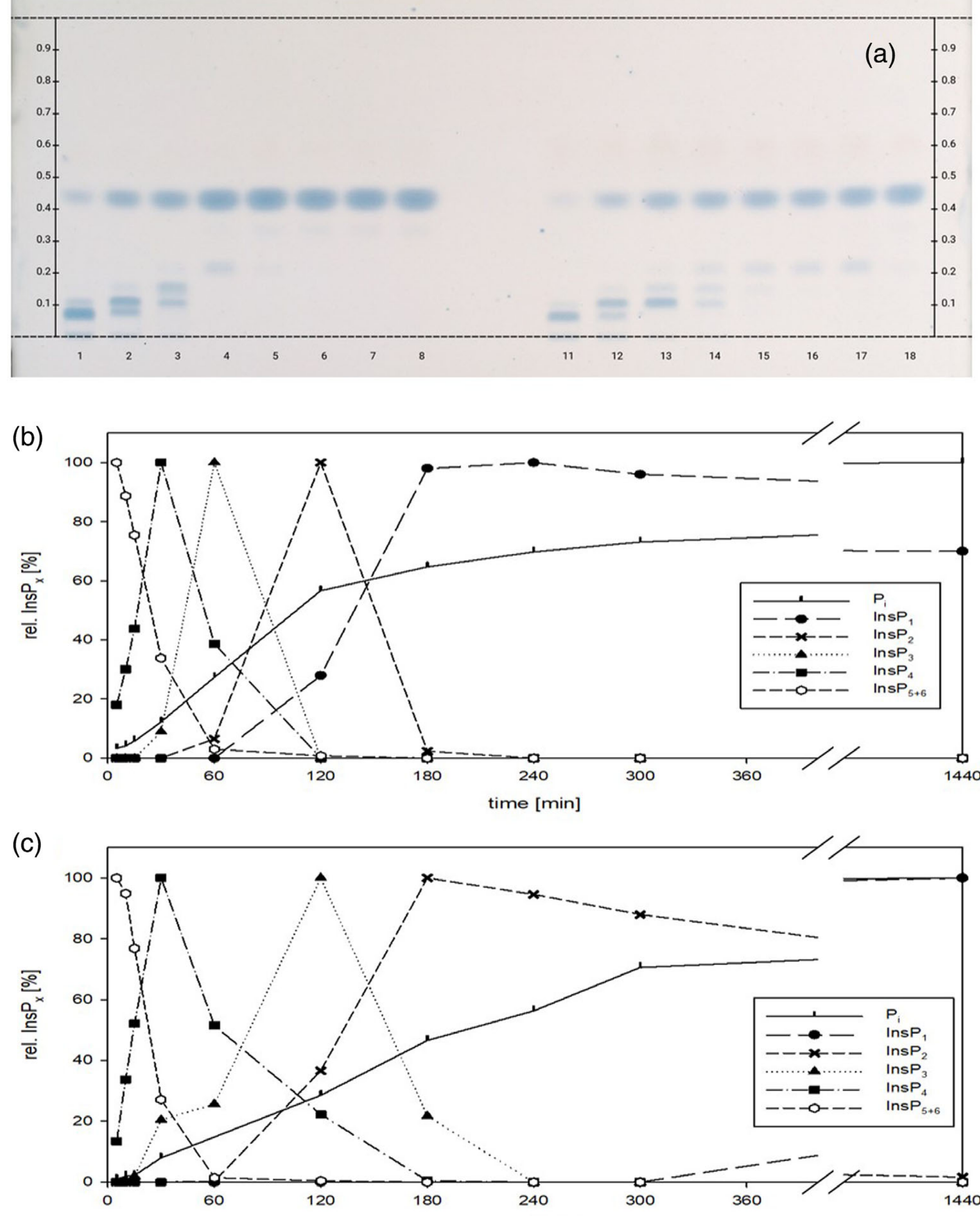

FIGURE 2 (a) HPTLC plate with $InsP_x$ fingerprint (10 U/L, 37°C) of the phytase Quantum® Blue at pH 3.6 (lanes 1–8) and pH 5.5 (lanes 11–18) at the time points 5, 30, 60, 120, 180, 240, 300 min and 24 h in ascending order (captured with Visualizer 2, VC v3.1, RemissionVis, Exposure 0.045 s, contrast 2.0). (b) (pH 3.6) and (c) (pH 5.5) show relative amounts of $InsP_x$ and P_i during enzymatic hydrolysis according to the $InsP_x$ fingerprint.

was only hydrolyzed at pH 3.6 whereby the concentration of $InsP_1$ at pH 5.5 increased continuously until the hydrolysis was terminated after 24 h. In contrast to the *E. coli* wild-type based phytases, the phytic acid hydrolysis deploying Axtra® PHY required comparatively longer to degrade $InsP_4$, $InsP_3$, and $InsP_2$, indicating a lower substrate specificity for lower inositol phosphates. At pH 3.6, traces of $InsP_4$ and a majority of $InsP_3$ and $InsP_2$ were still present after 24 h. At pH 5.5, $InsP_4$ and $InsP_3$ were completely degraded and traces of $InsP_1$ could be detected after 24 h. A summary of the $InsP_x$ degradation patterns for different commercial phytases is shown in Table 2.

A general accumulation of $InsP_1$ for commercial phytases could not be observed. At pH 3.6, Quantum® Blue and Enzy Phostar partially degraded $InsP_1$. This is in contrast to the previous assessment that phytases are only able to partially release phosphate groups from phytate.[23,24] The results of the $InsP_x$ fingerprint indicate that inositol monophosphate can be further hydrolyzed by phytases albeit at a very low rate. Furthermore, during the HPTLC method development, Quantum® Blue was applied to hydrolyze two $InsP_1$ standards (Ins(3)P_1 and Ins(1)P_1). Both standards were detected via the liberated free phosphate after the enzymatic hydrolysis using the molybdate

TABLE 2 $InsP_6$ degradation products produced by Quantum® Blue, Axtra® PHY, and Enzy Phostar at pH levels 3.6 and 5.5 evaluated as relative amounts.

		$InsP_x$ [%] at pH 3.6						$InsP_x$ [%] at pH 5.5					
	Time [min]	$InsP_{5+6}$	$InsP_4$	$InsP_3$	$InsP_2$	$InsP_1$	P_i	$InsP_{5+6}$	$InsP_4$	$InsP_3$	$InsP_2$	$InsP_1$	P_i
Quantum® Blue	5	100	18				3	100	13				1
	30	34	100	9			12	27	100	20			8
	60	3	39	100	7		27	1	51	26			17
	120	1			100	28	57		22	100	37		29
	180				2	98	65			22	100		47
	240					100	70				95		56
	300					96	73				88		71
	1440					70	100				2	100	100
Axtra® PHY	5	100					3	100	5				7
	30	80	34				9	45	100	10			20
	60	28	100	4			25	7	94	33			37
	120	3	73	41	1		50	6	58	59	2		48
	180	3	41	63	5		57	5	31	100	30		71
	240	2	20	73	16		72	3	7	92	74		75
	300		11	100	45		89	2		81	100		77
	1440		1	67	100		100				62	100	100
Enzy Phostar	5	100	43				9	100	78	7			27
	30	4	58	100	3		57	4	30	36	2		45
	60			13	100		72	3	20	100	14		53
	120				50	34	89	1		25	79		77
	180				45	39	94				100	10	83
	240				43	100	95				99	24	85
	300				43	50	96				88	35	89
	1440				38	43	100				68	100	100

reagent, indicating the enzymatic hydrolysis of $InsP_1$ at least by Quantum® Blue 5G.

Different studies have dealt with the degradation of phytic acid; however, enzymatic hydrolysis was often performed only at one pH level or a continuous assay was used.[5,16] Pontoppidan et al.[25] used a similar approach for an in vitro evaluation of enzymatic $InsP_6$ degradation by a phytase from *Citrobacter braakii*. The hydrolysis was carried out using an enzyme activity of 1 U/mL at 37°C for a period of 2 h in a buffered system at pH 5.5 and 4.0, respectively. Inositol phosphates were analyzed by means of ion chromatography whereby isomers of $InsP_3$-$InsP_6$ and a pool of $InsP_2$ could be analyzed. Pontoppidan et al. did not detect any differences between the $InsP_6$ degradation at the two pH levels studied. This may be related to the 100-fold higher enzyme concentration in the study. The analysis of the $InsP_1$ pools was not conducted by Pontoppidan et al. Menezes-Blackburn et al.[23]conducted a study to compare commercially available phytases based on their biochemical properties. The application of seven phytases using different initial phytase activities ranging from 0 to 1000 U/kg animal feed was assessed in an in vitro assay. Menezes-Blackburn et al.[23] analyzed the $InsP_x$ composition at the end of the in vitro assay and observed an incomplete $InsP_x$ degradation for all commercially available phytases. In the present study, a fast degradation of $InsP_6$ was observed. However, the degradation rate for lower $InsP_x$ differed between the commercial phytases as well as between pH 3.6 and 5.5 (Table 2). The resulting accumulations of the lower inositol phosphates may be caused by reduced degradation rates of lower inositol phosphates, for example, $InsP_4$. The different observations may be explained by the application of phytic acid in our experiments, which does not contain any inhibitors compared with the study from Menezes-Blackburn et al.[23]

Phosphate release

To evaluate the efficiency of the commercial phytases, the released phosphate was quantified and referenced to the maximum amount of phosphate released after acidic hydrolysis in 0.5 M HCl at 100°C for 5 days (Figure 3). Despite the standardized enzyme activity of 10 U/L, the increase in phosphate content over time differs for the individual phytases due to the varying substrate affinities to lower inositol phosphates.

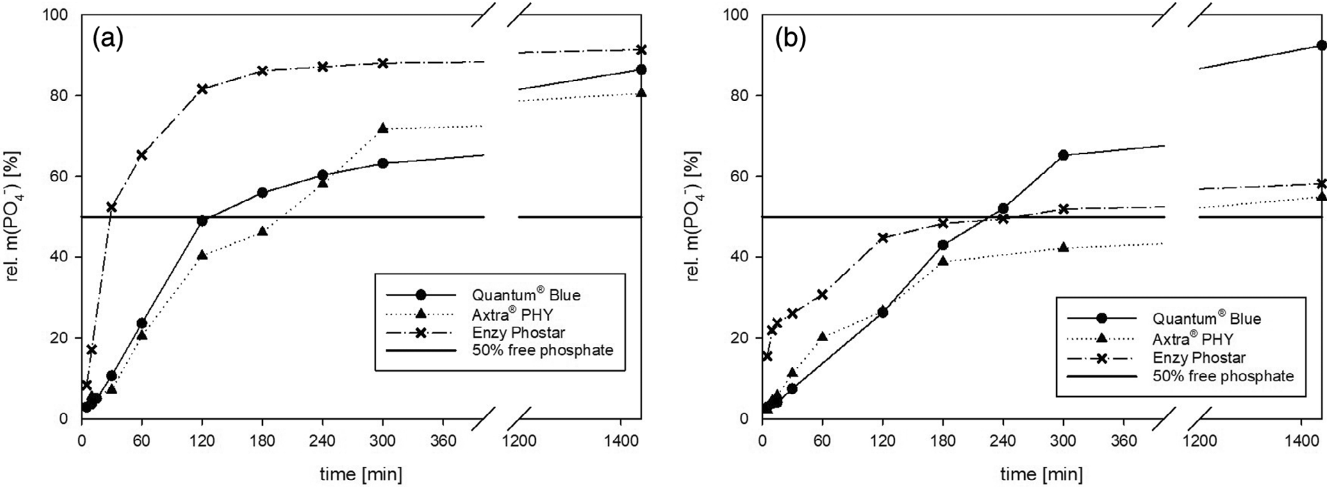

FIGURE 3 Phosphate release during phytase (EA of 10 U/L[1]) hydrolysis of phytic acid (1.66 g/L) at pH 3.6 (a) and pH 5.5 (b) respectively over 24 h relative to the total phosphate content for the phytases Quantum® Blue, Axtra® PHY and Enzy Phostar.

In general, the phosphate content increases more slowly during the hydrolysis at pH 5.5 compared with the hydrolysis at pH 3.6. At pH 5.5, Quantum® Blue and Axtra® PHY initially released phosphate at a similar rate, with Axtra® PHY slowing down after 3 h while hydrolyzing $InsP_4$ and $InsP_3$ and releasing ~55% of the total phosphate after 24 h. Quantum® Blue reached the 50% phosphate release mark after 4 h of hydrolysis time and achieved a P_i liberation of ~92% in total. Similar to Quantum® Blue, Enzy Phostar released phosphate initially with the highest rate, also reaching 50% free phosphate after 4 h. Subsequently, the phosphate release proceeded more slowly and a total of ~58% phosphate was released.

During hydrolysis at pH 3.6, Quantum® Blue and Axtra® PHY released phosphate at comparable rates. The 50% mark was reached after 2 and 3 h and a total of ~80% and ~ 86% of the total phosphate was released, respectively. Enzy Phostar released 50% of the total phosphate within the first 15 min. After 2 hours, the phosphate content had increased to ~80%, after which the phosphate liberation slowed down. Enzy Phostar was able to release ~90% of the total phosphate amount after 24 h.

Other studies often focused on the decrease in phytic acid content to evaluate the suitability of a phytase as a feed additive. However, for an initial evaluation of the ability of phytases to degrade phytic acid and thus releasing phosphate, quantification of free phosphate during in vitro assays seems to be more appropriate. Menezes-Blackburn et al.[23] focused on the phytase activity needed to achieve a 50% reduction in $InsP_6$ and an increase in P_i of 50% of the maximum reachable value, showing that 3–10 times higher phytase doses are needed to release 50% P_i compared with decreasing $InsP_6$ content by 50%.

CONCLUSION

The presented HPTLC method is able to simultaneously separate $InsP_{5+6}$ to $InsP_1$ as well as free phosphate. A baseline separation of P_i, $InsP_1$, $InsP_2$, and $InsP_3$ has been established, whereas $InsP_4$ was quantified by peak splitting and $InsP_{5+6}$ were quantified as a sum parameter. The combination of enzymatic hydrolysis and phosphate staining makes the method specific to inositol phosphates. Our results show that the HPTLC method is suitable for a rapid screening of inositol phosphates with a semi-high sample throughput. Accumulation of isomers can be detected as well as a quantitative phosphate release. Furthermore, it was shown that phytases are capable of completely hydrolyzing phytic acid to inositol, albeit with a very slow degradation rate of $InsP_1$. The presented HPTLC method is a useful tool for a fast, visual evaluation of phytases and their $InsP_x$ fingerprint.

ACKNOWLEDGMENTS

The authors would like to thank CAMAG® (Muttenz, Switzerland) for the assistance in the use of their CAMAG® TLC Visualizer 2 as well as Regina Brämer for assisting in the ICP-OES measurements. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

Kaesler Nutrition GmbH partially funded this work.

CONFLICT OF INTEREST STATEMENT

Kaesler Nutrition GmbH has a commercial interest in the sales of Enzy Phostar.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

ORCID

Katrin Ochsenreither https://orcid.org/0000-0002-5797-2789
Thomas Eisele https://orcid.org/0000-0002-5190-8233

REFERENCES

1. Ashley K, Cordell D, Mavinic D. A brief history of phosphorus: from the philosopher's stone to nutrient recovery and reuse. Chemosphere. 2011;84:737–746.
2. Graf E. Applications of phytic acid. JAOCS. 1983;60:1861–1867.
3. Shastak Y, Zeller E, Witzig M, Schollenberger M, Rodehutscord M. Effects of the composition of the basal diet on the evaluation of mineral phosphorus sources and interactions with phytate hydrolysis in broilers. Poult Sci. 2014;93:2548–2559.
4. Jondreville C et al. Modeling the fate of dietary phosphorus in the digestive tract of growing pigs 1. J Anim Sci. 2011;89:3596–3611.
5. Sommerfeld V, Schollenberger M, Hemberle L, Rodehutscord M. Modification and application of an in vitro assay to examine inositol phosphate degradation in the digestive tract of poultry. J Sci Food Agric. 2017;97:4219–4226. https://doi.org/10.1002/jsfa.8297
6. Lei XG, Porres JM, Mullaney EJ, Brinch-Pedersen H. Phytase: source, structure and application. Ind Enzymes. 2007;29:505–529. https://doi.org/10.1007/1-4020-5377-0_29
7. Shivange AV, Schwaneberg U. Recent advances in directed phytase evolution and rational phytase engineering. *Directed enzyme evolution: advances and applications*. Switzerland: Springer International Publishing; 2017. p. 145–172. https://doi.org/10.1007/978-3-319-50413-1_6
8. Dersjant-Li Y, Awati A, Schulze H, Partridge G. Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. J Sci Food Agric. 2015;95:878–896.
9. Marolt G, Kolar M. Analytical methods for determination of phytic acid and other inositol phosphates: a review. Molecules. 2021;26:145–172.
10. Oates K, de Borba B, Rohrer J. Determination of inositol phosphates in dried distillers grains with Solubles. 2014.
11. Skoglund E, Carlsson N-G, Sandberg A-S. *High-performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns*. 1998 https://pubs.acs.org/sharingguidelines
12. Sandberg A-S, Ahderinne R. HPLC method for determination of Inositol tri-, tetra-, Penta-, and Hexaphosphates in foods and intestinal contents. J Food Sci. 1986;51:547–550.
13. Spangenberg B, Seigel A, Brämer R. Screening of orange peel waste on valuable compounds by gradient multiple development diode-array high-performance thin-layer chromatography. JPC. 2022;35:313–330. https://doi.org/10.1007/S00764-022-00176-2
14. Spangenberg B. Quantitative Dünnschicht-chromatographie Eine Anleitung für Praktiker. 2014.
15. Malowan SL. Molybdänblau und seine Bedeutung für die analytische Chemie. Zeitschrift für Analytische Chemie. 1931;64:209–214.
16. Greiner R, Konietzny U, Jany K-D. Purification and characterization of two Phytases from Escherichia coli. Biochem Biophys. 1993;303:107–113.
17. Deutsche Norm. *DIN EN ISO 30024*. www.din.de 2009.
18. Lehrfeld J. High-performance liquid chromatography analysis of phytic acid on a pH-stable, macroporous polymer column. Cereal Chem. 1989;66:510–515.
19. Hatzack F, Rasmussen SK. High-performance thin-layer chromatography method for inositol phosphate analysis. J Chromatogr B Biomed Sci Appl. 1999;736:221–229.
20. Guiochon G, Siouffi A. Study of the performances of thin layer chromatography II. Band broadening and plate height equation. J Chromatogr Sci. 1978;16:470–481.
21. Poole CF. The essence of chromatography. Detroit, MI, USA: Elsevier Science Ltd; 2003.
22. Sommerfeld V, Santos RR. In vitro assays for evaluating phytate degradation in non-ruminants: chances and limitations. J Sci Food Agric. 2021;101:3117–3122.
23. Menezes-Blackburn D, Gabler S, Greiner R. Performance of seven commercial Phytases in an in vitro simulation of poultry digestive tract. J Agric Food Chem. 2015;63:6142–6149.
24. Hirvonen J, Liljavirta J, Saarinen MT, Lehtinen MJ, Ahonen I. Effect of Phytase on in vitro hydrolysis of Phytate and the formation of myo-inositol phosphate esters in various feed materials. J Agric Food Chem. 2019;67:11396–11402. https://doi.org/10.1021/ACS.JAFC.9B03919
25. Pontoppidan K, Glitsoe V, Guggenbuhl P, Quintana AP, Nunes CS, Pettersson D, et al. In vitro and in vivo degradation of myo-inositol hexakisphosphate by a phytase from Citrobacter braakii. Arch Anim Nutr. 2012;66:431–444.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Henninger C, Spangenberg B, Schmidt M, Ochsenreither K, Eisele T. Enzyme-assisted HPTLC method for the simultaneous analysis of inositol phosphates and phosphate. JSFA Reports. 2023;3(4):170–9. https://doi.org/10.1002/jsf2.109