

# Amino Acid Profile and Fortification Potential of Protein Fraction from *Samanea saman* Seed

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**Abstract.** The amino acid composition and fortification potential of the protein fraction from *Samanea saman* seed were evaluated. This study determined protein content, extracted proteins under alkaline conditions, fractionated them at pI 5 and pI 6, and analyzed essential amino acids (EAAs). Protein content was measured by the Kjeldahl method, while protein fractions were identified using RP-HPLC (Shimadzu 10A, Japan) with a fluorescent detector. *Samanea saman* seeds contained 34.95% crude protein. Protein recovery reached 18.70% at pI 5 and 11.39% at pI 6. The pI 5 fraction contained 38.16% EAAs, while the pI 6 fraction contained 25.26%. According to FAO/WHO/UNU (2007) requirements, the pI 5 fraction met the needs for histidine, valine, leucine, isoleucine, lysine, and aromatic amino acids, whereas the pI 6 fraction only fulfilled histidine and aromatic amino acids. These results indicate that the pI 5 fraction possesses stronger potential for food fortification, given its higher total EAA content and broader amino acid adequacy compared to the pI 6 fraction. Overall, *Samanea saman* seed protein, particularly the pI 5 fraction, can serve as a promising alternative protein source for improving nutritional quality in food formulations.

## 1 Introduction

Food consumption has shifted from animal-based to plant-based diets due to changes in consumers' preferences for more ethical, religious, and healthier dietary choices. Seeds have been extracted as an attractive alternative to traditional protein sources [1]. Compared with animal-derived proteins, plant proteins exhibit a less diverse amino acid composition. However, plant proteins are abundant enough to meet the growing population's protein requirements. Plant proteins have lower production expenses and high nutritional values, which meet the functional characteristics required for humans [2].

Grain legumes represent a valuable source of plant-based protein, characterized by low saturated fat content and high levels of dietary fiber, essential minerals, and vitamins. Regular consumption of legumes has been associated with a reduced risk of cardiovascular diseases [3]. *Samanea saman*, belonging to the legume family, is an important species in Indonesian forestry due to its substantial biomass and ecological significance. In addition to its role in reforestation, the pods and seeds of *Samanea saman* are utilized as feed for ruminants, with the seeds containing a notably high protein content of approximately 39.40% [4].

Protein is an essential macronutrient required for maintaining vital physiological functions. Protein deficiency arises from inadequate intake or poor-quality protein sources. The quality of dietary protein is primarily determined by its essential amino acid (EAA) profile, as EAAs cannot be synthesized by the human body and must be obtained through diet. Beyond their nutritional value, EAAs also serve as potential sources of functional protein fractions with applications in the food and biomedical industries [5]. Protein isolation is a technique used to obtain proteins with higher concentration and purity. This process often involves fractionation based on differences in isoelectric points (pIs), as proteins exhibit minimal solubility at pH values near their pI, facilitating their precipitation. Protein isolates are advantageous due to their higher protein content, balanced EAA composition, and reduced levels of seed-derived anti-nutritional factors such as phytate, oxalate, tannin, and saponin [6].

Fortification is an effective strategy to enhance the macro- and micronutrient content of food products. It is relatively cost-efficient and, when properly applied, does not significantly alter the physicochemical or sensory properties of the food [7]. This study aimed to fractionate the protein from *Samanea saman* seeds at specific pIs to obtain protein fractions with higher concentrations and abundant EAAs. Furthermore, the study evaluated the potential of these protein fractions as a novel protein source for use in food fortification.

## 2 Experimental Details

### 2.1 Sample preparation

Approximately 200 g of *Samanea saman* seed was separated from the seed coat and ground to produce 100 mesh powder. The defatted sample was prepared using the Soxhlet method. The sample was extracted at 40 - 60°C for 3 h using petroleum ether. The free fat sample was dried for 1 h at 60°C. The powder was stored in a sealed jar at 4°C until analysis.

### 2.2 Protein content

The protein content of the defatted samples was analyzed by the Kjeldahl method. This method was applied to the total nitrogen content (%N) or crude protein. Protein content was determined according to a previously reported method [8] with some modifications. About 1 g of fat-free seed powder was added to a Kjeldahl flask and mixed with 2.5 g of Na<sub>2</sub>SO<sub>4</sub> anhydrous, 0.05 g of CuSO<sub>4</sub>.5H<sub>2</sub>O, and 10 mL of H<sub>2</sub>SO<sub>4</sub>. First, the mixture was heated on a heating mantle at low heat. After the smoke appeared, the heat was increased until the solution was clear, and some smoke disappeared. The result of the digestion process was diluted to 100 mL of distilled water. About 50 mL of solution was transferred into a distillation flask. A few drops of PP indicators, boiling stones, 50% NaOH solution, and 200 mL of distilled water were then added to the solution. The distillate was captured using a 0.1 M HCl solution (25 mL). The distillation process was performed until the distillate drops were neutral. The distillate was titrated with 0.5 M NaOH as a standardized solution. A blank solution without a sample was prepared using the treatment described above. The crude protein content was determined using Equation 1:

$$\% \text{Protein} = \frac{(V_{\text{NaOH blank}} - V_{\text{NaOH sample}}) \times 14,008 \times M_{\text{NaOH}} \times A \times B}{\text{sample (g)} \times 1000} \times 100\% \quad (1)$$

A = dilution factor (2)

B = factor to convert N to protein (6,25)

### 2.3 Protein fractionation

Protein fractionation was performed as described previously [9] with some modifications. About 10 g of the free fat sample was dissolved in water (1:10) and stirred at 50°C. The solution was maintained at a pH of 11 to extract the protein by adding a 1 M NaOH solution, and the supernatant was separated by centrifugation (Biofuge primo R SORVALL®, USA) at 4°C. Protein precipitation was performed at pI 6 and pI 5. The acid solution, HCl 1 M, was added to the supernatant until pH 6 and stored for 24 h at 4°C. The pI 6 protein fraction was precipitated and centrifuged. The remaining supernatant was added to 1 M HCl until pH 5 and stored to obtain the protein precipitate at pI 5. Both pIs were washed several times with water to remove the contaminants formed during the fractionation process. The protein fractions were dried using a freeze dryer (Alpha 1-2 LO Plus CHRIST®, Germany). The weight of proteins was measured, and the percentage of protein in each pI was calculated using Equation 2:

$$\% \text{ Protein fraction} = \frac{\text{protein fraction (g)}}{\%N \times 10 \text{ g sampel}} \times 100\% \quad (2)$$

### 2.4 Protein hydrolysate

The acid hydrolysis process was performed following the method described in [2] with some differences. Approximately 60 mg of the protein fraction in pI 5 was hydrolyzed in a reflux system after adding 5 mL of 6 M HCl. The process was conducted at 108°C for 24 h. The hydrolysis product was neutralized by adding NaOH. The filtrate was filtered using Whatman's method 42. The same method was applied to the pI6 protein fraction. The total final volume was recorded after the hydrolysis process.

### 2.5 Amino acid profile

The amino acid profile was determined using a reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu 10A, Japan) column with a fluorescence detector (Shimadzu RF-138, Japan,  $\lambda$  excitation 340 nm dan  $\lambda$  emission 460 nm). About 25  $\mu$ L of the filtrate was derivatized with 300  $\mu$ L ortho-phthalaldehyde (OPA) and 1-2 drops of 2-mercaptoethanol. A total of 14 amino acid standards were used. The standards were prepared in three different concentrations: 50, 100, and 250 ppm. The concentration (mg/L) of each amino acid was determined by analyzing the chromatograms. The weight of detected amino acids (mg) was calculated by multiplying the concentration (mg/L) by the total volume of hydrolyzed amino acids. The percentage of amino acids was determined using Equation 3 as follows:

$$\% \text{ Amino acid} = \frac{\text{amino acid (mg)}}{60 \text{ mg protein}} \times 100\% \quad (3)$$

The analysis was conducted a single time. The sample matrix was straightforward and necessitated no additional examination to attain sufficient separation and resolution of the analyte of interest, and a solitary run was sufficient for the quantitative analysis.

## 2.6 Amino acid score

The chemical score was determined to confirm that the food source is potentially as food fortification. The EAA score must reach more than 1,00 FAO compared to the FAO/WHO/UNU (2007) standard for protein fortification [10]. The amino acid score shows the effectiveness with which absorbed dietary nitrogen can meet the EAA requirement at a safe level of protein intake. This chemical score can be calculated by Equation 4:

$$\text{Amino acid score} = \frac{\text{amino acid sample (g/100g)}}{\text{amino acid standar (g/100g)}} \quad (4)$$

## 3 Results and Discussion

### 3.1 The protein content

The protein content of *Samanea saman* seeds was about 34.95%. The protein content was higher compared to the other legumes, such as chickpea (22.7%), mungbean (27.2%), lentil (28.6%), peanut (30%), dry pea (25.7%), cowpea (27%), but the protein content was after soybean (35 – 50%) [11]. Thus, *Samanea saman* seed, due to its high protein content, is potentially a valuable source of food protein.

### 3.2 Protein fractionation

Protein can be obtained at high concentrations through two main processes: extraction and precipitation. These processes are typically conducted under varying pH conditions to optimize protein solubility and recovery. Proteins aggregate and precipitate at their pIs during fractionation depending on their specific amino acid composition. This separation technique, known as protein fractionation, allows the isolation of distinct protein fractions with different physicochemical characteristics.

This process was conducted using a defatted sample to facilitate efficient protein fractionation. The presence of fat during extraction typically leads to the formation of three separate layers—fat, dissolved protein, and undissolved residues—which complicates the fractionation process. To address this, the sample was subjected to aqueous extraction. Protein solubility during extraction is influenced by the nature of amino acid side chains and the pH of the solvent. Under alkaline conditions, protein solubility increases due to the disruption of the cellular matrix and enhances dissociation of protein–protein and protein–lipid interactions, thereby improving extraction efficiency [12].

The alkaline extraction at pH 11 was aimed to promote maximum protein solubilization. Theoretically, the alkaline extraction increases the negative charge of the protein side chain. The carboxyl ion and deprotonated amine chains induce a higher repulsive force between protein molecules that contain negative charges. This process weakens intermolecular interactions such as aggregation or hydrophobic association, thereby enhancing the dispersion of proteins in the aqueous medium [9]. Consequently, it improves the hydrophilic interaction between protein and solvent (water) and leaves other compounds that are not soluble in water.

Alkaline extraction can reduce tannin content. Tannin is a polyphenol compound that complexes with proteins to reduce their digestibility [13]. Tannin dissolves in water. Therefore, it can be separated during fractionation. The extraction process should be under pH 11.5 due to amino acid racemization and the undesirable compounds, such as nephrotoxic lysinoalanine, resulting from lysine and dehydroalanine, followed by cysteine and serine

degradation. Strong alkalinity affects protein quality by causing protein denaturation, hydrolysis, and discoloration [1].

Protein fractionation was carried out to isolate protein fractions rich in EAAs. The process was conducted at pH 4 and 5, which are close to the reported isoelectric point range of many legume proteins [14]. However, several studies have also reported that the isoelectric point of legumes is around pH 6 [15]. After the protein was solubilized in alkaline conditions, the pH was adjusted to the isoelectric point, where the proteins have a zero charge, resulting in protein aggregation [1]. The hydrophobic interaction among proteins increases, whereas the interaction between water and protein decreases. Proteins form aggregates that stick together and solidify during coagulation. This process required approximately 24 h at 4°C to maximize the precipitation.

The precipitate was separated from the supernatant by centrifugation. The precipitate was filtered and washed to remove the salt due to the addition of NaOH and HCl. The protein was dried using a freeze-dryer to maintain its quality. The freeze-drying process is performed for organic substances that are easily deteriorated by chemical, biochemical, and microbial processes during storage. [17].

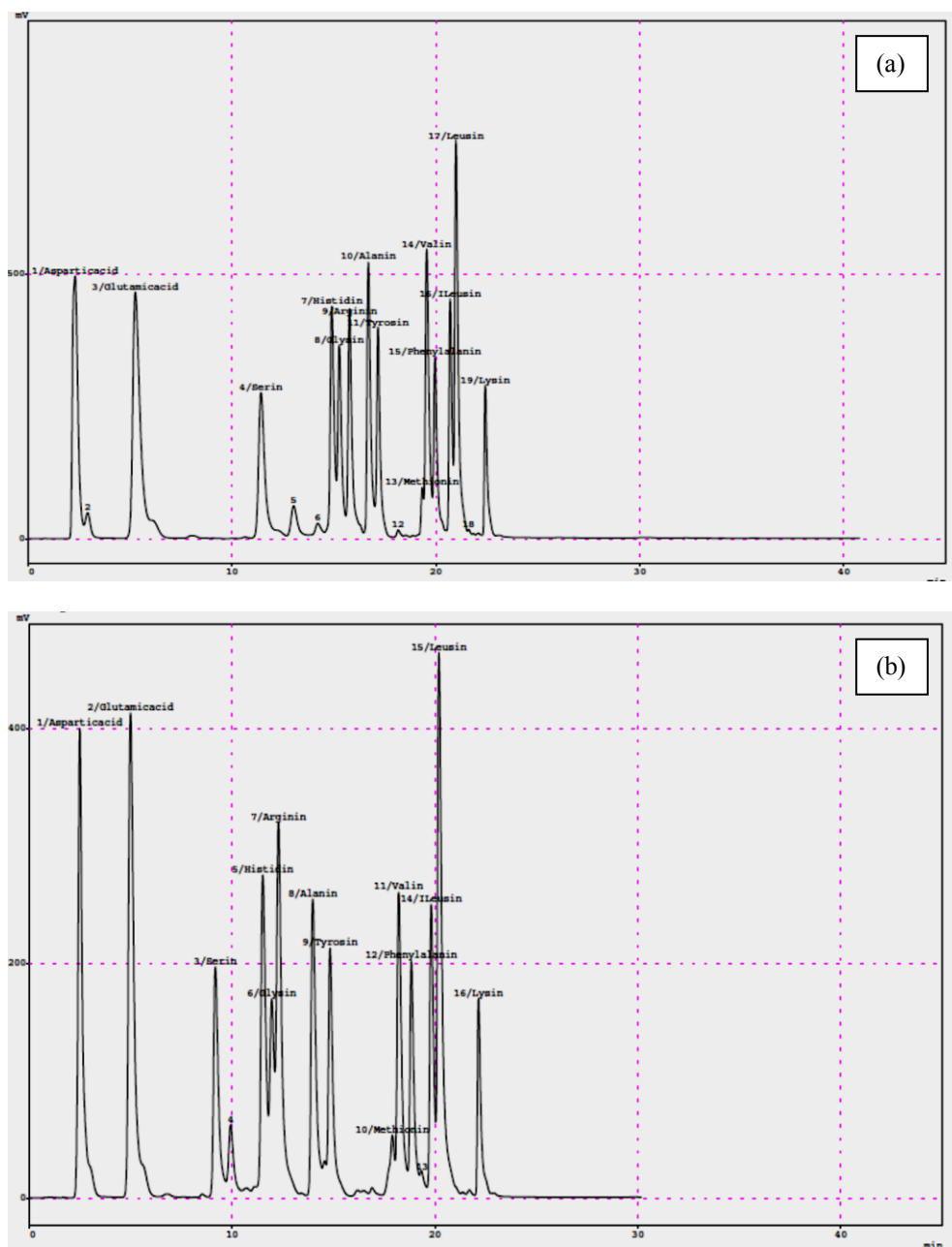
Table 1. The result of protein fractionation in pI 5 and pI 6

Protein fraction yield	pI 5	pI 6
Weight	0.647 g	0.394 g
%w/N content	18.70%	11.39%

The results presented in Table 1 were obtained after comparing them with the N content obtained from the Kjeldahl process. From the fractionation process, isolation in pI 5, 18.70% delivers a higher yield than in pI 6, 11.39%. After the extraction process, the fractionated protein weight following precipitation decreased by more than 80%, likely due to process losses, incomplete precipitation, and the selective nature of isoelectric fractionation. Therefore, it could not be concluded that *Samanea saman* seed could be optimally fractionated at pI 5. Further research on other pIs is required.

### 3.3 Amino acid profile at pI 5 and pI 6

Hydrolysis was performed for 24 h at a high temperature using a strong acid reflux system. Hydrolysis of 60 mg protein fractions resulted in 10.5 mL hydrolysate volume of pI 5 and 9 mL for pI 6. Acid hydrolysis is the most popular technique that was implemented to cleave the peptide bond chain, resulting in free amino acids [16]. Free amino acids were identified by reversed-phase HPLC. The HPLC method is not only for qualitative analysis, but also for determining free amino acids. Every peak detected in the chromatogram was identified by comparing it to the amino acid standards. The more polar compounds will come out first with the eluent, but the more nonpolar compounds will bind to the eluent. Thus, the chemical compound with the highest retention time has the lowest polarity.



**Fig. 1.** Chromatograms (a) the fraction of pI 5 dan (b) the fraction of pI 6

There were about 19 peaks observed for the pI 5 fraction and 16 peaks for the pI 6 fraction (Figure 1). About 14 amino acid standards were implemented to identify the samples. However, the chromatograms detected more than 14 peaks. Some impurities or other amino acid compounds may be present during the identification process. Amino acid standards had concentrations of 50, 100, and 250 ppm. The mass of the amino acid in each pI was calculated by multiplying the concentration obtained from the standard graph by the volume of hydrolysis results. The amino acid content could be determined by calculating the mass

percentage of the calculation results with the mass of the analyzed sample. This quantitative calculation is presented in Table 2.

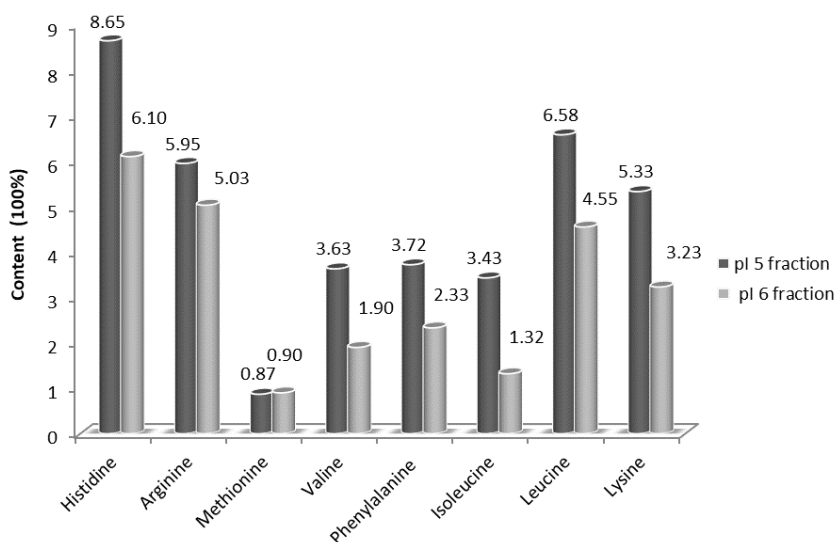
Table 2. Amino acid content in fractions of pI 5 and 6

Amino acid	Concentration (mg/L)		Mass (mg/60mg protein)		Percentage (%)	
	pI 5	pI 6	pI 5	pI 6	pI 5	pI 6
Aspartic acid	447.341	302.003	4.69	2.72	7.82	4.53
Glutamic Acid	696.472	530.614	7.31	4.78	12.18	7.97
Serine	198.205	122.301	2.08	1.10	3.47	1.83
Histidine*	494.165	407.019	5.19	3.66	8.65	6.10
Glycine	124.709	61.602	1.31	0.55	2.18	0.92
Arginine	339.842	335.655	3.57	3.02	5.95	5.03
Alanine	198.220	132.247	2.08	1.19	3.47	1.98
Tyrosine	260.709	204.151	2.74	1.84	4.57	3.07
Methionine*	49.734	59.832	0.52	0.54	0.87	0.90
Valine*	207.810	126.405	2.18	1.14	3.63	1.90
Phenylalanine*	212.820	148.464	2.23	1.34	3.72	2.33
Isoleucine*	196.147	88.861	2.06	0.79	3.43	1.32
Leucine*	376.171	303.408	3.95	2.73	6.58	4.55
Lysine*	305.077	215.394	3.2	1.94	5.33	3.23
Total amino acid			43.11	27.34	71.85	45.56
Total essential amino acid			22.90	15.16	38.16	25.26

Note: \*Essential amino acids

Fractionation in pI 5 resulted in a higher total amino acid content (71.85%) than fractionation in pI 6 (45.56%). The amino acid content of glutamic acid occupied the first position at approximately 12.18% and 7.97% at pI 5 and 6, respectively. The highest glutamic acid concentration results from a deamination reaction during acid hydrolysis that converts glutamine to glutamic acid [16].

The pI 5 fraction showed a higher EAA content of about 38.16%, whereas the pI 6 fraction contained only 25.26%. The comparison of EAA content between pI 5 and pI 6 is depicted in Figure 2. The pI 5 protein fraction consisted of 8.65% histidine, 5.95% arginine, 0.87% methionine, 3.63% valine, 3.72% phenylalanine, 3.43% isoleucine, 6.58% leucine, and 5.33% lysine. In contrast, the pI 6 protein fraction consisted of 6.10% histidine, 5.03% arginine, 0.90% methionine, 1.90% valine, 2.23% phenylalanine, 1.32% isoleucine, 4.55% leucine, and 3.23% lysine.



**Fig. 2.** The comparison of the EAA of pI 5 and pI 6 fractions.

Furthermore, the amino acids in the pI 5 fraction were generally present in higher proportions than those in the pI 6 fraction. Both fractions exhibited the highest histidine concentration, followed by leucine, whereas methionine accounted for the lowest proportion among the EAAs. The methionine content in the legume grains was inherently limited (Table 3), and hydrolysis further contributed to its reduction. Although acid hydrolysis represents a cost-effective approach, it may degrades tryptophan and results in partial methionine loss [18].

Table 3. The comparison of the EAAs between the *Samanea saman* seed fraction in pI 5 to the other legumes [17]

%EAAs	<i>Samanea saman</i> (pI 5)	Chickpea	Soybean	Lentil	Cowpea	Pea
Histidine	8.65	2.7–3.2	2.4–3	2.2–2.5	3.06–3.19	1.92–2.94
Valine	3.63	3.9–5.2	4.4–5.245	4.5–5	4.68–5.1	3.97–5.11
Methionine	0.87	0.8–1.6	1.1–2.72	0.8	1.46–2.11	0.72–1.6
Lysine	5.33	6.7–7.8	6–6.58	7–7.3	5.74–7.5	3.41–8.1
Isoleucine	3.43	4.1–5.2	4.2–5.9	3.8–4.1	3.75–3.84	3.09–4.5
Leucine	6.58	7.7–9.5	7.095–7.9	7.8	7.65–7.7	6.7–7.84
Phenylalanine	3.72	5–6.2	3.88–5.8	4.5–5	5.75–5.51	4.02–5.2

When compared with data on the EAAs of chickpea, soybean, lentil, cowpea, and pea that had been collected by [19], *Samanea saman* seed outperformed histidine values. At the same time, the other EAA contents were relatively the same and lower. This indicates that *Samanea saman* seeds have a unique nutritional profile that is rich in histidine, a crucial

amino acid. Therefore, *Samanea saman* seeds are a fairly high-protein source, particularly to meet the need for histidine.

### 3.4 The potency of protein fraction for food fortification

Certain ingredients can serve as potential sources of protein fortification in foods, depending on the quality and quantity of EAAs that meet the standards established by the FAO/WHO/UNU (2007). The evaluation of protein quality is based on the amino acid-to-protein requirement ratio (mg amino acid/g protein), which reflects the scoring pattern for protein assessment. The requirement pattern estimates the dietary indispensable amino acid needs for adults, corresponding to a protein intake of 0.66 g/kg body weight per day [10]. Quantitative analysis of amino acids provides insight into the fortification potential of a protein source by examining its amino acid score AAS, where a value greater than 1.00 indicates adequacy. This chemical score represents the effectiveness of a protein in meeting essential amino acid requirements. The calculation of the amino acid score is presented in Table 4.

Table 4. Identification of amino acid score in pI 5 and pI 6 fractions

EAAs	FAO/WHO/UNU 2007 (g/100g)	pI 5 fraction		pI 6 fraction	
		EAA content	Amino acid score	EAA content	Amino acid score
Isoleucine	3.00	3.43	1.14	1.32	0.44
Leucine	5.90	6.58	1.12	4.55	0.77
Lysine	4.50	5.33	1.18	3.23	0.72
Aromatic amino acids (Phenylalanine + Tyrosine)	3.80	(3.72 + 4.57)	2.18	(2.33 + 3.07)	1.42
Valine	3.90	3.63	0.93	1.90	0.49
Histidine	1.50	8.65	5.77	6.90	4.07
Methionine	1.60	0.87	0.54	0.90	0.56

The amino acid score evaluates the abundance of each EAA in food materials. *Samanea saman* seed demonstrated a higher content of histidine and aromatic amino acids (phenylalanine and tyrosine), as both were present in greater amounts across the evaluated fractions. In comparison, valine and methionine were the limiting EAAs in *Samanea samani* seed. The pI 5 fraction also fulfilled the requirements for isoleucine, leucine, and lysine. These findings suggest that *Samanea saman* seed has potential as a protein-rich ingredient for food fortification, particularly due to its relatively high levels of histidine and aromatic amino acids [20]. Given that histidine is an essential amino acid in infant nutrition, *Samanea saman* seed may contribute to complementary protein sources in diets for young children, though further studies are needed to confirm its suitability and safety for this age group. In addition, histidine can preserve cells through the reduction of reactive oxygen species generation that is related to red blood cell destruction [1].

## 4 Conclusion

The protein content of *Samanea saman* seed was about 34.95%. It was quite high, but the protein content was still lower than that of soybeans. The fractionation process revealed that the pI 5 fraction resulted in a higher yield than pI 6. Similar results were described by HPLC analysis that the fraction of pI 5 presented a higher EAA content than pI 6. The fractionate of *Samanea saman* seed could be a good source of protein since it consists of an abundance of EAAs. Compared to a fraction of pI 6, the fraction of pI 5 could be more potent as a source of protein fortification due to the numerous EAAs that met the requirements. Both fractions had potential scores for fortification to fulfill the need for histidine and aromatic amino acids (phenylalanine and tyrosine), but were limited in valine and methionine. In addition, the fraction of pI 5 also achieved a higher amino acid score (>1) for isoleucine, leucine, and lysine. Given its high histidine content, *Samanea saman* seed shows its potential for application in infant food formulations where histidine is nutritionally critical. It should be noted that acid hydrolysis may degrade tryptophan and partially destroy methionine, potentially underestimating their contents. Future studies could use alkaline hydrolysis or alternative methods to better assess tryptophan and provide a more complete evaluation of the protein quality of *Samanea saman* seed.

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