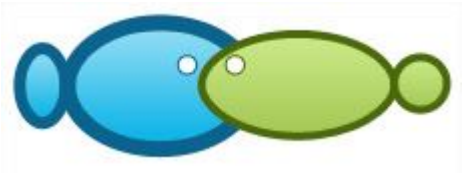

Phytate analysis and phytase application in *Jatropha curcas* kernel meal for use in aquaculture feeds

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Abstract. Phytate is the main phosphate storage molecule in plants. Phytate phosphorus is not available to monogastric animals, in addition, phytate may reduce the digestibility of proteins and minerals. Studies on different photometrical methods for phytate analysis as well as phytase application *in vitro* and *in vivo* for *Jatropha curcas* kernel meal (JKM) have been conducted with the following outcomes: 1) several frequently-used methods for phytate analysis involving the Wade-reagent as a photo indicator severely overestimate the true phytate content of JKM, predominantly because of oxalate, and should not be used despite their comparative convenience; 2) *in vitro* incubation of JKM with 2000 U kg^{-1} phytase at pH 4.5 completely eliminates phytate, as well as other inositol phosphates (IPs), from the meal. Incubation at the same pH without phytase addition also led to complete phytate eradication from the meal, but left significant amounts of IP5 and IP4 (0.43% and 0.47%, respectively); 3) feeding Nile tilapia (*Oreochromis niloticus*) with a phytase containing JKM-based diet leads to a significant reduction of about 56% of phytate, 31% of IP5 and 100% of IP4 in the feces compared to a control diet. This is associated with significantly lower P-, Ca- and Zn-content in the feces and therefore presumably better assimilation of these minerals. Though significant reductions were observed, considerable amounts of IPs were still found in feces despite phytase addition. To achieve maximum availability of minerals from JKM, phytase pre-incubation seems a necessary step.

Key Words: *Jatropha curcas*, phytate analysis, phytase, aquafeeds, mineral digestibility.

Introduction. **Introduction**. *Jatropha curcas* is an oilseed plant with increasing plantation area throughout the tropical world and the press cake left behind after oil extraction called *Jatropha curcas* kernel meal (JKM) may be used – after detoxification – as a protein-rich feedstuff with a well-balanced amino acid profile (Makkar et al 1997; Makkar & Becker 2009). However, studies conducted with fish or poultry have shown that there are limits to JKM application in feeds and these limits have been attributed to antinutritional factors, predominantly phytate (Montoya-Mejia et al 2017). Phytate (myo-inositole-hexa-kisphosphate or IP6) is the main phosphorus storage molecule in plants and their seeds (Raboy 2003), it is therefore also abundant in plant feedstuffs. Phosphorus bound to phytate is either not available or poorly utilized by monogastric animals; the concentration of phytate in most feedstuffs is proportional to its total phosphorus content (Eeckhout & de Paepe 1994).

Various authors have measured the concentration of phytate in JKM and results obtained in these studies were between 7 and 10% (Aderibigbe et al 1997; Makkar et al 1997; Martinez-Herrera et al 2006; Kumar et al 2010, 2012a, 2012b; Saetae & Suntornsuk 2011; Xiao et al 2011; Harter et al 2011; Akinleye et al 2012; Pradhan et al 2012; Saeed et al 2017). This is extraordinarily high compared to other oilseed plants or plant feedstuffs (Ravindran et al 1994). The photometric method applied in these works was developed by Latta & Eskin (1980), and refined by Vaintraub & Lapteva (1988). It involves the decoloration of a Fe^{2+}- sulfosalicylic acid complex (Wade reagent, Wade & Morgan (1953)) as the phytate replaces the sulfosalicylic acid at the binding of the Fe^{2+}. The benefits of these methods are time efficiency and simplicity, however, apart from

generating phytate values more than twice those of comparable feedstuffs, the ratio between phytate and total phosphorous (Eeckhout & de Paepe 1994) deviates strongly from typical values, calling for further studies.

This present study is structured in four parts:

1. Evaluation of different photometric methods for phytate determination: three photometric methods all based on the decolorisation of the Wade reagent are compared in order to evaluate their applicability with respect to analysis of phytate in JKM. As a reference method, high pressure ion chromatography (HPIC) is applied;

2. Identification of factors influencing the photometric determination of phytate: the content of various anions of JKM extract is measured by HPIC. The most predominant anions of the extract are evaluated in terms of interference with the Wade reagent and therefore their influence on accurate phytate analysis;

3. In vitro degradation of phytate through phytase in JKM; the degradation of phytate by phytase releases phosphate, which is thereby made available for digestion in farmed animals. It is therefore possible to minimize crystalline phosphate addition and lower phosphate release through feces to the environment, significantly decreasing environmental pollution. Commercial phytase products are established in the markets of poultry and swine farming and are also increasingly applied in aquaculture. For JKM, the efficiency of phytase in release of phosphate has not yet been demonstrated;

4. Effect of phytase application on the phytate and mineral composition of feces of Nile tilapia (*Oreochromis niloticus*) fed JKM-based diets. This trial tests whether the effects of phytase degradation observed in vitro are also valid in vivo and to what extent this has an impact on mineral composition of the feces.

Material and Method. The main work was carried out between October 2013 and March 2014 at Ahrensburg Branch of Thuenen Institute of Fisheries Ecology. Analysis of anions by HPIC was carried out at Wuppertal University and of different inositol-phosphates (IP 1 – IP 6) was carried out at Hohenheim University. Mineral analysis of feeds and feces was carried out at Institute of Aquaculture, Stirling University.

Evaluation of different photometric methods for phytate analysis

Preparation of JKM extracts. Detoxified JKM was supplied by Jatrosolutions GmbH, Hohenheim, Germany. If not stated differently, samples were treated as follows: 0.5 g of JKM was added to 10 mL of 3.5% HCl and shaken for 2 h in a magnetic stirrer at room temperature to extract all soluble anions. The sludge was centrifuged at 3 000 rpm for 10 min. An aliquot of 2 mL of the supernatant was centrifuged again at 10 000 rpm for 10 min. Subsequently, 1 mL of the supernatant (2 mL for method II) was diluted 1:5 times and this solution served as the basis for all following analysis. All extractions were made in triplicate.

Preparation of samples for different analytical methods of phytate determination

Method I (Vaintraub & Lapteva 1988): one mL of Wade reagent (0.03% $FeCl_3.H_2O$ and 0.3% sulfosalicylic acid in distilled water) was added to 0.1 mL of the sample solution and 2.9 mL of water. Samples were vortexed and centrifuged at 3000 rpm for 10 minutes.

Method II (Gao et al 2007): one g of NaCl was dissolved in the sample, vortexed and left at 4°C for 60 min. This treatment was supposed to dispose of matrix components, which might disturb the Wade reaction. The sample was then centrifuged at 10°C and 6 000 g for 10 min. One mL of the supernatant was diluted 1/25 and 3 mL was mixed with 1 mL of Wade-reagent.

Method III (Latta & Eskin 1980): the sample solution was again diluted 1:5 times and adjusted to pH 6 with 1 M NaOH. Five mL plastic syringes containing 0.5 g of anion exchange column (Dowex 1X8 200-400 (CI), Alfa Aesar GmbH, Karlsruhe, Germany) held by a glass fiber were prepared. Five mL of the sample solution were run through the column at a maximum speed of 5 mL per hour. The column was washed with 10 mL of

0.1 M NaCl solution. Phytate was subsequently extracted with 15 mL of 0.7 M NaCl solution. Three mL of the extraction solution were mixed with 1 mL of Wade reagent and analyzed in the photometer against a standard curve.

For methods I-III, absorption was measured at 500 nm in a Helios Beta photometer (ThermoScientific) measured against a standard curve containing sodium phytate (Sigma, P-8810).

Identification of factors influencing the photometric determination of phytate.

Anion and inositol phosphates (IP) analysis with High Performance Ion Chromatography (HPIC). Anions and IP were extracted from JKM with HCl (as described above). One half of the samples were adjusted to pH 6 with NaOH before analysis. Concentrations of organic and inorganic anions were analysed by ion chromatography (DX500, Dionex, Idstein, Germany) using an IonPac anion exchange column (AS11,4x200 mm, Dionex, Idstein, Germany) connected with a conductivity detector module (CD 20, Dionex, Idstein Germany). Anions were eluted with an NaOH gradient from 4 to 77% in 33 min (Lohaus et al 2000). Anion standards (containing IP6, oxalate, malate, citrate, sulfate, and phosphate) were run daily. As a reference material commercially available soybean meal was used. No standards for IP3, IP2 and IP1 were available, so these components could not be measured.

Evaluation of potential interferences of other anions with Wade-reagent. The photometric reagent was prepared (Wade-reagent, 30 mg $FeCl_3*6H_2O$ + 300 mg sulfosalicylic acid filled up with 100 mL distilled water). Series of 3 mL aqueous solutions containing 0, 20, 40, 80, 20 1and 200 µg of sodium phytate, disodium oxalate, citrate monohydrate and sodiumdihydrogenphosphate, respectively were prepared and added to 1 mL of Wade reagent. Absorptions at 500 nm were measured. A linear curve was fitted through the results.

In vitro degradation of phytate through phytase in JKM. Three treatments each with n = 3 replicates were prepared. Treatment one ("Phytase") was 0.5 g JKM spiked with 2000 U kg^{-1} phytase (Ronozyme P (L), DSM). Then 0.3 mL 2 M HCl was added to provide a pH of around 4.5 and filled up to 5 mL with distilled water. The samples were then incubated in a shaking device for 24 h at 45°C. Treatment two ("Control") was the same as "Phytase" only without the phytase. Treatment three ("JKM") was prepared the same way as "Phytase", but not incubated. Extracts were centrifuged, diluted and analyzed via HPIC. For all treatments standard samples containing 80 mg sodium phytate in 5 mL distilled water were run along with the samples.

Effect of phytase application on the phytate and mineral composition of feces of Nile tilapia (Oreochromis niloticus) fed JKM-based diets

Feeding trial. Nile tilapia, *Oreochromis niloticus* (n = 3 per treatment) were selected with a starting weight of 40 g. They were held individually in 40 L aquaria in a recirculation system at 26°C. Two diets were prepared with JKM as the main protein source: diet "Phytase" contained 2000 U kg^{-1} phytase (Ronozyme P (L), DSM), diet "Control" was of the same composition only without phytase (Table 1). In both diets, deficient amino acids were complemented by adding crystallized amino acids. Fish were fed daily ratios of 5 times their metabolic maintenance requirement calculated according to Richter et al (2002). After one week acclimation period for the fish, freshly deposited feces were collected by siphoning (typically within 5 min after defecation) every day until a sufficient amount for analysis was gathered. Feces were pooled, freeze-dried and homogenized. Phytate and other inositol phosphates in diets and feces were analyzed with HPIC.

Table 1

Composition of experimental diets fed to experimental fish

Ingredient (%)	*Phytase*	*Control*
JKM	60.4	60.4
Wheat meal	21.7	21.7
Sunflower oil	4.5	4.5
Fish oil	5.2	5.2
Cellulose	0.4	0.4
Vitamin premix[1)]	2.0	2.0
Mineral premix[2)]	2.0	2.0
Histidine	0.1	0.1
Methionine	0.8	0.8
Lysine	2.1	2.1
Threonine	0.2	0.2
Valine	0.6	0.6
Phytase (U kg^{-1})	2000	0.0
Proximate analysis (%)		
Dry matter	88.4	91.9
Crude protein	43.7	42.4
Crude lipid	11.6	10.2
Ash	9.7	10.2
Phytate	2.05	2.83
P	1.35	1.36
Ca	0.94	0.95
Mg	0.79	0.80
Zn (mg g^{-1})	0.09	0.09

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg kg^{-1}, unless otherwise stated): Vitamin A: 500 000 I.E. kg^{-1}; Vitamin D3: 50 000 I.E. kg^{-1}; Vitamin E: 2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2: 5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25 000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100 000; Inositol: 25 000; Vitamin C: 20 125; [2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg kg^{-1} mix): Calcium: 122 160; Phosphorus: 83 670; Magnesium: 14 960; Sodium: 18 180; Potassium: 210,250; Sulfur: 15 460; Chlorine: 29 720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

Statistical analysis. Statistical analysis was conducted with Statistica 8 software. For the evaluation of different photometric methods for phytate analysis, results were compared against control values measured with HPIC. Here, Dunnett´s test against a control was applied. Student´s t-test was used to detect differences between two single samples. Percentages were arcsine transformed before analysis. Statistical significance was assumed for p-values lower than 0.05. In case of multiple tests on the same data set, Bonferroni-Holm correction (Holm 1979) on p-values was applied.

Results

Evaluation of different photometric methods for phytate analysis. Figure 1 shows the results of the phytate measurement with different photometric methods. All photometric methods show significantly higher values than the control value measured with HPIC (Method I: 7.83±0.44%; Method II: 7.99±0.47%; Method III: 5.86±0.85%) and the control method (HPIC: 2.42±0.38%).

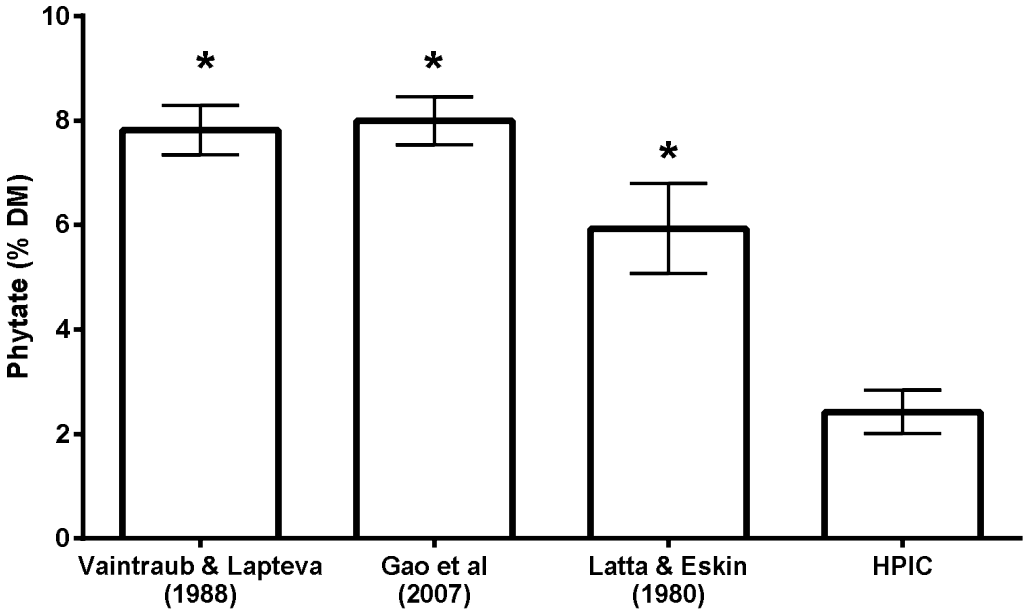

Figure 1. Phytate contents of JKM measured with different methods. All photometric methods provide significantly higher values than the control method (HPIC).

Identification of factors influencing the photometric determination of phytate. The anion composition of the JKM acid extract is shown in Table 2. The phytate, phosphate and oxalate are higher in JKM than in soybean meal, while citrate is lower and malate and sulfate are similar.

The standard curves of the Wade-reagent show a significant interaction of oxalate and citrate with the Wade-reagent (Figure 2). The slope of the oxalate curve is similar to the slope of phytate, while the slope of citrate is lower. Phosphate concentration had a non-significant influence on absorption of the Wade-reagent, malate and sulfate did not show any effect at all.

Table 2

Anion content of JKM and soybean meal acid extract (values in %)

	JKM	*Soybean meal*
Phytate	2.58	0.63
Phosphate	1.12	0.29
Citrate	1.79	2.95
Malate	0.40	0.42
Sulfate	0.46	0.40
Oxalate	2.44	0.20

Samples measured with HPIC.

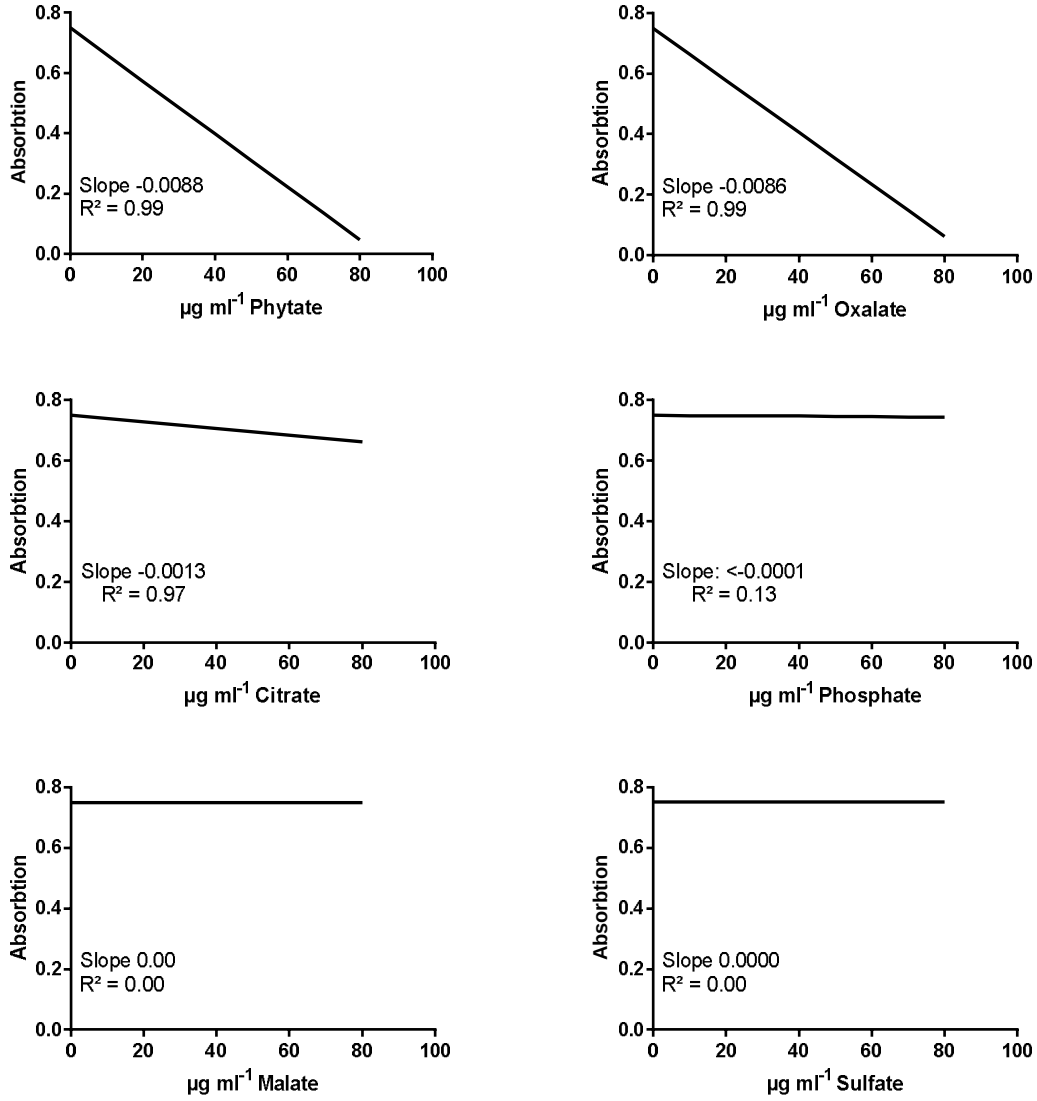

Figure 2. Interference of selected anions of JKM-extract on absorption of the Wade-reagent.

In vitro degradation of phytate through phytase in JKM. *In vitro* results show that untreated JKM-extract contains 2.42% phytate and 0.57% IP5 (Table 3), but no IP4. Samples incubated with phytase did not contain any IPs. Incubation of JKM without phytase (control) reduced the phytate content to zero, but increased the content of IP4. Available P content was increased from 0.17% in untreated samples to 0.37% in control and 1.14% in incubated samples.

Table 3

IP-values of acid extract of JKM with no incubation ("JKM") or after *in vitro* treatment with ("Phytase") and without phytase ("Control", values in %)

Treatment	*IP4*	*IP5*	*IP6*	*IP-P*[1)]	*Available P*[2)]	*IP-P + Available P*
JKM	-	0.57	2.42	0.84	0.17	1.01
Phytase	-	-	-	-	1.14	1.14
Control	0.47	0.43	-	0.24	0.37	0.61

[1)] IP-P determined with following formula: IP-P (%) = IP6 (%) * 0.282 + IP5 (%) * 0.274 + IP4 (%) * 0.264;
[2)] Available P measured as inorganic phosphate (PO_4^{3-}) in the JKM-extract.

Effect of phytase application on the phytate and mineral composition of feces of Nile tilapia (Oreochromis niloticus) fed JKM-based diets. Table 4 shows the composition of IP4, IP5 and IP6 in feces fed the two different experimental diets to Nile tilapia. Fish fed the phytase containing diet had significantly lower IP4, IP5 and IP6 values than control fish. This was accompanied by lower content of minerals in the feces, phosphorus was reduced by 19.1%, calcium by 9,0% magnesium by 7.6% and zinc by 19.2% (Table 5).

Table 4

IP-concentration in feces of Nile tilapia fed diets with ("Phytase") and without phytase ("Control", values in µmol g^{-1})

	IP6		*IP5*		*IP4*	
Control	12.0±0.79	p' = 0.007	4.60±0.07	p' = 0.018	0.17±0.06	p' = 0.045
Phytase	5.27±0.80		3.18±0.37		0.00±0.00	

p' = p-values with Bonferroni-Holm correction for multiple comparisons (Holm 1979). p' values lower than 0.05 indicate statistically significant differences between samples.

Table 5

Feces mineral analysis of Nile tilapia fed JKM-based diets with ("Phytase") and without phytase ("Control")

	P (%)	*Ca (%)*	*Mg (%)*	*Zn (‰)*
Control	3.61±0.32	7.14±0.29	0.66±0.14	0.78±0.09
Phytase	2.92±0.29	6.50±0.27	0.61±0.03	0.63±0.03
	p' = 0.176	p' = 0.176	p' = 0.570	p' = 0.176

p' = p-values with Bonferroni-Holm correction for multiple comparisons (Holm 1979). p' values lower than 0.05 indicate statistically significant differences between samples.

Discussion

Evaluation of different photometric methods for phytate analysis. The amount of phytate (myo-inositol-hexa-kisphosphate, IP6) in JKM was determined using three photometric methods and those were compared to a reference method using HPIC. The photometric methods originated from Vaintraub & Lapteva (1988), Latta & Eskin (1980) and Gao et al (2007). It was shown that the photometric methods overestimate the true phytate content by 400% (Vaintraub & Lapteva 1988; Gao et al 2007) and 250% (Latta & Eskin 1980) respectively. These photometric methods are thus not appropriate for determining phytate in JKM. Previous results, as considered in the introduction, therefore require correction for true phytate content. Overestimation of phytate has been documented before, however, the problems were associated with lower IPs interfering with correct analysis (Lehrfeld & Morris 1992). Lower IP5 and lower IPs also interfere with the Wade-reagent (Rounds & Nielsen 1993). Lower IPs do not have the negative effect on nutrient absorption resulting from IP6 (Lönnerdal et al 1989) and therefore need to be omitted from measurement if an accurate impact of phytate content on nutrient availability is to be assessed. Naturally 97% of all IPs are present in form of IP6 (Kasim & Edwards 1998) and only a small percentage as IP5. Processed plant meals have higher percentages of IP5, but also contain no IP4 or lower IPs (Kasim & Edwards 1998). This was confirmed by our measurements of JKM-extract (Table 2), which contained 2.42% of IP6 and 0.57% of IP5 and no lower IPs.

Identification of factors influencing the photometric determination of phytate. It was shown that the cause of phytate overestimation with methods involving the Wade-reagent lies in other anions present in the JKM-extract, predominantly oxalate. The equation for oxalate decoloration of the color-complex of the Wade reagent shows the same slope as does phytate. The interference of citrate with the Wade reagent is less pronounced. It is known that iron (Fe^{2+}) forms insoluble complexes with oxalate in

solutions (Noonan & Savage 1999; Savage et al 2000). The magnitude of the error of a phytate determination involving the Wade-reagent therefore depends on the concentration of these compounds in the respective plant meal. For example, soybean meal only contains about 10% of oxalate compared to JKM and therefore the error deriving from this substance will be less for this meal. Citrate content is relatively equal in both meals, but due to its lower affiliation to iron has a smaller effect on measurement accuracy. Unlike Vaintraub & Lapteva (1988), we found no influence of phosphate on the decoloration of the Wade reagent, possibly due to different concentration ranges tested. Generally speaking, it may be said that despite their practicability the photometric methods involving the Wade reagent are prone to errors because of other anions binding to Fe^{2+} of the Wade reagent and therefore this method should only be applied if the concentration and their degree of influence on the analysis is known. Although severely interfering with the phytate analysis, oxalates themselves did not significantly affect growth of carp or tilapia (Krome et al 2014; Focken et al 2015).

In vitro degradation of phytate through phytase in JKM. Phytase addition is a well established and cost-effective method to improve protein digestibility and mineral availibility in plant-based feeds for pigs and poultry, however, results in fish are not yet conclusive (Kumar et al 2012a, b; Lemos & Tacon 2017) and specific studies in JKM are missing so far. JKM was incubated in a phytase (2000 U kg^{-1}) solution to determine to what extent phytase pretreatment may reduce phytate content. After 24 h incubation, there was no phytate or other IPs left in the samples. Nwanna et al (2007) pre-incubated a diet made of various plant-based feedstuffs at 40°C for 15.5 h and found phytate to be reduced from 0.41% to 0.02% while an incubated non-phytase control reduced phytate content only to 0.25%. In the present experiment, even the non-phytase treated control diet had no phytate left, however, there were considerate amounts of IP5 and IP4 left in the sample. IP-P plus available P should be equal in all treatments. While with some variability attributable to the measurement technique, this could be considered true for treatments “phytase” (1.14%) and “JKM” (1.01%). For “Control”, total P was lower (0.61%). The amount of available phosphorus only slightly increased in this treatment due to incubation (0.37%) compared to the untreated treatment “JKM” (0.17%). It is likely that the difference in total available phosphorus is due to incomplete hydrolysis of IP6 and IP5, with high concentrations of IP1-IP3 remaining in treatment “Control”. It may be said that pre-incubation of JKM with phytase completely erases all IPs from the sample. Phytase pre-incubation of JKM may be useful when feed for fish which do not possess a low-pH stomach reducing phytase activity *in vivo* (e.g. carp) is produced,.

Effect of phytase application on the phytate and mineral composition of feces of Nile tilapia (Oreochromis niloticus) fed JKM-based diets. Nile tilapia were fed a JKM-based diet containing phytase (2000 U kg^{-1}). Phytate, IP5 and IP4 content of feces was measured and compared to feces from fish fed a control diet without phytase. Unlike in vitro pre-incubation of JKM with phytase, it was shown that phytate in feces was reduced only about 56% in vivo. Nevertheless, mineral content of feces were lower for phytase-containing diets, suggesting a better availability of the minerals for fish fed this diet. Improved mineral availability in feeds supplemented with phytase has been previously established (Cao et al 2007). In the present trial, however, it is interesting to see that a large portion of phytate is not completely eliminated despite phytase addition. Therefore, it may be cost efficient to pre-incubate JKM not only for stomach-less fish, but for all fish fed with high-phytate diets in order to maximize available phosphate and other minerals and minimize the need to supply minerals in form of premixes.

Conclusions. The present trial clarifies inaccuracies in measurements previously conducted for phytate in JKM. The reason for this is high amounts of oxalate in the meal interfering with the Wade-reagent, therefore no phytate measurements of any substance containing oxalate should be conducted with this technique. Further, we show that complete eradication of phytate, IP5 and IP4 is possible when JKM is pre-incubated with phytase. In vivo studies, however, show that only 56% of phytate is eliminated in the

animal, this likely leading to improved mineral availability. A future experiment could involve a comparison between feed made with pre-incubated JKM compared to a feed made of unincubated, phytase-containing JKM and observation of mineral availability to be able to quantify the potential additional value of pre-incubated meal.

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