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A New Rapid and Quantitative Assay to Determine the Phytase Activity of Feed

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Cite This: *ACS Omega* 2022, 7, 5292−5299

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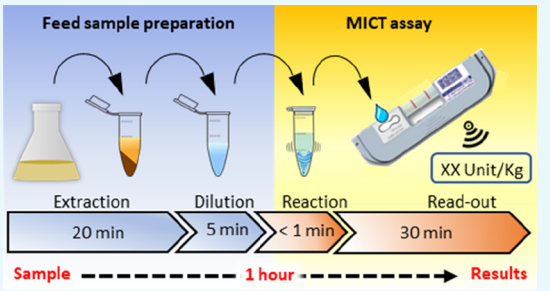
ABSTRACT: The fortification of animal feed with enzymes in order to optimize feed utilization has become a standard for the meat production industry. A method for measuring levels of active enzymes that can be carried out quickly would ensure that feed has been supplemented with the appropriate amount of enzyme. Phytase is the most widely used feed enzyme and is routinely quantified with an activity assay in a limited number of specialized laboratories. As an alternative, we report here the development of a rapid and easy method to perform a quantitative assay for the phytase from *Citrobacter braakii*. The method is suitable for use at local sites with a minimum lab setup and will reduce delays and potential interferences due to improper sample storage and shipment. The new assay is based on a lateral flow immunoassay that utilizes magnetic immune-chromatographic test (MICT) technology to quantify the phytase content of a feed extract. After extraction of the phytase from the feed, the sample is simply diluted and added to a reaction tube containing a specific anti-phytase antibody coupled to superparamagnetic particles. The mixture is then applied on an assay cassette, where the formed particle−antibody−phytase complexes are captured by immobilized antibodies on a nitro-cellulose strip housed in a cassette. The cassette is placed in the MICT reader that measures the magnetic signal of the captured particles. Using the calibration information stored in the cassette barcode, the signal is converted to a phytase concentration, given as phytase activity (FYT) per kilogram of feed. The accuracy and robustness of the assay compared to the ISO phytase activity assay were demonstrated through a large validation study including real feed samples from different compositions and origins. The MICT assay is the first quantitative assay for feed enzymes that is fast, reliable, and simple to use outside of a specialized reference laboratory and that is suitable for use in place of the current ISO assay.



INTRODUCTION

The fortification of animal feed with enzymes in order to optimize feed utilization is a standard for the meat production industry. Phytase is the most widely used feed enzyme,[1] and farmers increasingly rely on phytase supplementation to release phosphate from the feed phytate. As a result, the supplemented phytase activity covers more than 50% of the animals' requirement in phosphate.[2,3] Consequently, the importance of using a correct phytase amount in feed has increased. For this reason, the quantification of the feed phytase content, especially after the pelleting process, which can be harmful to the enzyme, is crucial for feed producers and farmers. In addition, to avoid introducing delays and interferences in the interpretation of results (due to sample shipment and storage), a rapid and easy method that could be performed on site or locally would be of huge advantage. Phytase amounts are currently measured with the ISO activity assay (ISO 30024:2009).[4,5] In this assay, samples containing phytase are incubated with sodium phytate at 37 °C for a precise amount of time, and released inorganic phosphate is detected using a molybdate-vanadate reagent that reacts with free phosphate to produce a colored complex. A phosphate standard is prepared each time the assay is performed to calibrate the assay and thereby maximize the accuracy of the feed results. This method, while a reference, presents several important drawbacks. As is typical for activity assays, the conditions under which the phytase ISO assay is performed must be well controlled since slight changes in incubation temperature or time can influence the results. The need for well-trained personnel as well as standardized equipment makes the ISO assay impractical for routine use in many feed laboratories. Due to the time required to submit samples to a qualified lab for testing and to receive the results, use of the ISO assay limits

Received: November 17, 2021
Accepted: January 18, 2022
Published: February 3, 2022

the nutritionist's or feed mill manager's ability to verify the level of active phytase in the feed after pelleting. Moreover, this assay is not specific to a phytase of interest and several interferences can occur. Factors that limit the accuracy of the ISO assay include the forms and purity of the phytate that serves as a substrate in the assay and the presence of compounds in the feed sample that interfere with the assay. Phytate has six phosphate groups, and phytases hydrolyze the phosphate bonds in a stepwise manner. Upon cleavage of each phosphate, various phytate degradation products are generated that also serve as substrates but that are hydrolyzed at different rates by the phytase.[6−8] As the ISO assay progresses, a complex mixture of substrates develops, which can affect the rate of phosphate release and skew the assay result. Additionally, commercial sources of phytate can be contaminated with varying amounts of partially hydrolyzed phytate and free phosphate that can likewise affect the accuracy of the assay result.[9,10] The presence of other phosphatases and sources of phosphate in the feed extract also needs to be considered when using the ISO assay as they can considerably vary between samples. Alternative assays such as antibody-based assays for measuring specific commercial phytases in feed are available. An ELISA-based assay was developed to determine an engineered *Escherichia coli* phytase in feed. A semiquantitative but easy-to-use lateral flow assay is also used for the same enzyme.[11] Quantitative lateral flow immunoassays provide a convenient and cost-effective mean to determine the concentration of a specific protein in complex liquid samples and are used widely in medical and veterinary diagnostics.[12] The MICT assay platform, which is based on a calibrated single-use lateral flow assay device and a small benchtop reader, relies on superparamagnetic nanoparticles for signal generation and detection and on advanced flow features for improved sensitivity and reproducibility compared to other lateral flow devices.[13] This paper presents the development of a rapid and easy method based on the magnetic immunochromatographic test (MICT) platform for the specific quantification of a supplemented phytase from *C. braakii* activity in feed samples. This is, to our knowledge, the first example of a commercially available lateral flow immunoassay for the quantitative determination of a feed enzyme. We showed that the assay can be conducted without the need for advanced laboratory facilities or extensive training and provides reliable results, independent of feed composition, both within an hour after starting the assay and from hours to weeks afterward.

RESULTS AND DISCUSSION

Specificity of the Antibody Used in the MICT Enzyme Assay. A key step in the development of the MICT assay was to verify that the used rabbit polyclonal antibody was specific to the enzymatically active phytase from *C. braakii*. For this purpose, mash feed samples were processed into pellets under significantly harsher conditions (60 s at 100 °C) than normal to obtain samples with attenuated phytase activity. The mash samples were used as reference for samples with full phytase activity. The active enzyme content of those samples was determined using both the Ab-based method (ELISA format) and the ISO phytase activity assay.[4] As shown in Figure 1, the enzyme content determined by the ELISA method agreed well with the activity assay, underlying the specificity of the antibody toward the active form of the phytase. The observed decrease in the activity and antigenicity of denatured phytase in pellet samples reflects the mutual dependence of catalysis and antibody binding on the enzyme structure. Extensive denaturation of enzymes can lead to further loss of activity by rendering the enzyme insoluble under experimental and physiological conditions.

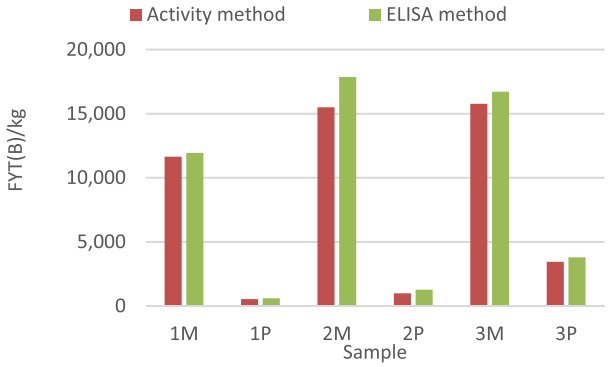


Figure 1. Comparison of phytase activity assay and immunoassay determinations of phytase content of feed samples. Three different feed samples were fortified with phytase and were processed as mash (M) and pelleted feed under harsh conditions (P) to inactivate the enzyme. The results for duplicate determinations of mash and pellet are shown.

The cross-reactivity of the anti-phytase antibody with other phytases was investigated by testing other commercially available phytase products in the MICT assay. The MICT assay did not report measurable phytase with any of the products at concentrations equivalent to 2000 FYT/kg feed (data not shown). Likewise, the MICT assay did not detect endogenous plant phytase in any of the feed types tested (data not shown).

Calibration of the MICT for Phytase Quantification. The assay device was calibrated by testing samples of known phytase concentration with conjugate and cassettes as described in the method. Plots of the calibration data for samples within the reportable range are shown in Figure 2. Polynomial regression analysis by the least absolute deviation method was applied to each calibration plot and, to assess assay performance, the equations of the curves were used to back-calculate calibration sample concentrations. Replicate concentrations were averaged, and accuracy and precision were calculated for each sample. Average sample accuracy was 100% with a range from 96 to 103% and precision CV was ≤10% within the reportable range of the assay (Table 1). The dynamic range of the calibration curves (0.0005−0.025 FYT/mL, Figure 2) established the detection limit (100 FYT/kg) and upper limit (5000 FYT/kg) of the assay.

Characterization of the MICT Devices (Read Time Window, Hook Effect, and Shelf-Life). Testing was done to determine the time windows during which the Rapid and Standard reads may be made. Assay devices were run with a control sample, and the cassettes were read repeatedly starting at 25 min after addition of the sample/conjugate mixture to the cassette. For the Rapid read, the change in concentration did not exceed 1% for reads from 25 to 32 min, while the Standard read result was essentially unchanged after 3 h (Table 2). Based on these results and the results from the calibration testing, a Rapid read window of 30 ± 2 min and a Standard read window of 4 h to 14 days were established.

Immunoassays can experience a hook effect that occurs when analyte concentrations exceed the binding capacity of the

https://doi.org/10.1021/acsomega.1c05917
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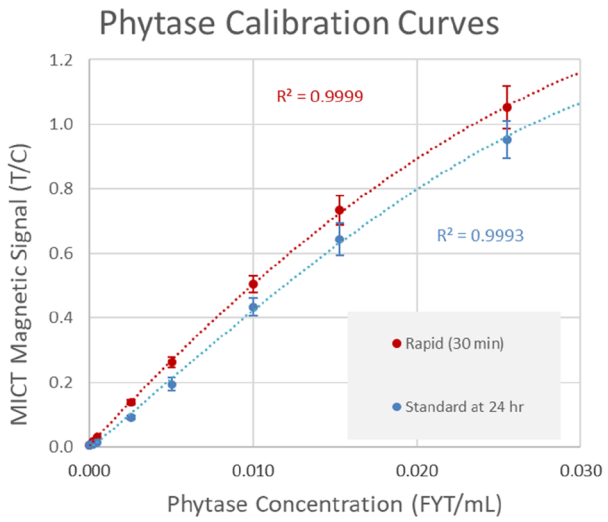

Figure 2. Response of the MICT assay signal to enzyme concentration and read time. The MICT device was calibrated by testing buffer-based samples of known phytase concentration and reading the assay cassettes 30 min and 24 h after starting the assay. Each sample was tested on replicate devices and testing was done over multiple days. The average result was plotted against the concentration of each sample and fitted with a polynomial curve. Separate curves were generated from the 30 min (Rapid read, $y = 0.0084x^3 - 0.0042x^2 + 0.0197x - 0.00012$) and 24 h (Standard read, $y = 0.0195x^3 - 0.0217x^2 + 0.029x + 0.00010$) data. The resulting calibration curve data were stored in the barcode of the MICT assay cassette and used by the reader to determine the phytase concentration of an unknown sample.

Table 1. MICT Assay Accuracy and Precision Calculated from Calibration Data[a]

	MICT assay calibration results			
	rapid read		standard read	
#	recovery	CV	recovery	CV
lot 1	100%	8%	100%	8%
lot 2	100%	9%	100%	10%
lot 3	100%	9%	100%	9%
average	100%	9%	100%	9%

[a]The Rapid and Standard read calibration curves derived from the data summarized in Figure 1 were used to back-calculate the concentration of the calibration sample replicates; accuracy was determined by dividing the average calculated concentration by the gravimetrically assigned concentration and multiplying by 100%; precision was expressed as the coefficient of variation (CV), which is calculated by dividing the standard deviation by the average of the calculated concentrations. For samples in the 0.00046 to 0.026 FYT/mL concentration range, the accuracy was 98−102% and the precision was ≤10% for both read types; repeating the Standard read at 14 days gave equivalent results.

assay and lead to a drop-in assay signal.[14,15] To determine the effect of excess free enzymes binding to the antibody on the assay result, control samples with high phytase concentrations were prepared (0.3 and 1.0 FYT/mL) and run on the device. No drop of signal was observed, even for a phytase concentration 40-fold higher than the assay reportable range, indicating that the hook effect should not be a concern. (Supporting Information, Table S1).

The stability of the cassettes was tested over time. Test devices showed an 18 month shelf-life when stored at 2−8 °C without a significant impact on recovery or precision (Supporting Information, Tables S2 and S3).

Comparison of MICT and ISO Assays in Buffer. Control samples were prepared in the ISO assay buffer at phytase concentrations that covered the reportable range of the MICT assay (see the methods section). Each sample was made in duplicate and analyzed by the MICT and ISO assays. The assay results and linear regression analysis are shown in Figure 3. A slope of 0.99, a coefficient of correlation (R^2) of 0.998, and a mean absolute error (MAE) of 53 indicate excellent agreement between the two methods.

Comparison of MICT and ISO Assays in Real Feed Samples. The MICT and ISO assays were also compared in a ring-test-style study using feed samples supplemented at six different phytase concentrations by adding the formulated phytase from *C. braakii* to a typical European diet and processing the samples into mash and pelleted feed forms. The samples were sent to two laboratories where they were extracted and analyzed using the same procedures. For mash samples, the results of the ISO assay were corrected by subtracting the endogenous activity determined for feed samples that had not been supplemented with phytase. No activity was detected in pellet samples prepared without phytase. With the MICT assay, results for each read type were recorded and analyzed separately. The combined 133 results from both laboratories and for both feed forms are plotted in Figure 4. A good agreement between the MICT and ISO results was found: a slope of 0.98 and a correlation coefficient of 0.94 were determined for both MICT reads. The good concordance between the two methods was confirmed by the Bland−Altman analysis (Supporting Information, Figure S1).

To compare the two MICT read types, the results for the Rapid read and Standard read at 4 h were analyzed by linear regression, which yielded a slope value of 0.99, a correlation coefficient of 0.98 when forced through zero, and a mean absolute error of 77 (data not shown). The stability of the Standard read was evaluated by repeating the Standard read at 14 days after the start of assay and comparing the results with the Standard read at 4 h: the slope of the fitted line and correlation coefficient were both 1.00 (data not shown).

Impact of Feed Composition on the MICT Assay. The influence of the feed composition on the agreement between the MICT and ISO assays was investigated by analyzing a diverse collection of commercial feedstuffs from the US, Mexico, Brazil, Great Britain, France, Germany, Denmark, and Australia for phytase content (73 samples analyzed by one lab). The results are presented in Figure 5. A correlation coefficient of 0.99 and a slope of 1 demonstrate a very good agreement between the two methods for the most widely used feed compositions.

The presence of a protease that is sometimes used in feed to improve the availability of amino acids at commercial levels (i.e., 15,000 PROT of Ronozyme ProAct protease/kg feed) did not affect the MICT and ISO results (Supporting Information, Table S4).

Precision of the MICT Assay with Feed Samples. Data from the analysis of the control feed samples prepared for the ring test were used to compare the within-day variation (repeatability), day-to-day variation (intermediate precision), and laboratory-to-laboratory variation (reproducibility) of the MICT and ISO assays. The results for both assays were comparable (Table 3). As the coefficients of variation for repeatability were only slightly lower than the values for

https://doi.org/10.1021/acsomega.1c05917
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Table 2. Effect of Read Time and Read Type Selection on MICT Result[a]

MICT phytase assay result, FYT/mL						
25 to 35 min				1 to 24 h		
read time	rapid		standard	read time	rapid	standard
25 min	0.0116		0.0135	1 h	0.0107	0.0126
26 min	0.0116		0.0135	2 h	0.0102	0.0121
27 min	0.0117		0.0135	3 h	0.0097	0.0116
28 min	0.0117	read window	0.0136	4 h	0.0098	0.0117
29 min	0.0116	↓	0.0135	5 h	0.0098	0.0117
30 min	0.0116		0.0135	6 h	0.0098	0.0117
31 min	0.0115		0.0134	7 h	0.0098	0.0117
32 min	0.0115		0.0134	24 h	0.0098	0.0117
33 min	0.0114		0.0133			
34 min	0.0114		0.0132			
35 min	0.0113		0.0132			

[a]The times over which the Rapid and Standard reads may be used were determined by repeatedly reading assay cassettes at timed intervals after adding the sample to the cassettes; for each read time, the result was calculated using the Rapid and Standard calibration curves; for readings between 28 and 32 min, the Rapid result was within 1% of the 30 min read; the Standard read results made at 4 h and later were also within 1% of the 30 min Rapid result.

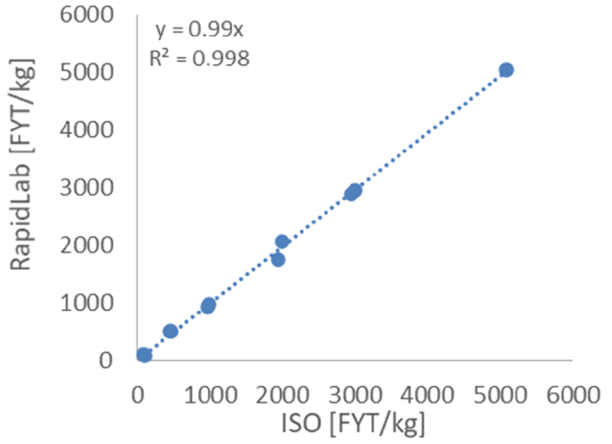

Figure 3. Acetate buffer used in the ISO assay was spiked with a phytase standard to a range of concentrations, and the samples were analyzed by the MICT and ISO assays. Samples were diluted into MICT dilution buffer prior to testing by the MICT, and the Rapid read result was recorded. The results were converted from FYT/mL to FYT/kg upon applying a feed sample dilution factor. The *y*-intercept of the regression line was set to zero to compare the dose response of the two methods. When not set to zero, the intercepts were well below the level of detection of the assays (data not shown) (n = 2).

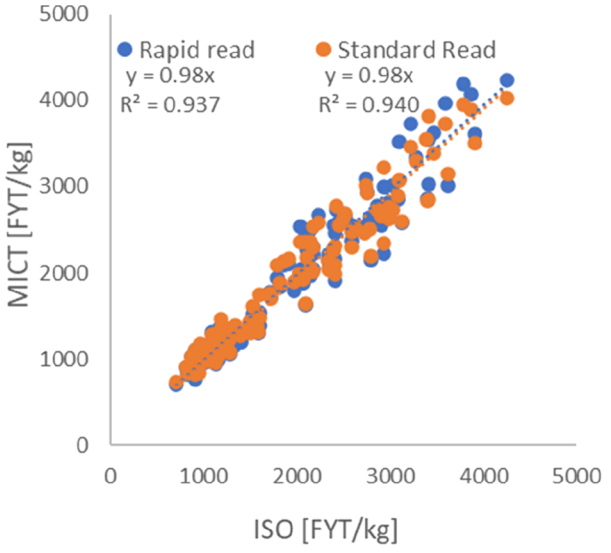

Figure 4. Determination of the phytase content of control feeds by MICT and ISO assays. Control feed samples prepared as mash and pellets were extracted, and the extracts were analyzed for phytase content both by the MICT assay and by the ISO assay. ISO results for mash samples were corrected for endogenous phytase activity by subtracting the activity determined for a feed sample prepared without phytase. MICT results were recorded for both the Rapid (blue) and Standard (orange) read types by reading assay cassettes at 30 min and again at 24 h after the start of assay. Determinations of identical samples were performed at two independent laboratories on four different days. The *y*-intercepts of the regression lines shown were set to zero to compare the dose response of the two methods. When not set to zero, the intercepts were near or below the level of detection of the assays (data not shown).

intermediate precision and reproducibility, the variability due to the use and performance of the assays contributed relatively little to the overall imprecision of the feed analyzes. The more significant source of imprecision is the variability among the samples extracted and analyzed, which can be caused by sample-to-sample differences stemming from feed inhomogeneity or an inconsistent sampling technique. The higher coefficient of variation values observed with the MICT assay can be attributed to the difference in precision of the analytical methods themselves: 10% for the MICT assay (Table 1) and 5% for the ISO assay (data not shown). Neither the phytase product formulation (M or GT) nor the feed presentation (mash or pellet) influenced reproducibility of the MICT and ISO assays (data not shown).

CONCLUSIONS

The MICT assay introduced herein makes in-feed quantitative analysis of phytase faster and easier. Neither the MICT assay device nor the reader requires calibration at the time of use, as both are calibrated by the manufacturer during production. Since the antibody used in the device is specific for the active

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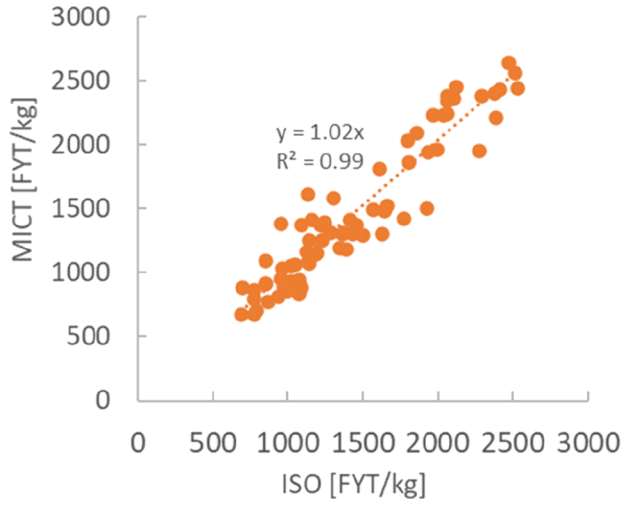

Figure 5. Determination of the phytase content of feed from worldwide sources. Different feedstuffs fortified with phytase and pelletized were received from the US, Germany, Great Britain, Denmark, France, Mexico, Brazil, and Australia and analyzed by the MICT and ISO assays. MICT results are for the Standard read. The *y*-intercepts of the regression lines were set to zero to compare the dose response of the two methods. When not set to zero, the intercepts were well below the level of detection of the assays (data not shown).

Table 3. Precision of the MICT and ISO Assays with Feed (%)[a]

precision level	MICT rapid read	MICT standard read	ISO
repeatability (within-day variation)	17.6	17.0	14.8
intermediate precision (day-to-day variation)	18.1	17.1	15.0
reproducibility (lab-to-lab variation)	18.8	18.4	16.0

[a]Data from analyses of control feed samples conducted in multiple laboratories and over multiple days were used to determine precision of the MICT and ISO assays (the same data set as presented in Figure 3); single extracts were used for analysis by both assays; MICT results were recorded for both the Rapid and Standard read types by reading assay cassettes at 30 min and again at 24 h after the start of assay; precision levels were based on the ICH Guideline Q2(R1) definition and are expressed as %.

form of phytase, the result is not influenced by endogenous phytases from the feed components or by denatured phytase that may arise during processing of the feed. The assay is performed at room temperature with basic laboratory equipment and does not require extensive training or regular maintenance, which makes it accessible for use wherever feed is produced and used. To increase the versatility of the MICT assay, the device was calibrated to report a result both within half hour of starting the assay (Rapid read) and from 4 h to 14 days afterward (Standard read). The Rapid read is most convenient when a relatively small number of samples are to be analyzed at once, whereas the Standard read is appropriate for analyzing large quantities of samples throughout the day. The performance characteristics of the MICT assay compare favorably with those of the ISO assay. The new assay is, to our knowledge, the first quantitative assay for feed enzymes that is fast, reliable, and simple to perform outside of a specialized reference laboratory, and that may be used in place of the current ISO assay. We believe that the MICT assay will allow for the improved use of phytase in the feed, which in turn will lessen the reliance on inorganic phosphate addition and increase industry sustainability. Lastly, the easy assay principle makes the platform very versatile and readily adaptable to the measurement of any other feed enzyme or biomarker of interest.

MATERIALS AND METHODS

Anti-Phytase Antibody. Purified phytase from *Citrobacter braakii* (HiPhos)[3] provided by the producer (Novozymes) was used to generate anti-phytase antibody in rabbits. The rabbit polyclonal antibody was IgG-enriched and protein A-purified prior to use. For ELISA, 96-well plates were coated with the rabbit antibody to capture phytase. Bound phytase levels were quantified using a secondary anti-phytase antibody raised in goats and bovine anti-goat IgG antibody conjugated with horseradish peroxidase. For the MICT assay device, the rabbit antibody was dialyzed into an appropriate buffer and either conjugated to carboxyl-modified superparamagnetic nanoparticles using conventional primary-amine-reactive cross-linking reagents or immobilized on a lateral flow nitrocellulose membrane using a BioDot system (Irvine, CA, USA). The cross-reactivity of the anti-phytase antibody with other commercial phytases was investigated by testing the following products in the MICT assay: different mutants of the phytase from *E. coli* expressed in *Saccharomyces pombe* (Phyzyme XP, Dupont), *Trichoderma reesei* (Quantum and Quantum Blue, ABVista), and *Pichia pastoris* (Optiphos, Huvepharma), the phytase from Buttiauxella spec. expressed in *Trichoderma reesei* (Dupont), and the fungal phytase from *Aspergillus niger* (Natuphos 5000,BASF).

MICT Assay Reader. The MICT reader has a barcode scanner, a magnetic-particle detection module, and updatable software. Upon inserting a cassette, the reader scans the barcode and retrieves the lot specific information. With the assay cassette, the reader software allows the user to select between "Feed" and "Other" sample types and between "Rapid" and "Standard" read types. Assay results are displayed on the reader screen and printout as well as stored in the reader memory. "Other" results are reported as FYT per milliliter of sample added to the assay device used with buffer-based control samples. "Feed" sample results are reported in units of FYT/kg, which is the FYT/mL value determined from the calibration curve multiplied by a conversion factor of 200,000 mL/kg. The conversion factor was calculated from the amounts of feed and water used to extract the sample and from the dilution of extract into buffer (see below). The reader is supplied with a verification cassette that, when inserted into the reader, initiates a self-test procedure to confirm a proper reader function.

MICT Assay Device for Phytase (DSM RapidLab HiPhos). As shown in Figure 6, the MICT assay device is composed of a stoppered tube of freeze-dried antibody−superparamagnetic particle conjugates and an assay cassette that contains a strip of the lateral flow membrane with immobilized antibodies. On the cassette is a 2D barcode with lot specific information such as calibration data and expiration date. The MICT assay device performs two assays in parallel: a test assay to measure phytase in the sample and a control assay to ensure that the reported phytase concentration is reliable. Both assays are initiated upon addition of the diluted feed sample extract to the tube containing a lyophilized preparation of two antibody−superparamagnetic particle conjugates.

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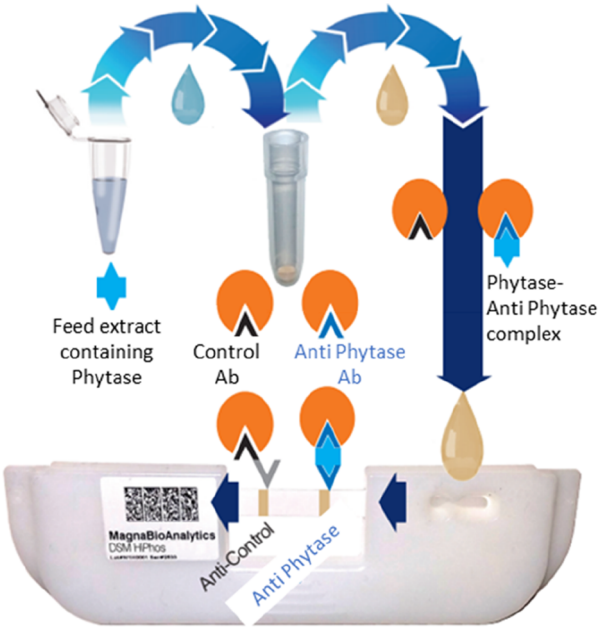

Figure 6. Schematic of the MICT use. A feed extract sample containing phytase is added to a tube containing freeze-dried superparamagnetic particle conjugates of the anti-phytase antibody (blue chevron) and control IgG (black chevron). Phytase in the sample is bound by the anti-phytase particle. Upon transfer to the assay cassette, the sample/particle mixture flows down the membrane, past lines of immobilized anti-phytase antibody and anti-control IgG antibody (gray chevron). The amount of particle bound to the anti-phytase line increases with the sample phytase concentration, whereas the particle bound to the anti-control IgG line does not. The 2D barcode on the cassette has assay calibration data used by the MICT reader to convert the measured magnetic signal to the phytase concentration of the sample

Phytase in the sample binds to the anti-phytase conjugate (Test particle); upon transfer to the cassette, the phytase−conjugate complex is captured in a line of the immobilized anti-phytase antibody (Test line), as the sample flows through the membrane. The control assay is based on the direct binding of the goat IgG-conjugate (Control particle) to the donkey anti-goat IgG antibody immobilized on the membrane (Control line). The magnetic signal from the Test line depends on the phytase concentration of the sample, whereas the Control line signal does not. The Control assay improves assay reproducibility and identifies procedural errors that could yield an inaccurate result. Measurable changes in the Control signal due to minor differences between assay devices can occur. The Test signal is likewise affected, and using a ratio of the two signals (Test/Control) improves assay device precision compared to the use of the Test signal alone. The assay device is calibrated by testing samples of known phytase concentration with conjugate and cassettes prepared with the anti-phytase antibody and measuring the magnetic signal on the assay cassette Test and Control lines using the MICT reader. The calibration samples are prepared by dissolving and diluting phytase standard with known activity determined by the ISO assay in MICT dilution buffer (PBS with 0.5% BSA and 0.023% Brij) and using weight measurements to calculate the phytase concentration. Assay devices are dual calibrated to enable the reporting of results both shortly after initiating the assay (Rapid) as well as hours after (Standard) the transfer of sample to the cassette.

Control Feed Samples. A diet, composed of 624 g/kg of wheat meal, 275 g/kg of soybean meal, 50 and 30 g/kg of soybean oil, 51 g/kg of corn gluten meal, and 20 g/kg of a commercial premix, was supplemented with phytase from *C. braakii* to prepare positive control samples for determining MICT assay performance. For precision testing, a formulated and salt-coated *C. braakii* phytase (Ronozyme HiPhos 20,000 (GT), batch HK930006, 20,000 FYT/g) and a *C. braakii* phytase of different formulation and coating (Ronozyme HiPhos (M), batch HK805009, 50,000 FYT/g) were added at 1000, 2000, and 3000 FYT/kg. A feed sample without added phytase served as the negative control sample. The control feed samples were prepared both as mash and in pelleted form. To obtain samples with attenuated phytase activity, some mash samples were processed into pellets under unusually harsh heat conditions (100 °C for 60 s in a pilot facility). The enzyme content of each sample was determined by the ISO method of Gizzi et al. For mash samples, the ISO assay result for the negative control sample was subtracted from the results for the samples supplemented with phytase. In this way, the results could be directly compared to the MICT results, for which the activity of the feed phytases is not included. Phytase product and samples were stored at 4−8 °C prior to use. Commercial feeds containing *C. braakii* phytase were obtained from producers in eight countries (Brazil, Mexico, United States, France, Great Britain, Denmark, Germany, and Australia) representing four different continents. Pelleted samples of these regional feed mixtures, for monogastric animals, were analyzed by the MICT and ISO assays to verify the correlation of the two methods with diets that are relevant in the field.

Extraction of Feed Samples. Feed (100 g) is added to a 1 L Erlenmeyer flask followed by the addition of distilled water (1.0 L), 0.500 mL of 20% Tween-20 solution, and an egg-shaped stir bar (50 × 20 mm). The flask is mixed on a magnetic stirring plate for 20 min at 600−700 rpm. Two mL of extract is removed and spun for 3 min at 14,000 rpm in a microcentrifuge to pellet and remove undissolved feed components. All steps are conducted at room temperature. The same clarified extract is analyzed for phytase by MICT and ISO assays as described below.

Analysis of Feed Extract by MICT Assay. Extract is diluted 20-fold (50 μL + 950 μL) with PBS containing BSA, Brij, and the preservative provided in the MICT assay kit. A pouched assay device from the kit is opened, the conjugate tube and cassette are taken out, and the conjugate tube stopper is removed. A pipettor is used to add 100 μL of the diluted extract to the conjugate tube, mix briefly, and then transfer the mix to the cassette sample well. The cassette is placed in the MICT reader either 28−32 min (Rapid read) or 4 h−2 weeks (Standard read) after transfer of the conjugate mix, and the read is initiated. All steps are conducted at room temperature. The reportable range of acceptable precision was found to be from 100 to 5000 FYT/kg feed.

Analysis of Feed Extract by ISO Phytase Activity Assay. Phytase activity of extracts was measured according to the ISO assay described by Gizzi et al.

Precision of MICT Assay with Feed Samples. Repeatability (within-day variation), intermediate precision (day-to-day variation), and reproducibility (between-laboratory variation) were determined by analyzing a set of control feed samples at three independent laboratories. The samples were prepared as described above using a single feed composition fortified with the phytase product at one or

https://doi.org/10.1021/acsomega.1c05917
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more dosages. Each sample was analyzed in triplicate on four different days using both the "Standard" and the "Rapid" read types. The data were pooled, and the three levels of precision were calculated based on the ICH Guideline Q2(R1) using standard statistic methods.

Comparison of MICT and ISO Assays. A set of buffer-based phytase control samples were prepared at 0.01, 0.05, 0.10, 0.20, 0.30, and 0.50 FYT/mL by adding the phytase from *C. braakii* extracted from its commercial product (HiPhos 20,000 (GT)) to 0.250 M sodium acetate buffer, pH 5.5, including 0.01% Tween-20. For MICT analysis, the samples were diluted into MICT dilution buffer before adding to the assay device, and the "Other" sample and "Rapid" read type were selected on the reader. For the ISO assay analysis, the above sodium acetate buffer was used for further dilutions. For comparison of the methods with real-world feed samples collected from Europe, the Americas, and Australia, one laboratory analyzed extracts of pelletized feedstuff by the MICT assay and ISO assay using the same extract for both determinations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05917.

Effects of excess enzyme on MICT assay results, within-run precision of MICT device stability data, linear regression analysis of MICT device stability data, Bland–Altman plot analysis to compare results of ISO and MICT methods in feed, interference of commercial protease in the feed extract on MICT results (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Authors are employed by the manufacturers of products described in this publication.

▽Retired

■ ACKNOWLEDGMENTS

The authors thank Marieke Svoboda, Robert Darter, and David Pratt of MagnaBioAnalytics for contributions to the development and production of the MICT assay, Pernille Miller and Birger Stjernholm Madsen of Novozymes for sample and data analyses, Philipp Liebsch, Stefan Kuehnel, and Christine Stadelmeyer of Biopract for sample analyses, and Karin Wertz and Aaron Cowieson of DSM for expert assistance in preparing the manuscript.

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https://doi.org/10.1021/acsomega.1c05917
ACS Omega 2022, 7, 5292−5299