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Electrophoretic analysis of proteins from raw materials and commercial diets used in animal feed

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Abstract: In aquaculture, balanced feed accounts for 50% to 70% of the operational costs of cultivating aquatic organisms. This feed is made from various raw materials that serve as protein sources, which significantly influence the final cost of the feed. Therefore, an electrophoretic study was conducted to compare the protein content in different raw materials and commercial diets used in animal feed. Electrophoretic profiles with standard concentration gels (SDS-PAGE) were used to separate protein fractions from animal-origin raw materials, including Earthworm Meals of Eisenia andrei (HLF) and Eisenia fetida (HLT), meat-bone meal (MBM), and fish meal (FM), as well as plant-origin meals, such as Leucaena leaf meal (LLM), corn meal (YCM), and wheat bran (WBM). Commercial diets (A, B, and C) were also evaluated. The protein content of the samples was determined. For electrophoretic analysis, a protease inhibitor cocktail was used, along with a molecular weight standard with a range from 250 to 4 kDa. The molecular weights of the diets ranged between 31.1 and 234.1 kDa, 36.3 and 190.4 kDa, and 4.281 and 95.685 kDa. Some of the protein bands in the analyzed samples (HLF, HLT, FM, YCM, and WBM) were very close to the reference protein bands at 148, 98, 64, 50, and 36 kDa, corresponding to the molecular weight standard used. Electrophoretic characterization provided insight into the different protein fractions, offering valuable information about their potential functional and nutritional properties. Electrophoresis is an essential analytical tool for assessing both the quality and safety of raw materials and foods.

Keywords: *Electrophoresis, Feed, Fish, New raw materials, Proteins.*

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1. Introduction

Alternative protein sources are employed in food formulations for various species such as fish (*Oreochromis spp*., *Dormitator latifrons*, *Oncorhynchus mykiss*, *Colossoma macropomum*), crustaceans (caridea), and echinoderms. Several authors suggest substituting animal-derived protein sources with other plant-based protein ingredients, focusing not only on the protein quantity but also on the diversity of protein types, thereby providing a broad amino acid profile to meet the essential amino acid needs of the target species [1] [2]. There are various traditional raw material sources of animal and plant origin with nutritional potential, such as fish meal, meat-and-bone meal, blood meal, bone meal, among others; and plant sources like corn meal, rice meal, wheat bran meal, and soybean meal. However, there are also alternative sources like leucaena meal and earthworm meal (*Eisenia andrei*); the latter being a protein source with a concentration greater than 60% w/w on a dry basis [3], essential amino acids and fatty acids [4] [5], and nutrients such as minerals and lipids (>11%) for both human and animal consumption [6] [7] [8]. These constitute an alternative due to their composition and low cost, especially for cold-water fish, which have high demands and require between 40-50% protein, amino acids, essential fatty acids, vitamins, and minerals for their development [9] [10].

Electrophoretic analysis not only helps identify the individual protein fractions of a sample but also clarifies the complexity and overlap between various protein fractions, their size, and solubility [11]. The SDS-PAGE electrophoretic technique plays an essential role in protein studies. However, this analytical method may present some limitations, such as poor separation, signal reduction, or the complete absence of bands; despite this, it is widely used in protein analysis studies [12]. The SDS-PAGE electrophoresis technique is commonly used to separate proteins according to their molecular mass. It corresponds to polyacrylamide gel electrophoresis with sodium dodecyl sulfate [13] [14]. In this method, proteins lose their native structure, i.e., they denature, first through a Laemmli sample buffer containing SDS, leaving polypeptide chains with a uniform negative charge. Subsequently, in a polyacrylamide gel matrix, the negatively charged proteins separate by applying an electric current. This gel is beneficial because it more significantly limits the migration of smaller molecules compared to larger ones. The negative charge on the polypeptide chains moves towards the positive electrode when the current is applied. The smaller the proteins, the faster and closer they move to the end of the gel [13] [15].

Previous works have performed electrophoretic analysis of an amaranth protein isolate using a 4-12% polyacrylamide gel, resulting in globulins and albumins as the main protein fractions in the seeds of this plant, with the predominant storage proteins being 7S and 11S globulins [16]. Protein extracts from barley seeds have also been evaluated using electrophoresis (SDS-PAGE) to establish the molecular weight distribution, showing tWBM barley proteins were largely composed of ~70% low molecular weight fractions with values < 20 kDa [17]. This is why this technique becomes an important alternative for evaluating protein fractions of various matrices. The technique's basis lies in using the dye Coomassie Brilliant Blue G-250, which is in its unstable red cationic form. When proteins are present in the dye-containing solution, hydrophobic and ionic interactions between the proteins and the dye stabilize the latter, transforming it into its stable anionic form [12].

The objective of this work was to perform a quantitative comparison of the proteins present in different animal, plant raw materials, and commercial diets through an electrophoretic study with SDS-PAGE gels, as a possible alternative to develop new formulations useful in animal feed.

2. Materials and Methods

Samples of animal based flours (earthworm *Eisenia andrei*) were obtained from the Faculty of Pharmacy and Bioanalysis, ULA-Mérida (HLF); earthworm (*Eisenia fetida*) from the state of Trujillo (HLT), produced by a local manufacturer, as well as meat and bone meal (MBM) and fish meal (FM), provided by the National Institute of Agricultural Research (INIA)-Cumaná. Plant-based flours included: *Leucaena* leaf meal (LLM), yellow corn meal (YCM), and wheat bran meal (WBM), sourced from the market; and three commercial diets, two domestic (A and B) and one imported (C).

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For the electrophoretic analysis, a protease inhibitor cocktail (SIGMA) was used. A Sonic Dismembrator (Fisher Scientific, model 500) and a centrifuge brand RG5 were employed. Polyacrylamide gels were used as the denaturing medium with SDS-2-mercaptoethanol [18], and an Invitrogen Corporation NuPAGE® NOVEX molecular weight standard (250-4 KDa) was used as a reference.

2.1. Determination of Protein Content

Before preparing protein lysates from the flour samples, the protein percentage was determined using the micro-Kjeldahl method [19]. For this analysis, 0.1 grams of each homogeneous sample were weighed in triplicate and transferred to 30 mL micro-Kjeldahl flasks with 3 glass beads. To each flask, 1 gram of sodium sulfate (Na_2SO_4), 0.01 grams of copper sulfate ($CuSO_4$), and 2 mL of concentrated sulfuric acid (H_2SO_4) were added. The flasks were placed in the digestion apparatus and heated at a low temperature until foam formation ceased. Then, the temperature was progressively increased, avoiding nitrogen loss through volatilization. At the end of the digestion, the digestive changed from black to light green, and once cooled to room temperature, it was dissolved with 10 mL of distilled water for distillation. For the distillation, a 100 mL Erlenmeyer flask containing 5 mL of 4% boric acid (H_3BO_3) solution and 3 drops of indicator solution was placed at the outlet of the distiller, ensuring tWBM the tip of the condenser remained submerged in the solution. The diluted digested was transferred to the distillation chamber, and 5-6 washes with 2 mL portions of distilled water were performed. Then, 10 mL of NaOH-$Na_2S_2O_3$ solution (60 g NaOH + 5 g $Na_2S_2O_3$/100 mL) was added, the funnel was washed with distilled water, and the equipment valves were closed. The steam generator was turned on, and distillation was carried out until 50 mL of the sample was collected, which was titrated with 0.02 N hydrochloric acid (HCl) using a graduated burette until a color change from light green to pink was observed. The nitrogen percentage was calculated according to the following formula:

%N = VNE/10a

V: Volume of 0.02 N HCl used during titration.
N: Concentration of HCl (0.02 N).
E: Nitrogen equivalent.
a: Weight of the sample (g).

The protein percentage of samples of animal origin was calculated by applying a conversion factor of 6.25 to the nitrogen percentage. For samples of plant origin, a factor of 5.7, which is the conversion factor for wheat, was used. The protein percentages obtained in triplicate for each sample were averaged, and their standard deviation was calculated in the same manner.

2.2. Preparation of Protein Lysates from Analyzed Flours

Based on the obtained protein percentages, the following amounts of each sample were weighed: 0.325 g HLF; 0.425 g HLT; 0.351 g MBM; 0.371 g FM; 0.752 g LLM; 1.174 g YCM; 0.620 g WBM; and 0.476, 0.418, and 0.381 g for commercial diets A, B, and C, respectively. These were transferred to 50 mL Eppendorf centrifuge tubes, and 5 µL of a protease inhibitor cocktail and 10 mL of distilled water were added. The tubes were placed in an ice bath and sonicated for 3 minutes with 10-second intervals of sonication and 10-second intervals of rest using a Sonic Dismembrator. Finally, they were centrifuged at 3500 g in a high-speed refrigerated centrifuge for 15 minutes. These samples were then used for analysis in standard concentration gels (8 cm wide by 7.5 cm long).

2.3. Electrophoretic Analysis with Standard Concentration Gels (SDS-PAGE):

For the characterization of proteins present in the analyzed samples, including HLF, HLT, MBM, FM, LLM, YCM, WBM, and commercial diets A, B, and C, 12.5% polyacrylamide gels were prepared. Electrophoresis was conducted using the EC 120 Mini Vertical Gel System (Thermo EC) at 20 mA for 1 hour in a Tris-glycine-SDS buffer at pH 8.3, with a power supply EC 105 (Thermo EC). The gels were stained and destained following the method described by [20], similar to the Bradford assay, which is

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recognized as the most sensitive colorimetric method for measuring protein concentration based on the absorbance of Coomassie Brilliant Blue G-250 dye [21]. This change converts the dye from red to blue. Therefore, the intensity of the blue color in the samples increases with higher protein concentrations [12]. To quantify the molecular weights of the proteins using SDS-PAGE, a standard curve ($y = -63.078\text{Ln}(x) - 16.315$, $R^2 = 0.9824$) was created for each gel. This curve was generated by measuring the Rf values (distance traveled in cm by the protein relative to the distance traveled by the bromophenol blue dye), for both the standard and the samples [22].

3. Results and Discussion

3.1. Determination of the Analysis of Protein Bands of Raw Materials of Animal and Vegetable Origin Commonly Used in the Production of Balanced Foods.

In the electrophoretic analysis with standard SDS-PAGE gels, approximately 13 protein bands were observed in the HLF and HLT samples, 4 in the MBM sample, and 3 in the FM sample Figure 1.

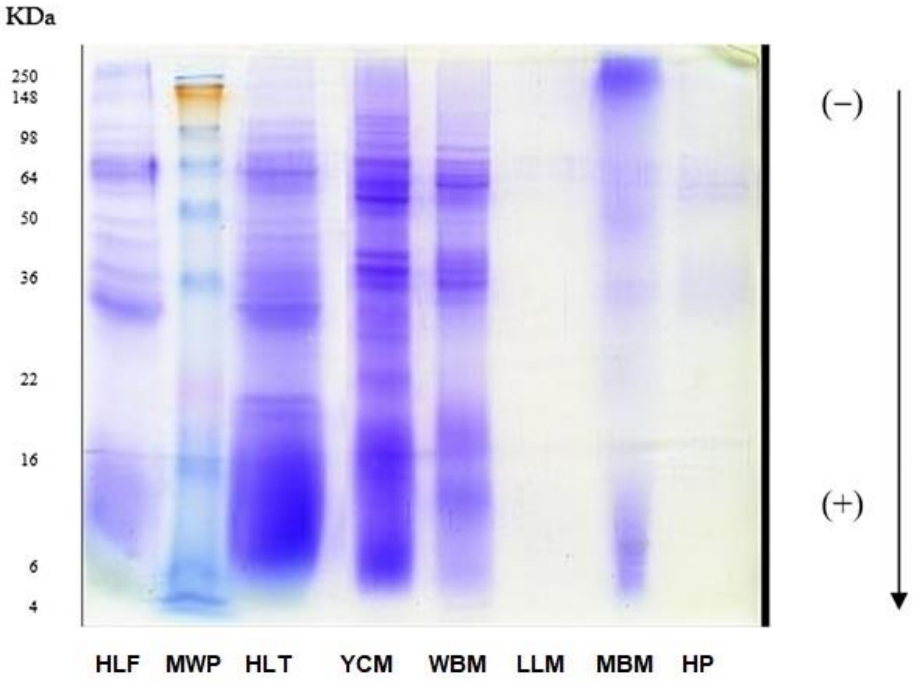

Figure 1.
SDS–PAGE of animal derived flours (HLF, HLT, MBM, FM) and plant derived flours (YCM, LLM, WBM), molecular weight standard (MWP).

All these protein bands were compared with the molecular weight standard. A molecular weight range between 31.1 and 234.1 kDa was observed (Table 1), and protein bands with molecular weights of 103.0 kDa and 190.4 kDa were present in most of the analyzed samples. The MBM and FM samples exhibited the fewest protein bands, which could be attributed to possible protein denaturation due to processing and improper drying conditions [23]. The denaturation or folding occurs when samples are handled unstably, affecting the analyzed structure due to temperature fluctuations [24].

The HLF and HLT samples differed in the number of protein bands obtained when compared with the 24 protein bands detected in raw earthworm (*Eisenia foetida*) and 17 protein bands in oven-dried earthworm flour. Molecular weights of 31.1, 45.2, 55.4, 67.7, 88.9, 95.5, 103.0, 121.1, and 139.2 kDa were obtained, which align with the trends presented in the literature [25]. Additionally, a protein with a molecular weight of 234.1 kDa was observed (Table 1), approaching the molecular weight of the S-

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globulin subunit of 250 kDa from the molecular weight standard used. In this regard, [26] state tWBM proteins with a molecular weight greater than 200 kDa are difficult to separate in polyacrylamide gels due to distortion caused by the high acrylamide content. Some of the protein bands from the HLF, HLT, and FM samples were located close to the 98, 64, and 50 kDa regions, corresponding to the molecular weights of bovine serum albumin (BSA), glutamate dehydrogenase, and alcohol dehydrogenase, respectively (MWP), which match the commercial molecular weight standard used in this study.

Table 1.
Molecular weights (kDa) of animal-derived proteins (HLF, HLT, MBM, FM) on SDS-PAGE electrophoresis gel (calculated from Figure 1).

HLF	HLT	MBM	FM
234.1	234.1	190.4	190.4
164.8	190.4	103.0	103.0
132.6	139.2	77.4	95.5
121.1	121.1	55.4	
130.0	111.4		
95.5	103.0		
82.9	88.9		
72.3	74.8		
67.7	72.3		
63.3	59.2		
55.4	53.6		
50.1	50.1		
45.2	31.1		

Note: HLF: Earthworm flour, Faculty of Pharmacy, ULA-Mérida.
HLT: Earthworm flour.
MBM: Meat and bone meal.
FM: Fish meal

Vegetal samples (YCM, WBM, and LLM) showed 15, 14, and 5 protein bands, respectively. In this case, a molecular weight range between 36.3 and 190.4 kDa was obtained (Table 2), with three protein bands having molecular weights of 82.9, 88.9, and 190.4 kDa, which were present in all three analyzed raw materials. The LLM sample exhibited the fewest protein bands, which could be attributed to the manufacturing process of this flour. Certain protein bands from the YCM and WBM samples were found to be very close in the regions of 148, 98, 64, 50, and 36 kDa, corresponding to the protein bands of serum phosphorylase, bovine serum albumin (BSA), glutamate dehydrogenase, alcohol dehydrogenase, and carbonic anhydrase, respectively (MWP), due to their similarity to the bands of the molecular weight standard used. This observation is consistent with the fact tWBM protein samples are indeed composed of subunits, normally the species of vegetable origin represent this protein quality [27].

Table 2.
Molecular weights (kDa) proteins of vegetable origin (YCM, WBM, and LLM) on SDS-PAGE electrophoresis gel (calculated from Figure 1).

YCM	WBM	LLM
190.4	190.4	190.4
146.7	146.7	88.9
132.6	121.1	82.9
121.1	111.4	55.4
111.4	103.0	48.4

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YCM	WBM	LLM
103.0	95.5	
95.5	88.9	
88.9	82.9	
82.9	72.33	
77.4	63.3	
67.4	59.2	
63.3	55.4	
59.2	48.4	
51.8	36.3	
45.2		

Note: YCM: Yellow Corn flour
WBM: Wheat Bran flour
LLM: Leucaena leaf flour

3.2. Determination of Protein Band Analysis of Commercial Feeds

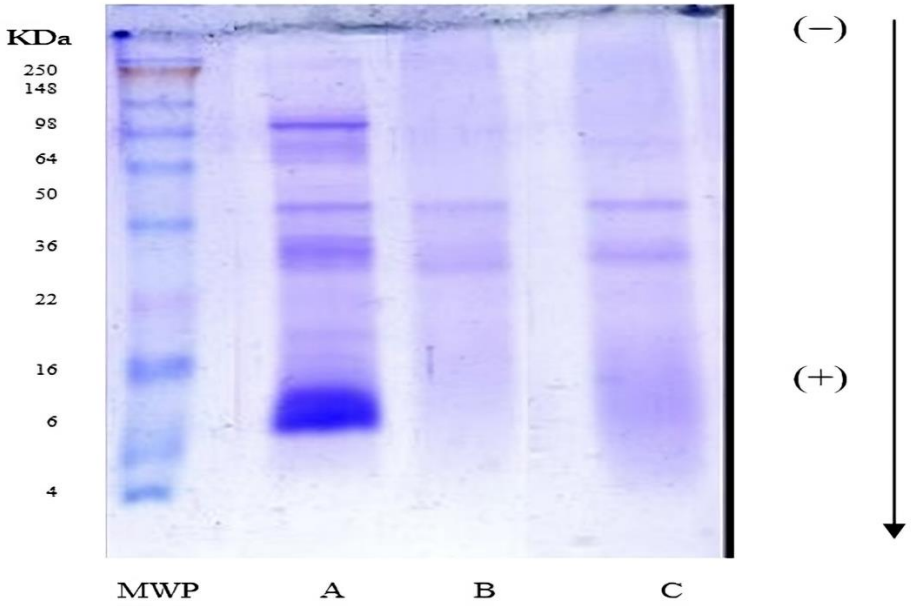

Figure 2.
SDS–PAGE of commercial diet flours: national (A and B), imported (C), and molecular weight standard (MWP).

Table 3.
Molecular weights (kDa) of proteins in commercial diets: National (A and B), imported (C), on SDS-PAGE electrophoresis gel (calculated from Figure 3).

A	B	C
47.751	95.685	49.947
32.320	80.253	32.320
28.946	67.645	19.712
11.567	47.751	14.177
4.281	32.320	
	28.946	
	12.860	

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Figure 2 shows the electrophoretic profiles of the protein lysates from the national commercial diets (A and B), the imported diet (C), and the MWP. Five, seven, and four protein bands were observed for diets A, B, and C, respectively. This indicates tWBM the national and imported feeds differ in their protein composition, with feed B exhibiting the greatest abundance of protein bands.

The molecular weights obtained from the analyzed diets (Table 3) ranged between 4.281 and 95.685 kDa. Diet A had five protein bands (4.281; 11.567; 28.946; 32.320; and 47.751 kDa), diet B had seven (12.860; 28.946; 32.320; 47.751; 67.645; 80.253; and 95.685 kDa), and diet C had four (14.177; 19.712; 32.320; and 49.947 kDa), which are similar to the standard proteins in the molecular weight pattern used.

When comparing the animal and plant-based raw materials (Tables 1 and 2) with the commercial diets (Table 3), it was found tWBM the highest number of protein bands was present in the YCM, WBM, HLF, and HLT samples.

The thickness of a protein band produced by electrophoresis indicates protein concentration, classifying bands as major (principal) or weak (minor) [28]. Regarding band thickness and intensity, this condition is related to the number of migrating molecules, with bands carrying higher ionic charges migrating further than those with lower ionic charges [29].

In protein electrophoretic studies, major bands are thicker and exhibit greater color intensity, indicating higher concentrations compared to weaker (minor) bands [28]. Changes in the protein pattern of SDS-PAGE electropherograms indicate modifications in protein composition and thinning or loss of protein bands signify a change in the nature of the proteins. This may be related to the band changes or reductions observed in some of the samples analyzed in this study [30].

4. Conclusions

The earthworm meals (*Eisenia andrei*) had more protein bands than the other animal-origin sources, confirming their potential for inclusion in diet formulations used for feeding various commercially important aquatic species.

Corn meal and wheat bran exhibited a higher number of protein bands compared to *Leucaena* leaf meal.

The animal and plant based raw materials provided quantitatively more information about their potential functional and nutritional properties when compared to the evaluated commercial diets.

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