Reprinted from

Aquaculture

Aquaculture 157 (1997) 251–262

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Accepted 17 February 1997

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Abstract

In aquaculture, feeding trials are the most dependable method of measuring nutritive value of feeds. However, these methods are time consuming, expensive, and the results may be affected by environmental factors. In vitro methods to evaluate protein digestibility are rapid and allow close observation of how far bonds are cleaved using only small amounts of raw materials. We developed a pH-stat assay using an enzyme fraction from shrimp hepatopancreas. We used the assay to evaluate the degree of hydrolysis (DH) and estimate protein quality in menhaden, anchovy, white fish, tuna waste, soybean protein, and langostilla meals. Data obtained by the pH-stat method were compared with those obtained by chemical analysis and an in vivo apparent protein digestibility (APD) assay. The pH-stat method predicted the protein quality better than chemical analysis. The APD of the experimental diets ranged from 64–91%. A significant correlation ($P < 0.05$) between DH and APD was observed ($r^2 = 0.77$ for enzymes from shrimp hepatopancreas and $r^2 = 0.71$ for a commercial enzyme mixture). The pH-stat method has the potential for estimating the digestibility of alternative protein sources for shrimp feeds. © 1997 Elsevier Science B.V.

Keywords: Degree of hydrolysis; In vitro; In vivo; Protein digestibility; pH-stat; Shrimp

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PII S0044-8486(97)00058-6

1. Introduction

Studies on the nutrition of aquatic organisms are necessary for profitable aquaculture. Effective feeding depends on a knowledge of how organisms use various diet components. In aquaculture, feeding trials have been used to measure the nutritive value of feeds for growth performance. However, these methods are expensive, time consuming, and the results may be affected by environmental factors (Lan and Pan, 1993).

Classical methods for the determination of protein quantity and quality include Kjeldahl analysis and determination of amino acid composition. These methods involve reactions harsher than those occurring during natural digestion, which release nutrients otherwise unavailable to the animal (Anderson et al., 1993). Considerable attention has been focused toward a rapid and reliable method of assessing protein digestibility (for references see Dimes and Haard, 1994). The properties of enzymes associated with the digestive tract determine the protein hydrolysis capabilities of the organism under study (Pedersen and Eggum, 1983). In vitro methods for evaluating protein digestibility are needed, because they are usually faster and less expensive than in vivo methods and allow a more detailed evaluation of the percentage of total peptide bonds hydrolyzed of food protein using only small amounts of raw materials (Grabner, 1985).

A strong correlation between in vitro and in vivo values exists only when the proteins are compared according to their origin, animal or plant. In samples containing both plant and animal proteins, the in vitro enzymatic procedures would yield only an approximate estimate of protein digestibility (Pedersen and Eggum, 1983).

Use of a pH-stat method to monitor protein digestibility offers some advantages: (1) constant pH during the digestion process without the use of a physiological buffer concentration and (2) the rate of protein hydrolysis can be estimated quickly during the digestion process by evaluating the automatically recorded titration curve (Pedersen and Eggum, 1983). Recently, Dimes and Haard (1994) developed an in vitro method using an enzyme fraction from trout pyloric ceca and estimated protein digestibility by a pH-stat assay. They concluded that the method was an accurate means to estimate the protein digestibility of alternate protein sources for salmonid feeds. Lan and Pan (1993) determined the in vitro digestibility of protein ingredients for grass shrimp (*Penaeus monodon*) using an extract from the shrimp hepatopancreas. However, because no in vivo digestibility data was provided the method was not validated.

Titration using the pH-stat is a method that determines the degree of protein hydrolysis (DH) using digestive enzymes. The aim of the present study was to evaluate the digestibility of protein by the white shrimp, *Penaeus vannamei*, with an in vitro pH-stat assay using an enzyme preparation from the hepatopancreas of the shrimp. The results of the in vitro assay are compared with in vivo apparent protein digestibility values by using statistical analysis.

2. Materials and methods

Reagents including the trypsin Type IX from porcine pancreas, a chymotrypsin Type II from bovine pancreas, peptidases from porcine intestinal mucosa (50–100 units/g),

pronase Type XIV from *Streptomyces griseus*, casein, and azocasein were purchased from Sigma Chemical (St. Louis, MO). The protein sources, listed in Table 1, were feed ingredients provided by commercial suppliers: Zapata Protein-USA, National Marine Fisheries Service, NMFS-Kokiak, and Productos Pesqueros de La Paz (PROPEPAZ). The langostilla (*Pleuroncodes planipes*) meal was prepared from the whole langostilla in the nutrition laboratory of CIBNOR, La Paz, B.C.S. (Goytortúa, 1993).

Enzymes were extracted from hepatopancreas of 10 to 12 g white shrimp (ca. 200 hepatopancreas) from CIBNOR. Enzyme preparation was done according to García-Carreño and Haard (1993). The hepatopancreas was removed from decapitated animals, frozen, and stored at −20°C prior to use. The thawed tissue was homogenized at 10°C. To eliminate lipid and tissue debris, the homogenate was centrifuged at 11,300 *g* for 20 min, at 10°C. The aqueous supernatant was freeze-dried and stored at −20°C.

Triplicate samples of the fish meals, as received, and diets were analyzed following the standard methods of AOAC International (1990). Dry matter was evaluated by weight loss after drying at 105°C for 24 h. Ash was evaluated after ignition at 650°C for 24 h. Crude protein (%N × 6.25 and %N × 5.71 for soybean protein) was measured by the Kjeldahl method, lipid by the Soxhlet method, and soluble protein content of enzyme preparations by the method of Bradford (1976).

The protease activity was assayed according to García-Carreño and Haard (1993) using the following assay mixture: 500 ml of 1% azocasein in 50 mM Tris · HCl, pH 7.5; 500 ml of 50 mM Tris · HCl buffer, pH 7.5; and 10 ml of the enzyme extract. The reaction mixture was incubated at 25°C for 10 min. The reaction was stopped by adding 500 ml of 20% trichloroacetic acid (TCA). After 10 min at 4°C, the reaction mixture was centrifuged at 14,500 *g* for 5 min and the absorbance was measured at 366 nm. Protease activity was expressed as units per mg of protein.

Table 1
Chemical composition of the test ingredients[1]

Test ingredients	Moisture[2]	Crude protein[2]	Crude fat[2]	Ash[2]
Anchovy meal[3]	10.4 ± 0.3^{d}	60.2 ± 0.1^{d}	12.5 ± 0.6^{e}	14.8 ± 0.1^{b}
Tuna waste meal[4]	4.9 ± 0.4^{a}	61.3 ± 0.4^{d}	6.4 ± 0.4^{b}	21.5 ± 0.1^{e}
Deboned white fish[5]	5.4 ± 0.3^{a}	75.7 ± 0.9^{e}	7.2 ± 0.1^{c}	9.3 ± 0.05^{a}
Menhaden fish meal[6]	7.8 ± 0.5^{c}	63.8 ± 1.0^{d}	11.9 ± 0.6^{d}	18.1 ± 0.5^{d}
Atlantic menhaden fish meal[6]	8.3 ± 0.2^{c}	60.9 ± 1.0^{c}	14.5 ± 0.2^{f}	15.9 ± 0.4^{c}
Langostilla meal[7]	5.9 ± 1.0^{b}	$39.4 \pm 1.0^{8,a}$	2.9 ± 0.6^{a}	42.6 ± 0.1^{f}
Soybean Protein[4]	9.3 ± 0.5^{d}	$49.5 \pm 1.0^{9,b}$	5.3 ± 0.7^{b}	21.5 ± 0.3^{e}

[1] Values with different letter for each column, are significantly different ($P < 0.05$).
[2] Precent dry matter basis.
[3] National Marine Fisheries Service.
[4] Productos Pesqueros de La Paz (PROPEPAZ).
[5] NMFS-Kokiak.
[6] Zapata Protein-USA.
[7] Nutrition Laboratory of CIBNOR, La Paz, B.C.S.
[8] 10% of the N is from chitin.
[9] N×5.71

The degree of hydrolysis (DH) of the protein sources was evaluated by pH-stat titration using shrimp hepatopancreas enzymes (García-Carreño et al., 1997) or an enzyme mixture made of commercial trypsin, chymotrypsin, aminopeptidase, and pronase (Satterlee et al., 1979). The evaluation of DH was done in a pHstat 718 Stat Titrino (Metrohom Ion Analysis, Switzerland), interfaced to an Acer 433s computer with a Metrodata Menu Program 718 STAT Titrino-PC. The apparatus is the lab-scale version of a full-scale pH controller. It was set on the following parameters: mode, TIP; end point, 8.0; dynamics, 1; maximum rate, 10 ml/min; minimum rate, 25.00 μl/min; stop type, drift; drift, 2 ml/min; and report, assign full. The meals were homogenized in water using a Waring blender. The amount of crude protein (the substrate) from the meals was adjusted to 8 mg/ml. Ten grams of the substrate suspension were poured into the hydrolysis vessel and the pH was adjusted to 8.0 with 0.1 M NaOH. The reaction was started by addition of 1 ml of shrimp hepatopancreas enzyme (10 mg/ml, adjusted to pH 8.0 with 0.1 M NaOH) or 0.3 ml of the enzyme system (1.6 mg/ml trypsin, 3.1 mg/ml chymotrypsin, 1.3 mg/ml aminopeptidase and 7.95 mg/ml pronase in water adjusted to pH 8.0 with 0.1 M NaOH). Protease activity was similar for both enzyme preparations. The pH was maintained at 8.0 and the volume of 0.1 M NaOH consumed was recorded. The DH was evaluated at 25°C. The protein hydrolysis was calculated from the following algorithm:

$$DH\% = \left[\left(B * N_b * 1.5/M * (S\%/100)\right)/8\right] * 100$$

where B = ml of standard alkali (0.1 N NaOH) required to maintain the pH of the reaction mixture at 8.0; N_b = normality of titrant; M = mass (g) of reaction mixture; S = protein concentration in the reaction mixture (Adler-Nissen, 1986).

P. vannamei juveniles weighing 3.5 to 4 g were obtained from the CIBNOR farming facilities. Ten shrimp per tank (three tanks per treatment) were randomly distributed in 80-l ($0.7 \times 0.4 \times 0.35$ m) rectangular, plastic indoor tanks equipped with a system supplying seawater and air by stone. Seawater was pumped from Ensenada de La Paz, filtered using a sand filter and UV treated. Over the course of the experimental period (two weeks), the water temperature was maintained at 27 ± 1°C, salinity at 35‰, and oxygen at 6 mg/l. Eighty percent of the water in the tanks was exchanged daily.

Animals were fed to satiation twice per day. After three days of acclimation, feces were collected daily for two weeks and pooled for each experimental tank. Samples were freeze-dried and stored at −20°C for subsequent protein analysis by Kjeldahl (%N × 6.25), and chromic oxide by wet acid digestion (Bolin et al., 1952).

Each diet was prepared by 15% substitution of the fish meal in a reference diet (Table 2). This type of substitution was described by Cho and Slinger (1979) and involves adding a test ingredient to a reference diet. The resulting diet contains: 84% of the reference diet, 1% chromic oxide as an inert indicator (Tacon and Rodrigues, 1984), and 15% of the test ingredient. All diets were formulated and prepared as described by Goytortúa (1993). The dry ingredients were finely ground and then mixed in a food mixer with the liquid ingredients. Tap water was incorporated at 50% vol/weight until a stiff dough was formed before slow extrusion through a 1-mm grill in a meat grinder. Pellets of 12.45-mm length were then dried at 60°C for a total of 12 h and stored frozen.

Table 2
Composition of the reference diet used to determined digestion coefficients for juvenile white shrimp (% dry matter)

Ingredient	%
Ingredient premix[a]	92.0
Mineral premix[b]	3.5
Vitamin premix[c]	0.5
Fish oil	1.5
Soy lecithin	1.5
Chromic oxide	1.0

[a] Ingredient premix (percentage in premix): 17.9, fish meal (tuna waste); 10.75, shrimp meal; 19.35, soybean meal; 25.8, wheat meal; 21.5, sorghum meal; 4.7, gelatin.
[b] Mineral premix (percentage in premix): 14.30, KCl; 14.30 $MgSO_4 \cdot 7\ H_2O$; 2.57, $ZnSO_4 \cdot 7\ H_2O$; 0.67, $MnCl \cdot 4\ H_2O$; 0.14, $CuCl_2 \cdot 2\ H_2O$; 0.14, KI; 0.07, $CoCl_2 \cdot 2\ H_2O$; 67.81, $NaHPO_4$.
[c] Vitamin premix (mg or IU/kg diet): 15,000 IU vitamin A; 7500 IU vitamin D_3; 400 mg vitamin E (tocopherol); 20 mg vitamin K_3 (menadione sodium bisulphite); 150 mg thiamin; 10 mg riboflavin; 50 mg pyridoxine; 100 mg pantothenic acid; 300 mg niacin; 1 mg biotin; 500 mg inositol; 0.1 mg cyanocobalamine; 20 mg folic acid; 1000 mg cellulose; 2000 mg ascorbic acid; 400 mg choline chloride.

Apparent protein digestion coefficients for the test ingredients in the reference diet was calculated by the equation:

$$APD = 100/15(Dtd - (0.85 \times Drd))$$

where: APD = apparent protein digestibility; Dtd = digestion coefficient of the test diet; Drd = digestion coefficient of the reference diet (Cho and Slinger, 1979).

The digestion coefficient of the diet (Dtd) and the reference diet (Drd) were calculated by the equation:

$$D = 100(100 - (\%Nf/\%Nfe * \%Cr_2O_3 \text{ in f}/\%Cr_2O_3 \text{ in fe}))$$

where: D = digestibility; Nf = nutrient in feces; Nfe = nutrient in diet; f = feces; and fe = diet (Forster and Gabbot, 1971).

Data were analyzed using STATISTICS software (Microsoft, Tulsa, OK) for PCS. Analysis of variance and the Tukey HSD test were used to compare the chemical composition, DH and APD. A multiple regression was made for the correlation coefficients and standard error of estimates to determine the correlation of the chemical analysis, DH and APD.

3. Results

There were significant differences ($P < 0.05$) in the composition of the protein sources (Table 1). The protein content, expressed on a moisture free basis, ranged from 39.4 to 75.7%. The menhaden fish meal had 18.8% ash, the highest within the fish meals, although the highest value of ash among all meals was for langostilla meal with 42.6%. The highest lipid content was for Atlantic menhaden meal. Protein and ash concentration were inversely related with an r^2 of 0.82 ($P < 0.05$). For example, the

Table 3
Total activity of enzyme preparation for the DH evaluation

Enzyme preparation	Protease activity (units/mg protein)	Casein DH%
White shrimp hepatopancreas	1.7	24.6
Four enzyme mixture[a]	7.2	30.7

[a]1.6 mg/ml trypsin, 3.1 mg/ml chymoptrypsin, 1.3 mg/ml peptidase and 7.95 mg/ml protease.

sample with the lowest percentage protein had the highest percentage ash (langostilla meal), whereas the sample with the highest percentage protein had the lowest percentage ash (deboned white fish meal). The correlation between protein and the other chemical compounds analyzed was not significant ($P > 0.05$) with an r^2 of 0.32.

A comparison of the enzymatic activity of hepatopancreas extract from white shrimp and the four-enzyme mixture is shown in Table 3. The DH of casein by the four-enzyme mixture was higher than that for shrimp hepatopancreas enzymes (30.7% vs. 24.6%).

The DH values of the seven protein sources analyzed by the pH-stat method are summarized in Table 4. There were significant differences ($P < 0.05$) among the meals. The DH of meals by shrimp enzymes and the four-enzyme mixture were similar, except for langostilla meal. In both cases, the deboned white fish meal displayed the highest DH together with anchovy meal and soybean protein when using hepatopancreas enzymes, but the highest alone when using the four-enzyme mixture. The lowest DH was for the tuna waste meal when using the hepatopancreas enzymes, and for the langostilla meal when using the four-enzyme mixture. Hydrolysis curves for the meals using the enzyme extract from shrimp hepatopancreas are shown in Fig. 1.

Table 4
Degree of hydrolysis (%) and in vivo protein digestibility of the test ingredients using the hepatopancreas enzyme extract and four-enzyme mixture[1]

Test ingredients	In vitro				APD In vivo[3]
	Hepatopancreas enzymes		Four-enzyme mixture		
	DH%	Digestibility[2]	DH%	Digestibility[2]	
Anchovy meal	31.7[d]	83.1	30.3[d]	80.1	83.6[c]
Tuna waste meal	23.1[a]	59.8	19.0[a]	63.1	63.6[a]
Deboned white fish meal	33.8[d]	88.7	36.8[e]	89.9	86.6[c]
Menhaden fish meal	26.3[b]	68.5	24.2[b]	70.9	67.1[b]
Atlantic menhaden fish meal	28.8[c]	75.2	27.8[c]	76.3	67.1[b]
Langostilla meal	26.4[b]	68.7	19.6[a]	64.1	66.4[b]
Soybean protein	31.1[d]	81.4	30.8[d]	80.9	90.9[d]
Correlation of DH with APD (r^2)	0.77[4]		0.71[4]		–

[1]Values with different letters for each column are significantly different ($P < 0.05$).
[2]Obtained by the equation shown in Table 5.
[3]In vivo digestibility was determined by chromic oxide method. $N = 3$ tanks/treatment. Data are average of triplicate determinations for each of three groups of shrimp for each diet group. The standard deviation was between 1.1 to 3.4.
[4]Significant ($P < 0.05$).

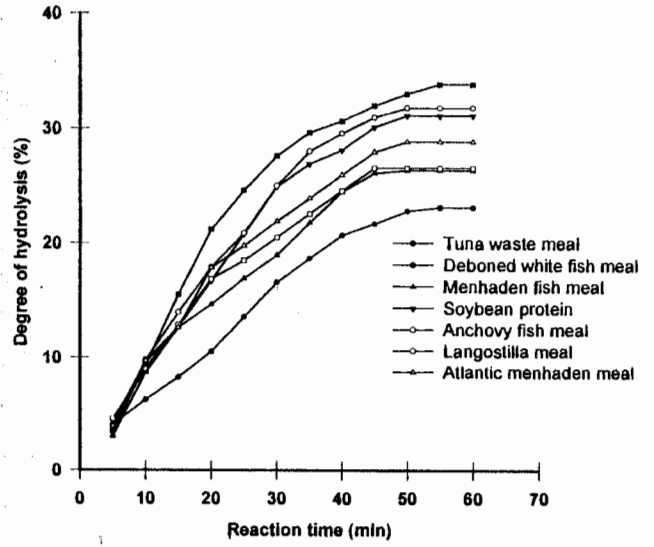

Fig. 1. Degree of hdyrolysis of protein sources using white shrimp hepatopancreas enzyme preparation. Data are average of triplicate determinations. The standard deviation was between 0.08 to 1.8.

The in vivo apparent protein digestibility values for the seven meals are summarized in Table 4. The soybean protein had a higher APD than all other meals. The lowest APD was seen for tuna waste meal. There were no differences ($P > 0.05$) among menhaden meal, Atlantic menhaden meal, and langostilla meal. These meals together with tuna waste meal, exhibited APD values lower than 70%.

Linear regression analysis showed no significant ($P > 0.05$) correlation between APD and chemical analysis ($r^2 = 0.09$ to 0.13). Regression equations and the corresponding correlation coefficient between in vitro and in vivo data are given in Table 5. The significant ($P < 0.05$) correlation between DH and APD was $r^2 = 0.77$ for the shrimp enzymes, and $r^2 = 0.71$ for the four-enzyme mixture. These data were used to construct

Table 5
Regression analysis of pH-stat assay after 60 min vs. in vivo protein digestibility

Regression output	Enzyme system	
	Hepatopancreas enzyme	Four-enzyme mixture
r^2	0.77	0.71
Constant	−2.7	34.5
Standard error of Y estimate	6.0	6.8
No. of observations	7	7
Degrees of freedom	5	5
X coefficient(s)	2.7	1.5
Standard error of coefficient	0.7	0.4
Regression equation	$Y = -2.7 - 2.7X$	$Y = 34.5 + 1.5X$[a]

[a]Where X = DH% by pH-stat method at 25°C and 60 min of reaction.

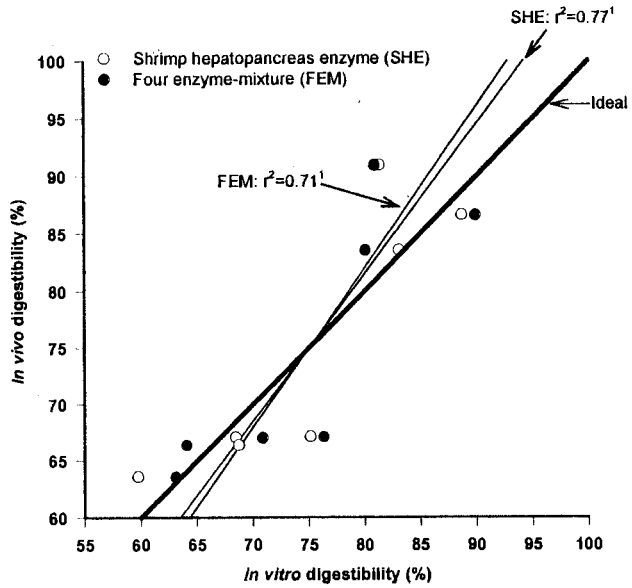

Fig. 2. Comparison of in vitro digestibility using pH-stat method and in vivo digestibility. [1]Significant $P < 0.05$.

the equations shown in Table 5. The linear regression curve describing the equation using the pH-stat values is shown in Fig. 2.

4. Discussion

The use of protein is affected by the nature of the dietary protein source, the level of protein intake, and the ability of an organism to use other dietary components. Protein quality is affected by the freshness of the raw material, amount of residual lipid, drying process, and whether the meal was made from whole fish or the waste from some other processing operation (Anderson et al., 1993).

The high ash content of the menhaden fish meal indicates that it was prepared from low quality waste from filleted fish, which has high scale and fishbone content. In the case of the langostilla meal, the high ash content is caused by the high content of chitin and minerals of the carapace. Because chitin is similar to protein in nitrogen content (Castro-González et al., 1995), langostilla protein could be overestimated. The differences in the chemical composition of the various meals was caused by the amount of lipid and moisture retained by these meals, although some differences can be attributed to fish species (Anderson et al., 1993). The inverse relation between protein and ash percentage has previously been reported by Dong et al. (1993).

Although protease activity in the enzyme preparations used in the pH-stat method

was controlled, the composition of hepatopancreas enzymes was different from that in the four-enzyme mixture. The specific enzymes present in the hepatopancreas of white shrimp are under study. Chymotrypsin as well as trypsin activity have been detected (unpublished data).

The results of the higher DH of casein with the four-enzyme mixture than with shrimp hepatopancreas extract were different from those obtained by Dimes and Haard (1994), who found that a pyloric ceca extract from trout gave a higher DH% of casein than the four-enzyme mixture. Differences were possibly caused because of the type of casein and the temperature of reaction.

The low DH of the tuna waste meal is attributed to the poor quality of the raw material or the type of processing used. The menhaden fish meal, although it had a high content of scale and fishbone, did not have a low DH. It appears that the protein digestibility of commercial fish meals is independent of fat and ash content, as long as the content of protein and amino acids are kept at the same level (Lan and Pan, 1993).

Chromic oxide has been reported to be an inadequate indirect digestibility indicator for *P. serratus* and *Pandalus platyceros* (Forster and Gabbot, 1971). However, in the present study its use was adequate because of the amount of feces collected, the high digestibility rate, the low standard deviation among replicates (reproducibility), and the homogeneous mixing of chromic oxide into the diet. A similar observation has been reported by Akiyama et al. (1989). It appears chromic oxide is an appropriate digestibility indicator for penaeid shrimp.

Knowledge of protein digestibility is important in diet formulation, because animals must consume dietary protein to supply amino acids. The APD values for the fish meals and soybean protein are similar to those reported by other investigators (Akiyama et al., 1989; Anderson et al., 1993). However, langostilla meal values were lower than reported by Goytortúa (1993). The low APD of tuna waste, menhaden fish, Atlantic menhaden fish, and langostilla meals indicate that these meals have limited nutritional value to shrimp, although a growth assay is still needed to confirm this. Our results indicated soybean protein was better digested than fish and langostilla meals. However, other authors like Forster and Gabbot (1971), working with *P. serratus* and *P. platyceros*, and Fenucci et al. (1982), with *Penaeus stylirostris*, have reported protein from animal sources are better digested than plant proteins. These conflicting observations concerning APD are possibly related to the species examined, ingredient quality, or diet composition (Akiyama, 1991).

Chemical properties for fish meals used in feed production for aquaculture include chemical analysis. Although it has limited sensitivity, it does help to indicate the general quality of the ingredient. As an example, the moisture content of fish meals should be between 6 and 10%, ash should not exceed 17%, and fat concentration should be between 6 and 12% (dry weight basis) (Lazo, 1994). The tuna waste meal had low moisture and a high ash content, which is an indication of over heating and high bone and scale content in the raw material. The over heating during drying could cause reactions of certain amino acids, reducing protein digestibility. Lysine could be involved in the Maillard reaction and cysteine:cystine ratio may be affected by heat. These changes may reduce protein use by animals (Anderson et al., 1993). The lack of correlation between the chemical analysis and APD data indicates the chemical analysis

data were not adequate as a predictor of the nutritional value of the meals. Although chemical analysis of the meals, such as proximate analysis, assists in determining the general quality of the meal, it does not always correlate with nutritional evaluation results obtained from growth and survival trials or from in vivo digestibility studies (Hardy and Masumoto, 1991), as in this study.

The pH-stat procedure is advantageous because it is a kinetic method that directly measures the percentage of peptide bonds hydrolyzed in the protein (Dimes and Haard, 1994). The in vitro protein digestibility by pH-stat method detects the effect of heat processing on enzyme inhibitors in raw material (García-Carreño et al., 1997). The in vitro protein digestibility values of soybean protein and Atlantic menhaden meal were overestimated as deduced from the digestibility values obtained by the in vivo assay. However, considering the linear regression coefficient value (r^2) of 0.77 ($P < 0.05$), the pH-stat method, using enzymes from shrimp hepatopancreas, appears to be a viable alternative to in vivo assays of white shrimp diets. Lan and Pan (1993) reported in vitro protein digestibility values similar to those obtained by us for soybean meal and white fish meal. They measured the absorbance (A_{280}) of a trichloroacetic acid supernatant after the hydrolysis of feed protein, with a good correlation ($r^2 = 0.99$) between in vitro protein digestibility and lysine and arginine concentration. However their work lacks any in vivo data that could be important to validate the method.

5. Conclusion

The pH-stat in vitro assay method employed in this study produced results that were, for the most part, in good agreement with in vivo apparent protein digestibility of white shrimp.

The in vitro assay using shrimp hepatopancreas extract has potential as a promising tool in estimating the digestibility of alternate protein sources for white shrimp diets.

Additional in vivo data are needed to establish a clear understanding of their relationship with in vitro digestibility. Other new diets, shrimp size, and species need to be tested.

We think the stability of the hepatopancreas enzyme extract should be further studied. The enzyme extract can possibly be used as a routine assay.

Acknowledgements

The authors thank Dr. Guillermo Arteaga and Dra. Ana Ma. Ibarra for their suggestions, M.C. Lucía Ocampo, Ings. Sonia Rocha, Mayra Vargas, Ernesto Goytortúa, and B. Guillermo Portillo for their technical assistance, and CONACyT for the grant given to author Fernando Luis García-Carreño and scholarship given to J. Marina Ezquerra B. Thanks to Dr. Ellis Glazier for editing of the English language manuscript.

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