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Short Communication

Sampling duration and freezing temperature influence the analysed gastric inositol phosphate composition of pigs fed diets with different levels of phytase

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ABSTRACT

This experiment was conducted to determine the effects of time and freezing temperature during sampling on gastric phytate (*myo*-inositol [MYO] hexakisphosphate [$InsP_6$]), lower inositol phosphates ($InsP_{2-5}$) and MYO concentrations in pigs fed diets containing different levels of phytase. Forty pigs were fed 1 of 4 wheat-barley diets on an *ad libitum* basis for 28 d. The diets comprised a nutritionally adequate positive control (PC), a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg, respectively (NC), and the NC supplemented with 500 (NC + 500) or 2,000 (NC + 2000) FTU phytase/kg. At the end of the experiment, chyme were collected from the stomach, thoroughly mixed and 2 subsamples (30 mL) were frozen immediately: one snap-frozen at −79 °C and the other at −20 °C. The remaining chyme were left to sit at room temperature (20 °C) and further subsamples were collected and frozen as above at 5, 10 and 15 min from the point of mixing. There were linear reductions in gastric $InsP_6$ concentration over time during sampling ($P < 0.001$), irrespective of diet or freezing temperature. Moreover, $InsP_6$ concentration was influenced by a diet × freezing temperature interaction ($P < 0.05$), with less $InsP_6$ measured in chyme frozen at −20 °C than at −79 °C; however, this difference was greater in the control diets than the phytase supplemented diets. Freezing chyme at −79 °C recovered more $\sum InsP_{2-5}$ + MYO than freezing at −20 °C in pigs fed phytase supplemented diets; however, this difference was not apparent in the diets without phytase (diet × freezing temperature, $P < 0.01$). It can be concluded that significant phytate hydrolysis occurs in the gastric chyme of pigs during sampling and processing, irrespective of supplementary phytase activity. Therefore, to minimise post-slaughter phytate degradation and changes in the gastric inositol phosphate profile, chyme should be snap-frozen immediately after collection.

1. Introduction

Super doses of phytase have been shown to improve the growth efficiency of monogastric animals, often beyond that expected due to improved phosphorus (P) bioavailability (Cowieson et al., 2011; Santos et al., 2014). However, despite much research, the 'extra-phosphoric' effects of phytase remain inconsistent. Factors known to influence *in vivo* phytase efficacy include phytase source, phytate concentration, dietary calcium (Ca) to phosphorus ratio and species (Dersjant Li et al., 2015). Furthermore, although it has received less attention, it seems reasonable to assume that the lack of standardised inter-laboratory sampling and analytical methodology within the scientific community has played a major role in generating these inconsistencies. Clearly, identifying the factors governing the phytase response presents a tremendous opportunity to further improve the economic and ecological value of phytase supplementation.

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The development of superior inositol phosphate (InsP) quantitation methodologies has seen a rise in the number of studies measuring phytate and its degradation products in the digesta of monogastrics, as a means of determining phytase efficacy. At present, there is no standardised method for the sampling of digesta for subsequent InsP analysis. Ostensibly, the most common practise is to freeze the digesta immediately at −20 °C (Kemme et al., 1999; Schlemmer et al., 2001; Kühn et al., 2016; Walk et al., 2018). However, this method is not shared by all, for example Blaabjerg et al. (2010) chilled the digesta on ice prior to freezing at −20 °C, whereas Laird et al. (2018) froze the digesta at −79 °C. Many others have not disclosed the freezing temperature (Blaabjerg et al., 2011; Walk et al., 2014; Beeson et al., 2017).

Therefore, the aim of this study was to determine if differences in sampling methodology, in particular freezing temperature and time taken to freeze the sample, influence phytate ($InsP_6$), $InsP_{2-5}$ and *myo*-inositol (MYO) content in pig gastric chyme. Moreover, chyme were obtained from pigs fed diets containing differing levels of phytase activity to determine if the response to different processing methods varies with phytase inclusion rate. Gastric chyme were the focus of this study as the stomach is the primary site of phytase activity in the pig (Kemme et al., 1998), and it is clear that the rapidity and extensiveness of phytate hydrolysis occurring here is key in determining the magnitude of the phytase response (Adeola and Cowieson, 2011).

2. Material and methods

This protocol was approved by the University of Leeds Animal Welfare and Ethical Review Body.

2.1. Animals and management

As part of a larger experiment, 160 crossbred (Large white × Landrace × Maxgro) finisher pigs (~12 weeks of age; initial BW ± SE = 36.7 ± 0.3 kg) were blocked into pens of 4 balancing for weight, sex and litter. Pens within a replicate were randomly allotted to 1 of 4 dietary treatments ($n = 10$). Pigs were housed in an indoor finisher facility with rooms thermostatically maintained at 21 ± 2 °C for the duration of the 28 d experiment. All pens (230 cm × 220 cm) had fully slatted plastic floors and were equipped with a single spaced trough feeder, 2 nipple drinkers and a ball and chain for enrichment. Feed and water were provided on an *ad libitum* basis. On d 28, 40 mixed sex pigs (one per pen; mean BW ± SE = 58.7 ± 0.6 kg) were slaughtered via captive bolt penetration followed by exsanguination for the collection of gastric chyme. The pigs selected for slaughter had a BW that closely matched that of the pen average, and where possible, those within a replicate were littermates.

2.2. Dietary treatments and experimental design

This randomised complete block experiment was designed to determine the effect of time and freezing temperature during sampling on gastric $InsP_{2-6}$ and MYO concentrations in pigs fed wheat-barley based diets containing different levels of phytase. The 4 dietary treatments included: a positive control (PC) formulated to meet or exceed the BSAS (2003) nutrient recommendations for all nutrients; a negative control (NC) similar to the PC but with reductions in Ca (1.6 g/kg), P (1.24 g/kg) and NE (0.170 MJ/kg), in accordance with the matrix values for 500 FTU/kg of the tested phytase; and the NC diet supplemented with phytase at 500 (NC + 500) or 2,000 (NC + 2000) FTU/kg. The phytase doses were selected to represent a standard (500 FTU/kg) and a super-dose (2,000 FTU/kg) of phytase commonly used in pig production. The phytase enzyme used was Quantum Blue 5G (AB Vista, UK), which is a modified *E. coli* derived phytase. One FTU denotes the amount of enzyme activity necessary to liberate 1 μmol of inorganic phosphate/min from an excess of Na-phytate at 37 °C and pH 5.5. All diets were pelleted through a 3-mm die at a temperature of 62 ± 2 °C. A detailed composition of the diets and formulated nutrient content is presented in Table 1.

2.3. Gastric chyme collection

Following the confirmation of death, clamps were positioned at the pyloric sphincter and the lower oesophageal sphincter and the stomach was excised from the abdominal cavity. The total gastric contents were mixed by massaging and inverting the stomach. A subsample of the gastric contents was collected into a glass beaker, mixed further and the pH recorded. Two representative subsamples of the mixed chyme (*ca.* 30 mL) were decanted into separate polypropylene screw topped tubes and frozen immediately; one at −20 °C and the other snap-frozen at −79 °C (on dry ice). Thereafter, the remaining chyme were left to sit at room temperature (20 °C) before a further 2 subsamples were collected and frozen as above at 5, 10 and 15 min from the point of mixing. It should be noted that the mixing of chyme occurred at approximately 4 min following the confirmation of death. Within replicate, sampling was conducted in a random fashion in order to equalise for variance introduced due to post-prandial time between the dietary treatments.

2.4. Laboratory analyses

Chyme were freeze dried, ground to pass a 1-mm sieve, and frozen at −20 °C pending subsequent analyses. Representative feed samples were sent to Sciantec Analytical Services Ltd. (Stockbridge Technology Centre, UK) for Ca and P analyses by ICP-OES (SOP S1015). Phytate and phytase activity in the feed were analysed by Enzyme Services and Consultancy (Ystrad Mynach, Wales, UK). Phytase was analysed according to the internal manufacturer's assay for Quantum Blue (Standard Analytical Method 020; AB Vista), whereas phytate was analysed by near-infrared spectroscopy (NIR). Chyme and feed were analysed for $InsP_{2-6}$ and MYO.

Table 1
Composition and nutrient specifications of experimental diets (as-fed basis, %).[1]

Item	PC	NC
Ingredient		
Wheat	48.1	48.5
Barley	15.0	15.0
Wheat	10.3	12.0
Rapeseed meal	10.0	10.0
Sunflower seed extract	7.0	7.4
Soybean meal	3.6	2.7
Soya oil	2.7	1.9
Dicalcium phosphate	0.99	–
Limestone flour	0.62	0.91
Vitamin-mineral premix[2]	0.25	0.25
Titanium dioxide	0.50	0.50
Calculated content		
Net energy, MJ/kg	9.30	9.13
Crude protein	16.0	16.0
Ca	0.72	0.56
Total P	0.61	0.45
Digestible P	0.25	0.13

[1] PC, a nutritionally adequate positive control; NC, a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg, respectively.
[2] Vitamin and trace mineral premix provided per kilogram of diet: 7,500 IU vitamin A, 1,650 IU vitamin D_3, 35 IU vitamin E, 2 mg vitamin K, 1.5 mg thiamine (B_1), 3 mg riboflavin (B_2), 2 mg pyridoxine (B_6), 15 μg vitamin B_{12}, 8 mg pantothenic acid, 20 mg nicotinic acid, 50 μg biotin, 0.3 mg folic acid, 15 mg $CuSO_4$, 1 mg iodine, 80 mg $FeSO_4$, 25 mg manganese, 0.25 mg selenium, 65 mg $ZnSO_4$.

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Inositol phosphates were analysed by anion-exchange HPLC with post-column addition of ferric nitrate in $HClO_4$ according to Lee et al. (2018). For MYO measurement, extracts were diluted 50-fold in water and analysed by pulsed amperometric detection on a gold electrode after 2 d separation on CarboPac PA1 and CarboPac MA1 columns (Lee et al., 2018).

2.5. Statistical analysis

Data were analysed as a 4 × 2 × 4 factorial using a three-way mixed ANOVA with the individual pig serving as the experimental unit (SPSS Statistics, Version 22; SPSS Inc., Chicago IL, US). The model included the effects of diet, freezing temperature, time and all appropriate interactions, with both time and freezing temperature included as repeated factors. No three-way interactions were observed for any of the parameters measured. Data displaying non-normal residuals or heteroscedasticity were log transformed log10 ($x + 1$) prior to statistical analysis. Polynomial contrasts were used to test for linear and quadratic effects of time. Differences were classed as significant if $P < 0.05$, or a trend if $P < 0.10$. Significantly different means were separated using the Tukey's honest significant difference (HSD) test.

3. Results

The recorded temperature of the freezer used to freeze chyme at −20 °C throughout the experiment was −26 °C. The analysed nutrient composition of the experimental diets is presented in Table 2. Diets contained moderate amounts of phytate which are in line with those reported in other wheat-barley based pig diets (Blaabjerg et al., 2010, 2011). The mean pH of the gastric chyme from slaughtered pigs receiving the PC, NC, NC+500 and NC+2000 treatments were similar at 4.1, 4.0, 3.6 and 3.7, respectively (SEM = 0.28, $P = 0.516$), and thus gastric pH was not deemed a confounding factor for phytate hydrolysis.

3.1. Gastric phytate concentration

Phytate ($InsP_6$) was continuously hydrolysed over time during sampling (linear $P < 0.001$), irrespective of diet or freezing temperature (Fig. 1). This equated to a 13.6% reduction in gastric $InsP_6$ concentration from 0 to 15 min at a constant rate of approximately 30.9 nmol/g DM per minute. Delaying the freezing of the gastric contents by 5 min from collection resulted in significant phytate hydrolysis (3,413 vs. 3,262 nmol/g DM; $P < 0.05$). Chyme $InsP_6$ concentration was also influenced by a significant diet × freezing temperature interaction ($P < 0.05$), as presented in Fig. 2. Less $InsP_6$ was hydrolysed in chyme frozen at −79 °C compared with that frozen at −20 °C; however, the difference between the two freezing temperatures was greater in diets devoid of added phytase. Moreover, within freezing temperature, diets with added phytase had significantly less $InsP_6$ than those without added phytase ($P < 0.001$); however, there was no difference between the two phytase diets or the two control diets.

Table 2
Analysed chemical composition of the experimental diets (as fed-basis, nmol/g).[1]

Item	PC	NC	NC+500	NC+2000
Phytase, FTU/kg	85	<50	751	2,420
Ca, %	0.71	0.58	0.57	0.61
Total P, %	0.60	0.43	0.41	0.43
$InsP_6$	9,532	10,748	10,565	10,000
$InsP_5$	1,464	1,782	2,047	2,284
$InsP_4$	145	228	259	290
$InsP_3$	154	189	263	294
$InsP_2$	1,205	1,662	1,743	1,713
MYO	488	483	566	572

$InsP_6$ = *myo*-inositol hexakisphosphate; $InsP_{2-5}$ = lower inositol phosphates; MYO = *myo*-inositol.
[1] PC, a nutritionally adequate positive control; NC, a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg, respectively; NC+500, NC supplemented with 500 FTU phytase/kg; NC+2000, NC supplemented with 2,000 FTU phytase/kg.

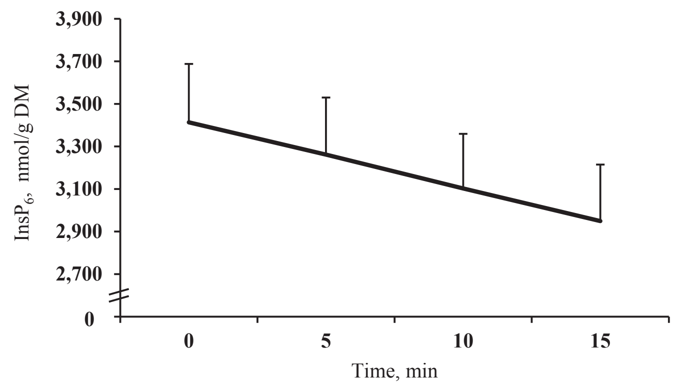

Fig. 1. Effect of time from sampling to freezing on $InsP_6$ concentration in pig gastric digesta. $InsP_6$ = *myo*-inositol hexakisphosphate. Values are the means of 40 observations + SEM. Trend analysis: linear, $P < 0.001$; quadratic, $P = 0.985$.

3.2. Gastric concentrations of phytate hydrolysis products

Time had no influence on the total concentration of measured phytate hydrolysis products in chyme, or on individual $InsP_5$, $InsP_4$, $InsP_2$ or MYO concentrations. The concentration of $InsP_3$, however, increased in a linear manner over time in the PC, NC and NC + 500 diets ($P < 0.05$), but remained relatively constant in the NC + 2000 diet, resulting in a tendency for a diet × time interaction ($P = 0.06$; data not presented).

The effect of diet and freezing temperature on the concentration of $InsP_{2-5}$ and MYO is presented in Fig. 3. As with $InsP_6$, the effect of freezing temperature on the sum of measured phytate hydrolysis products ($\sum InsP_{2-5}$ + MYO) was dependent on the diet fed, resulting in a significant diet × freezing temperature interaction ($P < 0.01$). In diets with no added phytase, freezing temperature had no effect on $\sum InsP_{2-5}$ + MYO concentration; however, in diets

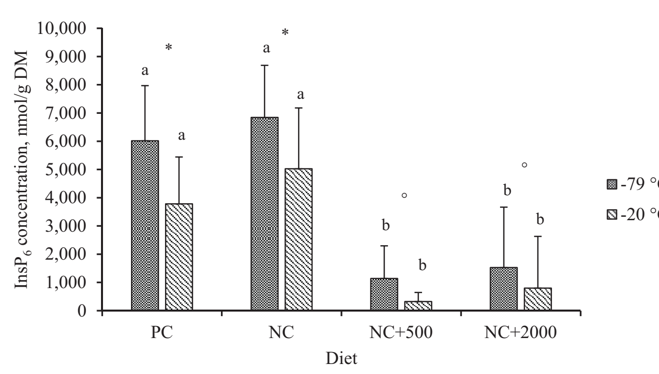

Fig. 2. Interactive effects of diet and freezing temperature on $InsP_6$ concentration in pig gastric digesta. $InsP_6$ = *myo*-inositol hexakisphosphate; PC, a nutritionally adequate positive control diet; NC, a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg, respectively; NC+500, NC supplemented with 500 FTU phytase/kg; NC+2000, NC supplemented with 2,000 FTU phytase/kg. Values are means of 10 observations + SD. Significance: diet × freezing temperature, $P < 0.05$; diet, $P < 0.001$; freezing temperature, $P < 0.001$. Within a diet, an asterisks (*) denotes a significant difference ($P < 0.001$) between freezing temperatures, whereas a circle (°) denotes a trend ($P < 0.10$). [a, b] Within freezing temperature, mean values that do not share a common superscript are significantly different ($P < 0.01$).

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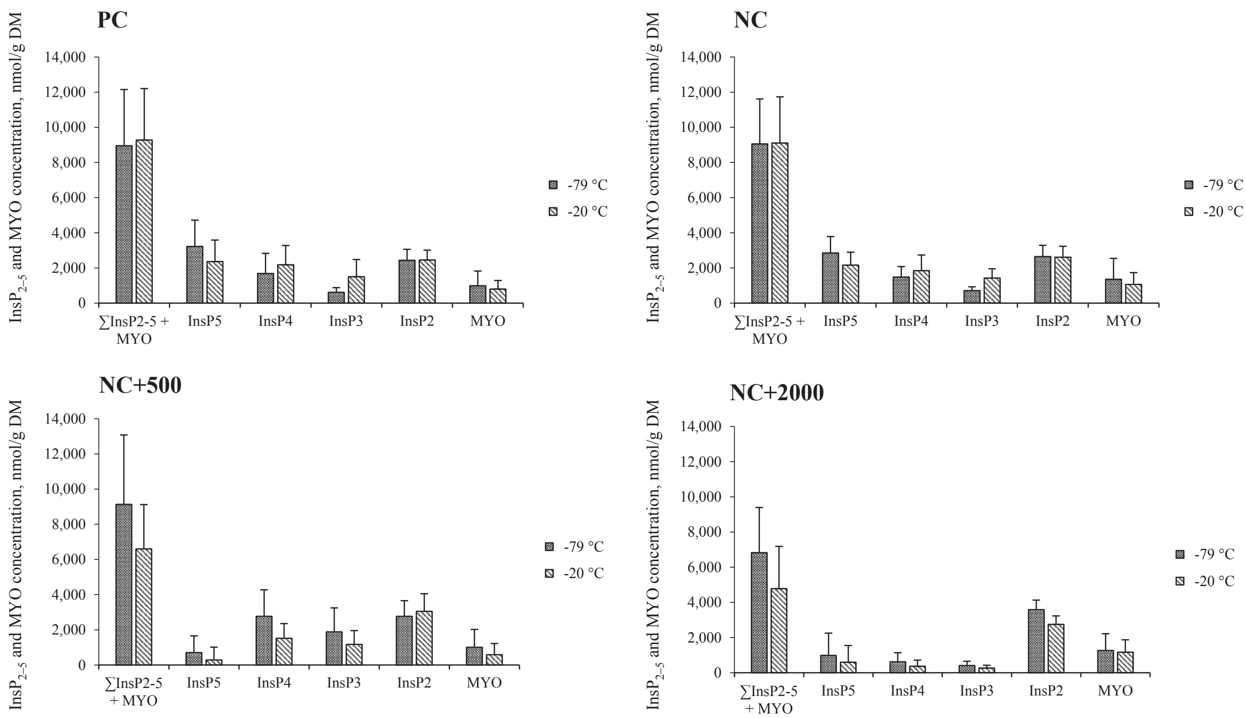

Fig. 3. Interactive effects of diet and freezing temperature on inositol pentakisphosphate ($InsP_5$), inositol tetrakisphosphate ($InsP_4$), inositol trisphosphate ($InsP_3$), inositol bisphosphate ($InsP_2$), *myo*-inositol (MYO) and total $InsP_{2-5}$ + MYO concentrations (nmol/g DM). PC, a nutritionally adequate positive control diet; NC, a similar diet but with Ca and P reduced by 1.6 and 1.24 g/kg, respectively; NC+500, NC supplemented with 500 FTU phytase/kg; NC+2000 = NC supplemented with 2,000 FTU phytase/kg. Values are means of 10 observations + SD. Significance: $\sum InsP_{2-5}$ + MYO, diet (D) × freezing temperature (FT) = $P < 0.01$, D = $P < 0.001$, FT = $P < 0.01$; $InsP_5$, D × FT = not significant (NS), D = $P < 0.001$, FT = $P < 0.01$; $InsP_4$, D × FT = $P < 0.001$, D = $P < 0.01$, FT = NS; $InsP_3$, D × FT = $P < 0.1$, D = $P < 0.01$, FT = NS; $InsP_2$, D × FT = $P < 0.01$, D = $P < 0.05$, FT = NS; MYO, D × FT = NS, D = NS, FT = NS.

with added phytase, more $\sum InsP_{2-5}$ + MYO were measured in chyme frozen at −79 °C.

Within freezing temperature, the composition of measured $InsP_6$ hydrolysis products between the PC and NC fed pigs did not differ. Adding phytase at either level reduced $InsP_5$ content ($P < 0.001$), though there was no difference between the two doses tested. Chyme $InsP_5$ content was also influenced by freezing temperature ($P < 0.001$): chyme frozen at −20 °C contained 30% less $InsP_5$ than that frozen at −79 °C (1,353 vs. 1,941 nmol/g DM). Gastric concentrations of $InsP_4$, ($P < 0.001$) $InsP_3$ ($P < 0.10$; trend) and $InsP_2$ ($P < 0.01$) were each influenced by a diet × freezing temperature interaction. In the PC and NC diets, chyme frozen at −20 °C tended to have higher levels of $InsP_4$ than that frozen at −79 °C ($P < 0.10$). Conversely, within the NC + 500 treatment, chyme frozen at −20 °C had lower levels of $InsP_4$ ($P < 0.05$) than that frozen at −79 °C. In the NC + 2000 fed pigs, however, gastric $InsP_4$ concentration was similar irrespective of freezing temperature. The diet × freezing temperature trend observed for $InsP_3$ concentration was similar to that described for $InsP_4$. Within freezing temperature, increasing phytase activity from 500 to 2,000 FTU/kg reduced chyme $InsP_4$ and $InsP_3$ concentrations ($P < 0.01$). Inositol bisphosphate ($InsP_2$) concentration was similar between PC, NC and NC + 500 treatments irrespective of freezing temperature; however, in the NC + 2000 treatment, $InsP_2$ concentration was higher in chyme frozen at −79 °C. Gastric MYO concentration was not influenced by any of the treatments.

4. Discussion

In the present study, the analysed inositol phosphate composition of pig gastric chyme was influenced by time taken to freeze the chyme after sampling. Phytase induced phytate hydrolysis is a time-dependent process, which in the pig is often limited by the relatively short retention time of the digesta in the stomach (Blaabjerg et al., 2011). Therefore, it was unsurprising that this enzyme catalysed reaction continued in the chyme after sampling from pigs fed diets with added phytase. Interestingly, phytate continued to be hydrolysed after sampling in chyme from pigs fed steam-pelleted diets without supplementary phytase. These data are contrary to the results of Kemme et al. (2006), who found that almost no phytate was degraded in the stomach of pigs fed a low phytase (35 FTU/kg) corn-soybean meal based diet. The reason for the discrepancy between the findings of these two studies is unclear, but may be due to differences in diet composition. Both wheat and barley possess much higher levels of intrinsic phytase activity than corn (Eeckhout and De Paepe, 1994); however, their contribution to phytate hydrolysis is commonly disregarded as this activity is generally lost during the pelleting process. Given the degree to which phytate was hydrolysed in both unsupplemented dietary treatments, an alternative source of phytase cannot be excluded. It is known that certain species of lactic acid bacteria reside within the pig stomach (Cranwell et al., 1976; Chow and Lee, 2006); however, whether these bacteria are capable of producing and secreting extracellular phytase remains a contentious issue (Reale et al., 2007).

Another key finding of the present study was that analysed $InsP_6$ concentration in the chyme, irrespective of initial phytate concentration, was influenced by freezing temperature, with samples frozen at −20 °C containing less $InsP_6$ than that snap-frozen at −79 °C. This study is the first to demonstrate that phytate continues to be hydrolysed throughout the freezing process. It is, therefore, clear that chyme must be frozen as quickly as possible in

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order to terminate the enzyme catalysed reaction and prevent possible erroneous estimation of *in vivo* phytate hydrolysis. Although the analysed gastric $InsP_6$ content was consistently lower when frozen at −20 °C, this difference was more apparent in chyme collected from pigs fed diets without added phytase. This interaction between freezing temperature and diet was not expected and is likely the result of phytase induced differences in initial phytate concentration. Phytate concentration was considerably higher in chyme obtained from pigs fed diets without supplementary phytase than those fed diets with added phytase, and therefore, the scope for continued phytate hydrolysis during the processing of such samples was greater. These findings suggest, whatever the initial phytate content at the point of collection, both sampling duration and freezing temperature are influential in subsequent phytate estimation, even in diets without supplementary phytase.

The gastric InsP and MYO profiles in the chyme of pigs fed diets without added phytase did not differ. This suggests that the phytate from these diets is likely being degraded by the same mechanism, through similar phytases and phosphatases with similar specificities and reaction kinetics. It can also be inferred that small reductions in dietary Ca and P concentrations have no influence on *in vivo* gastric phytate hydrolysis, which is in agreement with the findings of Kühn et al. (2016). Supplementing the control diet with 500 FTU/kg changed the InsP composition of the chyme, with reductions in $InsP_5$ leading to small increases in $InsP_4$ and $InsP_3$ content. Adding 2,000 FTU/kg to the diet effectively reduced levels of $InsP_4$ and $InsP_3$, resulting in more complete phytate hydrolysis. These findings are in agreement with those of Laird et al. (2018) who, using the same phytase enzyme, found that higher doses of phytase (2,500 FTU/kg) are needed to reduce levels of $InsP_4$ and $InsP_3$ in the ileal digesta of weaner pigs.

Interestingly, the more complete phytate hydrolysis achieved with the super-dose was not met with clear changes in $InsP_2$ or MYO concentration. Moreover, the sum of phytate hydrolysis products in the chyme from these treatments was lower than that of the controls. These results are suggestive of $InsP_1$ formation. Unfortunately, it was not possible to measure $InsP_1$ using the InsP quantitation methodology used in this study. $InsP_1$ is considered a transient compound that is present in minute quantities in the digesta, as it is rapidly dephosphorylated by endogenous phosphatases, or possibly absorbed directly across the brush border membrane of the small intestine (Adeola and Cowieson, 2011). Therefore, $InsP_1$ is frequently dismissed as having minor quantitative importance in ileal digesta; however, the results presented herein suggest that this is not the case in gastric chyme. These data are consistent with the view that most microbial phytase enzymes are unable to completely dephosphorylate phytate (Wyss et al., 1999). Interestingly, results from in house studies, including the present study (as yet unpublished), have demonstrated that super-doses of phytase increase ileal and portal plasma levels of MYO in pigs (Laird et al., 2018). These findings indicate complete dephosphorylation via phytase supplementation by the terminal ileum. Therefore, it seems plausible that while exogenous phytase is unable to completely dephosphorylate phytate to MYO within the stomach, it is able to degrade the phytate molecule to lower molecular weight, more soluble phytate esters, which are available to the animal's own endogenous phosphatase enzymes for further degradation to MYO.

5. Conclusion

It can be concluded that significant phytate hydrolysis occurs in the gastric chyme of pigs during sample collection and processing. There were linear reductions in the chyme phytate content when left to sit at room temperature for 15-min post-sampling. Furthermore, freezing temperature during sampling was also influential, irrespective of initial phytate concentration, with greater phytate degradation occurring in chyme frozen at −20 °C than that snap-frozen on dry ice. It is, therefore, the authors' suggestion that future *in vivo* phytate quantitation assessments snap-freeze digesta on dry ice immediately after collection to minimise phytate degradation. Such measures would ensure that post-sampling changes in the gastric InsP profile are kept to a minimum and prevent possible erroneous determination of phytase efficacy.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature that could be construed as influencing the content of this paper.

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