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CONCENTRADO PROTEICO DE SEMENTE DE ABÓBORA (*Cucurbita moschata*) NA NUTRIÇÃO DO JUNDIÁ

TESE DE DOUTORADO

Naglezi de Menezes Lovatto

Santa Maria, RS, Brasil
2015

CONCENTRADO PROTEICO DE SEMENTE DE ABÓBORA (*Cucurbita moschata*) NA NUTRIÇÃO DO JUNDIÁ

Naglezi de Menezes Lovatto

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Orientador: Profª. Leila Picolli da Silva

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CONCENTRADO PROTEICO DE SEMENTE DE ABÓBORA (*Cucurbita moschata*) NA NUTRIÇÃO DO JUNDIÁ

elaborada por
Naglezi de Menezes Lovatto

como requisito parcial para obtenção do grau de
Doutor em Zootecnia

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Santa Maria, 20 de Fevereiro de 2015.

DEDICATÓRIA

Aos meus pais (Gelci e Odacir) e a

minha irmã (Franciele), dedico.

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“Ontem passado. Amanhã futuro. Hoje agora. Ontem foi. Amanhã será. Hoje é. Ontem experiência adquirida. Amanhã lutas novas. Hoje, porém, é a nossa hora de fazer e construir.”

(Chico Xavier)

“Sucesso é a soma de pequenos esforços, repetidos o tempo todo”.

(Robert Collier)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Zootecnia
Universidade Federal de Santa Maria

CONCENTRADO PROTEICO DE SEMENTE DE ABÓBORA (*Cucurbita moschata*) NA NUTRIÇÃO DO JUNDIÁ

AUTORA: NAGLEZI DE MENEZES LOVATTO
ORIENTADORA: LEILA PICOLLI DA SILVA
Data e Local da Defesa: Santa Maria, 20 de Fevereiro de 2015.

O presente estudo teve como objetivo avaliar os efeitos da adição de fontes proteicas advindas da semente de abóbora no crescimento, atividade de enzimas digestivas proteolíticas e respostas metabólicas de jundiás (*Rhamdia quelen*) alimentados com substituições crescentes de farinha de peixe por farelo de semente de abóbora (FSA) ou concentrado proteico fosforilado de semente de abóbora (CPPFSA). Para o desenvolvimento do concentrado proteico, o método foi otimizado pela metodologia estatística de superfície de resposta (MSR) e delineamento central composto rotacional (DCCR), que apontou como maior rendimento 50,04%, utilizando 4% de trimetafosfato de sódio (STMP) em pH 4,5. Cujo conteúdo proteico foi 62,56%. Posteriormente, o FSA e o CPPFSA foram utilizados como substitutos proteicos nas dietas de jundiás. Durante oito semanas experimentais, 400 jundiás, com massa corporal de 24 ± 0,46 g foram distribuídos em 24 caixas, alimentados com cinco dietas experimentais em quatro repetições. Para análise dos dados, quatro contrastes ortogonais foram aplicados: Dieta Controle vs.. Dietas FSA, Dieta Controle vs.. Dietas CPPFSA, Dieta Controle vs.. Demais dietas e Dietas FSA vs.. Dietas CPFFSA. Os grupos de tratamento substituíam 25 e 50 por cento de proteína da farinha de peixe pelo FSA e CPPFSA. Foram avaliados parâmetros de crescimento, índices digestórios, atividades das enzimas digestivas tripsina e quimotripsina, bem como parâmetros metabólicos em plasma e fígado. Os parâmetros de crescimento dos peixes mostraram diferença significativa ($P < 0,05$) para massa corporal final ao final do período experimental no contraste Controle vs. dietas FSA. A conversão alimentar foi superior para os peixes que receberam 25% ou 50% CPFFSA em comparação àqueles que receberam FSA ($P < 0,05$). Os peixes que receberam a dieta Controle apresentaram menor índice digestivo-somático nos contrastes Controle vs. dietas CPFFSA e Controle vs. demais dietas. A atividade das enzimas tripsina e quimotripsina foram menores nos peixes do tratamento Controle, quando comparada aos peixes que receberam as dietas FSA ($P < 0,05$). Menor atividade de quimotripsina também foi observada nos peixes que receberam as dietas FSA ($P< 0,05$), no contraste dietas FSA vs. dietas CPFFSA. Os peixes que receberam as dietas CPPFSA apresentaram maior taxa de eficiência proteica do que os peixes que receberam dietas contendo FSA ($P< 0.01$). Para albumina no plasma, os menores conteúdos foram encontrados nos peixes da dieta Controle ($P<0.05$). A atividade da enzima ALAT foi maior nos peixes que receberam a dieta Controle ($P<0.05$), nos contrastes dieta Controle vs. dietas FSA, dieta Controle vs. dietas CPFFSA e dieta Controle vs. demais dietas. A ALP apresentou maior atividade nos peixes que receberam as dietas CPFFSA ($P<0.05$), considerando os contrastes dieta Controle vs. dietas CPFFSA e dieta Controle vs. demais dietas. Dessa maneira, pode-se concluir que a substituição da proteína da farinha de peixe por 25% ou 50% de CPFFSA não altera o crescimento e promove melhoria na conversão alimentar de jundiá e taxa de eficiência proteica, sem prejudicar atividade de enzimas digestivas, parâmetros metabólicos intermediários e hepáticos dos jundiás, podendo ser utilizado na nutrição do jundiá.

Palavras chave: *Rhamdia quelen*, concentração proteica, nutrição de peixes, proteínas vegetais

ABSTRACT

Animal Science Doctoral Thesis
Post-Graduate Program in Animal Science
Federal University of Santa Maria

PROTEIN CONCENTRATE PUMPKIN SEED (*Cucurbita moschata*) IN SILVER CATFISH NUTRITION

AUTHOR: NAGLEZI DE MENEZES LOVATTO
ADVISER: LEILA PICOLLI DA SILVA
Date and Defense Place: Santa Maria, February 20th, 2015

This study aimed to evaluate the effects of adding protein sources coming from the pumpkin seed in growth, activity of proteolytic digestive enzymes and metabolic responses of silver catfish (*Rhamdia quelen*) fed with increasing replacement of fishmeal by pumpkin seed meal (PSM) or phosphorylated protein concentrate of pumpkin seed (PPCPS). For the development of protein concentrate, the method was optimized by statistical methodology of response surface (MRS) and central composite rotational design (CCRD). The CCRD pointed as the highest yield 50.04%, using 4% sodium trimetaphosphate (STMP) at pH 4.5. Those same experimental conditions the protein content was 62.56%. Subsequently, the PSM and PPCPS were used as substitutes in silver catfish protein diets. For eight experimental weeks, 400 silver catfish, with a body mass of 24 ± 0.46 g were divided into 24 fiber glass, fed five experimental diets in four replicates. For the data, four orthogonal contrasts were applied: Control Diet vs. PSM Diets, Control Diet vs. PPCPS Diets, Control Diet vs. Other Diets and PSM Diets vs. PPCPS Diets. Treatment groups replaced 25 to 50 percent protein fishmeal by PSM and PPCPS. Silver catfishes were evaluated for growth parameters, digestive indices, activities of digestive enzymes trypsin and chymotrypsin, as well as metabolic parameters in plasma and liver. The fish growth parameters showed a significant difference ($P < 0.05$) for final body weight at the end of the trial period in contrast Control Diet vs. PSM Diets. Feed conversion was higher for fish that received 25% or 50% PPCPS than those that received PSM ($P < 0.05$). The fish receiving the Control Diet had the lowest digestive-somatic index in contrasts Control Diet vs. PPCPS Diets and Control Diets vs. Other Diets. The control fish treatments showed lower activity of trypsin and chymotrypsin enzymes than fish fed the PSM diet ($P < 0.05$). Also, less chymotrypsin activity was observed in the fish that received the PSM diet ($P < 0.05$), in contrast PSM Diets vs. PPCPS Diets. The fish that received the PPCPS Diets had higher protein efficiency rate than fish fed the FSA ($P < 0.01$). For albumin in plasma, low contents were found in fish that received Control Diet ($P < 0.05$). The ALAT activity was high in fish that received Control Diet ($P < 0.05$), in contrasts Control Diet vs. PSM Diets, Control Diet vs. PPCPs Diets and Control Diet vs. Other Diets. The ALP was most active in fish that received the PPCPS diets ($P < 0.05$), in contrasts Control Diet vs. PPCPS Diets and Control Diet vs. Other Diets. Thus, it can be concluded that the replacement of fishmeal protein by 25 or 50% PPCPS not alter growth and improve the feed conversion catfish and protein efficiency ratio, without affecting the activity of digestive enzymes, metabolic parameters intermediate and liver of silver catfish, can be used in silver catfish nutrition.

Keywords: *Rhamdia quelen*, protein concentration, fish nutrition, plant protein.

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LISTA DE ABREVIATURAS

FSA- Farelo de semente de abóbora

CPFFSA- Concentrado proteico fosforilado de farelo de semente de abóbora

DCCR- Delineamento central composto rotacional

STMP- Sodium trimetaphosphate, trimetafosfato de sódio

MSR- Metodologia de superfície de resposta

ALAT- Alanina aminotransferase

ALP- Fosfatase alcalina

PSM- Pumpkin seed meal

PPCPS- Phosphorylated protein concentrate of pumpkin seed meal

CCRD- Central composite rotational design

RSM- Response surface methodology

ANFs- Antinutritional factors, fatores antinutricionais

NDF- Neutral detergent fiber

FDN- Fibra em detergente neutro

CP- Crude protein

FC- Feed conversion

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INTRODUÇÃO

No ano de 2009, pela primeira vez, o cultivo de peixes ultrapassou a pesca extrativa (BRINKER; REITER, 2011), fornecendo metade da demanda mundial de pescado para consumo humano. Neste cenário, a piscicultura brasileira vem aumentando expressivamente sua produção, com expectativa de atingir 20 milhões de toneladas/ano até 2030 (MPA, 2014), o que tornará o País um dos maiores produtores mundiais de pescado. Esse rápido crescimento já se reflete no mercado de ingredientes e rações para as diversas espécies cultivadas, que busca formulações de dietas nutricionalmente equilibradas e altamente digestíveis, que sejam economicamente viáveis e ecologicamente sustentáveis (WEBSTER; LIM, 2002).

Entre as frações de nutrientes, a proteína é considerada a mais nobre, por ser intensamente utilizada na renovação de tecidos e deposição de músculo, que é o produto final desejado (WILSON, 2002). Porém, o mercado atual tem vislumbrado um cenário preocupante no que se refere à disponibilidade de farinhas de peixes (principal fonte proteica utilizada) devido à baixa disponibilidade e surpreendente alta de preços. Aliado a este fato, a possibilidade do uso de outras fontes proteicas provindas de animais terrestres causam preocupação e receio público em muitos países, devido aos riscos associados às zoonoses e sua transmissão (FUERTES et al., 2013).

Com isso, a transição de uso das fontes proteicas de origem animal por outras ambientalmente amigáveis, como as proteínas de origem vegetal, é uma demanda iminente para garantir a sustentabilidade dos sistemas produtivos e estabilidade de mercado aquícola (FUERTES et al., 2013). Normalmente as fontes proteicas de origem vegetal apresentam restrições quanto a seu uso na nutrição das diversas espécies de peixes, o que está associada à baixa aceitabilidade, a presença de fatores antinutricionais e ao desequilíbrio no perfil de aminoácidos (MARIOD et al., 2010). No entanto, seu uso intensivo torna-se cada vez mais urgente em face da rápida expansão da piscicultura nacional, e consequentemente da demanda de rações. Nesse cenário, os estudos devem se voltar para a busca de soluções sustentáveis para tal problemática sobre o desenvolvimento da piscicultura nacional, em especial quando consideradas a exploração intensiva de espécies onívoras mais exigentes, como o jundiá (*Rhamdia quelen*).

O jundiá é uma espécie nativa da região Sul do Brasil e tem se destacado como uma espécie promissora para a piscicultura, apresentando rápido crescimento em condições de cultivo. Possui alta resistência ao manejo (BALDISSEROTTO; RADÜNZ NETO, 2004), alimentando-se inclusive nos meses mais frios (FRACALOSSI et al., 2002), sendo considerada uma espécie com grande potencial para região sul do Brasil, ainda mais onde busca-se por espécies de peixes que sobrevivam durante invernos rigorosos e que cresçam rapidamente em verão com elevadas temperaturas.

Por ter hábito alimentar onívoro, o jundiá apresenta potencial para o aproveitamento de alimentos de origem vegetal. Naylor et al. (2009) ressaltam que o aumento na produção de peixes onívoros é uma tendência já que adaptam-se melhor à fontes de origem vegetal. Coldebella e Radünz Neto (2002), em experimento com alevinos de jundiá, observaram que os animais cresceram melhor com dieta à base de levedura de cana e farelo de soja, quando comparada com farinha de carne e farelo de soja. Lazzari et al. (2006) trabalhando com fontes proteicas de origem animal e vegetal para o jundiá, verificaram menor crescimento dos peixes alimentados com dietas contendo apenas farelo de soja como fonte proteica, quando comparados aqueles em que as dietas possuíam farinha de carne ou farinha de peixe.

No Brasil existe uma ampla variedade de subprodutos e resíduos agroindustriais, que possuem grande potencial nutricional, podendo ser incorporados em rações destinadas à produção de pescados. Dentre eles, destacam-se os oriundos do despolpamento da abóbora (*Cucurbita moschata*) para fabricação de doces, composto principalmente por cascas e sementes, que normalmente são incorporados ao solo como adubo orgânico. Embora contenha elevados teores de proteína (cerca de 30%) e de ácidos graxos poli-insaturados (Oleico e linoleico) (ESUOSO et al., 1998; KIM et al, 2012; YOUNIS et al., 2000), o resíduo de despolpamento de abóbora também possui elevado teor de fibras e alguns antinutrientes (polifenóis, flavonoides, taninos e glicosídeos cianogênicos) que limitam seu uso *in natura* em formulações nas dietas (DEL VECHIO et al., 2005).

Neste cenário, algumas estratégias de concentração e modificação química podem ser adotadas a fim de minimizar fatores antinutricionais e melhorar o perfil biológico de resíduos visando seu uso como ingrediente em rações de peixes (MARIOD et al., 2010). A técnica de fosforilação com trimetafosfato de sódio (STMP) tem sido aplicada em fontes vegetais (ELLINGER, 1972) para concentração de proteína e modificação do seu perfil aminoacídico. Na fosforilação o fosfato inorgânico (Pi) pode ser transferido para as proteínas por reações de S- ou N-esterificação que reagem com a hidroxila primária ou secundária da tirosina e, no caso de N-esterificação, combina-se com grupo ε-amino da lisina, imadazol da histidina, ou

com o grupo guanidina da arginina. Estes grupos são facilmente hidrolisados abaixo de pH 7 (MATHEIS et al., 1983) , gerando baixo volume de resíduo.

O enriquecimento da composição nutricional de fontes vegetais é a melhor alternativa para intensificar o uso dessas proteínas, pois estudos demostram que a substituição da farinha de peixe por fontes proteicas vegetais provocam alterações metabólicas que se refletem sobre o desempenho dos animais. Esse fato evidencia a necessidade de se conduzir estudos sobre a capacidade digestória, atividade de enzimas digestivas (LUNDSTEDT et al., 2004; STECH et al., 2009), bem como, estudos relativos ao metabolismo proteico e enzimas envolvidas no catabolismo hepático.

2 OBJETIVOS

2.1.Objetivo geral

O presente estudo teve como objetivo avaliar os efeitos da adição de fontes proteicas advindas da semente de abóbora no crescimento, atividade de enzimas digestivas proteolíticas e respostas metabólicas de jundiás (*R. quelen*) alimentados com substituições crescentes de farinha de peixe por farelo de semente de abóbora ou concentrado proteico fosforilado de semente de abóbora.

2.2.Objetivos específicos

- Determinar a composição química de nutrientes e antinutrientes do farelo de semente de abóbora;

- Otimizar o processo de concentração proteica de farelo de semente de abóbora, através da metodologia de superfície de resposta, determinando os efeitos do pH de extração e teores de trimetafosfato de sódio (STMP).

- Estudar o efeito do farelo de semente de abóbora e concentrado proteico fosforilado sobre o desempenho, sobrevivência, enzimas digestivas, parâmetros bioquímicos sanguíneos e hepáticos de jundiás.

3. ORGANIZAÇÃO DO ESTUDO

Este estudo foi desenvolvido a partir da obtenção e caracterização do concentrado proteico de farelo de semente de abóbora através de fosforilação química (CPPFSA). Posteriormente foi realizada a análise nutricional e antinutricional do farelo de semente de abóbora (FSA) e do CPFFSA. Após essa etapa foi realizado ensaio biológico utilizando as fontes proteicas como substitutos da proteína advinda da farinha de peixe, na alimentação do jundiá. Foram avaliados parâmetros de crescimento, atividades de enzimas digestivas, parâmetros sanguíneos e hepáticos dos jundiás.

Os resultados estão apresentados na forma de artigos científicos. O artigo 1 corresponde a metodologia para obtenção e otimização do CPPFSA, e avaliação nutricional em comparação com FSA e farinha de peixe.

A inclusão do FSA e CPPFSA sobre a resposta nutricional de jundiás (crescimento, parâmetros zootécnico, índices digestórios e atividades de enzimas digestivas) compõe o artigo 2. O artigo 3 contempla dados de crescimento, índices zootécnicos, parâmetros plasmáticos e metabolismo hepático dos jundiás.

4. ARTIGO 1

Optimization and preparation of phosphorylated pumpkin seed (*Cucurbita moschata*) protein concentrate for application in fish nutrition using response surface methodology[1]

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[1] Artigo submetido à revista Animal Feed Science and Technology

Abstract- This study aimed to optimize the extraction of protein concentrate from pumpkin seed meal by the response surface methodology (RSM), to determine the effects of pH extraction and content of sodium trimetaphosphate (STMP), as well as nutritionally characterizing the product obtained for aquatic feeds. The protein concentrates were obtained using the isoelectric point of the proteins. STMP and phosphoric acid were used in phosphorylation. For the response surface methodology, the effects of STMP (1.88 – 6.12 w/w) and pH (2.67 – 6.05) were assessed by central composite rotational design, which indicated for the central point (4% STMP and pH 4.5) the best result for yield and crude protein concentration for pumpkin seed meal. The protein concentrate obtained under these conditions was compared to pumpkin seed meal and fish meal for nutrient contents, amino acid composition, anti-nutrients, functional properties and *in vitro* protein digestibility. The response surface methodology was effective to select the procedures adopted for phosphorylation, resulting in a product with a high protein concentration (62.56 g/100g) and digestibility (62.03 g/100g) , combined with a higher concentration of essential amino acids (27.26 g/100 g), and a lower concentration of polyphenols (13.11 g/100g) when compared to the pumpkin seed meal (23.19 g/100g). The results showed that the extraction of the phosphorylated protein concentrate from pumpkin seed meal was optimized by the response surface methodology. The protein concentrate obtained had amino acid profile and protein digestibility comparable to fish meal, which is the protein source of reference for fish.

Keywords: amino acids, protein digestibility, protein phosphorylation, aquafeeds.

Abreviations: ANFs, antinutriotional factors; RSM, response surface methodology; STMP, sodium trimetaphosphate; PSM, pumpkin seed meal; PPCPS, phosphorylated protein concentrate pumpkin seed meal; DM, dry matter; NDF, neutral detergent fibre; PITC , phenylisothiocyanate; HPLC, high performance liquid chromatography; WHC, Water holding capacity; OHC, oil holding capacity.

Introduction

In food processing plants, a large proportion of foodstuffs is either underused or discarded as agro-industrial waste (El-Adawy et al, 2000), discarding along with it a massive nutrient density with high potential for nutrient application, unexplored in the scientific and industrial communities (Gupta, 2013), which can be converted into nutrients for animals and/or humans, becoming important food resources (El-Adawy et al, 2000) and helping to reduce the environmental impact.

In Brazil there is a wide variety of agro-industrial by-products and waste, which have great nutritional potential and may be incorporated in aquatic feeds. Among them are those from the pulping of pumpkins (*Cucurbita moschata*) used for sweets and jams, mainly composed of pumpkin peels and seeds, which are usually discarded and incorporated into the soil as organic fertilizer, even though pumpkin peels and seeds contains high levels of protein (about 30%), polyunsaturated fatty acids (oleic and linoleic) (Esuoso et al., 1998; Younis et al., 2000; Kim et al, 2012) as well as high fiber content and some antinutritional factors (ANFs) such as polyphenols, flavonoids, tannins and cyanogenic glycosides, that limit its use in food formulations (Del Vechio et al., 2005).

In this context, some chemical modification strategies can be adopted in order to minimize antinutritional factors and enhance the biological profile of the product, enabling it to be use as an ingredient in fish feed (Mariod et al., 2010). The technique of phosphorylation with sodium trimetaphosphate (STMP) has been applied to plant food sources (Ellinger, 1972) for protein concentration and modification of amino acid profile. In phosphorylation, inorganic phosphate can be transferred to the proteins by S- or N-esterification reactions that react with a primary or secondary hydroxyl of tyrosine. In the case of N-esterification, it combines with ε-amino group of lysine, histidine imadazole or with the guanidine group of arginine. These groups are readily hydrolyzed at pH bellow 7.0 (Matheis et al. 1983), generating a low waste volume.

The procedure of chemical phosphorylation can be further optimized by statistical response surface methodology (RSM) (Myers and Montgomery, 2002), which allows us to obtain different plant-based protein sources. This opens a wide research topic to be developed, once nutritional and commercial value can be added to agro-industrial waste improving the nutritional profile of fish diets.

This study was carried out to optimize the obtainment of the protein concentrate from pumpkin seed meal, by response surface methodology, to determine the effects of pH extraction and sodium trimetaphosphate content (STMP), as well as nutritionally characterizing the obtained product for fish nutrition.

2. Materials and Methods

2.1. Preparation of the samples

The pumpkin seeds (*Cucurbita moschata*), courtesy of the company Ritter Alimentos S.A. from Cachoeirinha, RS, Brazil, were washed in distilled water to remove excess pulp, dried at 45 °C in recirculating air stove and ground in a cooled micro-mill (MA-630, Marconi®). The oil sample was extracted with Hexane (P.A. FMaia, Brasil) in the ratio 2:1 (hexane: sample) in three sequential washes, resulting in pumpkin seed meal used to obtain the protein concentrates. The fish meal is made with waste filleting Nile tilapia and was acquired from Cooperativa Agroindustrial de Piscicultura Pisces – Copisces from Toledo, PR, Brazil. The fish meal was milled in micro-mill and sieved at 600 µm.

2.2. Preparation of protein concentrates

Extraction of protein concentrates was performed according to Smith et al. (1946) with modifications. Phosphorylation was performed following the method of Yamada et al. (2003). The protein concentration results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), with a 95 % significance. The effects for sodium

99 trimetaphosphate (STMP) (1.88 – 6.12 v/p) and pH – phosphoric acid – H_3PO_4 (2.67 – 6.05)
100 in yield and crude protein content in phosphorylation were assessed by central composite
101 rotatable design (CCRD) with 8 runs and 3 central points.

102 The procedure of protein concentration and phosphorylation is shown in Figure 1.
103 STMP was added to the pumpkin seed meal (PSM) according to the statistical design and the
104 mixture was suspended in ten parts of distilled water. The solution was processed for three
105 minutes in a blender (model *LIQ789*, Cadence, Brazil) and sieved in a 140 µm mesh. The
106 fraction retained on the sieve was again suspended in distilled water at a ratio of 1:10. This
107 procedure was repeated until a ratio of 1:30 (PSM): distilled water was obtained at the end of
108 processing. The three aqueous fractions were combined to obtain a single sample, in which
109 NaOH 1 M was added until pH 9.5 to obtain solubilized protein. The solution was allowed to
110 stand for 30 minutes at room temperature, and then the pH was adjusted with H_3PO_4 (extra
111 pure, 85%) according to the statistical design. The solution was conditioned overnight at 8 °C
112 to settle dispersed protein fraction, soon after the supernatant was discarded and the
113 concentrated protein fraction was dried in an oven with air circulation at 50 °C
114 (approximately 24 hours).

115 *2.3. Determination of the dependent variables*

116 The yield of the samples was calculated by the mass (g) of pumpkin seed meal used
117 and the mass (g) generated after the concentration procedure and chemical modification of the
118 protein using the formula:

119

$$Y(\%) = initial\ product\ weight\ (g)\ x\ protein\ concentrate\ weight\ (g)/100$$

120

121 Where Y (%) = Yield of the protein concentrate.

The crude protein was analyzed by determining total nitrogen by the micro Kjeldahl method using a conversion factor of 6.25 (N x 6.25), according to methodology no. 920.87 – AOAC (2000).

CCRD showed for the central point (4% STMP and pH 4.5) better results for extraction yield and crude protein concentration for pumpkin seed meal. The protein concentrate obtained under these conditions was compared to pumpkin seed meal (raw material for obtaining the protein concentrate) and fish meal for nutrients contents, amino acid composition, antinutrients, functional properties and *in vitro* protein digestibility.

2.4. Analyses of nutrients in products

The dry matter (105 ± 2 °C / 24 hours), ash (550 °C / 6 hours) and crude protein (determination of nitrogen by the micro Kjeldahl method – N x 6.25, nº. 920.87) were determined according to the methodologies described by AOAC (2000). The residual fat was extracted and quantified by the cold extraction method (Bligh and Dyer, 1959). The amount of neutral detergent fiber (NDF) was measured according to Van Soest et al. (1991). Digestion was performed with concentrated H_2SO_4 plus the catalytic mixture of copper sulfate and potassium sulfate (375 ºC for 4.5 hours) for the calcium and phosphorus analysis. The reading was held in atomic absorption spectrophotometry for calcium and in the area of the visible for phosphorus (colorimetric reaction of ammonium molybdate with phosphorus in the presence of reducing agent, using K_2HPO_4 as a standard (BAGINSKI et al., 1982).

2.5. Amino acid content

The amino acid profile was determined by high performance liquid chromatography (HPLC), with hydrolysis of samples in HCl 6M solution. The released amino acids were derivatized with phenylisothiocyanate (PITC) and separated with reverse phase column C18 (Pico-Tag – 3.9 x 300 mm) and UV detection at 254 nm, by the methodology proposed by White et al. (1986).

147 *2.6. Water holding capacity (WHC) and oil holding capacity (OHC)*

148 The water holding capacity (WHC) and oil holding capacity (OHC) of the samples
149 were determined as described by Macconell et al. (1974). Samples were hydrated in distilled
150 water (for WHC) or soy oil (for OHC). After rest (24 h) and centrifugation (1,300 *x g* for 20
151 minutes), excess supernatant was discarded. The results were expressed as the amount of
152 water/oil retained by the sample (in dry matter) per gram (g water/oil / g sample),
153 respectively.

154 *2.7. Antinutritional Factors (ANFs)*

155 The polyphenol content was measured by the Folin-Ciocalteu method described by
156 Chandra and Mejia (2004). Folin-Ciocalteu 2M reagent (0.5 mL) was added to 1 mL of the
157 sample. This blend was allowed to stand for 5 min before the addition of 2 mL of Na_2CO_3
158 20%. The solution was left standing during 10 min before measurements at 730 nm. The data
159 were expressed in mg of gallic acid equivalent (GAE) per g of crude extract, based on the
160 calibration curve of gallic acid.

161 The content of flavonoids was determined by the reaction with aluminum chloride
162 using the method described by Woisky and Salatino (1998). $AlCl_3$ 2% solution (0.5 mL) was
163 added to 1 mL of the sample. After 15 min, the absorbance was measured at 420 nm. The data
164 were calculated based on the calibration curve of rutin and expressed in mg equivalents of
165 rutin equivalents (RE) per g of crude extract.

166 The determination of condensed tannins was performed by the Morrison et al. (1995)
167 method, which uses vanillin as reagent. The absorbance of 500 nm was measured by
168 spectrophotometer. Data were expressed as mg of catechin equivalents (CE) per g of each one
169 of the fractions based on the calibration curve of catechin.

170 *2.8. In vitro digestibility of protein*

In vitro digestibility of protein assay was performed as proposed by Mauron (1973), with modifications proposed by Dias et al. (2010). The method is based on the digestion of the sample by the enzymes pepsin (1:10.000, Nuclear) and pancreatin (Sigma). The digestibility results from the relationship between the total nitrogen in the sample, the digested nitrogen, and the nitrogen produced by the auto digestion of the enzymes and the soluble nitrogen originally in the meal.

Ten mL of HCl 0.1 N and 4 mg of pepsin (dilution in HCl 0.1 N were added to the defatted sample (100 mg) at the ratio weight:volume 10:1). The samples were kept in a water bath at 37º under constant stirring for 1 hour. The solution pH was corrected at 7.0 (NaOH 0.4 N), 20 mg of pancreatin (diluted in sodium phosphate buffer 0.1 M pH 8.5 at the ration weight: volume 1:10) were added to each sample. The samples were kept at 37 ºC and stirred for 3 hours. The reaction was stopped with trichloroacetic acid (final concentration 5%). A 2-ml portion was removed for the determination of total nitrogen in the sample and the remaining fraction was centrifuged (10,000 rpm / 10 minutes) and digested nitrogen was analyzed.

For each test sample there is an equivalent white sample which does not receive the enzyme solutions, in order to verify the soluble nitrogen in the sample. So the nitrogen content from the enzyme auto-digestion can be considered, there is a sample containing only the enzyme solution. The nitrogen content of each fraction is determined by the micro Kjeldahl method. Casein was adopted as the standard (Synth, Brazil, purity of 90 %) to compare sample digestibility.

2.9. Statistical analysis

For the CCRD, a second order polynomial equation was used to adjust the data in Table 1, according to the model:

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2$$

Where Y_i $(i = 1 - 2)$ is the predicted response for the values of crude protein (CP %) and yield (Y %) of the protein concentrate. The term a_0 is the response obtained in the design central point, a_1 and a_2 are the linear terms, a_{12} is the interaction of the effects and a_{11} and a_{22} are the quadratic effects. The experimental and predicted responses were compared. The results were analyzed by analysis of variance (ANOVA) to validate the statistical model. Graphical and regression analysis were performed using the statistical software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA) with a significance level of 95 % ($P < 0.05$). Nutritional, ANFs, functional analysis data and in vitro digestibility protein were subjected to one way ANOVA and F test for comparison were run at the 5% probability level by Tukey's test. The mathematical model is described below:

$$Y_{ij} = m + t_i + e_{ij}$$

Where Y_{ij} = observed value of variable Y in the experimental unit that received treatment i in the repetition j; m = constant t_i = effect of the treatment i; e_{ij} = experimental error

3. Results

3.1. Optimizing protein concentrate by the RSM

CCRD showed 50.04 % as the higher yield of protein extraction, while the best concentration of the nutrient was of 65.28 g/100g (Table 1). STMP exerted a negative linear effect ($P < 0.05$), while the pH did not impact the protein concentration yield by chemical phosphorylation of PPCPS (Figure 2).

The influence of the independent variables in the phosphorylation procedure can be seen in Figure 3, where the highest yield was obtained under the condition of pH at level *-1* and STMP at level *1*. From these results the following equation (1) empirical model was obtained to yield the PPCPS (Y %):

$$Y\% = 49.67 - 3.03 \cdot pH^2 - 4.49 \cdot STMP^2 + 1.24 \cdot pH - 2.15 \cdot STMP + 2.32\, pH \cdot STMP \quad (1)$$

The model was validated by analysis of variance (ANOVA) and the coefficient of determination (r^2) was of 0.9242, which means that the model can explain 92.42 % of the total variability of the values studied. The quadratic effects of STMP and pH were significant ($P < 0.05$), although negative. The contour surface (Figure 2) shows higher yields in the area where the pH and STMP values are in the central point, indicating the point of maximum yield.

Figure 4 shows the effects of the independent variables on the content (CP obtained by PPCPS). Only the linear effects of the variables were statistically significant. Interaction between pH and STMP was significant, negatively impacting the crude protein content in the concentrate. The same for yield, an empirical model for percentage of crude protein was generated according to the values shown in Table 1; however, this model was not validated by analysis of variance (ANOVA), and is not presented in contour form.

3.2. Nutritional composition

With use of phosphorylation, a new ingredient was obtained with 53.5 % higher protein content than its original raw material and equivalent to the reference standard of animal-based protein sources (Table 2). The total fat content was lower ($P < 0.05$) for the pumpkin seed meal (2.9 %) and similar to PPCPS and fish meal (about 9 %). The fiber (NDF) content was 33.79% lower for PPCPS than its original raw material (PSM). The level of calcium was higher for the fish meal. The levels of phosphorus ($P < 0.05$) were higher for PPCPS. The increase in the ash content ($P < 0.05$) in PPCPS was observed.

3.3 Amino acids

PPCPS showed higher content of essential amino acids ($P < 0.05$) than fish meal and PSM (Table 3), and the content of arginine, tyrosine, phenylalanine and valine higher than fish meal.

3.4. *Water holding capacity (WHC) and oil holding capacity (OHC)*

Figure 5 shows the WHC and OHC of PSM, PPCPS and fish meal. The high WHC ($P < 0.05$) of fish meal and PSM were attributed to their higher content of polar amino acids when compared to PPCPS. The OHC was higher for PSM when compared to fish meal and PPCPS ($P < 0.05$), with similar behavior to the hydration capacity.

3.5. *Anti-nutritional factors – ANFs*

The phosphorylation procedure is ineffective in reducing flavonoids. However, it caused a decrease ($P <0.05$) of 56.53 % in total polyphenol content for PPCPS (13.11 mg/g) when compared to the PSM (23.19 mg/g) (Figure 6). In this study, condensed tannins compounds were not detected in samples (PSM and PPCPS).

3.6. In vitro digestibility of protein

PSM showed low protein digestibility *in vitro*. However, protein digestibility was similar for both PPCPS and fish meal ($P < 0.05$) (Figure 7).

4.Discussion

The results show that CCRD was efficient for obtaining PPCPS in studied conditions. Several methods of chemical modifications and concentrations of protein are used under different conditions. Sung et al. (1983) used STMP to modify serine and lysine of a soy isolate under alkaline conditions. About 40% of the serine residues were phosphorylated without protein cross-linking and the isolate showed enhanced solubility properties,

particularly under acidic conditions. Results like this have spurred interest in obtaining plant-based protein concentrates with high digestibility by chemical phosphorylation and that may be fed to fish. Thus, the models were prepared in order to obtain a product with high yield and protein content similar to protein sources commonly used in aquatic feeds, such as fish meal, soybean meal and soybean protein concentrate (NRC, 2011).

The RSM was used to identify the best conditions of pH and concentration of STMP, aiming to optimize the preparation PPCPS. The best results for yield were found when 4 g/100g of STMP and pH 4.5 were used. In this same condition (central point of each independent variable), the CP content (62.56 g/100g) was also satisfactory, being chosen for obtaining the protein concentrate.

Shen et al. (2008) investigated modifications in peanut protein to improve the emulsion ability, using STMP as a phosphorylating agent by means of CCRD. The optimum conditions were pH 8.5 and 8 % STMP at 25 °C. Peričin et al. (2008) used the response surface methodology to obtain the higher solubility of pumpkin seed globulins (*C. pepo*), obtaining better results at pH 7.69, concentration of 3.99 % of NaCl and temperature of 54 °C. Fann et al. (2014) used RSM to obtain antioxidant peptides from protein isolates of *C. pepo* pumpkin seed, where the best results (92.82 % of antioxidant activity for the isolated peptides) were achieved with a combination of pH 2.5, 6000 U/g of acid protease and incubation time of 5 hours at 50 °C. These studies show that the variability of conditions to achieve the maximum efficiency depends on the origin of the protein source and the purpose of application of the obtained product.

With use of phosphorylation, the new ingredient obtained (PPCPS) with 53.5 % of protein, lower NDF content and increased content of arginine, tyrosine, phenylalanine and valine, higher than fish meal, the results demonstrate that the protein concentration by chemical phosphorylation provides a satisfactory new product.

The amino acid composition of plant-based ingredients can vary depending on cultivars, culture treatments and industrial processing (Ledoux et al., 1998, Tavernari, 2008). But regardless of the variations, usually these sources are deficient in lysine and methionine, which are the most limiting amino acids to the growth and maintenance of the good health of fish (Tusche et al, 2011). The increase in levels of these two amino acids obtained by phosphorylation shows that the technique is effective not only for quantitative concentration but also to improve the protein quality of the sources used.

The functionality of proteins is defined by the physical and chemical properties that affect their behavior in food during processing, storage, and preparation (Araújo, 2008). Matheis et al. (1983), using chemical phosphorylation, also found a lower WHC in phosphorylated casein. This behavior occurs because the proteins are less hydrated in their isoelectric pH, wherein the increase in protein-protein interactions results in minimal interaction with water (Fennema, 2010). Dench et al. (1981) reported that absorption of fat varies depending on the number of protein hydrophobic groups exposed.

Other than the nutritional aspects, the physicochemical characteristics of the ingredients exert a decisive impact on the technological quality and digestibility of the aquatic feeds (Glencross et al., 2007). The parameters of hardness, stability in water, buoyancy and storage time of fish feed are influenced by the ability of the raw materials to hydrate and bind to fat (Draganovic et al., 2011). Ingredients with lower hydration capacity require less water during the feed extrusion procedure (Draganovic et al., 2014). The lower WHC and OHC observed for PPCPS can form pellets which require less water for extrusion, thus favoring energy expenditure and less waste to the environment (Draganovic et al., 2014)

In addition to measures of nutritional interest, plant-based protein sources should be properly assessed for the ANFs, which most often are the main factors that limit its use in aquatic feeds. Thus, knowledge of the levels of ANFs and the application of some processing

techniques can improve the nutritional value of the vegetable bran before its inclusion in diets (Moure et al., 2006; Drew et al., 2007). Most plant-based protein concentrates and isolates contain ANFs that decrease the complete hydrolysis of proteins from legumes and oilseeds by pancreatic proteases (Fennema, 2010). Phenolic compounds are present in the fibrous portion of different foods, being indigestible or poorly indigestible, causing effects in color, flavor and nutritional quality of ingredients(Silva and Silva, 1999) used in aquatic feeds. The reduction in fiber content during the procedure of protein concentration causes a decrease in the content of phenolic compounds, improving the nutritional and sensory characteristics of the ingredient.

The lower *in vitro* protein digestibility of PSM which is associated with the presence of ANFs and high fiber content, negatively influence the digestion and availability of protein for fish (Gatlin et al., 2007; Torstensen et al., 2008). The reduction of ANFs by phosphorylation reflected in protein digestibility similar for PPCPS and fish meal. This enhancement must also be attributed to partial hydrolysis of the protein and amino acids caused by the chemical phosphorylation procedure (Matheis et al. 1983).

Our results show that RSM was efficient in selecting the procedures adopted for phosphorylation (pH 4.5 and 4 % of STMP), resulting in a product with a high concentration and protein digestibility coupled with a higher concentration of essential amino acids. It should be noted that N-P binding that occurs in phosphorylation is acid labile. Thus, under the conditions prevailing in the stomach during the digestion process, the N-phosphorylated proteins are dephosphorylated and lysyl residues will be regenerated, without causing negative impact on the digestibility of lysine and other amino acids (Fennema, 2010).

5.Conclusion

The results obtained from the analyses show that the procedure for obtaining the phosphorylated protein concentrate of pumpkin seed meal was optimized by the response surface methodology, which showed the best experimental conditions using 4 % of sodium trimetaphosphate and pH 4.5. The protein concentrate obtained shows an amino acid profile and protein digestibility comparable to fish meal, which is the protein source of reference for this group of animals.

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Table 1. Central composite rotational design (CCRD) and responses of yield and crude protein content obtained in the different combinations of sodium trimetaphosphate (STMP) and pH.

Run	STMP (g/100g)[1]	pH	Y (%)[2]	CP (%)[3]
1	2.50 (-1)	3.20 (-1)	44.33	60.04
2	5.50 (1)	3.20 (-1)	40.80	65.28
3	2.50 (-1)	5.60 (1)	36.66	64.59
4	5.50 (1)	5.60 (1)	42.54	59.23
5	4.00 (0)	4.50 (0)	49.34	62.08
6	4.00 (0)	4.50 (0)	50.04	62.06
7	4.00 (0)	4.50 (0)	49.68	62.56
8	1.88 (-1.41)	4.50 (0)	42.0	64.14
9	6.12 (1.41)	4.50 (0)	47.36	59.98
10	4.00 (0)	2.67 (-1.41)	45.76	62.19
11	4.00 (0)	6.05 (1.41)	37.76	64.81

[1]STMP – sodium trimetaphosphate; ; [2]Yield; [3]Crude protein.

Table 2. Nutritional composition of pumpkin seed meal (PSM) and the phosphorylated protein concentrate of pumpkin seed meal (PPCPS) in comparison to fish meal.

Nutrient	PSM[1]	PPCPS[2]	Fish meal
	.....g/100g of the dry matter (DM).....		
Crude protein	44.48±1.34[b]	67.48±2.75[a]	67.37±0.30[a]
Lipid	3.06±0.06[b]	10.30±0.29[a]	10.22±0.60[a]
NDF	37.17±0.91[a]	12.53±1.88[b]	NA[3]
Calcium	0.32±0.09[c]	0.67±0.08[b]	6.34±0.16[a]
Phosphorus	1.00±0.13[c]	7.33±0.40[a]	2.59±0.07[b]
Ash	9.92±1.76[c]	27.78±2.26[a]	16.53±1.76[b]

Mean values followed by different letters in the row were considered significantly different using the Tukey's test ($P < 0.05$). [1] PSM:Pumpkin seed meal; [2] PPCPS:Phosphorylated protein concentrate of pumpkin seed meal. [3]No Analyzed.

563 Table 3. Total amino acid composition of meal (PSM) and phosphorylated protein concentrate
564 (PPCPS) of pumpkin seed, compared to fish meal.

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Amino acids	PSM[1]	PPCPS[2]	Fish meal
	.....g/100 g of the matter *in natura*.....		
Isoleucine	1.17	2.00	1.79
Leucine	2.07	3.67	3.30
Lysine	1.72	1.72	3.31
Methionine	0.64	1.12	1.83
Cystine	0.36	0.51	0.42
Tyrosine	2.21	1.99	1.36
Phenylalanine	1.51	2.78	2.00
Threonine	0.85	1.48	2.33
Valine	1.55	2.65	2.33
Tryptophan	NA	NA	NA
Histidine	0.82	1.10	1.09
Arginine	5.02	8.24	4.56
Total essential AA[3]	17.92	27.26	24.32
Asparagine	3.97	5.22	4.17
Glutamine	5.97	9.50	6.88
Serine	1.85	2.85	2.27
Proline	1.17	2.98	4.42
Glycine	2.98	4.75	7.13
Histidine	0.82	2.10	1.09
TOTAL	**34.68**	**54.56**	**50.28**

566 [1]Pumpkin seed meal; [2]Phosphorylated protein concentrate of pumpkin seed meal.
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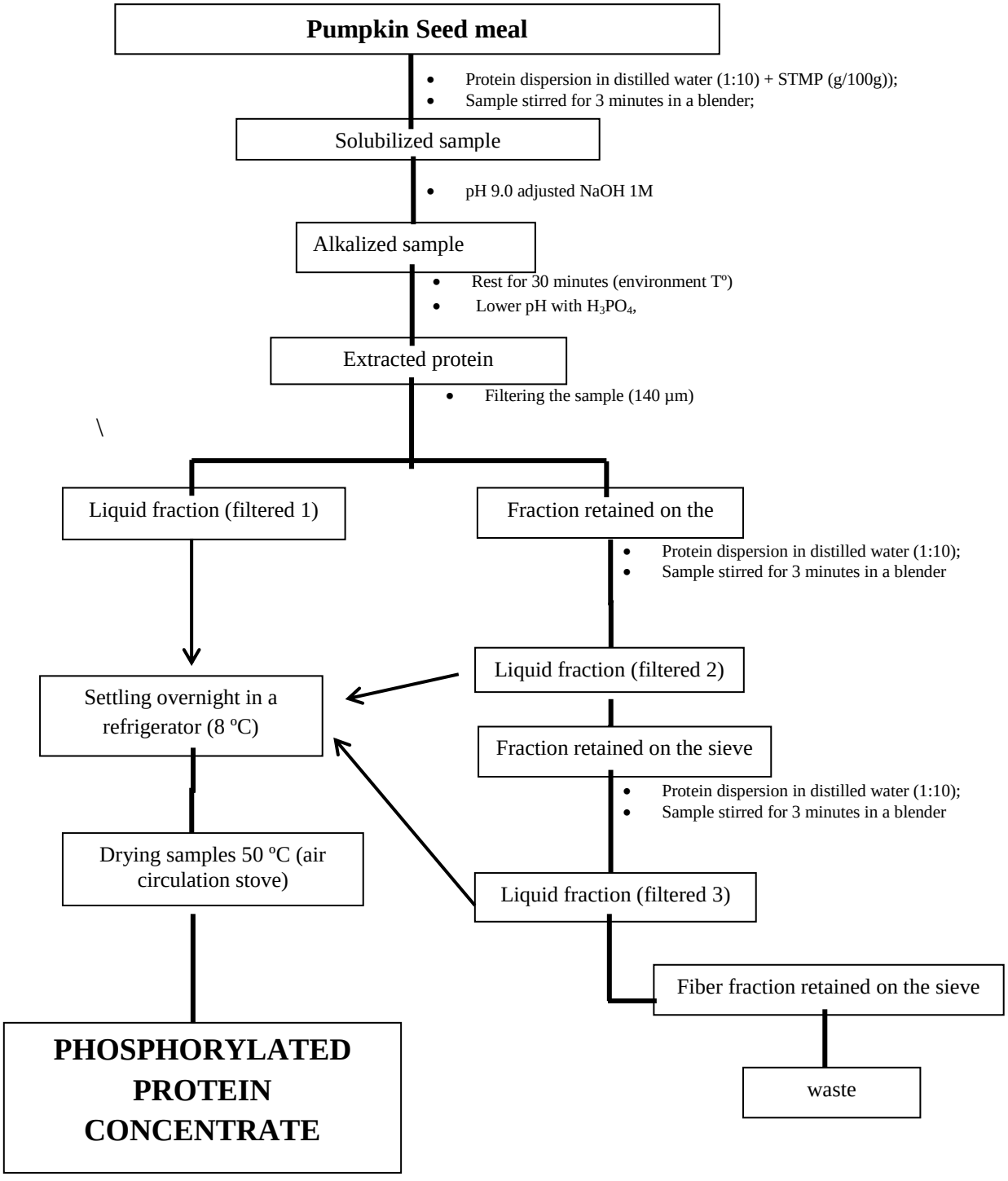


Fig.1. Organization chart of obtainment of the phosphorylated protein concentrate of pumpkin seed meal.

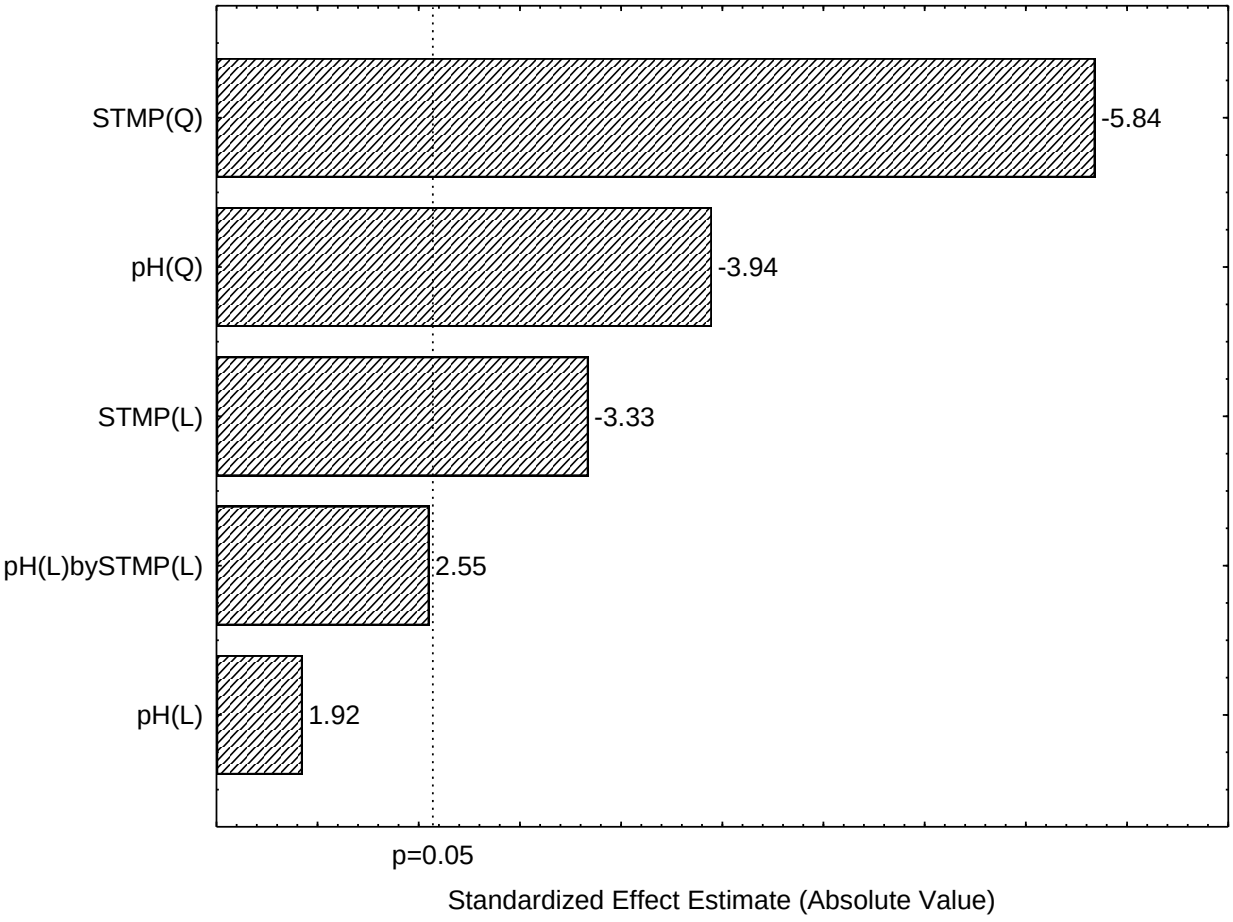


Fig.2 Pareto chart expressing the effects of variables to yield the protein concentration by phosphorylation of pumpkin seed; (Q): quadratic; (L): linear. STMP: Sodium trimetaphosphate (g/100g) x pH (phosphoric acid used).

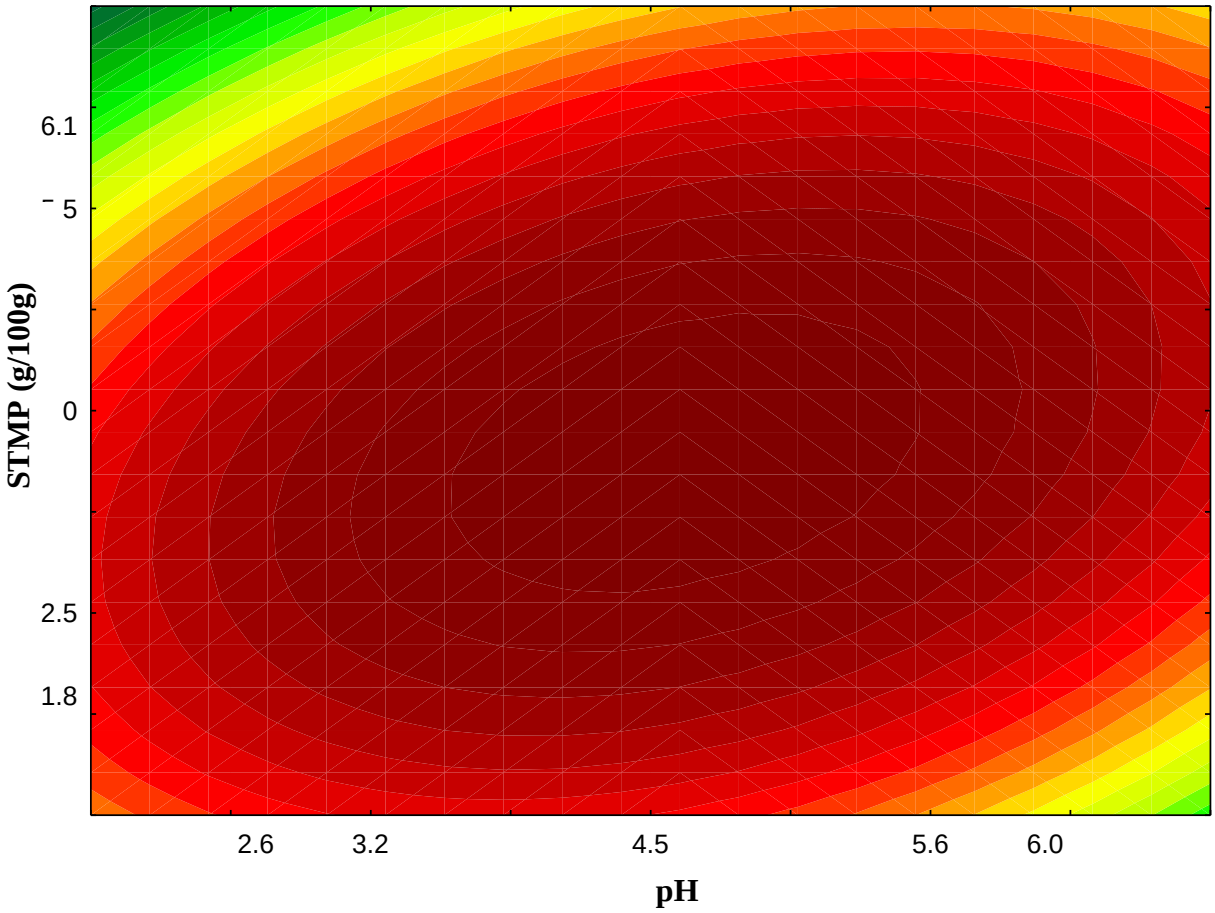


Fig. 3. Contour plot expressing the effects of variables on the yield of protein concentration by phosphorylation of pumpkin seed. STMP: Sodium trimetaphosphate (g/100g) x pH (phosphoric acid).

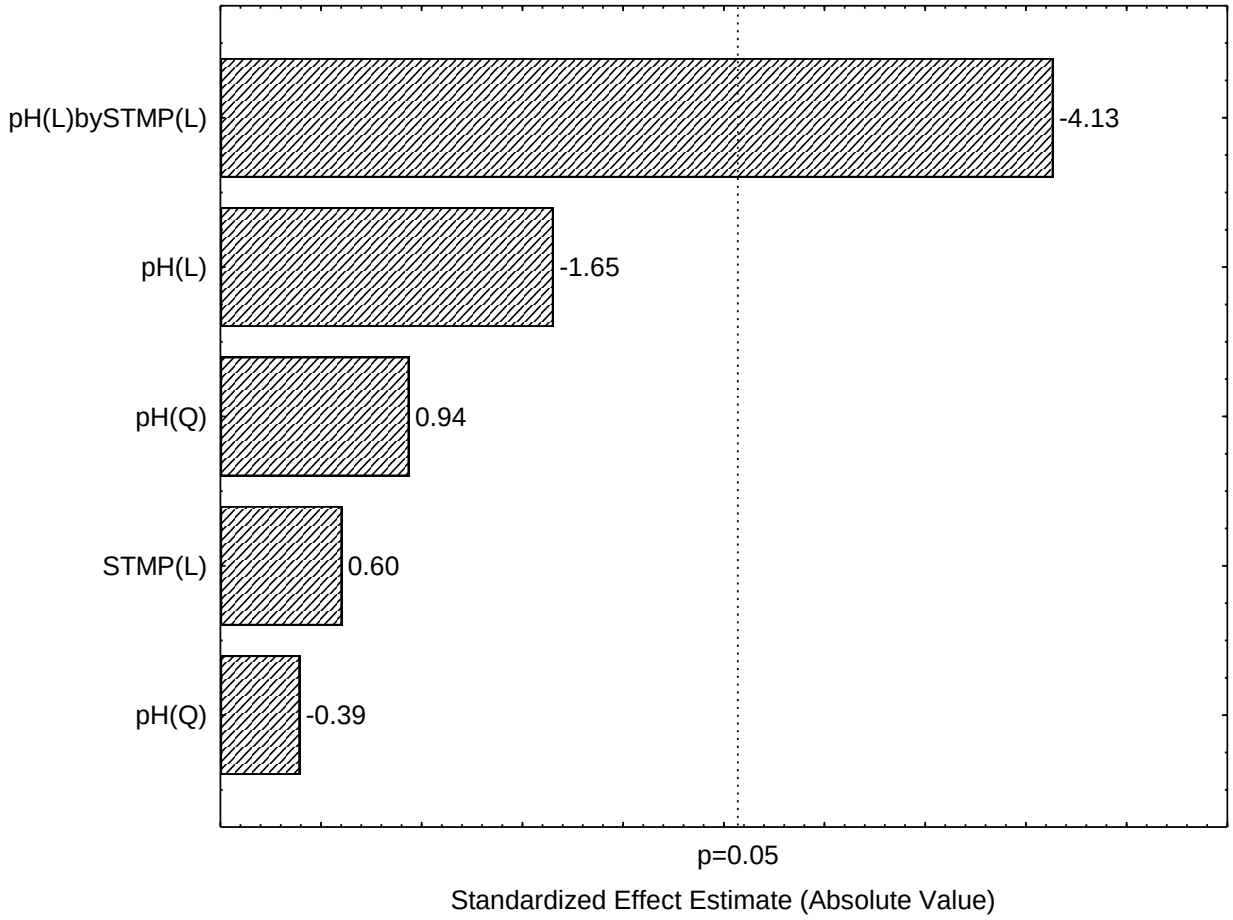


Fig. 4. Pareto chart expressing the effects of variables on the percentage of crude protein by protein concentration by phosphorylation of pumpkin seeds; (Q): quadratic; (L): linear; STMP: Sodium trimetaphosphate (g/100g); pH phosphoric acid.

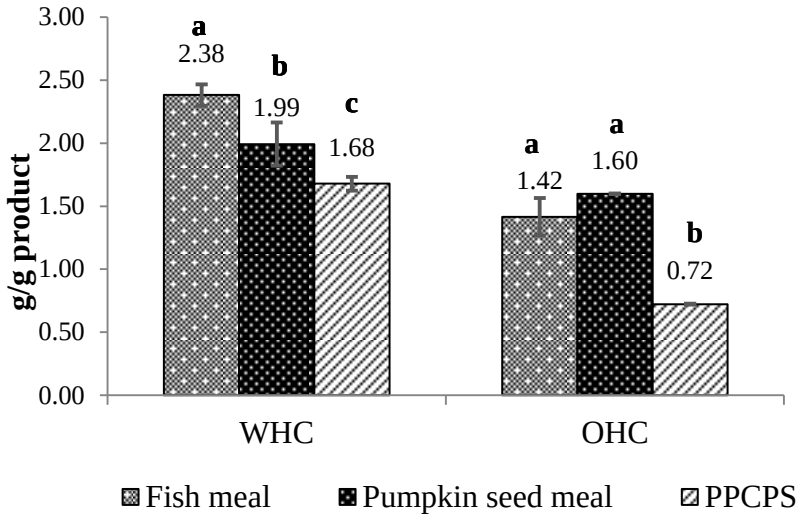


Fig. 5. Water holding capacity (WHC) and Oil holding capacity (OHC) of the pumpkin seed meal and the phosphorylated protein concentrate (PPCPS) of pumpkin seed in comparison to fish meal. Mean values followed by different letters were considered significantly different using the Tukey test ($P < 0.05$).

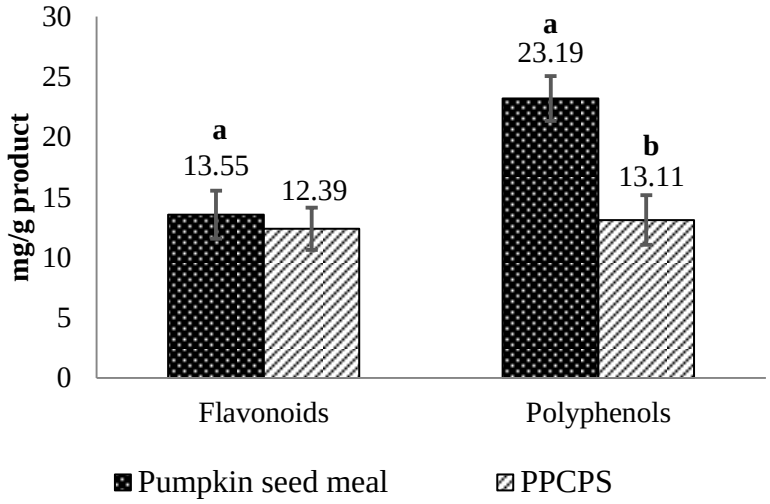
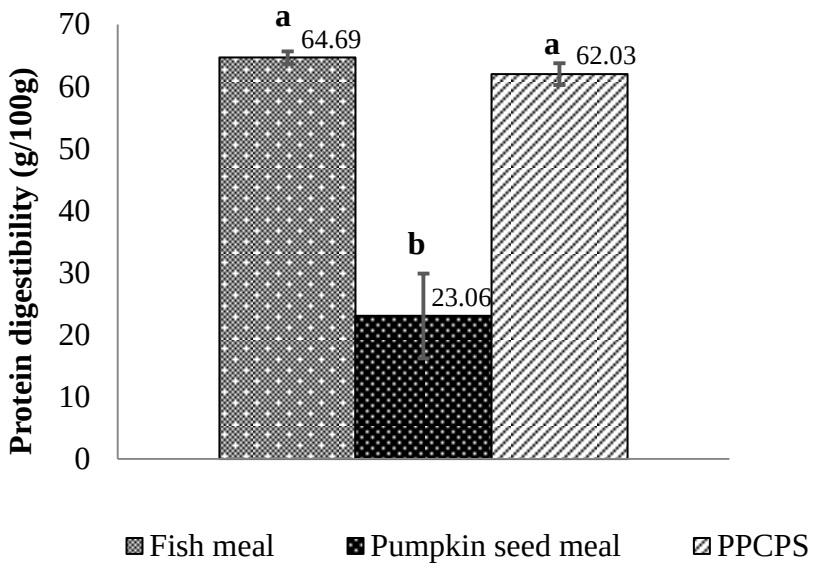


Fig. 6. Content of flavonoids and polyphenols of the pumpkin seed meal and of the phosphorylated protein concentrate of pumpkin seed (PPCPS). Mean values followed by different letters were considered significantly different using the Tukey test ($P < 0.05$).



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739 Fig. 7. *In vitro* digestibility of protein of pumpkin seed meal and of the phosphorylated
740 protein concentrate (PPCPS) of pumpkin seed, compared to the fish meal**.** Mean
741 values followed by different letters were considered significantly different using the
742 Tukey test ($P < 0.05$).

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4. ARTIGO 2

Effects of phosphorylated protein concentrate of pumpkin seed meal on growth and digestive enzymes activity of silver catfish (*Rhamdia quelen*)[2]

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Phosphorylated protein concentrate pumpkin seed in diet silver catfish

KEY WORDS: *Cucurbita moschata*, alternative protein, plant protein, trypsin, chymotrypsin.

[2] Artigo submetido à revista Aquaculture Nutrition

Abstract- The present study aimed to determine the growth and activity of proteolytic digestive enzymes of silver catfish (*Rhamdia quelen*) fed by increasingly replacing fish meal by phosphorylated protein concentrate (PPCPS) or pumpkin seed meal (PSM). Five experimental diets were formulated according to the requirements of 370 $g.kg^{-1}$ crude protein and 13.4 MJ Digestive $Energy.Kg^{-1}$. Were formulated to replace 0 (Control), 25 (25% pumpkin seed meal- PSM and 25% PPCPS) and 50% (50% PSM and 50% PPCPS). Crude protein from fishmeal was replaced by the PSM or PPCPS crude protein. Each diet was fed to four replicate groups of silver catfish (initial weight 24 ± 0.46 g) to apparent satiation during eight weeks. Four orthogonal contrasts were applied to biological assay data. The replacement of fishmeal by 25 or 50% of PPCPS does not change growth rates and promotes improvement in feed conversion of juvenile catfish. And the use of PSM as a protein ingredient is not recommended because it causes harm to fish growth. Thus, it can be concluded that the nutritional value of PSM can be improved by the simple chemical process of phosphorylation, yielding a protein concentrate (PPCPS) that can be used as a promising alternative ingredient.

1. Introduction

The Brazilian aquaculture production is estimated to significantly increase in the coming years, reaching 20 million tons / year by 2030 (MPA, 2014), when Brazil might become the world's largest producer of fish. Such expansion reflects upon the increased demand for qualitatively acceptable and economically feasible ingredients to be used in the formulation of aquaculture feed. Hence the common use of fish meal, as well as the possibility of using other protein sources from terrestrial animals, which give rise to some public concerns and fear in many countries, because of risks associated with zoonotic diseases and contamination (Fuertes *et al*. 2013).

Moreover, the transition from using animal protein sources to using other environmentally friendly sources, such as from plant, is an impending demand to ensure sustainability in production systems and stability in the aquaculture market (Fuertes *et al*. 2013). Plant sources such as sunflower meal and cake (Nyina-Wamwiza *et al.* 2010), soybean meal and soy protein concentrate (Robaina *et al.* 1995; Fasakin *et al*. 2005; Salze *et al*. 2010), cottonseed meal (Robinson & Li, 1994), and recently pumpkin seed cake (Murray *et al*. 2014) are currently being examined as potential ingredients for fish feed.

Pumpkin seed contains approximately 30% crude protein (Sharma *et al*. 1986, Esuoso *et al.* 1998) and high levels of polyunsaturated oleic and linoleic fatty acids (Esuoso *et al.* 1998; Younis *et al*. 2000). However, it has antinutritional and / or toxic factors, such as polyphenols, flavonoids, tannins and cyanogenic glycosides (Del Vechio *et al.* 2005), which constrain their use in fish feed.

Methods such as cooking, germination, roasting and extrusion are used to make the use of vegetable sources feasible, since they reduce anti-nutritional factors and improve protein digestibility (Nyina- Wamwiza *et al*. 2010). However, chemical methods can also be used to obtain protein sources with reduced antinutritional factors, to improve digestibility,

reduce fiber content and increase the content of amino acids. Among these methods, protein concentration by isoelectric point and by phosphorylation with sodium trimetaphosphate (STMP) stand out, promoting improved digestibility and modification of plant source amino acid profile.

Many studies have shown that replacing fishmeal by plant protein sources cause metabolic changes that are reflected on growth. This fact highlights the need for further studies on the digestive capacity and activity of digestive enzymes when vegetable protein ingredients are used as alternative sources in fish nutrition (Lundstedt *et al*. 2004; Stech *et al*. 2009).

There are no studies on the use of protein concentrates or pumpkin seed meal to feed catfish, a native species whose cultivation has been rapidly expanding in Brazil. Thus, this present study aimed to determine the growth and activity of proteolytic digestive enzymes of silver catfish (*Rhamdia quelen*) fed by increasingly replacing fish meal by phosphorylated protein concentrate or pumpkin seed meal.

2. Material and Methods

2.1.Ingredient gathering and preparation of the protein concentrate

The pumpkin seed (*Cucurbita moschata*) provided by Ritter Alimentos SA located in Cachoeirinha, RS, Brazil, went through a washing process for the removal of excess pulp, was dried at 45ºC and ground into a micro-cooled mill (MA-630, Marconi®). Sample oil was removed using Hexane (P.A. FMaia, Brazil) in a 2: 1 rate (hexane: sample) in three sequential washes, resulting in the pumpkin seed meal used to obtain protein concentrates.

The extraction and gathering of the protein concentrates were performed according to Smith *et al*. (1946), with adaptations from the isoelectric point of the protein. Phosphorylation was performed according to Yamada *et al*. (2003). STMP was added to the pumpkin seed

meal and the mixture was suspended in ten parts of distilled water. The solution was processed in a blender for three minutes (*LIQ789*, Cadence, Brazil) and sieved at a 140 µm mesh. The fraction retained on the sieve was resuspended in distilled water at a ratio of 1:10. This process was repeated until obtaining a ratio of 1:30 (product: solvent) at the end of processing. The three aqueous fractions were combined to obtain a single sample, when NaOH 1M was added up until pH 9.5, to obtain the solubilized protein. The solution was left to rest for 30 minutes at room temperature and then the pH was adjusted with concentrated phosphoric acid, according to the statistical design. The solution was conditioned under refrigeration (8 ° C) over night for the settling of the dispersed protein fraction, followed by supernatant discarding and drying of the concentrated protein fraction in an oven with recirculating air at 50 ° C (approximately 24 hours).

2.2. Antinutritional factors and in vitro digestibility of protein

The polyphenol content was determined by the Folin-Ciocalteu method described by Chandra & Mejia (2004). Afterwards, 2 N Folin-Ciocalteu (0.5 mL) was added to 1 ml of sample, followed by resting for 5 minutes and the adding of 2 ml of 20 % Na_2CO_3. The solution was left for 10 min, and readings were made at 730 nm. Data were expressed as mg of gallic acid equivalents (eq / mg GA) per g of crude extract, based on the calibration curve of gallic acid.

The flavonoid content was determined by means of the reaction containing aluminum chloride, through the method described by Woisky & Salatino (1998). A 2% solution of $AlCl_3$ (0.5 mL) was added to 1 ml of sample. After 15 min, the absorbance was measured at 420 nm. Data were calculated based on the calibration curve and expressed in terms of rutin equivalent (RE) per g of crude extract.

Condensed tannins were determined by the method of Morrison *et al.* (1995), using vanillin as a reactant. The absorbance of 500 nm was measured in a spectrophotometer. Data

were expressed as milligrams of catechin equivalent (CE) per g of each of the fractions, based on the calibration curve of catechin.

The *in vitro* assay was performed as proposed by Mauron (1973), with modifications proposed by Dias *et al.* (2010). The method is based on the digestion of the sample by the pepsin (1: 10,000, Nuclear) and pancreatin (Sigma) enzymes. The digestibility results from the relationship between the total nitrogen in the sample, the digested nitrogen, the nitrogen produced by the auto digestion of enzymes, and the soluble nitrogen originally in the meal.

2.3. Formulation and fabrication of the experimental diets

Pumpkin seed meal (PSM) and the phosphorylated protein concentrate of pumpkin seed meal (PPCPS) were assessed as partial substitutes for fish meal at different levels of catfish dietary replacement. The experimental diets were produced considering the percentage of crude protein and amino acids profile obtained from the investigated sources of protein. Diets were formulated according to the requirements of 370 $g.kg^{-1}$ crude protein established by Meyer & Fracalossi (2004), 13.4 MJ Digestive $Energy.Kg^{-1}$ and amino acids requirement by Montes-Girao & Fracalossi (2006).

Five experimental diets were prepared:

- Control: fish meal and soy protein concentrate (60%) as protein sources in the diet;

- 25% PSM: 25% crude protein from fishmeal was replaced by the PSM protein.

- 50% PSM: 50% crude protein from fishmeal was replaced by the PSM protein.

-25% PPCPS: 25% crude protein from fishmeal was replaced by the PPCPS protein.

-50% PPCPS: 50% crude protein from fishmeal was replaced by the PPCPS protein.

The diets were extruded in an EX-MICRO Lab Micro extruder, with production capacity of 15 kg of feed per hour.

2.4. Fish, Facilities and Experimental Design.

All procedures involving animals were conducted in compliance with the guidelines approved by the Committee on Research Ethics and Animal Welfare of Universidade Federal de Santa Maria, protocol number 23081.008738/2014-71.

This study included 400 fish with an average initial weight of 24 ± 0.46 g and total length of 13 cm ± 0.10 (mean ± standard deviation). The fish were randomly distributed into 20 tanks (experimental units), accounting for five treatments and four replications. A total of 20 fish were allocated per tank with capacity of 280 L each. Tanks had a water recirculation system with two biological filters and temperature control (electric resistances 2,000 W).

2.5. Experimental Monitoring and water quality

The fish were submitted to diets and to the experimental system for 10 days. The experimental diet was fed to apparent satiation three times a day (9am, 1pm and 5pm). The tanks were cleaned to remove feces twice daily (8am and 3pm). After that, fish received the experimental diets for seven weeks, maintaining the same schedule and routines mentioned, during the period of adaptation.

During the experimental period, water quality parameters were monitored and maintained as described below: Temperature: 23.60±1.47 ºC; dissolved oxygen: 7.15±0.53 ppm; pH: 7.37±0.23; total ammonia: 0.17±0.11 ppm; nitrite: 0.06±0.01 ppm; alkalinity: 40.12±3.87 mg $CaCO_3.L^{-1}$ and hardness: 38.75±10.63 $CaCO_3.L^{-1}$. All parameters remained within the range suitable for silver catfish (Baldisserotto & Silva 2004).

2.6. Growth development and fish use of diets

To determine animal growth, two biometric measures were made, at the beginning and at the end of the experimental period. Before the biometrics fish were subjected to a fasting period of 24 hours. For the managements during the biometric measuring, fish were anesthetized with benzocaine (100 $mg.L^{-1}$). The following measurements were recorded: Total length (cm) and weight (g), using digital calipers and scales.

To assess fish use of diets, we determined feed conversion – FC (total food consumed/ total weight gain); specific growth rate (% day) – SGR [(ln final body weight – ln initial body weight)/period]*100; and daily mean weight gain – DWG ($g.day^{-1}$).

At the beginning and end of the experiment, three fish per tank (12 per treatment) were captured. An incision was made to the spinal cord of fish in order to eviscerate, remove digestive tract for further analysis and fat to obtain data on the digestive somatic index (DSI). The length of the digestive tract was measured to determine the intestinal quotient (IQ).

2.7. Tissue homogenates and analysis of digestive enzymes

A 10 cm portion of the anterior intestine of fish was removed a frozen (-20 º C) for assessing the tripsin and chymotrypsin digestive enzymes. Each portion was dissected into Petri dishes containing saline (0.9 % NaCl) to remove any remaining intestinal contents and then homogenized. The homogenization was performed with buffer (0.02 M Tris / 0.01 M phosphate, pH 7.5 in 50 % glycerol) at 1:20 tissue: buffer ratio. We used Turrax tissue homogenizer (Marconi, Brazil, MA 102). The homogenates were centrifuged at 1200x g for 10 minutes and supernatants were used as source of enzymes.

Trypsin activity (E.C.3.4.21.4) was analyzed with α-*p*-toluenosulphonyl-L-arginine methyl ester hydrochloride (TAME). The extracts were incubated for two minutes (25 ° C) in 2 ml of buffer (0.2 M Tris / 0.01 M $CaCl_2$), pH 8.1. Chymotrypsin activity (E.C.3.4.21.1) was analyzed with benzoyl tyrosine ethyl ester (BTEE). Crude extracts were incubated for two minutes in 1 ml of buffer (0.1 M Tris / 0.1 M $CaCl_2$), pH 7.8. Both trypsin and chymotrypsin activities were assayed in duplicates and the enzymatic activities were read at 247 and 256 nm, respectively, according to protocols described by Hummel (1959). One unit of enzyme was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate (TAME or BTEE) / min / mg of protein.

2.8. Statistics

Data were subjected to analysis of variance by F test and for means comparison, Tukey test was used at 5% probability, for antinutritional factors and protein *in-vitro* digestibility data. Four orthogonal contrasts were applied to biological assay data: *Control vs PSM diets; Control vs PPCPS diets; Control vs other diets; and PSM diets vs PPCPS diets.*

3. Results

3.1. Protein concentration, antinutritional factors and protein in-vitro digestibility

PSM phosphorylation protein concentration provided a 52% increase in crude protein content (Table 2). Condensed tannins were not detected in samples of PSM and PPCPS. The processes of concentration and protein phosphorylation decreased by 56.53% total polyphenol content to PPCPS (13.11 mg. g^{-1}) in relation to the PSM (23,19 mg. g^{-1}), but were ineffective in reducing flavonoids (Table 2). The reduction of antinutrients by phosphorylation reflected in protein digestibility similar to PPCPS and fish meal ($P < 0.05$).

3.2. Performance on growth and fish use of food

The growth parameters of fish (Table 3) showed significant differences ($P < 0.05$) to body weight (BW) at the end of the trial period in the contrast *Control vs PSM diets*, where the greatest results were found in fish from the control diet, followed by fish fed 25% PSM and 50% PSM, respectively. In *Control vs other diets* and for *PSM diets vs PPCPS diets*, fish fed with PPCPS diets had greater body weight at the end of the experiment ($P < 0.05$). This same behavior is reflected in the CT and CP parameters of fish.

The FC was higher for fish that received 25% or 50% PPCPS compared to those who received PSM ($P < 0.05$) (Table 3), in contrast *PSM diets vs PPCPS diets.*

For SGR, differences ($P > 0.05$) were found for contrast *Control vs PSM diets*, where the highest value was obtained for fish receiving control diet. For the contrast *PSM diets vs*

PPCPS diets, significant differences were also found, since the fish that received 25% PPCPS diets and 50% PPCPS diets had higher SGR, respectively when compared to fish fed diets containing PSM.

Greater DWG was found for fish receiving the control diet in relation to fish receiving 25% PSM and 50% PSM diets ($P < 0.05$), in contrast *control vs PSM diets*. Fish fed 25% PPCPS and 50% PPCPS diets had greater DWG compared to those fed diets containing PSM ($P < 0.05$), in contrast *PSM diets vs PPCPS diets.*

Figure 1 shows results for catfish digestive rates. Fish fed the control diet had lower digestive-somatic index (DSI) (Figure 1a) in contrasts *Control vs PPCPS diets* and *Control vs. other diets*. The contrasts showed no differences for intestinal quotient (IQ) among fish fed different experimental diets (Figure 1b).

3.3. Analysis of digestive enzymes

Trypsin activity (Table 4) was lower for fish in the Control treatment, compared to fish fed diets with PSM ($P < 0.05$) (*Control vs PSM diets*) and also when comparing Control with other diets *(Control vs other diets*). Chymotrypsin activity was lower for fish that received PSM compared to Control, in *Control vs PSM diets* ($P<0.05$). Reduced chymotrypsin activity was also noted for fish that received PSM diet ($P < 0.05$), in *PSM diets vs PPCPS diets.*

4. Discussion

Our results showed that PSM presented relevant protein content for use in animal feed. However, the application of phosphorylation protein concentration on this ingredient afforded a new product (PPCPS) with greater protein content 52% and significantly reduced levels of

fibers and polyphenols associated with this fraction. Shamna *et al.* (2014) and Fuertes *et al.* (2013) also had high protein and reduced antinutritional factors when using isoelectric pH for protein concentration of *Jatropha* and pea, respectively. The best *in vitro* digestibility of PPCPS protein was both associated to the reduction in fiber content and to partial hydrolysis of the protein promoted by the phosphorylation process (Matheis *et al.* 1983).

Despite its significant protein content (44.5% of DM), inclusion of PSM in the diets caused damage in fish growth, which was expected due to its high content of polyphenols and fiber and lower in vitro digestibility of protein (around 23%). In contrast, fish fed diets containing PPCPS showed better growth rates than those fed diets with fishmeal and soy protein concentrate (Control diet).This fact confirms the efficiency of protein concentration by phosphorylation to obtain ingredients with proven nutritional efficacy for catfish.

Growth parameters significant greater results (BW, FC, SGR and DWG) for animals fed diets with PPCPS are explained by the improvement in the content of essential amino acids, increased availability of nutrients and reduced antinutritional factors of this concentrate in relation to PSM. Similar results were found by Shamna *et al.* (2014) when fingerlings of *Labeo rohita* were fed diets with different protein sources arising from *Jatropha*. However, these results contradict those found by some studies, where even the improved FC did not result in better growth due to deficiency in essential amino acids from vegetable protein concentrates (Penn *et al*., 2011, Zhang *et al*., 2012; Fuertes *et al*., 2013).

Trypsin greater activity in animals fed diets containing PSM is explained as an attempt of the body to increase protein digestibility, which is reflected in increased proteolytic activity. Alarcón *et al.* (2001) also reported an inverse relationship between the activities of intestinal trypsin and protein digestibility in *Lutjanus argentiventris* e *L. novemfasciatus* explaining that the inhibition of the enzymatic activity appears to be offset by increased secretion of proteolytic enzymes and increased absorption of the protein in distal portions of

the intestine. Animals fed PPCPS showed no difference in the activity of trypsin compared to control. These results are contrary to higher trypsin activity found for Atlantic salmon (*Salmo salar)* fed with pea protein concentrates (Penn *et al.*, 2011).

Song *et al.* (2014) also observed higher tryptic activity in *Platichthys stellatus* fed diets containing 15-70% replacement of fishmeal by soybean protein hydrolyzate. Greater trypsin activity when using vegetable protein concentrates can be associated with the induction of enteritis in fish (Krogdahl *et al*. 2003; Lilleeng *et al*. 2007; Penn *et al*. 2011;).

Chymotrypsin activity was lower for fish fed diets with PSM in both levels, and greater for the control and PPCPS diets. This is explained by Zambonino-Infante *et al*. (1997) who also found that chymotrypsin activity was induced by higher proportions of small peptides (di/tri peptide), that can be found in diets with fish meal or facilitated by protein phosphorylation. In addition, the high amount of free amino acids may have resulted in increased catabolism of amino acids (Espe & Lied, 1994).

The DSI was higher for animals receiving PPCPS, indicating increased production of intestinal cells (Furné *et al.*, 2008) and physiological well-being of the digestive system (Shamna, *et al.* 2014). Our results agree with those of Radünz Neto *et al.* (2006) who have found high DSI for piavas (*Leporinus obtusidens*) fed soybean meal compared with those who received meat and pork bone meal as a protein source. However, these results are contrary to those reported by Song *et al.* (2014) who found greater DSI for *Platichthys stellatus* fed diets that included only fishmeal as protein source, compared to fish fed diets with varying levels of soy protein hydrolysates.

For IQ scores, no differences were found among the studied diets, which were expected, considering that these changes are found when there is modification of the content of other nutrients such as fiber and carbohydrates.

The replacement of fishmeal by 25 or 50% of PPCPS does not change growth rates and promotes improvement in feed conversion of juvenile catfish. And the use of PSM as a protein ingredient is not recommended because it causes harm to fish growth. Thus, it can be concluded that the nutritional value of pumpkin seed meal can be improved by the simple chemical process of phosphorylation, yielding a protein concentrate (PPCPS) that can be used as a promising alternative ingredient for fish diet.

Acknowledgments

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Table 1. Formulation, nutritional composition and amino acids profile of experimental diets used during feeding trial (g kg^{-1})

	.....Diet code[1].....				
Ingredients ($g.kg^{-1}$)	CONTROL	PSM 25%	PSM 50%	PPCPS 25%	PPCPS 50%
PSM	0	146.3	292.6	0	0
PPCPS	0	0	0	106.4	212.7
SPC 60%[2]	200	200	200	200	200
Corn starch	226.4	227.1	154.6	226.7	226.9
Fish meal	408	306	204	306	204.3
Soybean oil	24.6	21	46.7	23.4	22.2
Vit. Min Mix[3]	30	30	28	30	30
Phosphate dicalcium	39	43.5	43	41	41
MSG[4]	5	5	5	5	5
BHT	0.1	0.1	0.1	0.1	0.1
Calcitic limestone	0	11.7	22	13.5	26.5
L-lisine	0	1.3	3.9	2.8	5.5
Inert	66.8	8.0	0	45.0	25.8
	.....Proximate composition[5] ($g.kg^{-1}$).....				
Crude Protein	371.5	370.3	365.8	368	362.3
Calculated energy (MJ kg^{-1})[6*]	13.4	13.4	13.4	13.4	13.4
Lipids	77.2	74.1	100	70.5	69.4
NDF[7]	87.5	105.4	123.3	85.5	83.5
CSDN[8*]	128.7	167.1	142.2	153.5	183.4
Total Ash	218.7	166.7	154.9	205.8	185.3
Calcium	33.3	33.3	31.6	33.5	33.1
Total phosphorus	17.8	17.8	16.9	17.9	17.7
Flavonoids (mg/g)	10.54	12.69	22.95	6.19	10.57
Polyphenols (mg/g)	13.22	12.42	20.57	8.96	12.74
	.....Amino acids[9] ($g.kg^{-1}$ of crude protein).....				
Histidine	41	45	37	26	41
Arginine	109	97	115	101	127
Threonine	48	45	52	46	58
Tyrosine	35	40	56	37	54
Valine	52	51	62	53	69
Methionine + Cysteine	45	42	48	42	54
Isoleucine	56	54	62	54	67
Leucine	35	40	56	37	54
Phenylalanine	52	51	62	53	69
Lysine	45	45	48	45	45

[1]Diets: PSM 25% and 50% PSM, pumpkin seed bran replacing 25 or 50% of the protein fishmeal. PPCPs 25% and 50% PPCPs: phosphorylated protein concentrate bran, pumpkin seed, replacing 25ou in 50% of protein from fish meal.

2 Soybean protein concentrate (60% crude protein). [3]Composition of vitamin and mineral mixture :. 300 mg, Ascorbic AC:. 15,000 mg, Pantothenic Ac:. 3,000 mg, Biotin: 0.06mg, niacin (B3): 9,000 mg Hill (B4): 103.500 mg, Vit.A: 1,000,000 IU, Vit B1:. 1,500 mg, Vit B2:. 1:50 mg, Vit B6. 1,500 mg, Vit D3:. 240 000 IU Vit. E: 10 000 mg , Vit K3:. 400 mg, Inositol: 10,000 mg Iron: 5.000mg, Manganese: 8.000 mg, Copper 1.000 mg Zinc: 14 000 mg Iodine: 45 mg, Cobalt: 60 mg, Selenium 60 mg, Magnesium: 5 mg., Mig Plus®.

[4] Monossodium glutamate.

[5]Composition analyzed.

[6]Digestible Energy = [(CP * 23.61 MJ / kg * 0.9) + Fat * 39.82 MJ / kg * 0.85) + CSDN * 17.21 MJ / kg *0.50)] (Jobling, 1983).

[7]NDF: neutral detergent fiber.

[8]CSND: Carbohydrates soluble in neutral detergent = 100- (moisture + crude protein + fat + ash + neutral detergent fiber).

[9]Calculated from the analysis of ingredients.

Table 2. Nutritional composition and *in vitro* digestibility of protein of pumpkin seed meal (PSM) and the phosphorylated protein concentrate of pumpkin seed meal (PPCPS)

Nutrient	PSM[1]	PPCPS[2]
	.....g.kg $^{-1}$ of the dry matter (DM)….....	
Crude protein	444.8±13.4[b]	674.8±27.5[a]
Lipid	30.6±0.6[b]	103.0±2.9[a]
NDF	371.7±9.1[a]	125.3±18.8[b]
In vitro digestibility of protein	241.6[b]	669.1[a]

Mean values followed by different letters in the row were considered significantly different using the Tukey's test ($P < 0.05$). [1] PSM:Pumpkin seed meal; [2] PPCPS:Phosphorylated protein concentrate of pumpkin seed meal.

Table 3. Growth index in silver catfish of different experimental groups fed different experimental diets.

Sources of variation	BW (g)[3]	FC[4]	SGR[5]	DWG[6]
	.....Control diet vs PSM diets.....			
Control	80.39±13.01	1.47±0.17	2.37±0.09	1.11±0.07
PSM 25%[1]	72.42±10.77	1.6±0.27	2.13±0.19	0.93±0.13
PSM 50%[1]	65.44±14.30	1.82±0.31	1.92±0.29	0.8±0.18
Test F	*	ns[6]	*	*
	.....Control diets vs PPCPS diets.....			
Control	80.39±13.01	1.47±0.17	2.37±0.09	1.11±0.07
PPCPS 25%[2]	83.33±16.54	1.29±0.11	2.37±0.17	1.11±0.14
PPCPS 50%[2]	81.04±16.74	1.39±0.14	2.28±0.23	1.10±0.19
Test F	ns	ns	ns	ns
Test F				
Control vs other diets	*	ns	ns	ns
PSM diets vs PPCPS diets	*	*	*	*

Means ± standard deviation compared as Orthogonal contrasts in the 5% level of significance
[1]PSM 25% and PSM 50%, pumpkin seed bran replacing 25 or 50% of the protein fishmeal.
[2]PPCPS 25% and PPCPS 50%: phosphorylated protein concentrate pumpkin seed, replacing 25or 50% of protein from fish meal.
[3] Body Weight (g)
[4] Feed Conversion.
[5] Specific growth rate.
[6]Dairy weight gain.
[7]non significative
[*]Significative

Table 4. Digestive enzymes activity in silver catfish of different experimental groups fed different experimental diets.

Sources of variation	Trypsin[3]	Chymotrypsin[4]
	.....Control diet vs PSM diets.....	
Control	3.88±1.70	4.06±0.99
PSM 25%[1]	4.58±1.48	3.05±0.69
PSM 50%[1]	5.68±1.35	3.31±1.20
Test F	*	*
	.....Control diet vs PPCPS diets.....	
Control	3.88±1.70	4.06±0.99
PPCPS 25%[2]	4.08±1.56	3.83±0.95
PPCPS 50%[2]	5.06±1.94	4.20±0.86
Test F	ns	ns
Test F		
Control *vs* other diets	*	ns
PSM diets *vs* PPCPS diets	ns	*

Means ± standard deviation compared as Orthogonal contrasts in the 5% level of significance
[1]PSM 25% and PSM 50%, pumpkin seed bran replacing 25 or 50% of the protein fishmeal.
[2]PPCPs 25% and PPCPS 50%: phosphorylated protein concentrate bran, pumpkin seed, replacing 25ou in 50% of protein from fish meal.
[3]trypsin=µmol TAME hydrolysed/min/mg protein;
[4]chymotrypsin=mmol BTEE hydrolysed/min/mg protein;
ns: non significative;
*Significative;

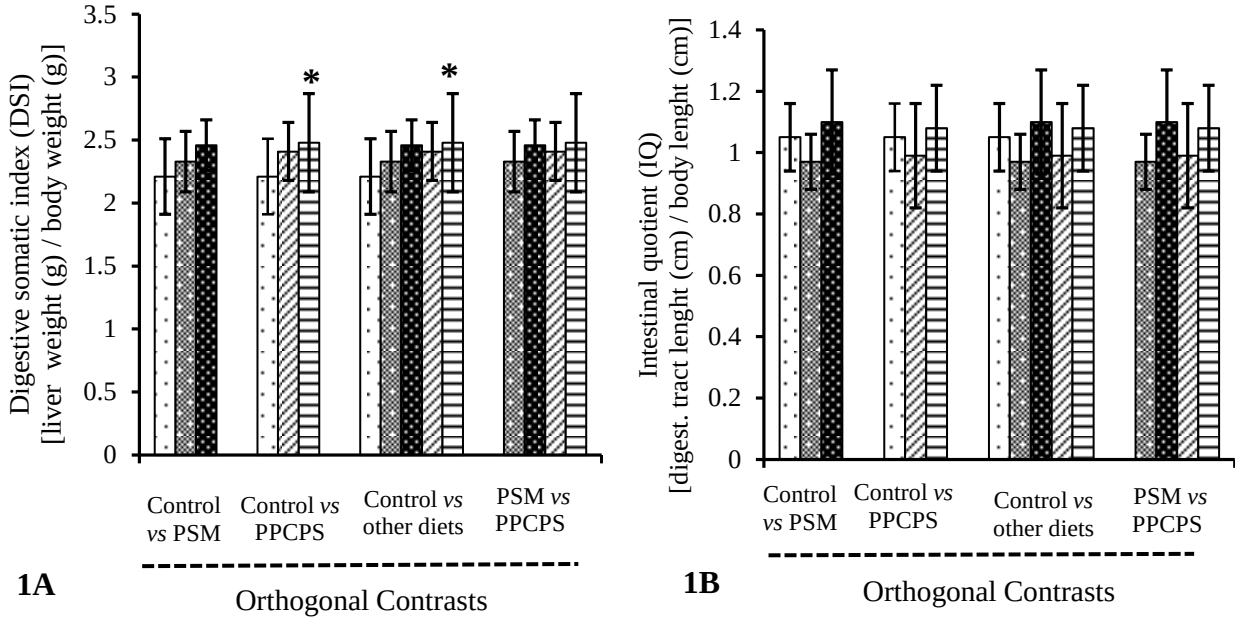

Fig. 1. Digestive somatic index (DSI)- 1A, Intestinal quotient (IQ)- 1B e, in silver catfish of different experimental groups fed different experimental diets. Means ± standard deviation compared as orthogonal contrasts in the 5% level of significance. The asterisk (*) shows a significant difference to the orthogonal contrast.

5. ARTIGO 3

Nutritional evaluation of phosphorylated pumpkin seed (*Cucurbita moschata)* protein concentrate in silver catfish *Rhamdia quelen* (Quoy and Gaimard, 1824)[3]

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Abstract- An 8-week feeding trial was conducted to evaluate the effect of replacing fish meal with pumpkin seed meal (PSM) or Phosphorylated protein concentrate of pumpkin seed meal (PPCPS) on growth and metabolic responses of silver catfish. Five isonitrogenous and isocaloric experimental diets were formulated. Control diet contained fish meal as the main protein source. The treatment groups contained 25 and 50 percent of either PSM or PPPCS protein replaced the fishmeal protein. A total of 400 silver catfish, with initial mean weight of 24 ± 0,46g, were distributed into 24 fiber glasses. For data four orthogonal contrasts were applied: *Control diet vs. PSM diets*; *Control diets vs. PPCPS diets*; *Control vs. other diets*; *PSM diets vs. PPCPS diets*. The results indicated that the fish fed PSM diets had lower weight gain when compared to either Control diet or PPCPS. The PPCPS don't affect growth and protein efficiency ratio. Lower albumin contents were found for the control diet fish for the contrasts Control diet vs. PPCPS diet and Control diet vs. other diets. The hepatic ALAT enzyme activity was higher in the fish fed the Control diet ($P < 0.05$). The hepatic ALP was most active in fish that received the PPCPS diets, when comparing Control diet vs. PPCPS diets and Control diet vs. other diets. The hepatosomatic index was higher for fish fed the PPCPS. Our results indicated that PPCPS presents relevant nutritional quality for fish and can replace the fish meal protein up to 50% without affecting growth, protein efficience ratio and intermediate metabolites in silver catfish.

Keywords: Jundiá, protein concentrate, hepatic metabolism, protein.

1.Introduction

In 2009 aquaculture achieved a landmark, supplying half of the total fish and shellfish demand for human consumption, and farmed fish consumption surpassed that of wild fish (Brinker and Reiter 2011) and for the first time worldwide farmed fish production topped beef production. The gap widened in 2012, with output from fish farming—also called aquaculture (Larsen and Roney 2013). This rapid growth in aquaculture production and the limitations in fish meal production forced the aquaculture sector to search for alternative protein sources as fish meal substitute in fish diets.

The need to substitute fish meal with plant-based products in fish feed was recently emphasized by publications on fish and shrimp nutrition, on the National Research Council (NRC 2011). One of the main alternatives for a continuous feed supply with high quality nutrients relies on the use of vegetable raw materials (Tusche et al. 2012). Among commercially available plant-based protein alternatives such as wheat gluten and soy bean meal have one of the best amino acid balances (Enami 2011; Tusche et al. 2012). However other vegetable sources should be studied to obtain new products with satisfactory amino acid profile able to satisfy the growing demands of the fish feeding industry for economical protein sources (Hardy 2010).

Moreover several potential protein replacements may be derived from vegetable sources such as agro industrial by-products or waste (FAO 2012), the most abundant, accessible, and sustainable source of proteins (Watson et al. 2012). However, most alternative sources also have antinutritional factors (ANFs), polyphenols, tannins, hormonal inhibitors like glucosinolates, unavailable phosphorus complexes such as phytate, high fiber levels, and complex carbohydrates may all have negative effects on palatability, digestibility and fish growth (Watson et al. 2012). Some effects of ANFs can be mitigated or eliminated through processing, thus producing acceptable feed from various plant sources (Watson et al. 2012).

In Brazil, pumpkin pulp is widely used for the manufacture of pumpkin jam and other culinary sweets and the seed is either discarded or underused. The pumpkin seed is a residue from the processing of the pumpkin pulp containing about 30% crude protein, rich in polyunsaturated fatty acids and complex vitamin A (Younis et al. 2000; Kim et al. 2012). However, *C. moschata* seeds also contain high fiber and some anti-nutrients (polyphenols, flavonoids, and tannins), limiting its use in aquatic feed (Del Vechio et al. 2005).

Some strategies for the concentration and chemical modification of proteins can be used to minimize anti-nutritional factors and improve the digestibility of such by-products, enabling it to be used as an ingredient in aquatic feed (Mariod et al. 2010). The technique of phosphorylation with sodium trimetaphosphate (STMP) has been applied to plant-based protein sources (Ellinger 1972) for protein concentration, and modification of the amino acid profile. Obtaining phosphorylated protein concentrates is feasible because in the conditions prevailing in the stomach during the digestion process, the N-phosphorylated proteins are dephosphorylated and lysyl residues will be regenerated, without causing negative impact on the digestibility of lysine and other amino acids (Fennema 2010). With this backdrop, a phosphorylated protein concentrate from pumpkin seed was prepared and standardized in our lab. This study aimed to evaluate the effects of adding phosphorylated pumpkin seed (*C. moschata)* protein concentrate from on the growth, nutrient utilization and metabolic responses of silver catfish (*Rhamdia quelen).*

2.Material and Methods

2.1. Preparation of the samples and protein concentrate obtaining

The pumpkin seed, courtesy of Ritter Alimentos S.A. Cachoeirinha, RS, Brazil, was washed in distilled water, to remove excess pulp, and then dried at 45 °C in recirculating air stove and ground in a cooled micro-mill (MA-630, Marconi®). The oil sample was removed with Hexane (P.A. FMaia, Brasil) in the ratio 2:1 (hexane: sample) in three sequential washes, resulting in pumpkin seed meal used to obtain the protein concentrates.

Extraction and obtainment of protein concentrates were performed according to Smith et al. (1946) for pH isoelectric protein. Phosphorylation was performed following the method of Yamada et al. (2003). Sodium trimetaphosphate was used as phosphorylated agent. The pH was adjusted with NaOH 1M and H_3PO_4 (extra pure, 85%).

2.2. I*n vitro digestibility protein*

In vitro digestibility of protein assay was performed as proposed by Mauron (1973), with modifications proposed by Dias et al. (2010). The method is based on the digestion of the sample by the pepsin enzyme (1:10.000, Nuclear) and pancreatin (Sigma). The digestibility results from the relationship between the total nitrogen in the sample, digested nitrogen, nitrogen produced by auto digestion of the enzymes and the soluble nitrogen originally in ingredient.

2.3. Diet formulation and preparation

Five iso-nitrogenous (about 370 $g.kg^{-1}$ crude protein) and iso-caloric (about 13 $MJ.\ kg^{-1}$) experimental diets were formulated (Table 1). Control diet (C) contained fish meal and soy protein concentrate as the main protein source, but did not contain pumpkin seed meal (PSM) and phosphorylated protein concentrate from pumpkin seed meal (PPCPS). The treatment

groups contained 25 and 50 percent of either PSM or PPPCS protein replaced the fishmeal protein.

Other ingredients such as corn starch, soybean meal were also included in the composition of the diet. The vitamin-mineral mixture, the antioxidant butylated hydroxytoluene (BHT), and monosodium glutamate, used for palatability, were included at the same dosage in all diets. The diets were formulated for amino acids requirements according to Meyer and Fracalossi (2004) and Montes-Giraos and Fracalossi (2006).

2.4. Procuring, acclimation of experimental animals and experimental set up and monitoring

This study was carried out at the Experimental fish farm and laboratory of the Federal University of Santa Maria (Universidade Federal de Santa Maria- UFSM) - RS, Brazil. All procedures involving animals were carried out in compliance with the guidelines approved by the Committee on Research Ethics and Animal Welfare of said university, protocol number 23081.008738/2014-71. The fish were acquired from Andrighetto Fish Farming, Ajuricaba, RS, Brazil. A total of 400 silver catfish, corresponding to 20 animals per tank, with initial mean weight of 24 ± 0,46g, were distributed into 20 tanks 280-liter-capacity of with individual water inlets and outlets, connected to a water recirculating system consisting of two biological filters with gravel, backwash system, and controlled temperature. The fish were acclimated for a ten- week period, during eight weeks were fed the experimental diets. In the two weeks prior to the experiment the fish which they were fed a commercial feed with 36% of crude protein.

2.5. Water quality

During the experimental period, the water quality parameters were monitored and maintained as follows: temperature of 23.60±1.47ºC; dissolved oxygen: 7.15±0.53 ppm; pH: 7.37±0.23; total ammonia: 0.17±0.11 ppm; nitrite: 0.06±0.01ppm; alkalinity: 40.12±3.87 mg $CaCO_3.L^{-1}$; and hardness: 38.75±10.63 $CaCO_3.L^{-1}$. According to Baldisserotto and Silva (2004) these parameters are within the optimum range for silver catfish *R. quelen* culture.

2.6. Data collection and assessed variables

In the early and late experimental period (eight weeks of treatment) biometrics was performed to collect data of the animals, which had fasted for 24 hours and were anesthetized with Benzocaine (100 $mg.L^{-1}$), to estimate the following: body weight gain (%); protein efficiency ratio (PER)= [net weight gain (g on the wet weight basis)/ protein feed (g on dry matter basis)]; survival (%)= [(Total number of fish harvested/total number of fish stocked) x 100] and hepatosomatic index (HSI) (%): [(weight of the liver/weight of the whole fish) x 100]; For the analysis, were used twelve animals from each treatment. Crude protein was determined by the micro-Kjeldahl method (method 960.52) using the N x 6.25 factor (AOAC 2000). The protein deposition was calculated according to the following equation:

$$-Total\ deposition\ of\ crude\ protein\ (TDP) = [FW * (\%\ FBP/100)] - [IW * (\%\ IBP/100)]$$

Where: FW = final weight; IW = initial weight; IBP = initial body protein; FBP = final body protein;

2.7. Plasma biochemistry and hepatic metabolism assay

In the late experimental period, twelve fish per treatment were captured. The animals fasted for 24 h. Blood was quickly collected from the caudal vein using heparinized syringes and the fish killed by spinal cord excision behind operculum and eviscerated to remove the liver. Thereafter, livers were quickly placed on ice and frozen at -20ºC for biochemical parameters analysis. Plasma aliquots were separated after blood centrifugation at room temperature for 10 min at 1200xg for posterior determination of plasmatic metabolic parameters. From the hepatics analyses, liver glycogen levels were determined according to Bidinotto et al. (1998). The liver tissue was weighed (50 mg) and KOH and ethanol (1 and 3 mL, respectively) were added for hydrolysis and glycogen precipitation. For protein analysis, the tissues were heated at 100°C with KOH and centrifuged at 1000x*g* for 10 min. Supernatant was used to estimate the total protein level by the method described by Bradford (1976), using bovine albumin serum as standard.

To measure hepatic amino acids and transaminases, liver samples were mechanically disrupted by adding 1 mL phosphate buffer 20 mM, pH 7.5 and the homogenate was centrifuged at 1000x*g* for 10 min. The neutral supernatant extract was used for amino acid colorimetric determination according to Spies (1957), using ninhydrin 1.5% in isopropyl alcohol as the color reagent.

This neutral extract was used to measure the hepatic transaminases concentration, but it was necessary to dilute the crude extract in homogenization buffer for the protein and alanine aminotransferase (ALAT) (EC 2.6.1.2) and alkaline phosphatase (ALP) (EC 3.1. 1.1) quantification, respectively, two and ten times. The enzymes were determined by using colorimetric procedures following the protocols described in the kits (Doles Reagents and

Laboratory Equipment Ltda. Goiania, Goiás, Brazil). ALAT concentration was expressed as UFR enzyme.mg^{-1} hepatic tissue.

For the (ALP) analysis, the sample was incubated with p-nitrophenyl phosphate salt and cyclohexylamine (substrate). Under the action of phosphatases, salt hydrolysis occurs with the release of p-nitrophenol. With the addition of NaOH and the reaction alkalization of the medium, the p-nitrophenol liberated turns yellow.

The phosphatase activity is proportional to the amount of p-Nitrophenol neo-formed. The ALP concentration was expressed as UI enzyme.mg^{-1} hepatic tissue. All hepatic extracts were prepared using the proportion 1:20 tissue:homogenization buffer. Colorimetric kits (Doles) were used for the plasmatic metabolic parameters analysis of triglycerides, albumin, total proteins and cholesterol and the procedures followed the specific protocols for each parameter.

2.8. Statistical analysis

Data were first analyzed for outlier identification and subjected to analysis of variance by F-test for the comparisons of the mean, at 5% probability. Tukey's test was used for antinutritional data factors and in vitro digestibility of protein. Biological assay data in four orthogonal contrasts were applied: *Control diet vs. PSM diets*; *Control diets vs. PPCPS diets*; *Control vs. other diets*; *PSM diets vs. PPCPS diets*.

3. Results

3.1. Protein concentrate obtaining and protein digestibility in vitro

The protein concentration by PSM phosphorylation increased in crude protein content 52% ($P < 0.05$) and decreased the fiber content by 66.29% ($P < 0.05$) (Table 2), obtaining a

protein concentrate with 674.8 $g.kg^{-1}$ crude protein and 125.3 $g.kg^{-1}$ NDF. The protein concentration by phosphorylation enabled more *in vitro* digestibility of protein to protein concentrate (669.1 $g.kg^{-1}$) when compared to PSM ($g.kg^{-1}$).

3.2. Growth, protein utilization and survival rate

The fish fed PSM diets had lower weight gain (Table 3) when compared to either Control diet or PPCPS fish ($P < 0.05$), in the contrasts Control diet vs. PSM diet, Control diet vs. other diets and PSM diet vs. PPCPS diet . No significant differences were found for Control Diet vs. PPCPS diets.

The fish fed PPCPS diets had higher protein efficiency ratio (PER) than fish fed the PSM diets ($P < 0.01$), in the contrasts PSM Diet vs. PPCPS Diet. For the total deposited protein (TDP) and survival (Table 3) no significant differences were found in the orthogonal contrasts tested.

3.3. Plasma biochemistry assay

The triglycerides and total protein contents (Table 4) did not differ for the orthogonal contrasts tested. Lower albumin contents were found for the control diet fish ($P < 0.05$) for the contrasts control diet vs. PPCPS diet and other diets vs. control diet. The fish fed the PSM diets had lower albumin content ($P < 0.05$) when compared to those fed the PPCPS diets, when comparing PSM diets vs. PPCPS diets. No significant differences were found for other contrasts.

3.4. Hepatic metabolism assay

There were no significant differences in the orthogonal contrasts studied for liver content of AA, protein and glycogen (Table 5). The ALAT enzyme activity was higher in the fish fed the control diet ($P < 0.05$) when comparing the control diet vs. PSM diet, control diet vs. PPCPS diets and the control diet vs. the other diets. The ALP was most active in fish that received the PPCPS diets ($P < 0.05$), when comparing control diet vs. PPCPS diets and control diet vs. other diets.

3.5 Hepatosomatic index

The HSI was higher for fish fed the PPCPs then fish fed the control and PSM diets to the diet in the contrasts Control vs. PPCPS diets and PSM diets vs. PPCPS diets (Fig. 1).

4. Discussion

The increase of 2.77 times *in vitro* digestibility of the protein, the greater weight gain and higher PER of fish fed diets containing PPCPS when compared to PSM were probably due to the decrease in fiber content (Table 2) and substances associated with this fraction, such as polyphenols and flavonoids (Table 1). Studies report that the fiber content and anti-nutritional factors present in plant-based proteins affect growth by impairing digestion and amino acid availability, which is reflected in lower PER in fish (Francis et al. 2001).

The similarity in the content of total proteins circulating in the plasma of fish fed experimental diets indicates that the protein was properly metabolized and used without impairing the hepatic synthesis (Marks et al. 2007). Fish fed 25% PPCPS diets had higher

plasma protein content (P = 0.062) and albumin (p> 0.05), confirming the superior quality of this diet.

Carter and Houlihan (2001) reported that the hepatic synthesis rate is sensitive to variations in the diet, because the liver is responsible for maintaining the body amino acid pool, which reflects on the levels of amino acids in the tissue (Yamamoto et al. 2000). The composition of the diets not only affects the growth and the amount of internal body nutrients, but also interferes with enzymatic activity of the liver (Correa 2002). In this study no difference was found in the evaluated hepatic metabolites (amino acids, protein and glycogen). However, higher liver ALAT activity in fish fed the control diet suggests increased protein catabolism and gluconeogenic activity (Metón et al. 1999).The lower ALAT activity observed in fish fed the PSM and PPCPS diets may indicate a lower rate of hepatic transamination (Hansen et al. 2007). However, it should be noted that the tested diets were formulated according to the amino acid requirements for the species (Montes Girao and Fracalossi 2006), there was no deficiency in glutamate synthesis (arginine, glutamine, histidine and proline) during the transamination process (data not shown).

Gómez - Requeni et al. (2004), replaced fishmeal with a mixtures of vegetable proteins at different levels in the diets of gilthead sea bream *(Sparus aurata)* and found lower ALAT activity per liver $IU.g^{-1}$, which was not observed when the values were normalized for the weight of the fish. This shows that the activity of this enzyme is related to the organ size and catabolic capacity for a particular animal and feed. Moreover, the same authors showed that body weight gain and PER were greater in fish fed diets containing plant-based protein. Our data for weight gain, PER and ALAT ($IU.g^{-1}$) corroborate those found by the mentioned authors.

The ALP is a non-specific enzyme that has its activity increased by liver injury (Kramer and Hoffmann, 1997), which is usually related to compensatory hypertrophy and

tissue hyperplasia (Bacila, 2003). Higher ALP activity was observed in fish fed PPCPS 50% diet, which may indicate liver injury. However, histological analyzes are recommended to confirm these results, since the replacement of fish meal by plant-based proteins is highly variable among species (Gómez-Requeni et al. 2004) and fish feeding habits. Increased HSI is usually observed for fish fed diets containing high levels of carbohydrates (Debnath et al. 2007) and accompanied by the increase in hepatic glycogen content. Paradoxically, our data showed higher HSI in fish fed PPCPS 25%, non-interference in hepatic glycogen content. Lee et al. (2012) found higher HIS in *Paralichthys olivaceus* fed different levels (10-40%) of Promate Meal® as a substitute for fish meal. The authors attributed this result to the greater non-protein nitrogen content of this ingredient, which is also common in plant-based protein sources.

Our results support the conclusion that PPCPS presents relevant nutritional quality for fish and can replace the fish meal protein up to 50% without affecting growth, PER and intermediate metabolites in silver catfish. However, studies on the apparent digestibility of the ingredient are needed in order to complement the satisfactory results found in the study.

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Table 1. Formulation, nutritional composition and amino acids profile ($g.kg^{-1}$) of experimental diets used during feeding trial for silver catfish.

	.....Diet code[1].....				
Ingredients ($g.kg^{-1}$)	CONTROL	PSM 25%	PSM 50%	PPCPS 25%	PPCPS 50%
PSM	0	146.3	292.6	0	0
PPCPS	0	0	0	106.4	212.7
SPC 60%[2]	200	200	200	200	200
Corn starch	226.4	227.1	154.6	226.7	226.9
Fish meal	408	306	204	306	204.3
Soybean oil	24.6	21	46.7	23.4	22.2
Vit. Min Mix[3]	30	30	28	30	30
Phosphate dicalcium	39	43.5	43	41	41
MSG[4]	5	5	5	5	5
BHT	0.1	0.1	0.1	0.1	0.1
Calcitic limestone	0	11.7	22	13.5	26.5
L-lisine	0	1.3	3.9	2.8	5.5
Inert	66.8	8.0	0	45.0	25.8
	.....Proximate composition[5] ($g.kg^{-1}$).....				
Crude Protein	371.5	370.3	365.8	368	362.3
Calculated energy ($MJ\ kg^{-1}$)[6*]	13.4	13.4	13.4	13.4	13.4
Lipids	77.2	74.1	100	70.5	69.4
NDF[7]	87.5	105.4	123.3	85.5	83.5
CSDN[8*]	128.7	167.1	142.2	153.5	183.4
Total Ash	218.7	166.7	154.9	205.8	185.3
Calcium	33.3	33.3	31.6	33.5	33.1
Total phosphorus	17.8	17.8	16.9	17.9	17.7
Flavonoids (mg/g)	10.54	12.69	22.95	6.19	10.57
Polyphenols (mg/g)	13.22	12.42	20.57	8.96	12.74
	.....Amino acids[9] ($g.kg^{-1}$ of crude protein).....				
Histidine	41	45	37	26	41
Arginine	109	97	115	101	127
Threonine	48	45	52	46	58
Tyrosine	35	40	56	37	54
Valine	52	51	62	53	69
Methionine + Cysteine	45	42	48	42	54
Isoleucine	56	54	62	54	67
Leucine	35	40	56	37	54
Phenylalanine	52	51	62	53	69
Lysine	45	45	48	45	45

[1]Diets: PSM 25% and 50% PSM, pumpkin seed bran replacing 25 or 50% of the protein fishmeal. PPCPs 25% and 50% PPCPs: phosphorylated protein concentrate bran, pumpkin seed, replacing 25ou in 50% of protein from fish meal.

2 Soybean protein concentrate (60% crude protein). [3]Composition of vitamin and mineral mixture :. 300 mg, Ascorbic AC:. 15,000 mg, Pantothenic Ac:. 3,000 mg, Biotin: 0.06mg, niacin (B3): 9,000 mg Hill (B4): 103.500 mg, Vit.A: 1,000,000 IU, Vit B1:. 1,500 mg, Vit B2:. 1:50 mg, Vit B6. 1,500 mg, Vit D3:. 240 000 IU Vit. E: 10 000 mg , Vit K3:. 400 mg, Inositol: 10,000 mg Iron: 5.000mg, Manganese: 8.000 mg, Copper 1.000 mg Zinc: 14 000 mg Iodine: 45 mg, Cobalt: 60 mg, Selenium 60 mg, Magnesium: 5 mg., Mig Plus®.

[4] Monossodium glutamate.

[5]Composition analyzed.

[6]Digestible Energy = [(CP * 23.61 MJ / kg * 0.9) + Fat * 39.82 MJ / kg * 0.85) + CSDN * 17.21 MJ / kg *0.50)] (Jobling, 1983).

[7]NDF: neutral detergent fiber.

[8]CSND: Carbohydrates soluble in neutral detergent = 100- (moisture + crude protein + fat + ash + neutral detergent fiber).

[9]Calculated from the analysis of ingredients.

Table 2. Nutritional composition and *in vitro* digestibility of protein of pumpkin seed meal (PSM) and the phosphorylated protein concentrate of pumpkin seed meal (PPCPS).

Nutrient	PSM[1]	PPCPS[2]
	.....g.kg^{-1} of the dry matter (DM).....	
Crude protein	444.8±13.4[b]	674.8±27.5[a]
Lipid	30.6±0.60[b]	103.0±2.9[a]
NDF[3]	371.7±9.1[a]	125.3±18.8[b]
In vitro digestibility of protein[*]	241.6[b]	669.1[a]

Mean values followed by different letters in the row were considered significantly different using the Tukey's test ($P < 0.05$). [1] PSM: Pumpkin seed meal; [2] PPCPS: Phosphorylated protein concentrate of pumpkin seed meal. [3]Neutral detergent fiber. [*] Casein was used as standard protein: digestibility of 769.2 g.kg^{-1}.

Table 3. Growth, protein utilization and survival rate in silver catfish of different experimental groups fed different experimental diets.

	Weight gain (%)	PER[3]	TPD[4]	Survival rate (%)
Sources of variation	.....*Control diet vs. PSM[1] diets*.....			
Control	235.59±14.62	1.78±0.06	8,43±1.53	100.00
PSM 25%	197.29±27.73	1.62±0.28	8.21±0.52	100.00
PSM 50%	168.60±38.12	1.57±0.11	7.91±1.97	99.00±1,15
Test F	*	ns	ns	ns
	.....*Control diets vs. PPCPS[2] diets*.....			
Control	235.59±14.62	1.78±0.06	8.43±1.53	100.00
PPCPS 25%	236.42±28.97	1.83±0.03	8.46±1.98	100.00
PPCPS 50%	222.55±39.54	1.87±0.15	9.64±2.53	100.00
Test F	ns	ns	ns	ns
Test F				
Control vs. other diets	*	ns	ns	ns
PSM diets vs. PPCPS diets	*	*	ns	ns

Means ± standard deviation compared as orthogonal contrasts in the 5% level of significance. The asterisk (*) shows a significant difference to the orthogonal contrast.
[1]PSM: Pumpkin seed meal; [2]PPCPS: Phosphorylated protein concentrate of pumpkin seed meal.
[3] Protein efficience ratio.
[4] Total protein deposited.

Table 4. Plasma biochemistry ($g.dL^{-1}$) values for silver catfish of different experimental groups fed different experimental diets.

Sources of variation	Triglycerides	Albumin	Total proteins	Cholesterol
	.....*Control diet vs. PSM[1] diets*.....			
Control	610.46±178.74	0.80±0.34	2.90±0.42	155.64±36.79
PSM 25%	566.59±191.35	1.02±0.59	2.94±0.23	151.63±26.74
PSM 50%	596.46±213.57	1.33±0.46	2.98±0.23	160.13±28.45
Test F	ns	ns	ns	ns
	.....*Control diets vs. PPCPS[2] diets*.....			
Control	610.46±178.74	0.80±0.34	2.90±0.42	155.64±36.79
PPCPS 25%	472.14±205.65	1.62±0.66	3.14±0.23	138.03±24.07
PPCPS 50%	628.20±43.74	1.61±0.54	2.95±0.24	140.09±30.08
Test F	ns	*	ns	ns
Test F				
Control vs. other diets	ns	*	ns	ns
PSM diets vs. PPCPS diets	ns	*	ns	ns

Means ± standard deviation compared as orthogonal contrasts in the 5% level of significance. The asterisk (*) shows a significant difference to the orthogonal contrast.
[1]PSM: Pumpkin seed meal; [2]PPCPS: Phosphorylated protein concentrate of pumpkin seed meal.

Table 5. Hepatic biochemistry values for silver catfish of different experimental groups fed different experimental diets.

	Free AA mM AA.g tec^{-1}	Protein mg. g tec^{-1}	Glycogen mMglic hidr.g tec^{-1}	ALAT UFR.mg tec^{-1}	ALP UI.mg tec^{-1}
Sources of variation	*.....Control diet vs. PSM[1] diets.....*				
Control	9.77±2.73	10.1±2.52	5.64±2.18	99.75±29.94	20.06±17.22
PSM 25%	10.51±2.88	9.35±3.15	6.34±1.63	72.51±13.91	34.33±8.04
PSM 50%	10.76±4.66	10.41±3.91	5.14±1.97	68.17±15.77	37.13±29.24
Test F	ns	ns	ns	*	ns
	.....Control diets vs. PPCPS[2] diets.....				
Control	9.77±2.73	10.1±2.52	5.64±2.18	99.75±29.94	20.06±17.22
PPCPS 25%	12.16±3.40	10.29±2.70	5.37±1.67	81.29±21.43	31.58±22.54
PPCPS 50%	9.43±3.79	8.22±2.07	4.97±1.81	78.3±24.60	74.01±40.67
Test F	ns	ns	ns	*	*
Test F					
Control vs. other diets	ns	ns	ns	*	ns
PSM diets vs. PPCPS diets	ns	ns	ns	ns	*

Means ± standard deviation compared as orthogonal contrasts in the 5% level of significance. The asterisk (*) shows a significant difference to the orthogonal contrast.
[1]PSM: Pumpkin seed meal; [2]PPCPS: Phosphorylated protein concentrate of pumpkin seed meal.

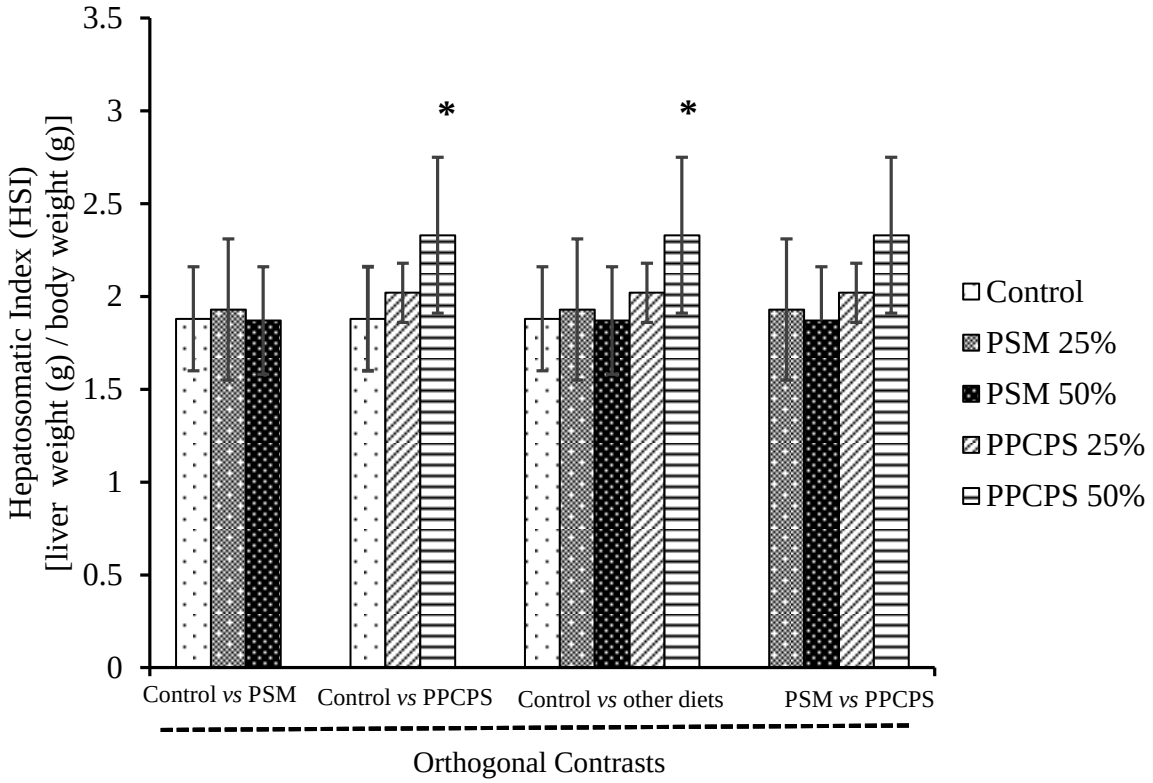

Figure 1. Hepatosomatic index values for silver catfish of different experimental groups fed different experimental diets. Means ± standard deviation compared as orthogonal contrasts in the 5% level of significance. The asterisk (*) shows a significant difference to the orthogonal contrast.

6. DISCUSSÃO GERAL

A tecnologia aplicada à agricultura desde a produção até o armazenamento tem garantido que uma gama de culturas de alto potencial produtivo se tornem substitutas naturais a ingredientes proteicos de origem animal, na nutrição de organismos aquáticos. Apesar disso, a presença de fibras, fatores antinutricionais e perfil de aminoácidos pouco satisfatórios quando comparados à farinha de peixe, ainda são fatores limitantes ao uso intensivo de proteínas vegetais.

Para utilizar fontes vegetais de maneira efetiva e competitiva à farinha de peixe são necessárias técnicas que minimizem os fatores antinutricionais e que permitam aumento no conteúdo de aminoácidos, o que pode ser atingido pelo processo de concentração proteica através de fosforilação química. Nesse estudo, a concentração proteica do farelo de semente se abóbora foi otimizado pela metodologia estatística de superfície de resposta (MSR). Utilizando a MSR, o delineamento experimental (DCCR) apontou como maior rendimento 50,04%, utilizando 4% de STMP em pH 4,5 (Figura 3- Artigo 1), nessa mesma condição experimental o conteúdo proteico foi de 62,56%.

O modelo foi validado pela análise de variância (ANOVA) e o coeficiente de determinação (r^2) foi de 0.9242, o que significa que o modelo pode explicar 92,42% da variabilidade total dos valores estudados. A superfície de contorno (Figura 2 – Artigo 1) mostra maiores rendimentos na região em que os valores de pH e STMP estão no ponto central, indicando o ponto de máximo rendimento.

Diversos métodos de modificações e concentrações químicas da proteína são utilizados sob diferentes condições. Nesse trabalho, os modelos foram preparados a fim de obter produto com elevada rentabilidade e teor proteico semelhante às fontes de proteína comumente utilizadas na alimentação animal, tais como farinhas de peixe, farelo e concentrado proteico de soja (NRC, 2011).

Quando o concentrado proteico fosforilado de farelo de semente de abóbora (CPPFSA) foi analisado nutricionalmente e comparado à farinha de peixe e ao farelo de semente de abóbora (FSA), o CPPFSA apresentou maior conteúdo de aminoácidos essenciais arginina, tirosina, fenilalanina e valina.

As mudanças no perfil de aminoácidos causados pela concentração proteica por meio de fosforilação podem alterar a funcionalidade das proteínas, modificando o comportamento

no alimento durante o processamento, o armazenamento e preparação (ARAÚJO, 2008). O CPFFSA apresentou menor capacidade de hidratação. Matheis et al. (1983) utilizando fosforilação química também encontraram menor capacidade de absorção de água em caseína fosforilada. Esse comportamento ocorre, pois as proteínas são menos hidratadas em seu pH isoelétrico, e o aumento das interações proteína-proteína resulta em mínima interação com a água (FENNEMA, 2010).

Além dos aspectos nutricionais, a concentração proteica pode modificar as características físico-químicas dos ingredientes, as quais exercem efeitos determinantes sobre a qualidade tecnológica e a digestibilidade das rações (GLENCROSS et al., 2007). Os parâmetros de dureza, estabilidade em água, flutuabilidade e tempo de armazenamento das rações para peixes são influenciados pela capacidade de hidratação e ligação à gordura das matérias primas utilizadas (DRAGANOVIC et al., 2011). Ingredientes com menor capacidade de hidratação exigem menor quantidade de água durante o processo de extrusão da ração. A menor capacidade de hidratação e de ligação à gordura observada para o CPFFSA pode formar *pellets* que exigem menor quantidade de água para a extrusão, favorecendo para menor gasto de energia e resíduos gerados no ambiente (DRAGANOVIC et al., 2014).

Além de medidas de interesse nutricional, as fontes proteicas de origem vegetal devem ser adequadamente avaliadas quantos aos antinutrientes, que na maioria das vezes são os principais fatores que limitam seu uso na nutrição animal. A maioria dos isolados e concentrados proteicos vegetais contém antinutrientes que prejudicam a hidrólise completa de proteínas de leguminosas e de sementes oleaginosas pelas proteases pancreáticas (FENNEMA, 2010). O processo de fosforilação foi eficiente na redução de polifenóis, causando queda de 56,53% no teor total de polifenóis para o CPFFSA (13,11mg/g) em relação ao FSA (23,19mg/g) (Figura 7 –Artigo 1). Contudo, foi ineficiente para redução de flavonoides.

A redução dos fatores antinutricionais do CPFFSA, pela fosforilação, se refletiu em digestibilidade proteica semelhante para CPFFSA e farinha de peixe ($P < 0,05$). Essa melhoria também deve ser atribuída a hidrolise parcial da proteína e aminoácidos, causada pelo processo de fosforilação química (MATHEIS et al., 1983). Deve-se ressaltar que a ligação N-P que ocorre na fosforilação é lábil a ácidos. Assim, sob as condições prevalentes no estômago durante o processo de digestão as proteínas N-fosforiladas serão desfosforiladas e os resíduos lisil serão regenerados, não causando impacto negativo sobre a digestibilidade da lisina e demais aminoácidos (FENNEMA, 2010).

Após a obtenção e avaliação nutricional do FSA e do CPFFSA, os mesmos foram avaliados como substitutos da farinha de peixe na alimentação do jundiá. Apesar de conteúdo proteico de 44,5% (na MS), a inclusão de FSA nas dietas causou prejuízo no crescimento dos peixes. Em contrapartida, os peixes que receberam as dietas contendo CPFFSA apresentaram índices de crescimento semelhantes do que aqueles que receberam a dieta Controle. Esse fato confirma a eficiência de concentração proteica por fosforilação para obtenção de um ingrediente com comprovada eficácia nutricional para jundiás.

Os resultados semelhantes de massa corporal final, conversão alimentar, taxa de crescimento específico e ganho de peso diário dos animais que receberam as dietas com CPFFSA são explicados pela melhoria no conteúdo de aminoácidos essenciais, maior disponibilidade de nutrientes e redução de fatores antinutricionais deste concentrado em relação ao FSA. Resultados semelhantes foram encontrados por Shamna et al. (2014), quando alevinos de *Labeo rohita* receberam dietas com diferentes fontes proteicas advindas de *Jatropha.* Porém, esses resultados se opõem aos encontrados por alguns trabalhos, que os peixes que receberam as dietas contendo fontes proteicas vegetais, apresentaram melhora na CAA, porém sem melhora no crescimento, devido à deficiência de aminoácidos essenciais dos concentrados proteicos vegetais (FUERTES et al., 2013; PENN et al., 2011; ZHANG et al., 2012).

A maior taxa de eficiência proteica do CPPFSA em comparação ao FSA foi reflexo da diminuição nos teores de fibra (Tabela 2 – Artigo 3) e substâncias associadas a essa fração, tais como os polifenóis e flavonoides (Tabela 1 – Artigo 3) . Estudos relatam que o conteúdo de fibras e fatores antinutricionais presentes em proteínas vegetais afetam o crescimento por prejudicar a digestão e a disponibilidade de aminoácidos, o que se reflete em menor taxa de eficiência proteica nos peixes (FRANCIS et al., 2001)

O índice digestivo-somático (IDS) (Figura 1-B, Artigo 2) foi maior para animais recebendo CPFFSA, indicando maior produção de células intestinais (FURNÉ et al., 2008) e bem estar fisiológico do sistema digestório (SHAMNA et al., 2014). Nossos resultados corroboram com Radünz Neto et al. (2006) que encontraram elevado IDS para piavas (*Leporinus obtusidens*) alimentadas com farelo de soja, quando comparado aquelas que receberam farinha de carne e ossos suína como fonte proteica na dieta.

A maior atividade da enzima tripsina (Tabela 4, Artigo 2) nos peixes alimentados com dietas contendo FSA é explicada como tentativa do organismo em elevar a digestibilidade proteica, o que se reflete em maior atividade proteolítica. Alarcón et al. (2001) também relataram relação inversa entre as atividades da tripsina intestinal e digestibilidade proteica

em *Lutjanus argentiventris* e *L. novemfasciatus*, explicando que a inibição da atividade enzimática parece ser compensada pelo aumento da secreção de enzimas proteolíticas e maior absorção de proteína nas porções distais do intestino.

Os animais alimentados com CPFFSA não apresentaram diferença na atividade de tripsina em relação à dieta Controle. Esses resultados são contrários a maior atividade de tripsina encontrada para salmão do Atlântico (*Salmo salar*) alimentados com concentrados proteicos de ervilha (PENN et al., 2011). Song et al. (2014) também observaram maior atividade tripsínica para *Platichthys stellatus* que receberam dietas com substituição de 15-70% da farinha de peixes pelo hidrolisado proteico de soja. A maior atividade de tripsina quando são utilizados concentrados proteicos vegetais pode estar associada com indução de enterites nos peixes (KROGDAHL et al., 2003; LILLEENG et al., 2007; PENN et al., 2011).

Na avaliação dos parâmetros plasmáticos, a semelhança no conteúdo de proteínas totais circulantes (Tabela 4- Artigo 3) indica que a proteína ofertada foi utilizada e metabolizada adequadamente, para todas a dietas testadas, sem comprometimento da síntese hepática (MARKS et al., 2007). Os peixes alimentados com CPPFSA 25% apresentaram maior conteúdo de proteína plasmática ($P = 0,062$) e de albumina ($P > 0,05$), confirmando a qualidade superior dessa dieta.

Em nosso estudo não foram encontradas diferenças nos metabólitos hepáticos avaliados (Tabela 5- Artigo 3) (aminoácidos, proteínas totais e glicogênio). No entanto, maior atividade de ALAT nos peixes que receberam a dieta Controle sugere maior catabolismo proteico e maior atividade gliconeogênica (METÓN et al., 1999) devido a maior disponibilidade de aminoácidos na dieta. A menor atividade da ALAT dos peixes que receberam as dietas FSA e CPFFSA pode indicar menor disponibilidade de aminoácidos ligados ao processo de transaminação (HANSEN et al., 2007). Contudo, deve-se observar que as dietas avaliadas foram formuladas de acordo com as exigências de aminoácidos para a espécie (MONTES GIRAO; FRACALOSSI, 2006) e não houve deficiência de formadores do glutamato para o processo de transaminação.

Aumento da atividade da ALP no fígado indica crescimento do tecido hepático como um processo compensatório a injúrias teciduais (BACILA, 2003), Maior atividade de ALP foi observada nos peixes que receberam a dieta CPFFSA 50%, contudo análises histológicas são recomendadas para confirmar esses resultados, já que a substituição da farinha de peixe por proteínas vegetais é altamente variável entre as espécies (GÓMEZ-REQUENI et al., 2004) e hábito alimentar dos peixes.

Aumento do IHS é usualmente encontrado em peixes alimentados com dietas contendo elevados níveis de carboidratos na dieta (DEBNATH et al., 2007) acompanhado da elevação no conteúdo de glicogênio hepático, quando essas fontes são utilizadas como fonte energética em detrimento da proteína. Nossos dados mostraram maior IHS (Figura1- Artigo 3) nos peixes alimentados com CPFFSA 25% sem apresentar alteração no conteúdo de glicogênio hepático (Tabela 5- Artigo 3). Lee et al. (2012) encontraram maior IHS para *Paralichthys olivaceus* alimentados com diferentes níveis (10 a 40%) de Promate meal® como substituto da farinha de peixe, devido ao provável maior conteúdo de nitrogênio não proteico desse ingrediente quando comparado à farinha de peixe.

7. CONCLUSÕES GERAIS

Com base nos resultados obtidos neste trabalho, pode-se concluir que:

- A obtenção do concentrado proteico fosforilado de farelo de semente de abóbora foi otimizada pela metodologia de superfície de resposta, que indicou as melhores condições experimentais com o uso de 4% de trimetafosfato de sódio e pH 4,5.
- O valor nutricional do farelo de semente de abóbora pode ser melhorado com processo simples de fosforilação química, originando um concentrado proteico (CPFFSA) que pode ser usado como um ingrediente alternativo promissor na dieta de peixes.
- O CPFFSA obtido apresenta perfil de aminoácidos satisfatório e digestibilidade proteica comparável à farinha de peixe, que é fonte proteica de referência para este grupo de animais, o que permite concluir que o CPFFSA é uma fonte de qualidade superior. O CPFFSA apresenta menor conteúdo de polifenóis e fibras, fatores que são entrave do uso de fontes vegetais na alimentação de peixes.
- A substituição da proteína da farinha de peixe por 25 ou 50% de CPFFSA não altera o crescimento e promove melhoria na conversão alimentar de jundiá e taxa de eficiência proteica, sem afetar parâmetros metabólicos intermediários e hepáticos dos jundiás.
- O uso de FSA como ingrediente proteico não é recomendado, pois provoca prejuízos ao crescimento dos peixes.

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ANEXOS

ANEXO A – Normas de publicação da Revista Animal Feed Science and Technology

Introduction

Types of article

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Book Reviews

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles should cover subjects falling within the scope of the journal which are of active current interest.

Manuscripts describing the use of commercial feed products are welcome, but should include the following information: major components, contents of active ingredients (for example enzyme activities). Independent verification, as opposed to a manufacturers guarantee, is always desirable and often avoids difficulties in the review process, especially where there are no, or few, treatment impacts. The Editors reserve the right to reject any manuscript employing such products, wherein this information is not disclosed.

Submissions concerning feedstuff composition are welcome when published and/or accepted analytical procedures have been employed. However, unusual feedstuffs and/or a wide range of data are pre-requisites. Submissions concerning NIRS may be suitable when more accurate, precise or robust equations are presented. Mathematical, technical and statistical advancement may constitute the foundation for acceptance. For more details see the editorial in Vol. 118/3-4.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process.

Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance.All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trial.

Poorly written and/or presented manuscripts (relative to the journal's guidelines) may be returned to authors for upgrading by the editorial office, prior to a review for scientific merit. Before preparing their manuscript, it is suggested that authors examine the editorial by the Editors-in-Chief in Vol. 134/3-4, which outlines several practices and strategies of manuscript preparation that the Editors-in-Chief have found to be successful.

This editorial also outlines practices that can lead to difficulties with reviewers and/or rejection of the manuscript for publication. There is also an example of an Animal Feed Science and Technology manuscript available on the journal website at http://www.elsevier.com/locate/anifeedsci.

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PREPARATION

Use past tense for current findings, and the present tense for "truths" and hypotheses.

Article Structure

Manuscripts should have **numbered lines**, with wide margins and **double spacing** throughout, i.e. also for abstracts, footnotes and references. **Every page of the manuscript, including the title page, references, tables, etc., should be numbered**

continuously. However, in the text no reference should be made to page numbers; if necessary, one may refer to sections. Avoid excessive usage of italics to emphasize part of the text.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

If reference is made to AOAC, ISO or similar analytical procedure(s), the specific procedure identification number(s) must be cited. A number of references for neutral and acid detergent fibre (NDF, ADF) assays exist, and an alternative reference to the now out-of-print USDA Agriculture Handbook 379 must be used. There are many options for NDF and ADF assays (e.g. sodium sulfite, alpha amylase, residual ash), which must be specified in the text. For more details see the editorial in Vol. 118/3-4.

The following definitions should be used, as appropriate:

a.NDFom-NDF assayed with a heat stable amylase and expressed exclusive of residual ash.

b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash.

c.A NDF-NDF assayed with a heat stable amylase and expressed inclusive of residual ash.

d. NDF-NDF assayed without a heat stable amylase and expressed inclusive of residual ash.

e.ADFom-ADF expressed exclusive of residual ash.

f.ADF-ADF expressed inclusive of residual ash.

g. Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid.

h. Lignin (pm)-Lignin determined by oxidation of lignin with permanganate.

While expressions of NDF and ADF inclusive of residual ash will continue to be acceptable (i.e., the terms aNDF, NDF and ADF above), the Editors-in-Chief highly recommend reporting all fibre values, including digestibilities, on an OM basis. Silica is partially soluble in ND, is quantitatively recovered in AD, and so may contribute to the 'fibre' values and to subsequent digestibility coefficients.

Reporting 'hemicellulose' values as the difference between NDF and ADF is generally only acceptable if the analyses have been sequential on the same sample. Crude fibre (CF),

nitrogen-free extract (NFE) and total digestible nutrients (TDN) are not acceptable terms for describing feeds and should only be referred to in a historical context.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature. Combined 'Results and Discussion' sections are only acceptable for 'Short Communications', except under compelling circumstances.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems.

Avoid abbreviations and formulae where possible.

• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

The abstract should be clear, descriptive and not longer than 400 words. It should contain the following specific information: purpose of study; experimental treatments used; results obtained, preferably with quantitative data; significance of findings; conclusions; implications of results if appropriate.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUB: Biochemical Nomenclature and Related Documents: http://www.chem.qmw.ac.uk/iubmb/ for further information.

Authors and Editors are, by general agreement, obliged to accept the rules governing biological nomenclature, as laid down in the *International Code of Botanical Nomenclature*, the *International Code of Nomenclature of Bacteria*, and the *International Code of Zoological Nomenclature*. All biotica (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.

SI or SI-derived units should be used throughout (e.g. MJ and not Kcal for energy concentrations). Concentrations should be expressed on a 'per kg' basis (w/w); however, w/v, v/v, mol/mol or M may be accepted depending on the circumstances. In addition, 'units' and 'equivalents' are acceptable. Normality should be avoided, as it may be ambiguous for certain acids. If analytical standards have been used, they should be specified by name (e.g. yeast RNA) and form (e.g. lactose monohydrate). Percents should only be used when describing a relative increase or decrease in a response. Proportions should be maximum 1.0 or ≤1.0. For more details see the editorial in Vol. 118/3-4.

Percent is *only* used to indicate relative changes. For composition, both w/w (often solids composition g/kg) and w/v (e.g. g/L), v/v (e.g. m/L), mol/mol or M can be accepted depending on the circumstances. Specify units (e.g. g/L) and never as percent.

Digestibility/metabolisability and degradability should always be expressed as a coefficient (not %), and the content of, for example, the digestible component should be expressed as g/kg: thus, the coefficient of digestibility of dry matter is 0.8, while the content of digestible dry matter is 800g/kg. A distinction between true and apparent digestibility should be made, as well as between faecal and ileal (e.g. coefficient of total tract apparent

digestibility - CTTAD). The terms 'availability' and 'bioavailability' should be avoided without definition in context.

In chemical formulae, valence of ions should be given as, e.g. Ca^{2+}, not as Ca^{++}. Isotope numbers should precede the symbols e.g. ^{18}O. The repeated use of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g. phosphate as P_2O_5).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

If differences between treatments are statistically significant, this should be indicated by adding the actual 'P' value obtained. If $0.10 > P > 0.05$, then differences can be considered to suggest a trend, or tendency, to a difference, but the actual 'P' value should be stated. Further information on this issue can be found in *Animal Feed Science and Technology* Vol. 129/1-2.

Spaces should be used between all values and units, except for the following: Between the value and degrees or percent. In equations around * and /. In probability expressions ($P<0.05$). When probability values are given, the 'P' should be a capital letter.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

All publications cited in the text should be presented in a list of references following the text of the manuscript. The manuscript should be carefully checked to ensure that the spelling of authors' names and dates are exactly the same in the text as in the reference list. The accuracy of the references is the responsibility of the author(s).

References published in other than the English language should be avoided, but are acceptable if they include an English language 'Abstract' and the number of non-English

language references cited are reasonable (in the view of the handling Editor) relative to the total number of references cited.

In the text refer to the author's name (without initial) and year of publication, followed - if necessary - by a short reference to appropriate pages. Examples: "Since Peterson (1988) has shown that...". "This is in agreement with results obtained later (Kramer, 1989, pp. 12-16)".

If reference is made in the text to a publication written by more than two authors, the name of the first author should be used followed by "et al.". This indication, however, should never be used in the list of references. In this list names of first author and co-authors should be mentioned.

References cited together in the text should be arranged chronologically. The list of references should be arranged alphabetically on authors' names, and chronologically per author. If an author's name in the list is also mentioned with co-authors the following order should be used: publications of the single author, arranged according to publication dates - publications of the same author with one co-author - publications of the author with more than one co-author. Publications by the same author(s) in the same year should be listed as 2001a, 2001b, etc.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference

style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

References concerning unpublished data and "personal communications" should not be cited in the reference list but may be mentioned in the text.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations:http://www.issn.org/services/online-services/access-to-the-ltwa/.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more.

Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including

ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages athttp://www.elsevier.com/artworkinstructions.

ANEXO B- Normas de publicação da Revista Aquaculture Nutrition

Author Guidelines

Effective with the 2014 volume, this journal will be published in an online-only format. No printed issue of this title will be produced but authors will still be able to order offprints of their own articles.

Manuscript Submission

Manuscripts should be submitted online at http://mc.manuscriptcentral.com/anu. Full instructions and support are available on the site and a user ID and password can be obtained on the first visit. Support can be contacted by phone (+1 434 817 2040 ext. 167), e-mail (support@scholarone.com) or at http://mcv3support.custhelp.com. If you cannot submit online, please contact Anette Hatland in the Editorial Office by telephone (+47 55905200) or by e-mail (an@nifes.no).

A covering letter must be included, signed by the corresponding author (i.e., the author to whom correspondence should be addressed), and stating on behalf of all the authors that the work has not been published and is not being considered for publication elsewhere. Authors are encouraged to suggest four potential referees for their manuscripts.

Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve its grammar, spelling, punctuation, and clarity. Please visit the following website http://wileyeditingservices.com/en/ to learn about the options. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Copyright

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions http://exchanges.wiley.com/authors/faqs---copyright-_301.html

New: Online-only format

Effective with the 2014 volume, this journal will be published in an online-only format. No printed edition will be published. Your article will therefore appear online-only. All normal author benefits and services remain in place e.g. authors will continue to be able to order print reprints of articles if required. Furthermore, there will be no cost to authors for the publication of colour images in the online-only edition.

Page Charges

Original research articles exceeding 8 pages when in proof will be subject to a page charge of GBP 100 per additional page. The first 8 pages will be published free of charge. An average 8-page article will have approximately 6200 words in manuscript, with approximately 5 figures or tables and 50 references. An invoice will be sent to authors for these charges upon Online publication of their article. Invited and review articles are excluded from this rule. Download Page Charge Form.

Preparation of the Manuscript

All sections of the manuscript should be double-spaced and with 30mm margins. Articles are accepted for publication only at the discretion of the Editor(s). Authors will receive prompt acknowledgement of receipt of their paper and a decision will be reached within 3 months of receipt. A manuscript should consist of the following sections:

Title page

This should include: the full title of the paper; the full names of all the authors; the name(s) and address(es) of the institution(s) at which the work was carried out (the present addresses of the authors, if different from the above, should appear in a footnote); the name, address, and telephone and fax numbers of the author to whom all correspondence and proofs should be sent; a suggested running title of not more than fifty characters, including spaces; and six key words to aid indexing.

Main text

Generally, all papers should be divided into the following sections and appear in the order: (1) Abstract or Summary, not exceeding 150-200 words, (2) Introduction, (3) Materials and Methods, (4) Results, (5) Discussion, (6) Acknowledgements, (7) References, (8) Figure legends, (9) Tables, (10) Figures.

The Results and Discussion sections may be combined and may contain subheadings. The Materials and Methods section should be sufficiently detailed to enable the experiments to be reproduced. Trade names should be capitalized and the manufacturer's name and address given.

All pages must be numbered consecutively from the title page, and include the acknowledgements, references and figure legends, which should be submitted on separate sheets following the main text. The preferred position of tables and figures in the text should be indicated in the left-hand margin.

Units and spellings

Système International (SI) units should be used. The salinity of sea water should be given as g L-1. Use the form g mL-1 not g/mL. Avoid the use of g per 100g, for example in food composition, use g kg-1. If other units are used, these should be defined on first appearance in terms of SI units, e.g. mmHg. Spelling should conform to that used in the Concise Oxford Dictionary published by Oxford University Press. Abbreviations of chemical and other names should be defined when first mentioned in the text unless they are commonly used and internationally known and accepted.

Scientific names and statistics

Complete scientific names should be given when organisms are first mentioned in the text and in tables, figures and key words. The generic name may subsequently be abbreviated to the initial, e.g. Gadus morhua L., otherwise G. morhua. Carry out and describe all appropriate statistical analyses.

References (Harvard style)

References should be cited in the text by author and date, e.g. Lie & Hemre (1990). Joint authors should be referred to by et al. if there are more than two, e.g. Hemre et al. (1990).

More than one paper from the same author(s) in the same year must be identified by the letters a, b, c, etc., placed after the year of publication. Listings of references in the text should be chronological. At the end of the paper, references should be listed alphabetically according to the first named author. The full titles of papers, chapters and books should be given, with the first and last page numbers; journal titles should be abbreviated according to World List of Scientific Periodicals.

Lie, O., Lied, E. & Lambertsen, G. (1988) Feed optimization in Atlantic cod (Gadus morhua): fat versus protein content in the feed. Aquaculture, 69, 333-341.

Lall, S.P. (1989) The minerals. In: Fish Nutrition (Halver, J.E. ed.), 2nd edn, Vol. 1, pp. 219-257. Academic Press Inc., San Diego, CA, USA.

Work that has not been accepted for publication and personal communications should not appear in the reference list, but may be referred to in the text (e.g. A. Author, unpubl. observ.; A.N. Other, pers. comm.). It is the authors' responsibility to obtain permission from colleagues to include their work as a personal communication. A letter of permission should accompany the manuscript.

References in Articles

We recommend the use of a tool such as EndNote (http://www.endnote.com/) or Reference Manager (http://www.refman.com/) for reference management and formatting.

EndNote reference styles can be searched for here:

http://www.endnote.com/support/enstyles.asp

Reference Manager reference styles can be searched for here: http://www.refman.com/support/rmstyles.asp

Illustrations and tables

These should be referred to in the text as figures using Arabic numbers, e.g. Fig. 1, Fig. 2, etc., in order of appearance. Three copies of each figure should be submitted and each figure should be marked on the back with its appropriate number, together with the name(s) of the author(s) and the title of the paper. Where there is doubt as to the orientation of an illustration the top should be marked with an arrow.

Photographs and photomicrographs should be unmounted glossy prints and should not be retouched. Labelling should be clearly indicated on an overlay or photocopy. Colour illustrations are acceptable when found necessary by the Editor.

Line drawings should be on separate sheets of white paper in black indelible ink (dot matrix illustrations are not permitted); lettering should be on an overlay or photocopy and should be no less than 4 mm high for a 50% reduction. Please note, each figure should have a separate legend; these should be grouped on a separate page at the end of the manuscript. All symbols and abbreviations should be clearly explained.

Tables should be self-explanatory and include only essential data. Each table must be typewritten on a separate sheet and should be numbered consecutively with Arabic numerals, e.g. Table 1, and given a short caption. No vertical rules should be used. Units should appear in parentheses in the column headings and not in the body of the table. All abbreviations should be defined in a footnote.

All tables and figures that are reproduced from a previously published source must be accompanied by a letter of permission from the Publisher or copyright owner.

Acknowledgements

These should be brief and must include references to sources of financial and logistical support.

ANEXO C- Normas de publicação da Revista Fish Physiology and Biochemistry

Instructions for Authors

MANUSCRIPT SUBMISSION

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

TITLE PAGE

- Title Page
- The title page should include:
- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author
- Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

- Manuscripts should be submitted in Word.
- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).
- Manuscripts with mathematical content can also be submitted in LaTeX.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols. Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

REFERENCES

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

EndNote style (zip, 2 kB)

TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

ARTWORK AND ILLUSTRATIONS GUIDELINES

- Electronic Figure Submission
- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.
- Line Art

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,
- "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

When preparing your figures, size figures to fit in the column width.

For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.

For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

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If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)

Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)

Any figure lettering has a contrast ratio of at least 4.5:1

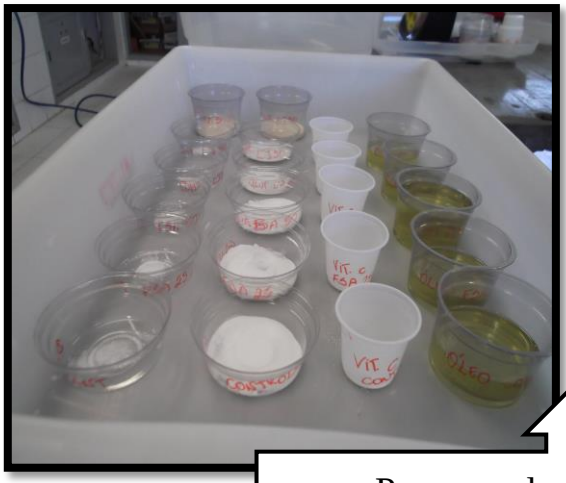
ANEXO D- Obtenção do concentrado proteico fosforilado de farelo de semente de abóbora

Semente de abóbora seca

A) Precipitação alcalina, B) precipitação ácida (H_3PO4) e C) decantação da proteína.

Concentrado proteico de farelo de semente de abóbora

ANEXO E- Confecção das dietas experimentais



Pesagem dos ingredientes

Mistura dos ingredientes

Extrusão das dietas

ANEXO F- Sistema de recirculação de água