Effect of Hydrothermal, Phytase, or Organic Acid Pretreatments of Canola Meal on Phytate Level of the Meal

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Abstract

The objective of this study was to investigate the effect of pretreatment of canola meal with hydrothermal processing, phytase, and organic acid on phytate degradation. Six experiments were conducted processing canola meal in a hydrothermal reactor with different moisture conditions (15-350%), acid additions (hydrochloric acid, citric acid, malic acid, and lactic acid), pH (pH 4, 5, and 6), incubation times (30, 45, and 60 min), and phytase enzymes ('Quantum Blue' and 'Finase'). The study revealed that although hydrothermal pretreatment of canola meal with phytase and higher moisture (200-350%) would allow 24.8-36% phytate breakdown, and a higher moisture addition (200%) combined with organic acid increased even further to 46.6%, lower moisture (50 and 100%) and organic acid was still effective in reducing phytate (by 7.9-19.4%). Optimal pH and incubation time in hydrothermal reactor for phytase efficacy were determined to be pH 4 and 5 and 30 to 60 min. Still, the results of the current study suggest that pretreatments should be further evaluated to optimize the efficacy of enzyme and organic acids as prebiotics to reduce anti-nutrients in canola meal, thus improving its utilization for livestock and reducing excretion to environment.

Keywords: canola meal, phytate, enzyme additive, hydrothermal reactor

1. Introduction

Canola meal is a commonly used feed ingredient for monogastrics, however, its nutritional value is limited by the high level of nonstarch polysaccharides and anti-nutrients, such as phytate [or phytic acid (PA)]. Canada's 13 crushing and refining plants have the capacity to crush about 9.0 million tonnes of canola seed, and produce about 3.6 million tonnes of canola oil and 5.4 million tons of canola meal (CM; 42.6% CP, 71.5% TDN in DM basis; Damiran, Lardner, Larson, & McKinnon, 2016) annually (Canola Council of Canada, 2014). Canola meal is considered a relatively high phytate feedstuff containing 3.1-6% PA (Cheryan, 1980; McCurdy & March, 1992; Al-Asheh & Duvnjak, 1994) in solvent extracted (commercial) canola meal (SECM). This is almost twice the phytate content of soybean meal (SBM). However, it also, contains 0.38% nonphytate (available) P, higher than SBM, cottonseed meal, wheat, wheat bran, corn, or barley, at 0.28, 0.23, 0.09, 0.26, 0.07, and 0.13%, respectively (Khajali & Slominski, 2012). Phytic acid decreases mineral, especially phosphorus (P) digestibility in monogastrics and can form complexes with proteins, thus reducing their availability for absorption (Al-Asheh & Duvnjak, 1994; Bedford, 2000; Maenz, 2001; Viveros, Brenes, Arija, & Centeno, 2002).

Phosphorus has become one of the major contributors to environmental pollution. It is estimated that about 80% of inorganic P is wasted and the buildup of bioavailable P contributes to eutrophication of ground water (Zvomuya et al., 2006; Cordell, 2010). For the sake of sustainability, P must be recovered in an uncontaminated and plant-available form (Schröder, Cordell, Smit, & Rosemarin, 2009). Phosphorus is mainly excreted in the feces; grow-finish pigs excrete 63% of the P consumed (van der Peet-Schwering, 1998). Moreover, supplementing inorganic P for livestock increases diet cost.

Utilization of CM can be improved using exogenous enzymes (Slominski, Campbell, & Guenter, 1992; Simbaya, Slominski, Guenter, Morgan, & Campbell, 1996). Supplementation of microbial phytase to swine diets improves the absorption and utilization of P in feed ingredients of plant origin (Simons et al., 1990; Cromwell, Stahly, Coffey, Monegue, & Randolph, 1993; Lei, Ku, Miller, & Yokoyama, 1993a; Lei, Ku, Miller, Yokoyama, & Ullrey, 1993b; Slominski, Cyran, Guenter, & Campbell, 1999) along with lowering P excretion by 20 to 50%

(Simons et al., 1990; Beers & Jongbloed, 1992; Lei & Stahl, 2000; Emiola, Akinremi, Slominski, & Nyachoti, 2009; Casas & Stein, 2015). Doses of phytase from 500 to 4000 U/kg diet have been reported to have efficacy (Beers & Jongbloed, 1992; Slominski et al., 1999; Adeola, Sands, Simmins, & Schulze, 2004; Beaulieu, Bedford, & Patience, 2007; Khan, Chaudhry, Butt, Jameel, & Ahmad, 2013; Dersjant-Li & Dusel, 2019), and as in an early study of superdosing by Nelson, Shieh, Wodzinski, and Ware (1971), phytate was hydrolysed to 94.4% with 7,600 FTU/kg of phytase produced by *Aspergillus ficuum* and to 100% with 4800 and 48 000 FTU/kg of two fungal phytases in a more recent study (Liu, 2014). Pretreatments of feed or feed ingredient, as Newkirk and Classen (1998) suggested, may increase phytate phosphorus hydrolysis before and during feeding. Processing of CM in hydrothermal reactors (HTR) used during conditioning of oilseeds and desolventising the meal with addition of feed enzymes, can be an effective treatment for breaking weak bonds between polysaccharides in the cell wall, opening the cell wall structure and reducing particle size, thereby increasing the surface area accessible for enzymes, both endogenous and feed added (exogenous) (de Vries, Pustjens, Kabel, Kwakkel, & Gerrits, 2014).

Moreover, exogenous enzymes and organic acids act as prebiotics, selectively stimulating the growth of beneficial bacteria. Additionally, organic acids may also improve gastrointestinal function by reducing, digesta pH, and improving digestive enzyme and microbial phytase activity (Jongbloed, Mroz, van Derweij-Jongbloed, & Kemme, 2000; Dibner & Buttin, 2002). Results from Li et al. (2008) suggested that the improved growth performance of weaned pigs fed a diet with a dry organic acid blend might be mainly by reducing pathogenic bacteria and increasing the beneficial bacterial population in the gut and feces. Also, Tsukahara, Koyama, Okada, & Ushida, (2002) noted that gluconic acid reaches the pig large intestine to stimulate lactic acid bacteria, hence may be useful as a prebiotic to stimulate butyrate production in the large intestine. Furthermore, a potential of significantly improving iron bioavailability with fermentation or addition of small amounts of lactic acid to maize products was proposed by Proulx and Reddy (2007).

Others have implied of a feasibility to replace inorganic P supplementation for pigs (calcium phosphate) adding wheat middlings (as a cereal phytase source), microbial phytase, and citric acid (1.5%) to corn-soybean meal (Han, Roneker, Pond, & Lei, 1998) or achieved some results with phytase alone as in Emiola et al. (2009). More on potential additive effects of organic acids and enzymes, as studies revealed, supplementation with EDTA, citrate, and phthalate increased the efficacy of microbial phytase in hydrolyzing phytic acid (Maenz, Engele-Schaan, Newkirk, & Classen, 1999), citric acid and phytase affected phytate-P utilization in chicks (Boling, Webel, Mavromichalis, Parsons, & Baker, 2000), and increased Ca, P, Fe, and Zn retention (Lei & Stahl, 2000; Viveros et al., 2002; Adeola et al., 2004), however, others found that the growth response to phytase was depressed by citric acid in chicks (Brenes et al., 2003).

Several factors may influence the efficacy of phytase, including the type and amount of phytase, method of inclusion, method of pretreatment, other ingredients, concentrations of nutrients (especially protein and minerals), and the animal species (Sajjadi & Carter, 2004). Effects of HTR processing conditions alone or in combination with enzyme and organic acids on chemical characteristics and nutritional quality of canola meal are less known and require better characterization to maximize effective use. The objective of this study was to determine optimal parameters of canola meal pretreatments, in particular, processing the meal in hydrothermal reactor with added phytase and organic acid and investigate the effects on phytate level of the meal.

2. Materials and Methods

2.1 Sample Preparation

Solvent extracted canola meal (SECM; var. *Brassica napus*) was obtained from the Canadian Feed Research Centre (North Battleford, SK, Canada), ground in a coffee grinder for even consistency and stored in plastic pails under dry and cool conditions (2-4 ℃). Samples from each treatment and replicate were dried in a forced air oven at 80 ℃ for 48 h and then ground using Retsch ZM200 grinder (Retsch, Haan, Germany) via 0.5 or 1 mm screens prior to further analysis.

2.2 Chemicals and Enzymes

Analytical grade reagents were purchased from Sigma-Aldrich Canada (Oakville, ON) and Fisher Scientific Canada (Ottawa, ON); hydrochloric acid (HCl) of 1M concentration, citric acid (CA) and malic acid (MA) in powder, of 100% purity and lactic acid (LA) in liquid form, of 80% purity were used for the experiments. Enzymes used for the experiments were phytases; Quantum Blue 5G (declared potency of 5000 FTU/g; Quantum® Blue; EC 3.1.3.26; AB Vista, Wiltshire, UK) and Finase EC 5P (declared potency of 5000 FTU/g; Finase® P, EC 3.1.3.8; AB Vista, Wiltshire, UK). Quantum Blue is an *E. coli*-derived 6-phytase and Finase is an *A. niger*-derived 3-phytase, that initiate dephosphorylation from position 6 and 3, respectively, on the

myo-inositol ring. These phytases developed specifically for use in swine and poultry feeds belong to the class of histidine acid phytases (Greiner & Bedford, 2010). Both enzymes are thermostable (maximum recommended temperature for Quantum Blue is 90.5 ℃), water soluble, pH-value is 4.5-5 (100 g/L), and were supplemented according to their activity determined at standard conditions (pH 5.5, 37 ℃, 5 mmol/L sodium phytate) (ABVista Feed Ingredients, ABVista, Inc.).

2.3 Experimental Design

Six HTR experiments were conducted designed to investigate the HTR processing parameters and feed additives: 1) moisture additions, 2) phytase addition, 3) pH, 4) incubation times, 5) acids, and 6) phytase types. Unless specified otherwise, parameters throughout the experiments were: 100% moisture addition [or 1:1 (wt/wt) as water/sample ratio], pH 4 (adjusted using HCl), incubation time 30 min, incubation temperature 40 ℃, and phytase type 'Quantum Blue' at a rate of 0.02% to contain 1000 FTU/g activity, where the latter two factors (incubation temperature and phytase inclusion rate) were kept constant throughout.

HTR experiment 1 (Exp. 1) aiming to evaluate the effect of moisture on PA level with different amounts of moisture addition (15 to 350%) and phytase to CM was conducted in completely randomized design (CRD) with moisture as the treatment.

In HTR experiment 2 (Exp. 2), phytase effect on PA level was evaluated in CRD where phytase was the treatment.

In HTR experiments 3, 4, and 5, pH (pH 4.0, 5.0, and 6.0) (Exp. 3; 3×3), incubation times (30, 45, and 60 min) (Exp. 4; 3×3), and phytase types ('Quantum Blue' and 'Finase') (Exp. 5: 2×3) with acids (hydrochloric acid (HCl), citric acid (CA), and malic acid (MA)) were tested to determine the optimal parameters for phytase efficacy in split plot design.

In HTR experiment 6 (Exp. 6), in an effort to further reduce the moisture in CM to better adjust for feed mill production condition, CM was tested with a lower amount of moisture addition (50% vs. 100%), phytase, and organic acid in 2×3 factorial arrangement.

Treatments were replicated (n = 4 to 10) and results were compared with untreated CM (Control 0). It should be noted that in Exp. 2, 5, and 6, a no-enzyme treatment (No Enzyme) was included to compare the effects of moisture or HCl alone, without or with phytase.

2.4 Hydrothermal Reactor Run Procedure

A bench-scale HTR with some modifications to adjust for the laboratory conditions were used (Figure 1) for this study. This equipment consisted of a water bath and heater/circulator (ANOVA, Anova Culinary LLC, Houston, TX, USA), a 12-cell double metal rack for the 12 cups/containers (500 mL capacity), 12 powered mixer-heads with removable spiral stirrers capable of generating speeds up to 450 rpm, and a power generator with corresponding cables.

After the water obtained the desired temperature, one hundred grams of CM was weighed into each cup followed by the addition of the required amount of water. The pH was measured while mixing the meal intensively with the power stirrer, using a pH-meter (H160, HACH, Loveland, CO, USA) and adjusted to the desired level using 1M HCl solution or organic acid.

The canola meal was conditioned in the pre-heated water bath for 15 min while stirring at ~450 rpm speed continuously until the meal temperature became 40 ℃, the enzyme solution containing phytase 1000 FTU/kg activity (or supplements) were then added. At the end of incubation, the reaction was stopped by increasing the pH to 9.0 with the addition of 20% sodium hydroxide solution. The sample was dried in a forced-air oven and ground using Retsch ZM100 grinder (Retsch, Haan, Germany) via 1 mm screen for chemical analysis and via 0.5 mm screen for phytate and SCP determinations.

Figure 1. A bench-scale hydrothermal reactor used in the study

2.5 Chemical Analysis

2.5.1 Moisture, pH, and Other Analysis

Moisture content (%) of hydrothermally treated sample was determined using a two-step method (Method 930.15; AOAC, 2000). Sample pH was measured using a pH-meter (H160, HACH, Loveland, CO, USA). Crude protein (CP) was determined by the combustion method using a LECO FP-528 total nitrogen analyzer (St. Joseph MI, USA) and calculated as N $\times$6.25 (Method 968.06, AOAC 2000). Determination of total soluble crude protein (SCP) involved an incubation of the sample in bicarbonate-phosphate buffer and filtration through Whatman #54 filter paper followed by Kjeldahl-N analysis as described by Roe, Sniffen, and Chase (1990).

2.5.2 Phytate Determination

Phytate determination was based on the colorimetric method of Gao et al. (2007) with some modifications described by Darambazar (2018). Briefly: samples weighing 0.5 $\pm$0.01 g were extracted in 10 mL, 2.4% (0.64N) hydrochloric acid (HCl) for 16 h with shaking at 300 rpm on a platform shaker (VWR Advanced Digital Shaker Model 3750), followed by centrifugation at 3000 rpm and 10 ℃ for 20 min in an Avanti J-E Centrifuge (Beckman-Coulter Mississauga, ON, Canada). The supernatant was filtered through Whatman #1 filter paper into a tube containing 1.0 $\pm$0.01 g of Sodium Chloride (NaCl) and the mixture was stirred until the salt dissolved, followed by continued mixing at 300 rpm for 20 min. The precipitate was allowed to settle at 2 - 4 ℃ for 60 min or at -20 ℃ for 20 min and then centrifuged at 3000 rpm and 10 ℃ for 20 min. One milliliter of supernatant was taken into a 25 mL - volumetric flask and diluted with 24 mL deionized water. Sodium phytate standard stock solution was prepared using phytic acid sodium salt hydrate (from rice, $C_6H_{18}O_{24}P_6$ xNa ɏH$_2$O; 79% purity and 20.6% P as is; Sigma Aldrich, St. Louis P8810; CAS 14306) and standards of 0, 5, 10, 30, 60, and 80 mg/mL concentrations were prepared for a calibration curve. In a test tube, 3 mL of the diluted sample and standards was combined with 1 mL of Wade Reagent (Ferric Chloride Hexahydrate (FeCL$_3$ 6H$_2$O), Fisher Scientific CAS F2877), mixed on a vortex and centrifuged at 3000 rpm and 10 ℃ for 10 min. Absorbance of color reaction was read at 500 nm on a spectrophotometer (Ultrospec III UV/Visible Spectrophotometer, Pharmacia Model 80-2097-62 LKB Biochrom, England). The analysis was done in duplicates.

2.6 Statistical Analysis

Experiment 1 was conducted using a completely randomized design (CRD) with moisture as the treatment. In Exp. 2, the effect of phytase enzyme on phytate level was tested in a CRD with phytase as the treatment. The model used for Exp.1 and 2 was: $Y_{ij} = \mu + T_i + e_{ij}$; where Y_{ij} was an observation of the dependent variable ij; μ was the population mean for the variable; T_i was the fixed effect of treatment (moisture and phytase for Exp. 1 and Exp. 2, respectively); and e_{ij} was the random error associated with the observation ij.

Experiments 3 to 6 had two factors and were analyzed as a split-plot design. The model used was $Y_{ij} = \mu + X_i + Z_j + (X \times Z)_{ij} + e_{ij}$, where Y_{ij} = response variable; μ = mean; main plot (X; Exp. 3: pH 4, 5, and 6; Exp. 4: incubation time 30, 45, and 60 min; Exp. 5: phytase type, 'Quantum Blue' and 'Finase'; Exp. 6: moisture, 50 and

100%), subplot (Z; Exp. 3, 4, and 5: HCl, CA, and MA; Exp. 6: HCl, CA, MA, and LA) were both fixed effects; and error was e_{ij}.

All experimental data were analyzed using the Proc Mixed Model procedure of SAS (2003). LSmeans were determined using the least squares means statement of SAS and were separated using Tukey's multi-treatment comparison method (Saxton, 1998). For all statistical analyses, significance was declared at $p < 0.05$. The regression between moisture and phytate concentration was analyzed using the PROC REG procedure of SAS (2003).

3. Results and Discussion

The canola meal contained on average 10.3% moisture, 37.4% CP, 6.6% SCP, 2.5% PA, and 0.65% PAP (as is basis), and the initial pH was 5.8 [2:1 (wt/wt) as water/sample ratio] (data not shown). Phytic acid content in CM in the current study was lower than the range (3.1-6% PA) reported elsewhere (Cheryan, 1980; McCurdy & March, 1992; Al-Asheh & Duvnjak, 1994), while PAP concentration was within the 0.40-0.80% range reported in CM and 00-rapeseed meal (NRC, 2012; Khan et al., 2013; Weightman et al., 2014), but lower than the 0.78-0.87% range in canola expeller meals and 00-rapeseed expeller meals (NRC, 2012; Slominski, Jia, Rogiewicz, Nyachoti, & Hickling, 2012) and 0.04% higher than 0.70% PAP observed in CM by Tahir et al. (2012).

Results of Exp. 1, presented in Figure 2, showed that there was a strong and positive correlation between PA degradation level and moisture (PA% = 2.36 − 0.00242 × Moisture%, $r^2 = 0.80$, $n = 46$, $p < 0.001$).
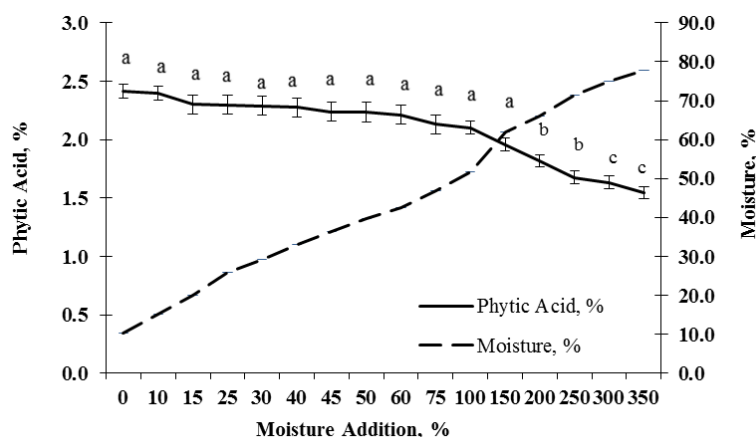

Figure 2. Phytate and moisture contents of canola meal with different moisture additions in a hydrothermal reactor (Exp. 1)

Note. [a-c]The different lowercase letters indicate significant difference at $p < 0.01$. 0 = Control 0, solvent extracted canola meal, untreated.

Specifically, with a gradual reduction of PA level with addition of increasing moisture amount, at 200 to 350% moisture additions (which corresponded to 66 to 77.7% of the moisture content of the meal) 24.8 to 36% decreases were observed ($p < 0.05$) compared to the untreated CM. This effect could be related to the high moisture providing favorable condition for the enzyme with increased substrate solubility and access of the enzyme to the substrate and to phytate-bound nutrients. This agrees with much of the literature as indicated in that phytase is inactive in dry conditions, but activated in wet conditions to degrade phytate (Blaabjerg, Carlsson, Hansen-Moller, & Poulsen, 2010), that moisture greatly influenced the efficacy of phytase and other enzymes increasing phytate hydrolysis in CM (Slominski et al., 1999), and that soaking in water for 2 h at 30°C before feeding increased the efficacy of phytase for apparent P absorption by 47.7% and reduced apparent fecal excretion by 18.3% (Liu, Bollinger, Ledoux, Ellersieck, & Veum, 1997). Conversely, others have shown that high moisture was deleterious for the intrinsic phytase, especially at high temperatures (Eeckhout, 2000, Esmaeilipour, Van Krimpen, Jongbloed, De Jonge, & Bikker, 2013).

The high moisture (>150%) favorable for PA breakdown in CM as shown in Exp. 1, would not be feasible in a feed mill condition due to high energy consumption and costs. Hence, an attempt to create conditions more analogous to a commercial feed mill, 100% moisture addition was applied to CM in Exp. 2 and others (unless

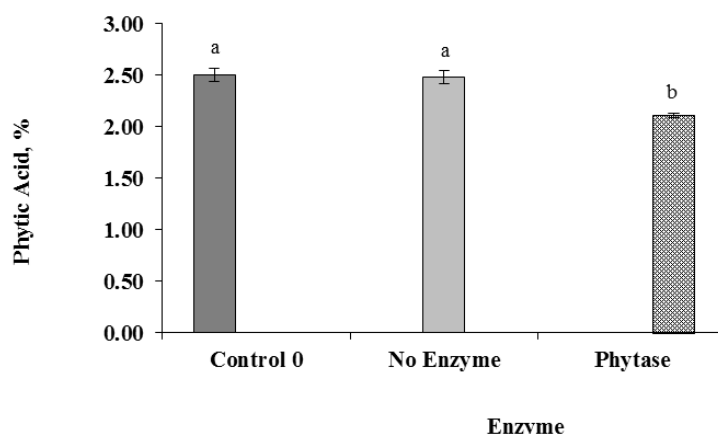otherwise specified). Results of Exp. 2 are displayed in Figure 3.

Figure 3. Phytate content of canola meal treated with and without phytase in a hydrothermal reactor (Exp. 2)

Note. [ab]Bars with different lowercase letters indicate significant difference at $p < 0.05$. Moisture condition was 100%. Control 0 = solvent extracted canola meal, untreated. No Enzyme = solvent extracted canola meal without phytase, but with 100% moisture addition.

Expectedly, phytate degradation was higher (15.5%) in CM with phytase (1000 FTU/kg) compared to both, untreated CM ($p < 0.05$) or without phytase, but with 100% moisture addition ($p < 0.05$) (Figure 3), which was similar to the increased P retention by 14.3% units, compared with the control diet with phytase, at the same rate as ours, obtained by Emiola et al. (2009), but was lower than the 38.9% increase by Nelson et al. (1971) with 950 FTU/kg fungal phytase in broiler chick diet.

In further optimizing HTR condition for CM with phytase and organic acid additions, the results of the experiments (Exp. 3-6) indicated that the enzyme action for breaking down phytate was most effective under pH 4.0 and 5.0 ($p < 0.05$) (Figure 4) and all three (30, 45, and 60 min) incubation times ($p < 0.05$) (Figure 5) and acids ($p < 0.05$) (Figures 4, 5, and 6).

The optimal pH values obtained in the current study were similar to the ranges recorded by others for improved efficacy of the *E. coli*-derived acidic type phytate-degrading enzymes to which the enzyme in the current study belongs to, i.e., between pH 3 and 5 (Newkirk & Classen, 1998; Menezes-Blackburn, Gabler, & Greiner, 2015), pH 2 and 4.5 (Adeola et al., 2004), and lower than the optimal pH ranges from 4.5 to 6.0 (Konietzny & Greiner, 2002). Phytases will differ in their phytate-degrading activities at other than their standard pH conditions (i.e., pH 5.5 at 37 °C), with microbial phytases performing better at pH values below pH 5.5 (Greiner & Bedford, 2010).
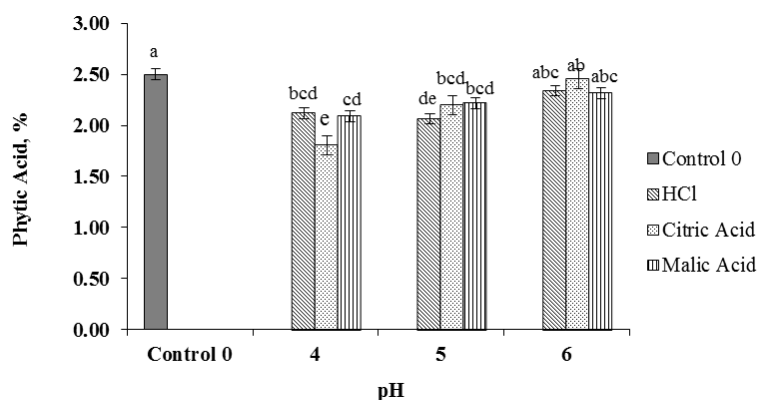

Figure 4. Phytate content of canola meal treated with phytase, at different pH and acids in a hydrothermal reactor (Exp. 3)

Note. [a-e]Bars with different lowercase letters indicate significant difference at $p < 0.01$. A pH × acid interaction was detected ($p < 0.01$). Moisture condition was 100%. Control 0 = solvent extracted canola meal, untreated.
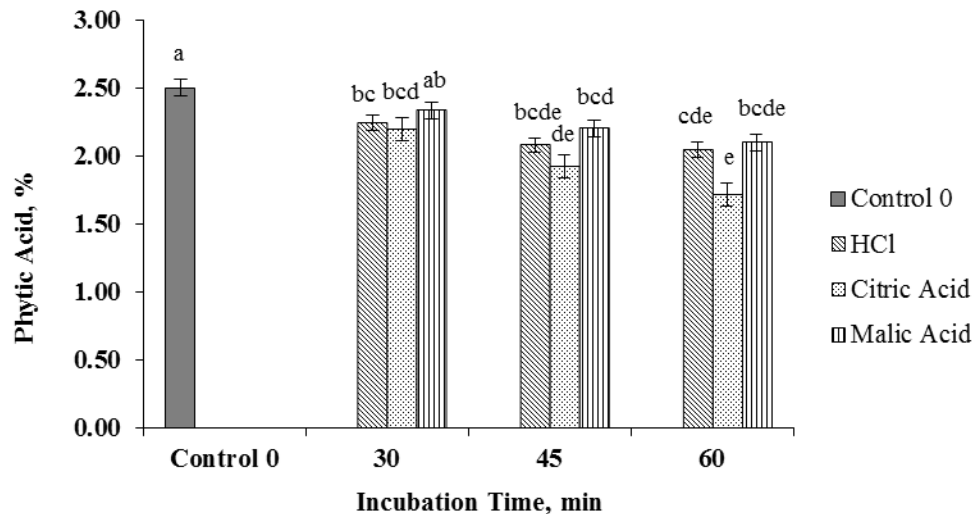
Figure 5. Phytate content of canola meal treated with phytase, at different incubation times and acids in a hydrothermal reactor (Exp. 4)

Note. [a-e]Bars with different lowercase letters indicate significant difference at $p < 0.01$. No time $\times$ acid interaction was detected ($p = 0.27$). Moisture condition was 100%. Control 0 = solvent extracted canola meal, untreated.

Results of the experiments 3-6, also, demonstrated that acid, pH, and incubation time affected phytate degradation ($p < 0.05$). Although, a significant acid $\times$ pH interaction ($p < 0.01$) was observed, an acid $\times$ time interaction was not detected ($p = 0.27$). Phytase in combination with organic acid and HCl, was more effective in degrading PA at pH 4.0 and pH 5.0 ($p < 0.05$) (Figure 4) and 30-60 min of incubation time of CM in HTR ($p < 0.05$) (Figure 5) with the exception of MA at 30 min ($p > 0.05$).

Even though, as the results in Figures 3-6 indicated, difference between the effects of HCl and organic acids on PA was negligible ($p > 0.05$), using organic acids is preferable due to their potential prebiotic benefits (Jongbloed et al., 2000; Dibner & Buttin, 2002; Tsukahara et al., 2002; Proulx & Reddy, 2007; Li et al., 2008). Most efficient in degrading PA was the CA treatment with phytase, at pH 4.0, and 30 and 60 min incubated in HTR (by 27.7 and 31.5%, respectively), compared to the untreated CM, with the highest decline in PA being detected with the CA treatment at 60 min of incubation time (by 31.5%, Exp. 4, $p < 0.05$).

Bressani, Turcios, de Ruiz, and de Palomo, (2004) reported a 14.9% loss of PA by cooking whole grain maize for 75 min with addition of 1.2% lime, the processing time of which was close to the highest effective time (60 min) detected in the current study. Adeola et al. (2004), in an experiment with *E. coli*-derived phytase, also, processed the sample in a water bath at 40 ℃ for 1 h, analogous to the conditions in the current study. Interestingly, it was observed during our experiments that longer incubation times (to 24 h) of CM in HTR with phytase and moisture could lead to 84% of PA hydrolyzed relative to the initial PA level (data not shown). Likewise, Adeola et al. (2004) observed that during a 24-h incubation with 500 FTU/kg of *E. coli*-derived phytase 81% of P was released in corn diet, and water steeping of maize for 8 h resulted in a 29% loss of PA regardless of temperature (Bressani et al., 2004). Furthermore, with superdosing fungal phytases, Liu (2014) documented a complete phytate hydrolysis in coproducts of ethanol production within 5-60 min of enzymatic treatment under optimal condition (pH 4.2-4.3; temperature 50-60 ℃). These findings support the current results indicating an influence of processing time on phytate degradation. The positive effect of adding organic acids to diets was documented by others, as in Hoehler and Pallauf (1993), the addition of 1.5% citric acid to a corn-soy diet for young pigs improved P availability and tended to improve the utilization of Ca, Mg, and Fe, and in Liem, Pesti, and Edwards Jr., (2008), the addition of citric and malic acids significantly increased the retention of P and decreased phytate P in broiler chicks. Interestingly, when fed a diet containing 6% citrate, CA primarily affected phytate-P utilization in chicks, but had smaller effects in pigs (Boling et al. 2000). As suggested by Han et al. (1998), there may be two possible mechanisms for CA promoting dietary P utilization, it may be enhancing the solubility of digesta P and the transit time of digesta in the small intestine by lowering digesta pH (Jongbloed et al., 2000), thereby improving total P absorption.

Phytases from different sources typically vary in their activity and recorded in the literature (Leske & Coon, 1999; Igbasan, M änner, Miksch, Borriss, Farouk, & Simon, 2000). Two phytase types were tested in HTR Exp. 5, 'Quantum Blue', *E. coli*-derived 6-phytase and 'Finase', *A. niger*-derived 3-phytase, and the results are presented in Figure 6.
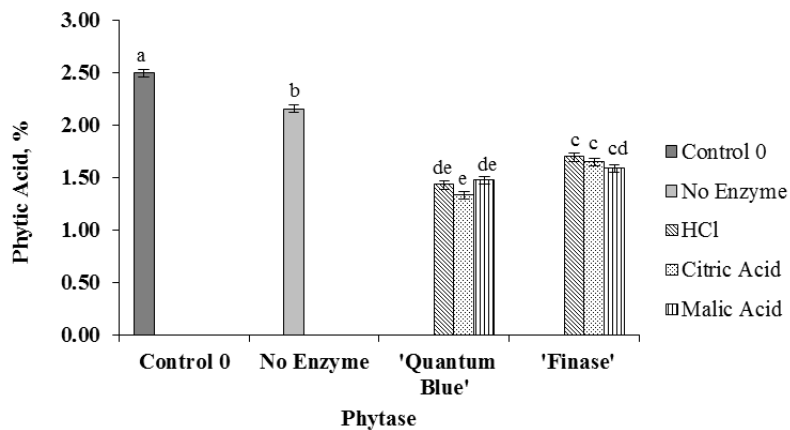

Figure 6. Phytate content of canola meal treated with different phytase types and acids in a hydrothermal reactor (Exp. 5)

Note. [a-e]Bars with different lowercase letters indicate significant difference at $p < 0.01$. A phytase $\times$ acid interaction was detected ($p < 0.05$). Moisture condition was 200%. Control 0 = solvent extracted canola meal, untreated. No Enzyme = solvent extracted canola meal without phytase, but with 200% moisture addition.

The results demonstrated that acids did not differ ($p > 0.05$), but phytases differed ($p < 0.05$) with 'Quantum Blue' displaying greater phytate hydrolysis (41-46.6%) than 'Finase' (32.1-36.4%), although an interaction of acid $\times$ phytase was detected ($p < 0.05$). Similarly, Wilcock (2012) in a study of phytases in pig diets, pointed out that Quantum Blue was more effective than a wild type *E. coli* phytase in phytate breakdown. Due to the difference in composition, levels and location of phytate (IP6), as well as the contribution of intrinsic phytase in some cereals and oilseeds, the rate of hydrolysis of phytate by microbial phytase can vary to a large extent in these plant-based ingredients (Leske & Coon, 1999), also, phytases of various microbial origins behave differently with respect to their in vitro properties (Igbasan, M änner, Miksch, Borriss, Farouk, & Simon, 2000). Moreover, the substrate phytate was reported to act as an inhibitor of many histidine acid phytases (Konietzny & Greiner, 2002) to which both of the phytases in the current study belonged. Therefore, substrate inhibition must be considered when determining phytate-degrading activity (Bedford, 2000). The greatest decline (46.6%) of PA in the present study was observed with 'Quantum Blue' phytase in combination with citric acid ($p < 0.05$) compared to control.

Again, differences in the declines in PA with the same phytase and citric acid treatments in Exp. 3 and Exp. 5 (27.7 vs. 46.6%) may be explained by the different moisture conditions in the meal (100 vs. 200%). Even though the high moisture during processing of CM in the HTR was favorable for PA breakdown in CM as the results in the present study suggested (Figures 2-6), in exp. 6, the amount of moisture addition was reduced further (from 100 to 50%) to make the treatment application industrially more feasible. As the results showed (Figure 7), PA was similarly influenced with phytase and organic acid under both 50 and 100% moisture additions with the highest declines in PA ($p < 0.05$) being found for CA treatment (11 and 19.4%, respectively).
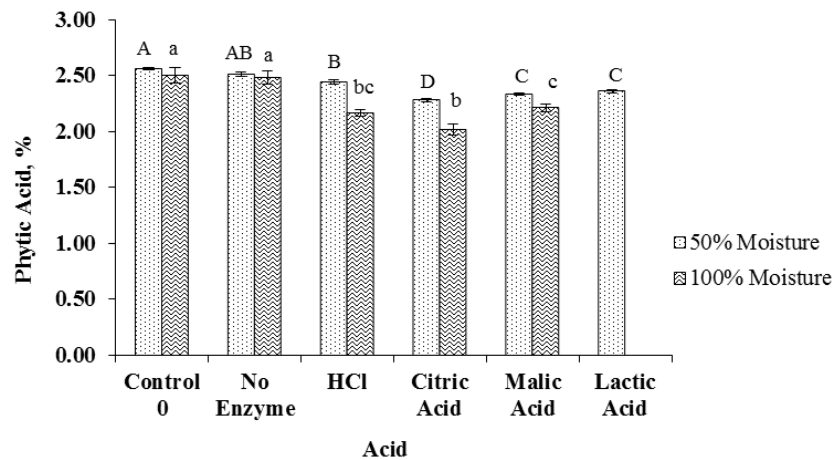
Figure 7. Phytate content of canola meal treated with phytase, different moisture and acid in a hydrothermal reactor (Exp. 6)

Note. [A-D]Bars with different uppercase letters indicate significant difference within the 50% moisture treatment at $p < 0.01$. [a-c]Bars with different lowercase letters indicate significant difference within the 100% moisture treatment at $p < 0.01$. Control 0, solvent extracted canola meal untreated. No Enzyme, solvent extracted canola meal without phytase, but with HCl.

Likewise, under 70-80% moisture conditions of CM with an enzyme cocktail that included phytase, Slominski et al. (1999) obtained the highest degree of phytate hydrolysis (22.5-29.8%) compared to the control, and 80% moisture was considered the optimal moisture conditions for enzyme treatment of canola meal. Overall, the results of the present study suggested that CM processing in HTR with a combination of moisture (50 to 100%), phytase, and organic acid (CA, MA, and LA) at pH 4, for 30 to 60 min would create an effective condition for phytate degradation in the meal, thus improving its quality and utilization for livestock.

4. Summary and Conclusions

Findings of the current study indicated that a greater efficacy of phytase can be achieved using hydrothermal pretreatment of CM and with increasing moisture addition (to 350%) greater phytate degradation was possible. Processing of high moisture feed ingredient, however, would not be feasible in industrial mills, especially in pelleted form (a standard form for monogastrics). Even with less moisture addition (50%), phytase in combination with organic acid can reduce the PA level (by 11%) compared to the untreated CM. The optimal condition for reducing phytate in CM with phytase was determined as pH 4 and pH 5 and 30-60 min incubation time, with temperature, moisture, and phytase rate at 40 °C, 100%, and 1000 FTU/kg, respectively. Under this condition, up to a 19.7% decrease in PA can be achieved, with longer incubation time favoring more reduction. Adding an organic acid provided more favorable condition for phytase as indicated by the higher phytate reduction (by up to 31.5%) when citric or malic acid was added in the meal. Overall, combination of hydrothermal, phytase, and organic acid pretreatments of CM would be an effective tool in improving the quality of the meal by reducing the phytate level, thus increasing availability of the nutrients for livestock and decreasing their release to the environment. Certainly, further investigation is crucial for optimizing the efficacy of phytase and other additives during hydrothermal processing of CM to better utilize CM for livestock diet.

Acknowledgements

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