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Extruded fish feed with high residual phytase activity and low mineral leaching increased *P. mesopotamicus* mineral retention

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ABSTRACT

This study attempts to provide valuable information about optimization of extrusion process in order to have a fish feed with maximum residual phytase activity, minimum mineral leaching and good mechanical characteristics. Also, the effects on mineral retention of extruded feed obtained in optimal condition, using a juvenile *Piaractus mesopotamicus* model was determined. In order to study the simultaneous effect of blend moisture (*M*) and extrusion temperature (*T*) on specific volume (SV), water resistance (WR), floatability (F), residual phytase activity (RPA), leached phosphorus (LP), calcium (LCa), zinc (LZn), and iron (LFe) a central composite design (3^2) was used. The levels of each variable were: *T*: 160–180–200 °C and *M*: 140–160–180 g/kg. A multiple response optimization of physicochemical properties of extruded feed (WR, F, RPA and mineral leaching) was performed using the Derringer's desirability function. The global desirability function value was 0.8990, and the obtained optimal conditions were 183.6 °C and 158 g/kg of moisture content. Phytase extruded feed (PEF) was obtained at such conditions and it had the following physical properties: WR: 81.8 ± 2.5%, F: 94.5 ± 0.72%, LP: 9.40 ± 0.61%, LCa: 2.20 ± 0.2%, LZn: 2.00 ± 0.15%, and LFe: 11.2 ± 2.8%. Fish consuming PEF with RPA of 3934.9 ± 47.7 UP/kg had higher iron, zinc, and phosphorus retention than those fed with control extruded feed (CEF) obtained under the same optimal conditions ($p < 0.05$). However, no significant difference in final body weight was detected between dietary treatments ($p > 0.05$) after 38 days of feeding trial at 25 °C. Extrusion process can be optimized to obtain fish feed based on vegetable meals with high residual phytase activity and low mineral leaching, increasing *P. mesopotamicus* mineral retention.

1. Introduction

Phosphorus (P) is the most important mineral required by fish, as its requirement is higher than that of other minerals (Rocha et al., 2014). If the diet does not supply sufficient bioavailable P, fish easily show deficiency signs such as poor growth and bone deformity. However, it has also been reported that excessing amounts of dietary P inhibit zinc utilization (Satoh et al., 1996). On the other hand, P is considered to be a major factor of eutrophication in aquaculture environment (Phillips et al., 1993). Thus, it is necessary that P excretions from cultured fish were kept at minimum level. Other minerals, such as iron and zinc are essential

Abbreviations: CEF, control extruded feed; PEF, extruded feed added with phytase enzyme; *M*, blend moisture; *T*, extrusion temperature; SV, specific volume; WR, water resistance; RPA, residual phytase activity; F, floatability; LP, leached phosphorus; LCa, leached calcium; LZn, leached zinc; LFe, leached iron; SMEC, specific mechanical energy consumption; TMR, total mineral retention; LPO, lipid peroxidation

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elements having fundamental roles in fish cellular biochemistry and metabolism (Rocha Aride et al., 2010; Aisen et al., 2001). Moreover, fish diets are commonly supplemented with inorganic iron and zinc sources to satisfy nutritional requirement of the specie. However, clinical signs of nutritional iron and zinc deficiency might be observed if the content of these minerals in the feed is inappropriate (Carriquiriborde et al., 2004; Do Carmo et al., 2005). Therefore, a loss of minerals in water decreases the nutritional quality of the food and can affect the fish growth, increasing production cost due to greater supplementation.

Fish feed can be produced by pelleting or extrusion cooking. Extrusion technology is commonly used to produce fish feeds, since physical properties, such as water stability, durability, hardness, and buoyancy control, usually are improved compared to steam pelleted diets (Sørensen et al., 2009). On the other hand, most of studies showed that partial replacement of fish meal by other protein sources such as soybean meal can be successful (Hossain and Koshio, 2017; Jirsa et al., 2015). In this regard, partial replacement of fish meal with soybean meal improved physical quality of feed in terms of breaking force and durability, reducing bulk density and increasing radial expansion (Sørensen et al., 2002). However, the direct use of the leguminous oilseed as a dietary ingredient is limited since the presence of phytic acid and other anti-nutritional factors such as tannin, glucosinolates, saponins, soluble no starch polysaccharides and gossypol, which are not destroyed or inactivated by processes involved with product manufacture or during extrusion pelleting. (Roy et al., 2014; Hardy, 2010).

Soybean meals contain around 14 g/kg phytic acid (Deak and Johnson, 2007). Up to 80% of total P content is in the form of phytate and is practically not available for monogastric or agastric aquatic animals (Hardy, 2010). Phytates present in plant meals are negatively charged. They can bind cations or positively charged functional groups of molecules. The complexes formed with minerals are not absorbed through the gastrointestinal tract and bioavailability of minerals is decreased (Albarracín et al., 2015). Therefore, it is important to find ways to improve mineral availability of minerals in extruded fish feeds based on vegetable meals. A promising alternative is using a microbial phytase. Phytase (myo-inositol hexakisphosphate phosphohydrolase) is a phosphatase enzyme that catalyzes the hydrolysis of phytate to inositol and inorganic P (Lemos and Tacon, 2017). Different studies have reported the use of phytase increases mineral bioavailability such as phosphorus, iron, magnesium, calcium, manganese, and zinc (Roy et al., 2014; Morales et al., 2014; Cao et al., 2007; Cheng and Hardy, 2003; Lemos and Tacon, 2017). However, the effects of extrusion conditions on phytase activity and mineral leaching have not been studied. Moreover, there are not reports about residual phytase activity after the extrusion process and even less about optimized extrusion conditions to obtain a fish feed with maximum residual phytase activity and minimal mineral leaching. In this context, the aims of this work were: (i) to develop a fish feed formula based on vegetable meals added with phytase enzyme, (ii) to optimize extrusion conditions in order to have a product with maximum water resistance, floatability, and residual phytase activity; but minimum mineral leaching, (iii) to assess P, Ca, Fe, and Zn retention of this feed using a juvenile *P. mesopotamicus* model.

2. Materials and methods

2.1. Production of experimental diets

Experimental diets (CEF and PEF) were formulated with commercial corn meal (613 g/kg), soybean meal (200 g/kg), bovine plasma protein concentrate (130 g/kg), corn starch (20 g/kg), vitamin-mineral mix (7 g/kg), and canola oil (30 g/kg) taking into account the nutrient requirement for *P. mesopotamicus* (Bicudo et al., 2009). Soybean meal and bovine plasma protein concentrate were donated by America Pampa Agroindustrial S.A. (America, Argentina) and Yeruvá S.A. (Esperanza, Argentina), respectively. Thermo-resistant microbial phytase (Ronozyme, NOVOZYME®) was added to PEF at 0.2 g/kg.

The ingredients were mixed using a Yelmo 2202 dough mixer and water was added in order to achieve moisture content. The blends were sealed in polyethylene bags and stored 1 h at room temperature before each run for moisture stabilization. Moisture content of blends was checked using AOAC (2000) methods.

The extrusion process was carried out with a Brabender 10 DN single-screw extruder, using a 3:1 compression ratio screw, a 3/20 mm (diameter/length) die and 175 rpm screw speed. A Central Composite Design (CCD) (3^2), with three replicates in the central point resulting in 11 runs, was used to study the simultaneous effect of blend moisture (*M*) and extrusion temperature (*T*) on specific volume (SV), water resistance (WR), floatability (F), residual phytase activity (RPA), leached phosphorus (LP), leached calcium (LCa), leached zinc (LZn), and leached iron (LFe) of PEF. The levels of each factor were: *T*: 160–180–200 °C and *M*: 140–160–180 g/kg. Experiments were randomized. While the extruder feeding section temperature was maintained by circulating water through the jacketed device, the metering and die section temperature were both kept at that corresponding to each run by using the heat control device of the extruder. The feeding rate of the extruder was at full capacity. Experimental samples were taken after stationary state was established, then torque (Brabender Units – BU) and mass output (g/min) were measured.

Extrudates were dried in an oven at 40 °C (Bioelec) until a moisture content of ~80 g/kg was reached, divided into several portions and kept in plastic bags hermetically sealed until evaluation. For chemical analysis, extruded feeds were ground with a cyclone sample mill (UDY Corp Boulder Colorado, USA) using a 1 mm sieve.

In order to evaluate the effect of PEF obtained in optimal extrusion conditions on mineral retention of juvenile *P. mesopotamicus*, an extruded feed without phytase (CEF) was obtained under the same extrusion conditions than PEF, and used as a control for *in vivo* assay.

2.2. Physical properties of feed added with phytase (PEF)

2.2.1. Specific volume (SV)

Specific volume (SV) was determined according to González et al. (2002). Diameters of extruded feeds were measured with a caliper (Vernier, 0–150 mm, Stronger Argentina) on 10 pieces of sample randomly selected. Each piece was weighed and SV was calculated as the ratio between the volume and the weight of the extruded piece (cm^3/kg).

2.2.2. Water resistance (WR) and mineral leaching

Extruded feeds were dispersed at 50 g/kg in distilled water (at 25 °C), stirred during 30 min and filtered through a 50-mesh sieve (0.297 mm). The retained solids (particle size > 0.297 mm), were weighed and the solid content was determined after drying (24 h at 105 °C). Water resistance (WR) was calculated as: (g retained solids × 100)/g dry extruded feed. All determinations were performed by triplicate.

Calcium, zinc, and iron contents in retained solids were determined by atomic absorption spectroscopy after dry mineralization. Also, phosphorus content was determined following AOAC (2000) methods. Mineral leaching was calculated as follow:

$$\text{Mineral leaching (\%)}: [(A - B)/A] \times (100)$$

Where, A: extruded feed weight (g) x mineral content of extruded feed (g/g); B: retained solid weight (g) x mineral content of retained solids (g/g).

All determinations were performed by triplicate.

Leached phosphorus, calcium, zinc, and iron percentages were named LP, LCa, LZn, and LFe, respectively.

2.2.3. Floatability (F)

Floatability (F) was determined according to De Cruz et al. (2015), with some modifications. Ten pieces of each extrudate were poured into a 100 mL beaker filled with distilled water at room temperature. The number of floating extrudates (Nf) suspended in the beaker was observed after 30 min and F was calculated as [(Nf/10) × 100]. All determinations were performed by triplicate.

2.2.4. Residual phytase activity (RPA)

Residual phytase activity (RPA) was tested according to Hassaan et al. (2013). One phytase unit (PU) was defined as the enzyme activity that releases 1 μmol phosphorus/min under the given reaction conditions. Results were expressed as PU/kg extruded feed. All determinations were performed by triplicate.

2.3. Optimization of extrusion process

Derringer's desirability function was used for multiple response optimizations according to Derringer and Suich (1980). The method involves transformation of each predicted response to a dimensionless partial desirability function (*di*). The global desirability function (D) is defined as the geometric mean of the different *di* values. A value of D different from zero implies that all responses are in a desirable range simultaneously and, consequently, for a value of D close to 1, the combination of the criteria is globally optimal. In this work, WR, F, and RPA were maximized, while mineral leaching was minimized.

The specific mechanical energy consumption (SMEC) of extruded feed obtained in optimal conditions was determined according to González et al. (2002).

2.3.1. Model validation

Samples of experimental extruded feed were obtained in the same way as it was previously described at extrusion conditions (*T* and *M*) given by the optimization procedure. The experimental data (WR, F, RPA and mineral leaching) were compared to values of these responses predicted by the models. Additionally, experimental data responses (WR, F, RPA and mineral leaching) obtained in each confirmatory experiment were compared to values predicted from the developed models by a *t*-test analysis.

2.4. Chemical analysis of diets obtained in optimal conditions

Chemical composition, phosphorus, and phytic acid of diets were determined using AOAC (2000) approved methods. Iron, zinc, and calcium contents of extruded feed were measured by atomic absorption spectroscopy after dry mineralization using an atomic absorption spectrophotometer analyst 300 Perkin-Elmer (Norwalk, CT, USA). Phytase activity of diets after extrusion was tested as mentioned before. Results were expressed as PU/kg diet. All determinations were performed at least in triplicate.

Chemical composition and phytase activity of diets are shown in Table 1. Chemical analysis confirmed diets supplied a similar amount of macronutrients. In agreement with diet formulation, phytase activity only was detected in PEF.

2.5. Effects of feeds obtained in optimal conditions on mineral retention of juvenile P. mesopotamicus

2.5.1. Fish and feeding trial

Juvenile *P. mesopotamicus* were obtained from a fish farm (Pez Campero Paraná, Argentina). The experiment was performed in the

Table 1
Chemical composition and phytase activity of extruded feeds (CEF and PEF).

Components[a]	CEF (g/kg)	PEF (g/kg)
Dry matter	900.6 ± 5.3	895.3 ± 4.7
Crude protein	285.5 ± 4.2	276.8 ± 4.7
Crude lipid	34.4 ± 2.6	35.7 ± 1.9
Total starch	444.2 ± 4.3	446.5 ± 0.3
Ash	23.6 ± 0.9	25.6 ± 0.43
Calcium	1.6 ± 0.1	1.8 ± 0.2
Phosphorous	2.2 ± 0.21	2.4 ± 0.2
Zinc	0.1 ± 0.0	0.1 ± 0.0
Iron	0.2 ± 0.0	0.2 ± 0.0
Phytic acid	8.1 ± 0.2	7.9 ± 0.1
Phytase activity (PU/kg)[b]	N.d	6080.4 ± 182.0

N.d: not detected.
[a] Chemical composition expressed as mean ± SD (n = 3).
[b] PU: μmol P/min.

Aquaculture Laboratory at the Instituto Nacional de Limnología (CONICET, Argentina) in a recirculating water system supplied with dechlorinated city (tap) water, and equipped with an external quartz-anthracite filter (Multiválvula Vulcano Filtro VC10). The water flow to the tanks was at 15.1 L/min with artificial aeration and 12/12 h light/dark photoperiod regime provided by artificial illumination. Physico-chemical parameters of the water remained within the values recommended by Urbinati et al. (2010) for *P. mesopotamicus* (temperature 25.0 ± 1 °C, dissolved oxygen 6.67 ± 0.63 mg/L, pH 6.15 ± 0.32, electrical conductivity 189.40 ± 25.93 μs/cm, and total ammonia nitrogen 0.24 ± 0.1 mg/L).

Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks. At the start of the feeding experiment, 24 juvenile *P. mesopotamicus* (initial body weight 10.7 ± 1.9 g) were randomly stocked into four conical tanks with 6 fish per tank. The diets obtained in optimal conditions, CEF (control) and PEF (phytase), were randomly assigned to duplicate tanks. Fish were fed twice a day with known feed weight, during 38 days at 25 °C. Uneaten diet was collected to prevent nutrient leaching, dried, and weighed.

The experiment was conducted in accordance with national and institutional guidelines (CONICET, 2005) for the protection of animal welfare.

2.5.2. Sample collection and morphometric indexes

At the end of the feeding trial, fish from each diet treatment were anaesthetized in benzocaine 0.1 g/L as described Parma de Croux (1990). Body weight (g), total and standard length (cm) were recorded for each individual. White muscle, liver, and intestine were dissected, quickly frozen in liquid nitrogen, and subsequently stored at −80 °C until lipid peroxidation (LPO) and enzymes involved in antioxidant system analysis.

2.5.3. Total mineral retention of fish fed with experimental feeds

Total mineral retention of diets was measured according to Satoh et al. (2003), using the juvenile *P. mesopotamicus* model. Whole fish was ground using a Moulinex AD5661AR meat mincer (Buenos Aires, Argentina) in order to obtain a homogenate. Phosphorus content of homogenates was determined following AOAC (2000) approved methods. Iron, zinc and calcium contents were measured by atomic absorption spectroscopy after dry mineralization. All determinations were performed at least in triplicate.

Total mineral retention (TMR) was calculated as the amount of mineral retained in whole fish expressed as a percentage of total amount of mineral consumed on experience:

$$TMR\ (\%) = [(ME - MB)/MC)] \times (100)$$

Where, TMR: total mineral retention, ME: mineral (mg) of fish at the end of experience (accumulated final weight x mineral content of fish at the end of experience), MB: mineral (mg) at the beginning of experience (accumulated initial weight x mineral content of fish at the beginning of experience), and MC: mineral (mg) consumed during experience (accumulative feed intake x mineral content of feed).

Each fish was analyzed individually and the results reported represent a mean of the values obtained from the duplicate tanks. All assays were performed by triplicate.

2.5.4. Lipid peroxidation and enzymes involved in the antioxidant system of liver, intestine, and muscle of fish fed with experimental feeds

Tissues extracts for the determination of lipid peroxidation (LPO) of liver, intestine, and muscle were prepared from each individual fish. Briefly, tissues were homogenized using phosphate buffer (pH 7.4; 30 mmol/L). The homogenate was centrifuged at 3200 *xg* (4 °C) for 30 min, and the supernatant was collected and stored at −80 °C. Plasma, liver, intestine, and muscle LPO levels were determined by measuring thiobarbituric reactive substances (TBARS), according to Yagi (1976). Protein content of each extract was determined according to Bradford (1976).

Extracts for the determination of antioxidant enzyme activities from liver, intestine, and muscle were prepared from each

Table 2
Central composite design responses for specific volume (SV), water resistance (WR), floatability (F), residual phytase activity (RPA), leached phosphorus (LP), leached calcium (LCa), leached zinc (LZn) and leached iron (LFe).

Extrusion conditions		SV (cm^3/g)	WR (%)	F (%)	RPA (PU/kg)	LP (%)	LCa (%)	LZn (%)	LFe (%)
Temperature (T ºC)	Moisture (M g/kg)								
160	140	3.1	61.5	91.0	3851.2	27.5	76.2	42.7	25.8
160	160	2.2	74.7	79.0	4069.5	13.5	8.7	7.8	3.2
160	180	1.6	20.3	65.0	4240.6	41.8	86.6	57.3	60.6
180	140	3.6	77.9	95.0	4125.7	7.0	9.8	7.6	7.6
180	160	3.6	83.5	93.0	3990.0	15.1	15.3	3.5	17.8
180	160	4.0	80.0	94.0	3905.6	9.4	4.9	11.0	10.4
180	160	3.6	75.5	95.0	3909.2	10.3	2.0	5.5	12.2
180	180	2.3	63.2	80.0	4093.1	29.9	29.0	36.3	43.3
200	140	3.1	86.0	90.0	3575.4	5.0	3.6	2.0	2.0
200	160	3.4	85.8	92.0	3902.1	6.8	7.3	4.8	7.6
200	180	1.9	79.5	71.0	3999.3	13.4	14.9	17.8	17.8

individual (not pooled), according to Bacchetta et al. (2014). The activity of glutathione-S-transferase (GST, EC 2.5.1.18) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR, EC 1.6.4.2) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, 1.15.1.1) activities were determined according to Beutler (1982) and Misra and Fridovich (1972), respectively. The enzyme activities were calculated in terms of sample protein content. All assays were carried out in triplicate.

2.6. Statistical analysis

STATGRAPHICS Centurion XV 15.2.06 (Statpoint Technologies, Inc., Warrenton, Virginia, USA) was used to perform ANOVA, to fit the second-order polynomial equations of experimental data (Table 2) and to obtain the coefficients of such equations. The significance of each term of the models was evaluated referred to pure error. For verification of the model adequacy, the lack of fit and the coefficient of determination (r^2) were calculated. STATGRAPHICS Centurion XV 15.2.06 was used as well for the numerical optimization procedure through the Derringer's desirability function. The statistical differences among samples were determined using the least significant difference (LSD) test with a level of signification $\alpha = 0.05$.

3. Results

3.1. Effect of extrusion conditions on physical properties of diet added with phytase (PEF)

Table 2 shows physical properties of experimental extruded feeds obtained at different extrusion conditions. Table 3 shows the degree of significance (p values) corresponding to each polynomial term of the regression model. Regression models can be considered adequate to describe the effects of T and M on each response since the lack of fit was not significant ($p > 0.05$), and the coefficient of determination (r^2) was acceptable for each one.

For specific volume (SV), the effects of T^2 and M were significant ($p < 0.05$). SV was inversely related to M and was observed a maximum at intermediate T (183 °C) and low M (Fig. 1a). The highest values corresponded to the extruded feed obtained at 180 °C and 160 g/kg M. In the case of water resistance (WR), all terms, except T^2, were significant ($p < 0.05$) (Table 3). A maximum in WR response surface at 184 °C and 157 g/kg M was observed (Fig. 1b). Regarding floatability (F), the effects of T and M were significant in

Table 3
Analysis of variance for the overall effect of the two variables on specific volume (SV), water resistance (WR), floatability (F), residual phytase activity (RPA), leached phosphorus (LP), leached calcium (LCa), leached zinc (LZn) and leached iron (LFe).

Source of variation		*P-values*						
	SV (cm^3/g)	WR (%)	F (%)	RPA (PU/kg)	LP (%)	LCa (%)	LZn (%)	LFe (%)
Temperature (T)	0.1023	**0.0105**	**0.0180**	**0.0280**	**0.0163**	**0.0136**	**0.0128**	**0.0224**
Moisture (M)	**0.0157**	**0.0239**	**0.0017**	**0.0217**	**0.0256**	**0.0104**	**0.025**	**0.0119**
T^2	**0.0327**	0.2250	**0.0056**	0.1232	0.6133	0.0869	0.2409	0.3114
T x M	0.4901	**0.0495**	0.0728	0.7520	0.4338	0.9560	0.8961	0.1329
M^2	0.0509	**0.0308**	**0.0096**	0.2631	**0.0383**	**0.0289**	**0.015**	**0.0202**
Lack of fit	0.2231	0.1103	0.1517	0.1620	0.1387	0.2062	0.1954	0.1746
r^2	0.9127	0.8793	0.9478	0.9433	0.8572	0.9178	0.8331	0.8194

Bold values indicates significant differences ($p < 0.05$). Degrees of freedom: n-1.

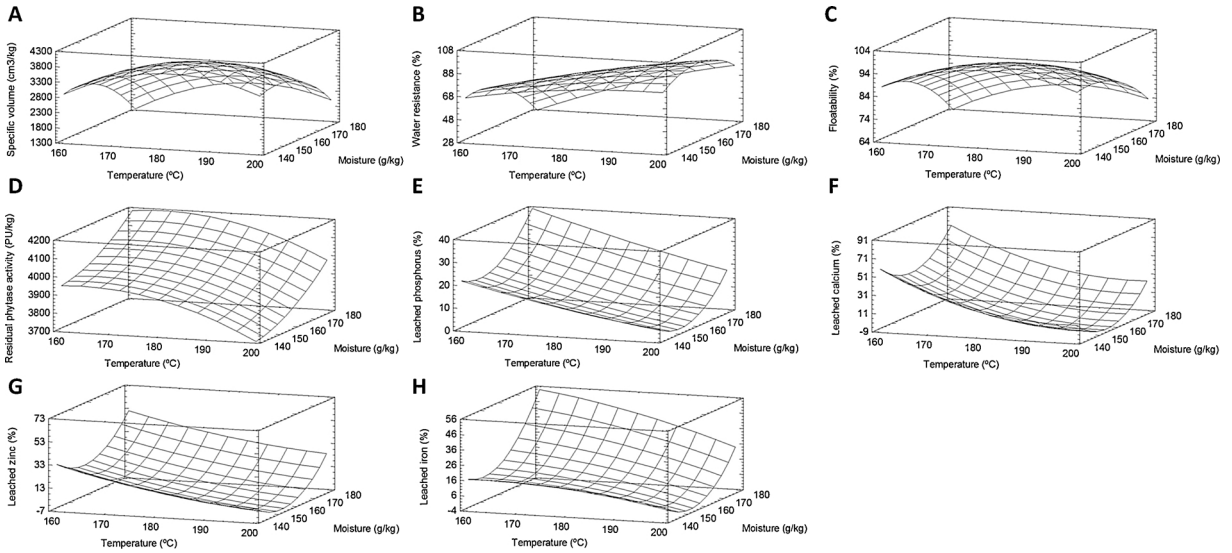

Fig. 1. Response surface plot corresponding to the effects of extrusion temperature and moisture content on specific volume (A), water resistance (B), floatability (C), residual phytase activity (D), leached phosphorus (E), leached calcium (F), leached zinc (G), and leached iron (H).

both, linear and quadratic terms (Table 3). F response surface (Fig. 1c) showed a maximum at intermediate values of *T* and *M* (182 °C and 151 g/kg). In this regard, a direct relationship between F and SV (r^2: 0.9288) was observed. The ANOVA results for residual phytase activity (RPA) showed that only the linear terms of *M* and *T* were significant. RPA was directly related with *M* and showed a minimum at 200 °C and 140 g/kg *M* (Fig. 1d). The reduction rate of phytase activity after extrusion ranged from 30.2 to 42.0%, the highest value corresponding to 200 °C and 140 g/kg *M*. Regarding leached phosphorus (LP), calcium (LCa), zinc (LZn), and iron (LFe), the effects of *T*, *M*, and M^2 were significant ($p < 0.05$) (Table 3). LP decreased with *T* and increased with *M*, showing a maximum at 160 °C and 180 g/kg *M* (Fig. 1e). As for LP, LCa was inversely related with *T*, for all the moisture range studied (Table 2). In this regard, the maximum value of LCa is obtained at the same extrusion conditions corresponding to the maximum value of LP (160 °C and 180 g/kg *M*) (Fig. 1f). Regarding LZn and LFe, the highest value was obtained at 161.2 °C and 183 g/kg *M* (Table 3). Moreover, LZn and LFe response surfaces (Fig. 1f and g) showed a minimum at 194.1 °C and 150 g/kg *M*. In all cases, mineral leaching was inversely related with WR, Pearson correlation being 0.8402, 0.8586, 0.8212, and 0.8065 for LP, LCa, LZn, and LFe respectively.

3.2. Optimization of extrusion process

A multiple response optimization of physical properties of diet added with phytase (WR, F, RPA, LP, LCa, LZn and LFe) was performed using the Derringer's desirability function. Optimization criteria were to maximize WR, F, and RPA ($W = 5$ and $I = 5$), and to minimize LP, LCa, LZn, and LFe ($W = 5$ and $I = 5$). The global desirability function value was 0.8990, and the obtained optimal conditions were 183.6 °C and 158 g/kg *M*. The specific mechanical energy consumption (SMEC) in this condition was 0.470 ± 0.030 MJ/kg. This result is in agreement with those reported by Cian et al. (2017) for fish extruded products based on vegetable meals.

The suitability of the generated mathematical model to predict maximum WR, F, and RPA and minimum LP, LCa, LZn, and LFe was experimentally validated using the conditions determined in the optimization. The experimental values as well as those predicted by the generated model are shown in Table 4. In this regard, the experimental and predicted values generated by the mathematical model showed adequate agreement ($p > 0.05$).

Table 4
Predicted and experimental mean values obtained at 183.5 °C and 158 g/kg of moisture content.

Model validation	WR (%)	F (%)	RPA (UP/kg)	LP (%)	LCa (%)	LZn (%)	LFe (%)
Predicted Value[a]	85.0	95.0	3950	8.31	2.23	1.86	8.53
Experimental value[b]	81.8 ± 2.5	94.5 ± 0.72	3934.9 ± 47.7	9.40 ± 0.61	2.20 ± 0.2	2.00 ± 0.15	11.2 ± 2.8

[a] Values obtained using the second-order polynomial equation ($y = a_0 + a_1 T + a_2 M + a_3 T^2 + a_4 M^2 + a_5 T x M$) and the corresponding regression coefficients ($a_0 - a_5$).

[b] Mean ± SD (n = 3).

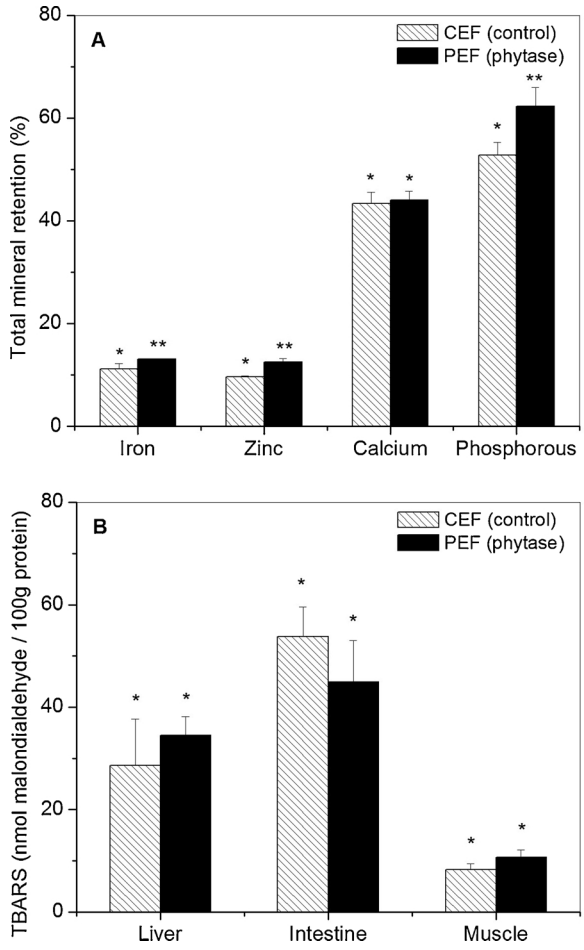

Fig. 2. Total mineral retention (A) and lipid peroxidation (B) of extruded feeds obtained at optimal conditions. Different symbols in each mineral or tissue mean significant differences between samples ($p < 0.05$).

3.3. Effects of diets obtained in optimal conditions on mineral retention of juvenile P. mesopotamicus model

Fish promptly accepted all diets, and no mortality occurred during the feeding trial. Final body weights were 22.0 ± 4.6 g and 19.5 ± 5.9 g for fish fed with CEF and PEF, respectively. No significant difference in final body weight was detected between dietary treatments ($p > 0.05$) after 38 days of feeding trial.

Fig. 2A shows mineral bioavailability measured as total mineral retention (TMR) from *P. mesopotamicus* fed with CEF and PEF diets obtained in optimal conditions. Fish consuming PEF had higher iron, zinc, and phosphorus retention than those fed with CEF diet ($p < 0.05$). However, there was no significant difference in calcium retention between diets. Moreover, there were not significant differences in lipid peroxidation (LPO) or enzymes involved in the antioxidant system (glutathione S-transferase, glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase) of liver, intestine, and muscle between diets (Fig. 2B and Table 5).

4. Discussion

4.1. Effects of extrusion conditions on physical properties of experimental feed added with phytase (PEF) and optimization of extrusion process

Specific volume of extruded feed is inversely related with bulk density (Cian et al., 2017), which is the best property to describe product porosity (Pastor-Cavada et al., 2011). Both properties are related with floatability of fish feed (Chevanan et al., 2009). In this sense, a direct relationship between SV and F was found (r^2: 0.9324). This result was also observed by De Cruz et al. (2015) for extruded fish pellets containing taro or broken rice starch. They observed that as die temperature increased from 125 °C to 170 °C, bulk density decreased. It was observed as *T* increased until 182.3 °C, SV increased and therefore the product was less dense. In the range of 160–200 °C, SV decreased with moisture level indicating a reduction of the degree of cooking. Note that SV is a good

Table 5
Activity of glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) in different tissues of extruded feeds obtained at optimal conditions.

		CEF[a]	PEF[a]
Liver	**GST** (mU/mg protein)	143.53 ± 8.31	156.24 ± 8.84
	GR (mU/mg protein)	41.17 ± 5.65	39.88 ± 1.76
	GPx (mU/mg protein)	862.74 ± 107.87	1012.76 ± 42.86
	CAT (U/mg protein)	39.74 ± 2.92	41.70 ± 2.95
	SOD (U/mg protein)	50.01 ± 6.82	43.00 ± 4.26
Intestine	**GST** (mU/mg protein)	95.32 ± 9.11	96.11 ± 7.23
	GR (mU/mg protein)	79.86 ± 9.11	91.07 ± 14.31
	GPx (mU/mg protein)	408.47 ± 23.74	339.62 ± 10.90
	CAT (U/mg protein)	11.19 ± 2.80	11.92 ± 2.23
	SOD (U/mg protein)	125.14 ± 14.64	128.03 ± 16.08
Muscle	**GST** (mU/mg protein)	29.80 ± 1.99	35.28 ± 1.49
	GR (mU/mg protein)	52.06 ± 4.52	46.11 ± 7.05
	GPx (mU/mg protein)	151.08 ± 11.02	176.85 ± 10.16
	CAT (U/mg protein)	1.03 ± 0.16	1.12 ± 0.22
	SOD (U/mg protein)	81.61 ± 10.98	75.06 ± 2.14

[a] Data expressed as mean ± SD.

indicator of degree of cooking for extruded products, which is directly related to granule structure destruction (Albarracín et al., 2015).

On the other hand, water resistance (WR) of the extruded feed is one of the most important properties because it helps to reduce nutrient leaching caused by disintegration of feed pellets (lower solubility) and improves the overall performance of an aqua feed (De Cruz et al., 2015). WR would be related with compaction in the range of 140 to 180 g/kg moisture content. On the other hand, the reduction of SV from 180 °C to 200 °C indicated an incomplete cooking process. It could be attributed to perturbation of the particle transport inside the extruder that not only could retard the cooking process of starch particles caused by friction, but also could broaden the residence time distribution of particles inside the extruder (Haller et al., 2012). Thus, some particles would reach the die very fast, without suffering much change, obtaining a compaction product (González et al., 2013). In agreement with this, mineral leaching was inversely related with WR, indicating a reduction of mineral losses by compaction at high *T* and low *M*. Thus a balance of WR (avoiding mineral leaching) and SV (directing related with F) is necessary for a feed with good physical properties.

Although the phytase used in this work was thermal-resistant, its activity decreased after extrusion. Reduction rate at high *T* and low *M* was more effective than at low *T* and high *M*. These results indicate reduction of phytase activity was dependent on the process conditions. Reactions taking place in the feed during extrusion process are largely determined by shear-forces, temperature, moisture, residence time, and pH (Sørensen et al., 2002). In addition, endogen phytase activity is sensitive to high extrusion temperature and pressure (Cao et al., 2007; Cheng and Hardy, 2003).

As can be seen, the maximum RPA does not match with the maximum WR and F, even less with the minimum mineral leaching. Therefore, in order to obtain a product with high WR, F, and RPA, and lower mineral leaching, it was necessary to optimize the extrusion conditions. For a single screw extruder the optimal conditions were 183.6 °C and 158 g/kg moisture.

4.2. Effects of experimental feed obtained in optimal conditions on mineral retention of juvenile P. mesopotamicus model

Mineral retention of *P. mesopotamicus* fed with CEF (control) and PEF (phytase) was used as mineral bioavailability indicator. Iron, zinc, and phosphorus retention was significantly higher for fish consuming PEF due to phytase reduced phytic acid in the gastrointestinal tract, improving mineral bioavailability. Sugiura et al. (2001) studied the effects of microbial phytase at levels of 0, 500, 1000, 2000, and 4000 PU/kg diet on the utilization of phosphorus, trace minerals, and protein by rainbow trout (*Oncorhynchus mykiss*) fed with soybean meal-based diets. They found phytase supplementation increased the apparent absorption of phosphorus, nitrogen (protein), magnesium, copper, iron, strontium, and zinc in low-ash diets containing soybean meal. The apparent absorption of phosphorus increased according to the level of phytase added, reaching 90% at 4000 PU/kg diet. In agreement with this, RPA of PEF obtained in optimal conditions was 3935 PU/kg, indicating the activity of enzyme was enough to exert an effect on phosphorus, zinc, and iron retention in juvenile *P. mesopotamicus* grown at 25 °C.

Although iron is an essential element, its metabolism is poorly described in fish and its homeostasis by *P. mesopotamicus* is unknown. We found that PEF obtained in optimal conditions increased iron bioaccessibility. This is an important effect because iron is an essential element having fundamental roles in cellular biochemistry and metabolism in fish (De Silva et al., 1996; Aisen et al., 2001). However, iron can also vary its redox state being rapidly oxidized from Fe^{2+} to Fe^{3+} (ferrous to ferric form) in the presence of oxygen (Drago, 2016). This reaction generates the superoxide anion, which through redox reactions leads to the generation of toxic hydroxyl radicals (Aisen et al., 2001). Thus, iron status in the body must be carefully regulated to provide enough amounts for biological functions, whilst avoiding overload, which can lead to oxidative stress (Carriquiriborde et al., 2004). For *P. mesopotamicus*, there is no information on maximum iron levels that produce toxicity and increase lipid peroxidation (LPO). Therefore, LPO in liver, intestine, and muscle of *P. mesopotamicus* fed with CEF and PEF was measured by TBARS. We found there were not significant differences in LPO or enzymes involved in the antioxidant system in these tissues between diets. These results agree with those found

by Carriquiriborde et al. (2004) who studied the effects of Fe-deficient, normal, and high-Fe diets (33, 175, 1975 mg Fe/kg feed, respectively) on growth, food conversion ratio, hematology, and lipid peroxidation (measured as TBARS) using rainbow trout (*Oncorhynchus mykiss*) model. They found a significant effect of dietary Fe on TBARS in intestine and liver. TBARS in the intestine of rainbow trout fed with high-Fe diet was significantly elevated by week 4 and remained above normal values for the entire experiment. Also, hepatic TBARS were positively correlated with Fe concentration in the liver at the end of the experience at normal iron level of diets (170 mg Fe/kg). The increase of iron bioaccessibility by phytase addition did not increase lipid peroxidation of different tissues from juvenile *P. mesopotamicus*.

5. Conclusion

The present study has documented for the first time the simultaneous effect of blend moisture and extrusion temperature on residual phytase activity and mineral leaching of fish feed added with phytase, extruded with single screw extruder. We optimized the extrusion process in order to obtain a product with high phytase activity after extrusion, proper physical properties of extruded feed and low mineral leaching. This is very important since the process has a high impact on the activity even though the phytase was thermal resistant. The feeds based on vegetable meals having approximately 8.0 g/kg of phytic acid were evaluated in a juvenile *P. mesopotamicus* model at 25 °C. It was demonstrated that feed with approximately 4000 PU/kg increased mineral retention respect to the control diet and the higher bioavailable iron did not affect oxidative status or lipid oxidation of fish tissues at the used iron level. Thus, extrusion process can be optimized to obtain fish feed based on vegetable meals with high residual phytase activity and low mineral leaching, which reduces eutrophication of the water environment, increasing mineral retention in *P. mesopotamicus*.

Conflict of interest

The authors declare that there are no conflicts of interest.

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