Proceedings of Science and Mathematics
Faculty of Science, Universiti Teknologi Malaysia
https://science.utm.my/procscimath/
Vol. 20 (2023)

Analyzing Nutritional Content and Essential Amino Acid Composition of *Perna Viridis* Mussels: Insights from Proximate Analysis and High-Performance Liquid Chromatography-Ultraviolet Analysis

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Abstract
Most consumers and cultivators of green mussels are aware that it contains high percentage of protein, but they are unaware that the protein's value is derived from essential amino acids. Proximate analysis of green mussels was carried out which resulted in 55.98 ± 0.95% of protein, 83.83 ± 2.09% of moisture, 7.28 ± 0.05% of ash, 6.86 ± 0.05% of fat and 0.09 ± 0.001% of crude fiber. In the present study, the hydrochloric acid hydrolysate of green mussels was derivatized with phenyl thiocyanate resulting phenylthiocarbamyl derivatives and separated on reverse-phase high performance liquid chromatography (HPLC) by isocratic elution with aqueous buffer and acetonitrile-water and detected in UV region. Under these conditions, the optimum HPLC method conditions were 80:20 ratio of mobile phase, pH of 6.0, 1.0 mL/min of flowrate at 254 nm. The concentration of threonine, histidine and arginine found in green mussels were 1.56µg/mg, 0.28µg/mg and 1.31µg/mg respectively.

Keywords: green mussel; proximate analysis; amino acids; HPLC

1. Introduction

Perna Viridis mussel or also known as green mussel is a commercially valued protein-rich shellfish that is mostly cultivated in the Straits of Johor (Sheng et al., 2021). The greater availability from wild and cultured settings has led to an increase in bivalve mollusk consumption in Malaysia, particularly in Johor. Mussels are one of the molluscan species that is most widely dispersed worldwide. They are simple to grow due to their quick growth, rapid reproductive rate, and robust adaptability. It is well known that eating mussels in general significantly increases intake of mineral, fatty acids, and amino acids (Bejaoui et al., 2021). Most consumers are aware that green mussels contain protein, but they are unaware that the protein's value is derived from essential amino acids. The essential amino acids are crucial and the creation of various secondary metabolites that engage in cellular responses, articulation of genes and homeostatic regulation, protein phosphorylation, hormone synthesis, and antioxidant ability begin with the essential amino acids, which are crucial for protein synthesis (Pandey et al., 2020). The synthesis of arginine, cysteine, glutamine, glycine, proline, and tyrosine may be constrained by several circumstances, making them conditionally necessary (Manta-Vogli et al., 2020). For instance, metabolic immaturity in premature newborns may restrict cysteine production (Manta-Vogli et al., 2020). Almost all essential amino acids are not natural chromophores, thus from the analytical point of view, it is difficult to detect them and requires derivatization with a UV-active chromophore to do so. The post-column approach holds the benefit of eliminating chemicals which interferes before the reaction takes place and is mostly on the separating of amino acid on cation-exchange resin (Ferré et al., 2019). Nevertheless, precolumn derivatization with revered phase-HPLC (RP-HPLC) is typically the method of choice, as it offers the benefits of quick reaction times, straightforward instrumentation, and an

affordable technique (Vojvodić Cebin et al., 2022). Derivatization has its functions to increase the hydrophobicity of amino acids and makes it possible to detect them using absorbance or fluorescence. Proline is one of the amino acids that phenyl isothiocyanate (PITC) interacts with to create phenylthiocarbamide-amino acids (PTC-AA), which when using UV-detector have an absorbance at 254 nm (Gamon et al., 2020). To date, there's no literature to be found on the nutritional content and amino acid analyses on green mussel reared in the Straits of Johor, even though Johor is the biggest rearing area in Malaysia. Thus, this study aims to investigate the nutritional and three essential amino acid content in green mussels reared in Pasir Gudang, Johor, Malaysia.

2. Materials and methods

2.1 Chemicals, apparatus, and instruments

Green mussels were obtained from Kampung Pasir Putih rearing site, Pasir Gudang (Johor, Malaysia). Proximate analysis includes the usage of drying oven, desiccator, furnace, water bath, furnace, hot plate, distillation unit and digestion block. All chemicals were the highest purity of analytical reagent grade. Hexane, hydrochloric acid (HCl), and amino acid standards were used. 1000 ppm of the stock standard mixture of amino acids was prepared and diluted down to 50 ppm. 0.14 M sodium acetate aqueous buffer and 60% of acetonitrile were used as mobile phase in HPLC analysis. Triethylamine (TEA), phenyl isothiocyanate (PITC), and HPLC grade ethanol were purchased from Sigma Aldrich (USA). An integrated process for pre-column derivatization of amino acids using PITC as derivatizing mixture were used to analyze amino acids. The derivatization mixture was made from ethanol, water, PITC, and TEA (7:1:1:1 v/v) (Pandey et al., 2020). The test tubes containing the derivatization mixture were stirred and left at room temperature for 20 mins. Sampler injector, degassing unit and solvent delivery pump make up the gradient HPLC unit (Agilent, USA). The reverse-phase column C_{18} with UV detector were used for the HPLC separation at a temperature of 40 °C and a wavelength of 254 nm.

2.2. Proximate analysis methodology

Proximate analysis was conducted in an accredited external analytical service laboratory (Johor, Malaysia). The methodology for proximate analysis were adopted from Association of Official Analytical Chemists (AOAC method) (Cunniff, 1998). For the determination of moisture, empty dish was heated at 95-100°C. The dish was cooled and weighed before use. Well mixed test portion was weighed into the dish. The dish was dried, covered and contented for 5 hours in oven provided with opening for ventilation and was maintained at 95-100°C. The dish was covered while still in oven, the dish was transferred to desiccator and weighed soon after reaching room temperature. The residue was reported as total solids and loss in weight as moisture. For the determination of ash, an empty crucible was ignited, cooled, and weighed before use. A well-mixed test portion was placed into the crucible. The sample was charred on a hot plate until it stopped smoking to prevent sample splattering. The crucible was then ignited in a furnace at approximately 550°C to 600°C until a light gray ash residue or a constant weight was achieved. The residue was cooled in a desiccator and weighed soon after reaching room temperature. For the determination of fat by acid hydrolysis, 10 ml of alcohol was added and stirred to moisten all particles and prevent lumping upon acid addition. Subsequently, 10-15ml of HCL was added and mixed well. A conical flask was placed in a water bath at 70-80°C for 30-40 minutes, stirring at frequent intervals. The digestion was considered complete when the solution turned dark brown. After cooling, 10 ml of alcohol was added, and the mixture was transferred to a separating funnel. The beaker was rinsed into the separating funnel with 25 ml of ethyl ether in three portions, and the flask was stoppered and shaken vigorously for 1 minute. Then, 25 ml of petroleum ether was added. The solution was allowed to stand until the upper liquid became practically clear. The ether-fat solution was filtered to allow ether to pass freely into a pre-weighed 25 ml conical flask containing boiling chips. The liquid was re-extracted in the separating funnel twice, each time with 15 ml of petroleum ether, and the clear ether solution was filtered into the same beaker, ensuring the tips of the funnel were washed. The ethers were evaporated slowly on a steam bath, and the fat was dried in an oven at 100°C until a constant weight was reached. The flask was removed, placed in a desiccator, and weighed. This weight was corrected by blank determination using the reagents used. For the determination of crude fiber, 2-5g of the sample was weighed and transferred to a 1 L beaker. Then, 200 ml of 1.25% H_2SO_4 was added. The flask was heated until the contents started to boil for exactly 20 minutes, with periodic checks to

prevent solids from adhering to the sides. The contents were filtered through pleated gauze placed in a filter funnel. The flask was rinsed thoroughly, and the residue was washed on the gauze until the washings were acid-free, as indicated by litmus paper. The gauze was allowed to drain well, and the residue was returned to the beaker. Subsequently, 20 ml of 1.25% NaOH was added, and the flask was heated until the contents started to boil, boiling for exactly 30 minutes. The flask was checked periodically to prevent solids from adhering to the sides. The contents were filtered through pre-weighed ashless filter paper placed in the filter funnel, rinsed thoroughly with hot water, and the residue was rinsed properly until the washings were acid-free, as indicated by litmus paper. The residue and the ashless filter paper were transferred to an ashing dish and dried for 2 hours at 130°C. The residue was cooled in a desiccator, weighed, and ignited at 550°C. After cooling in the desiccator, it was reweighed. Blank determination was performed on reagents used for correction. For the determination of protein, 0.5 g portions were weighed into a digestion flask. A tablet of catalyst, consisting of 3.5 g of K_2SO_4, and 15 ml H_2SO_4 were added to each flask. The digestion tubes were placed in a tube rack and heated to 420°C until a clear and blue-green solution was obtained. After completion of the digestion process, the contents were allowed to cool to room temperature, and 50 ml of deionized water was added. A distillation unit with tap water inlet outlet system was used. A 250 ml conical flask was placed in position, and the clamping device was secured. The digestion tube was placed into position with a tight fit against the Viton cone. The protection door was closed, and the distillation process was initiated. The display unit showed the distillation sequence of chemical additions, distillation time, and waste suction process during the run. Once the program had finished, the digestion tube was removed by lowering the quick clamping device. The 250 ml conical flask containing the distillate was also removed for titration. Correction was performed for blank determination on reagents, and boric acid solution was titrated with 0.1 M HCl using a burette.

2.3 Pre-column derivatization and HPLC-UV methodology

6 mL of 6 N HCL was used to dissolve 100 mg of dried mussels. At 110°C, the solution was digested for 20–24 hours. 4000 µL of sample (50 ppm) were mixed with 400 µL of the derivatizing solution and was vortexed before been stored in the duct for an hour. The excess PITC were removed with 1 mL of hexane, which was then vortexed and allowed to stand for 10 minutes. Hexane layer was eliminated after shaking. Filtering 0.125 µL of the bottom layer from the aqueous layer, followed by the addition of 0.375µL of diluents (0.1M HCL), proper shaking, and filtering through PTFE 0.45µ membrane. Additionally, 25µL of the filtrate were taken for HPLC analysis. The optimization of HPLC-UV method was carried out to enhance optimum conditions for the analysis of amino acids. Several experimental parameters such as flowrate, wavelength, and the pH of mobile phase were optimized and evaluated.
The optimized method was evaluated based on the linearity of the graph, the limit of detection (LOD), the limit of quantification (LOQ), precision as well as recovery in order to produce the reliability of analytical results.

3. Results and discussion

3.1 Proximate analysis of green mussel

Table 1 presents the proximate analysis results of green mussels, revealing key nutritional components. This information can be invaluable for both consumers and nutritionists seeking to make informed dietary choices. The moisture content, measuring at 83.83 ± 2.09%, holds significant importance in food products as it directly influences the final quality of the product. Maintaining an appropriate moisture level is crucial to ensure the desired texture, taste, and shelf life of the mussels if the mussels are canned. Moving on to the ash content, which was determined to be 7.28 ± 0.05%, this parameter provides insights into the total mineral or inorganic residue that remains after either ignition or the complete oxidation of organic matter in food items. Monitoring ash content is essential for assessing the nutritional composition and quality of the mussels. In the case of fat content, our analysis revealed a level of 6.86 ± 0.05%. It's noteworthy that a balanced diet necessitates a certain amount of fat, as it serves as a vital source of essential fatty acids. These essential fatty acids are critical for the body, as it cannot produce them independently. Hence, understanding the fat content in green mussels contributes to the broader perspective of their nutritional value. Turning to the crude fiber content, which measured at 0.09 ± 0.001%, this parameter plays a significant role in food, as it promotes the production

of beneficial intestinal bacteria and supports the digestive process. Recognizing the presence of crude fiber in green mussels underscores their potential health benefits beyond the basic nutritional components. Lastly, the protein content was found to be substantial at 55.98 ± 0.95%. Protein is the most abundant of all nutrients discovered in green mussels. It is a fundamental component of the human body, playing a crucial role in various physiological processes and the maintenance of overall health. Notably, the concentration of essential amino acids, which are indispensable for human nutrition, can be inferred from the protein content, shedding light on the comprehensive nutritional profile of green mussels.

Table 1. The results of proximate analysis of green mussel sample reared in Kampung Pasir Putih, Pasir Gudang (Johor, Malaysia)

Protein (%)	Moisture (%)	Ash (%)	Fat (%)	Crude Fiber (%)
55.98 ± 0.95	83.83 ± 2.09	7.28 ± 0.05	6.86 ± 0.05	0.09 ± 0.001

3.2 HPLC-UV analysis of selected essential amino acids in green mussels

An HPLC-UV method was developed and optimized for the analysis of three essential amino acids (i.e., threonine, histidine, and arginine) in green mussels. Figure 1a-c shows the graph of peak area when three parameters were optimized in the HPLC-UV instrument.

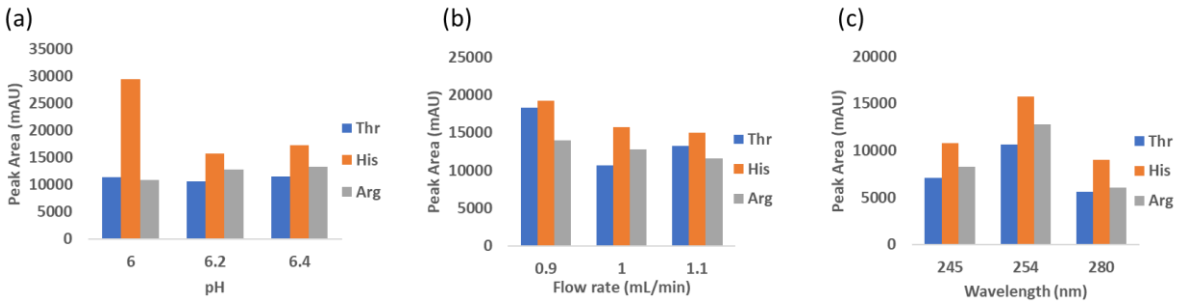

Figure 1. The graph of peak area for the optimization of (a) pH, (b) flow rate and (c) wavelength within HPLC-UV instrument.

Figure 1a represented the trend of the peak area for threonine, histidine, and arginine in the pH range within 6.0 – 6.4. The effect of mobile phase pH was an important variable in determining selectivity, peak shape, and retention time. During the pH adjustment, acetic acid was used to reduce the pH, while NaOH was added to increase the pH. pH 6.2 yielded the highest signal. pH values of 6.0 and 6.4 resulted in poor resolution and tailing, respectively. Peak tailing might have been caused by column “secondary interactions,” column contamination, column aging, or column loading. To address this, a test was conducted to determine if the column was dirty or damaged. Hence, pH 6.2 was selected for further experiments to rectify the compromised condition of the three essential acids. Flow rate was optimized within the range of 0.9 to 1.1 mL/min. A flow rate higher than the optimum could adversely affect chromatography quality by not allowing sufficient time for analyte interaction with the stationary phase. Conversely, a flow rate lower than the optimum might result in extended waiting for the peak to appear at the detector. From Figure 1b, it was observed that the retention time was inadequate at 0.9 mL/min, and resolution was suboptimal at 1.1 mL/min, indicating poor separation of peaks. As 1.0 mL/min provided the highest resolution compared to other flow rates, it was chosen for subsequent optimization steps. The wavelength of the UV detector was optimized to determine the wavelength at which the analytes exhibited maximum absorption. This minimizes spectral interference arising from detector measurement errors and maximizes method sensitivity. Figure 1c presented signals at wavelengths of 245 nm, 254 nm, and 280 nm. In this study, the retention times of the three essential amino acid peaks were nearly identical. The peaks at a wavelength of 280 nm had the highest resolution but the lowest peak area. At 245 nm, the peak area was larger compared to peaks at 280 nm, but there was less spacing between the peaks. At 254 nm, the peak area was the highest, and the peak was

narrower, indicating good efficiency, despite poorer resolution and selectivity, meaning that the peaks were not adequately separated. Hence, wavelength 254 nm was selected due to its superior efficiency and peak area.

The HPLC method was validated using all optimum conditions as established during the optimization process. These optimal conditions, including a mobile phase ratio of 80:20, a pH of 6.2, a flow rate of 1.0 ml/min, and a wavelength of 254 nm, were applied to the analysis of essential amino acid standards. Matrix-matched calibration was constructed for each essential amino acid, involving a series of spiked threonine, histidine, and arginine solutions in the concentration range of 2-10 ppm. The correlation in the calibration plots was linear (determination coefficient (R^2) values of 0.9938, 0.9904, and 0.9991 for threonine, histidine, and arginine, respectively). These high R^2 values indicate the accuracy and reliability of the HPLC method for quantifying these essential amino acids. Furthermore, the limits of detection (LODs) were determined to be 1.71 ppm for threonine, 2.95 ppm for histidine, and 0.71 ppm for arginine, demonstrating the method's sensitivity. The limits of quantification (LOQs) were calculated as 5.69 ppm for threonine, 9.85 ppm for histidine, and 2.36 ppm for arginine, indicating the lowest concentrations at which these amino acids can be reliably quantified. To assess the precision of the method, intra-day and inter-day studies were conducted. In the intra-day precision study, repeatability was assessed by analyzing three essential amino acid standard solutions at a concentration of 6 ppm five times within a single day (n=5). In the inter-day precision study, repeatability was evaluated by analyzing the same standard solutions at 6 ppm five times over the course of three days (n=5). The results of these precision studies are summarized in Table 2.

Table 2. Intraday and interday data for threonine, histidine and arginine standard analytes.

Amino acid		Intra day			Inter day		
	Concentration (ppm)	Observed (ppm)	Accuracy (%)	Precision (%)	Observed (ppm)	Accuracy (%)	Precision (%)
Threonine	6	4.46±0.28	25.67	6.23	4.54±0.3	24.33	6.57
Histidine	6	5.76±0.29	4	5.11	5.35±0.55	10.83	10.35
Arginine	6	4.41±0.73	26.57	16.57	3.72±0.65	37.94	17.43

When the validated HPLC method was applied to the green mussel sample, the concentrations of threonine, histidine, and arginine were determined to be 1.56 µg/mg, 0.28 µg/mg, and 1.31 µg/mg, respectively. These findings provide valuable insights into the nutritional content of green mussels, specifically in terms of these essential amino acids, which are essential for human health and well-being.

4. Conclusion

In conclusion, this study shows a comprehensive analytical approach for analyzing the nutritional content and the essential amino acid composition in green mussels. The proximate analysis revealed vital nutritional parameters, including protein, moisture, ash, fat, and crude fiber content, in green mussels. Subsequently, a meticulous optimization process fine-tuned the HPLC method, pinpointing the ideal conditions such as mobile phase pH, flow rate, and wavelength. Notably, the method exhibited robust linearity, with high determination coefficients (R^2) for threonine, histidine, and arginine, signifying its accuracy and reliability. The sensitivity of the method was demonstrated through low LODs and LOQs values, underscoring its capability to detect even trace amounts of these essential amino acids. Intra-day and inter-day precision assessments reinforced the method's repeatability and robustness.

Acknowledgement

A.S.A.K is grateful for the financial support of the work by the Ministry of Higher Education Malaysia (MOHE) and Universiti Teknologi Malaysia via Fundamental Research Grant Scheme (registration

number: FRGS/1/2022/STG04/UTM/02/1, cost centre number: R.J130000.7854.5F528, reference number: PY/2022/03336).

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