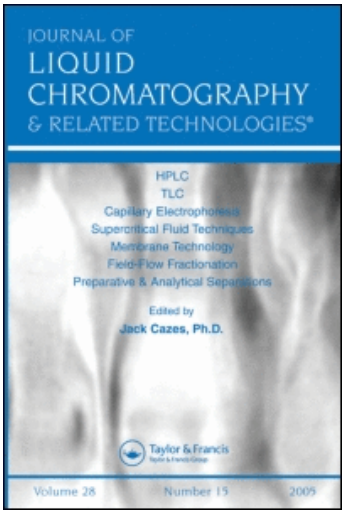

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[a] Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, Sonora, México [b] Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora, Hermosillo, Sonora, México [c] Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Sinaloa, México

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Quantitative HPLC Analysis of Riboflavin and Aromatic Amino Acids in Three Forms of Shrimp Hydrolysates

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[1]Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, Sonora, México
[2]Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora, Hermosillo, Sonora, México
[3]Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Sinaloa, México

Abstract: This paper presents a high performance liquid chromatography (HPLC) method for the determination of riboflavin and free aromatic amino acids. To determine riboflavin, the method includes an acid hydrolysis (HCl 0.1 N) followed by an enzymatic digestion and a protein precipitation with trichloroacetic acid. An analytical column, Chrom SEP SS C18 5 μm (4.6 mm × 150 mm) was used with an isocratic flow using a mobile phase consisting of 5 mM ammonium acetate-methanol (72:28, vv^{-1}), at a flow rate of 1.0 mL min^{-1} to 28°C. The method showed adequate linearity, repeatability, reproducibility, accuracy, and limits of detection. The method was used to quantify the cited analytes in three different forms of hydrolysate obtained from fermented shrimp remnants.

Keywords: Aromatic amino acids, Fluorescence detection, HPLC, Hydrolysate, Riboflavin, Shrimp remnants

Correspondence: D. I. Sánchez-Machado, Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, 5 de febrero 818 Sur, CP 85000, Cd. Obregón, Sonora, México. E-mail: dsanchez@itson.mx

INTRODUCTION

The aromatic amino acids, tyrosine, phenylalanine, and tryptophan, offer intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions.[1] Tyrosine is a non-essential amino acid that helps to regulate temperament and stimulates the nervous system, as well as being a precursor of dopamine and other catecholamines.[2] The body needs adequate supplies of tyrosine to generate many important brain chemicals that help regulate appetite, monitor pain sensitivity, and support the body's response to stress.[3] It was also found that if there is a lack or a low amount of tyrosine in the diet, the phenylalanine would be converted into tyrosine.[4] The second amino acid in the group of three is phenylalanine, whose requirements are influenced by the level of tyrosine in the diet. Phenylalanine is an essential amino acid that is required for normal functioning of the central nervous system.[5] Phenylalanine has been used successfully to help control symptoms of depression and chronic pain, as well as other diseases linked to a malfunctioning central nervous system.[6] The third, tryptophan, is indispensable due to its diversity of biological functions. Tryptophan is the precursor of the neurotransmitter serotonin and the vitamin niacin,[7] and is also responsible for many brain functions and plant growth regulation.[8] The determination of the quantity of tryptophan presents several analytical problems. This is due to the labile nature of tryptophan in the presence of light and hydrogen ions.[9]

Due to their natural fluorescence, free aromatic amino acids are usually analyzed without derivatization, which gives quick and easy detection.[10] Several methods for the quantification of these amino acids have been developed for pure protein and feed protein involving ion exchange column chromatography or reversed phase high performance liquid chromatography with fluorescence and UV detection, other methods used are gas chromatography and capillary electrophoresis.[11,12] Methods using fluorescence detection are generally more sensitive and detect fewer interfering compounds than those using UV detection. The use of HPCL coupled with fluorimetric detection has enabled specific and sensitive methods to be developed for the determination of these compounds.[13.14]

In comparison, riboflavin (vitamin B_2), a water soluble vitamin of complex B, is a precursor of coenzymes (flavin mononucleotide and flavin-adenine dinucleotide), which are involved in the reactions of intermediary metabolism related to energy production and redox.[15,16] These reactions are indispensable for normal growth, self maintenance, and good performance of human and animal bodies.[17] The extraction of riboflavin for analytical purposes normally involves an acid hydrolysis treatment (hydrochloric acid is the agent most often used).[15] This is

followed by enzymatic digestion (takadiastase, clarase, or amylase),[18] and the proteins are sometimes removed by precipitation with trichloroacetic acid.[19] The standard AOAC methods for the quantification of riboflavin are seldom used, due to the presence of interferences caused by nonvitamin compounds.[20] Fluorimetry, spectrophotometry, and microbiological methods have been used for the identification and quantification of riboflavin, but these methods are tedious, and time consuming.[21] Reversed phase liquid chromatography has been recommended for the determination of riboflavin. This process employs an ultraviolet detector or a fluorescence detector because of their intense fluorescence.[16] HPLC methods have the advantage of being more specific and non-destructive, having a shorter analysis time and having the capability of differentiating between different forms of a vitamin with varying biological activity.[18,22]

During the processing of shrimp, for its subsequent freezing and marketing, a large amount of remnants are generated due to the fact that 35% of the animal is inedible and discarded. These remnants are composed of cephalothorax and exoskeleton. Shrimp remnants have been identified as an animal protein source of great potential; and also identified as an important source of chitin and asthaxanthin.[23] Lactic acid fermentation has been reported to be a workable and cheap technique, which will stabilize and retain the nutritional quality of the remnants.[24] With this technique, protein hydrolysates, chitin, minerals, and lipids can be recovered.[25] Several studies in relation to protein hydrolysates from seafood waste have focused on the amino acid composition, however, there are no reported studies for riboflavin content in shrimp remnants.

The purpose of the present study was to develop and validate a method for the determination of riboflavin and aromatic amino acids in three forms of shrimp protein hydrolysate using liquid chromatography. The relatively simple analytical procedure requires an initial sample preparation step for liquid-liquid extraction and subsequent reversed phase HPLC analysis with fluorescent detection. Additionally, it is important to point out that the HPLC-FL method reported here employs the native fluorescence of those compounds with the same chromatographic conditions, only varying the wavelengths specific to each compound. Accordingly, the methodology described here is expected to be of interest not only for analysis food, but also to researchers interested in byproducts or natural products.

EXPERIMENTAL

Chemicals

Amino acid and riboflavin (vitamin B_2) standards were purchased from Sigma (St. Louis, MO, USA); HPLC grade methanol obtained

from EDM (Darmstadt, Germany), and ammonium acetate from Merck (Darmstadt, Germany). Acetic acid, hydrochloric acid, ethanol, and sodium acetate were supplied by Fermont (Monterrey, Nuevo León, México). Trichloroacetic acid was from Fluka (Seelze, Germany). Clara-diastase was from Fluka (Buchs, Switzerland). All aqueous solutions were prepared with ultrapure water purified with a NANOpure Diamond UV system (Barnstead International, Dubuque, Iowa, USA).

The concentrations of solutions used were as follows: hydrochloric acid, 0.1 N; ammonium acetate, 0.005 M; sodium acetate, 2.5 M; clara-diastase, 6% in water. The solutions were always prepared on the day of use, and stored under refrigeration.

Stock standard solutions of tyrosine (203 $\mu g\,mL^{-1}$), phenylalanine (204 $\mu g\,mL^{-1}$), and tryptophan (217 $\mu g\,mL^{-1}$) were prepared in 0.1 N hydrochloric acid and a stock standard solution of riboflavin (30 $\mu g\,mL^{-1}$) by dissolution in 0.02 N acetic acid. All stock solutions were protected from the light and stored in a refrigerator. Working solutions for amino acids and riboflavin were prepared daily from those solutions and diluted with 0.1 N hydrochloric acid and 0.02 N acetic acid, respectively.

Apparatus

The HPLC system was equipped with an auto injector 410, a solvent degasser MetaChem Technologies (Torrance, CA, USA), a system controller with Galaxie Workstation for chromatography data analysis, a pump 230, and a fluorescence detector 363, all of Varian (Melbourne, Victoria, Australia).

Chromatographic analysis was performed using an analytical scale (4.6 × 150 mm) Chromsep SS C18 column with ChromSep guard column SS 10 × 3 mm and a particle size 5 µm from Varian (Melbourne, Victoria, Australia). The HPLC conditions were as follows: the mobile phase was a mixture of 5 mM ammonium acetate-methanol (72:28 $v\,v^{-1}$) filtered through a 0.45 µm membrane and degassed at a flow rate of 1.0 $mL\,min^{-1}$, column temperature was 28°C. The volume of injection was 50 µL and 100 µL for aromatic amino acids and riboflavin, respectively.

For the detection of free tyrosine, phenylalanine, and tryptophan, the photomultiplier (PMT) voltage was low, while the voltage for riboflavin the PMT was medium. The fluorescence was recorded at the optimal wavelength for tyrosine ($\lambda_{ex} = 274$ nm and $\lambda_{em} = 304$ nm) for 2.2 min, followed by the optimal wavelength for phenylalanine ($\lambda_{ex} = 254$ nm and $\lambda_{em} = 282$ nm) for 2.9 minutes. Finally, the optimal wavelength for tryptophan ($\lambda_{ex} = 280$ nm and $\lambda_{em} = 348$ nm) was another 4.1 min, with a total time analysis of 7 min. The optimal wavelength for riboflavin was $\lambda_{ex} = 450$ nm and $\lambda_{em} = 525$ nm, the analysis time was 12 min.

Sample

For the production of liquid protein hydrolysate, shrimp (*Penaeus* spp.) remnant samples (heads and cephalothoraxes) were used. Slightly thawed, minced remnants were fermented at 30°C for 36 h. The silage was centrifuged (5°C) at 1250 rpm for 15 minutes to obtain the chitin-rich fraction (sediment), the liquid protein hydrolysate, and the lipid fraction.[26]

The analyzed samples were dry powder, concentrated paste, and liquid protein hydrolysate. In producing the dry powder, the liquid hydrolysate, rich in protein, was dehydrated using a spray dryer SD-04 Lab Scale Spray Drier (LabPlant, Huddersfield, West Yorkshire, England). The liquid hydrolysate was transferred to a conical flask and placed in an electric grill heated to a constant 80°C. The temperature of the air inlet was 180°C and the air outlet was 140°C. The speed of the peristaltic pump was minimized to produce a slow flow of fluid input ($1\,L\,h^{-1}$); the flow of air in the chamber was 100%. The dry sample was collected in glass bottles with lids. The concentrated paste was prepared in a design level laboratory, which consisted of two moisture retaining traps, prepared with silica and cotton. Both were connected to a pump to generate a vacuum. An 800 mL sample was placed in a conical flask to be heated in an electric grill at 80°C, which will reduce the volume by half in about 1 hour.[24] All samples were stored in amber bottles and were kept in the dark until use.

Sample Preparation

Extraction of Free Aromatic Amino Acids

For the determination of free tyrosine, phenylalanine, and tryptophan in the analyzed samples, 1 g of liquid protein hydrolysate, 500 mg of concentrate paste, and 100 mg of dry powder were each placed in a 50 mL volumetric flask and diluted with ultrapure water, then sonificated for 90 seconds. For the quantification of tyrosine in the concentrated paste, the filtrate was diluted to a concentration of $1\,mg\,mL^{-1}$.

Extraction of Riboflavin

To extract riboflavin, acid hydrolysis and enzymatic digestion was performed as instructed by Sánchez-Machado et al.[27] with some modification. For each specific analysis, 5 g of liquid protein hydrolysate, 500 mg of dry powder, and 600 mg of concentrate paste were placed in amber colored flasks. Then, 10 mL of 0.1 N HCl was added and the mixture was heated in a water bath at 100°C for 30 min. After being allowed to

cool to room temperature, it was brought to pH 4.3–4.7 with 2.5 M sodium acetate treated with 1.25 mL of 6% aqueous solution of clara-diastase. Following an incubation period in an oven at 50°C for 3 h, 1 g of 100% trichloroacetic acid was added and sonificated for 15 min. Next, the solution was centrifuged at 2400 rpm for 10 min and filtered through Whatman No.41 paper (Maidstone, England), and finally diluted to 25 mL in a volumetric flask with ultrapure water. The resulting solution was filtered with a 0.45 μm membrane and was injected into the column of the HPLC system.

Statistical Analysis

For statistical analysis, the computer program was SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). The relative standard deviation (RSD %) is the ration standard deviation/average expressed as a percentage.

RESULTS AND DISCUSSION

Sample Preparation

Free Aromatic Amino Acids Extraction

For free tyrosine, phenylalanine, and tryptophan extraction, the sample preparation method was simple and consisted of diluting the liquid protein hydrolysate, dry powder, and concentrated paste in water. To determine the amount of sample to be used in the analysis, the assays were made from different concentrations of liquid protein (10, 20 y 30 mg mL^{-1}), dry powder (2, 4, 20 mg mL^{-1}), and concentrated paste (6, 10, 20 mg mL^{-1}). Also, the lengths of time of sonification varied (0, 30 y 90 seg). The optimum amounts of sample used for the analysis were 20, 2, and 10 mg mL^{-1}, for liquid protein hydrolysate, dry powder, and concentrated paste, respectively.

Optimum Conditions of Riboflavin Extraction

In the extraction of riboflavin, several methods have been employed. For example, an acid hydrolysis (0.1 N or 5 N HCl; 0.1 N H_2SO_4),[21,28] followed by an enzymatic digestion (clara-diastase, papain, pepsin, takadiastase)[18,19] guarantees the complete hydrolysis of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to riboflavin. Some researchers recommend a protein precipitation at the end of the process with trichloroacetic acid.[15,18] To determine the optimal

conditions of extraction, preliminary assays were performed. For the determination of the amount of sample to be used in the extraction, different concentrations of liquid protein hydrolysate (40, 120 y 200 mg mL^{-1}), dry powder (10, 20, 40 mg mL^{-1}), and concentrated paste (12, 24 y 40 mg mL^{-1}) were used. The assay indicated that the amounts of 200, 20 y 24 mg mL^{-1} from liquid protein hydrolysate, dry powder, and concentrated paste, respectively, are adequate for the analysis.

The volumes of 0.1 N HCl (5, 10, and 15 mL) and 6% clara-diastase (1.25, 1.5, and 2 mL), the time of enzymatic digestion (3, 5, and 7 h), the concentration of 100% TCA (0.5 and 1 g), and the time of sonification after the addition of TCA (5, 10, and 15 min) were optimized. The temperature of hydrolysis and concentration of HCl (0.1 N) and clara-diastase (6%) have been recommended in early studies.[27] The best results were archived using 10 mL of 0.1 N HCl (100°C, and 30 min) and 1.25 mL of 6% clara-diastase (50°C for 3 h). The addition of 1 g of TCA 100% (sonificated for 15 min), and followed by centrifuging and filtration is adequate for a complete protein precipitation, which obtains an optimal riboflavin peak resolution.

Identification and Separation

The optimal chromatographic conditions were determined from the results of preliminary trials with standards. In the separation and quantification of the riboflavin, various researchers have used, as a mobile phase, acetonitrile-10 mM phosphate buffer pH 5.5 (13:87);[20] methanol-potassium phosphate buffer pH 7 (10:90)[18] and methanol-5 mM sodium acetate buffer pH 5 (28:72).[29] However, for aromatic amino acid identification, methanol-40 mM sodium acetate buffer (20:80)[26] and methanol-70 mM sodium acetate buffer pH 4.5 (20:80)[30] were used. The trials were performed using two buffer solutions, 40 mM sodium acetate and 5 mM ammonium acetate (pH 4.6, 5.2, and 6.9). Three different eluent proportions with methanol were tested (72:28, 75:25, 85:15) with three mobile phase flow rates (0.7, 1.0, 1.3 mL min^{-1}) and three different column temperatures (28, 32, 35°C). The best resolution was obtained with methanol-5 mM ammonium acetate pH 6.9 (28:72) at 28°C and a flow rate of 1.0 mL min^{-1}.

Riboflavin and aromatic amino acids were identified through the comparison of their retention times with those times obtained from stock standard solutions. When determining retention times, the stock solutions from tyrosine, phenylalanine, and tryptophan were injected, both individually and as a mixture. In contrast, the stock standard solution of riboflavin was injected individually. Figure 1 shows the chromatograms of free tyrosine, phenylalanine, and tryptophan of a liquid protein

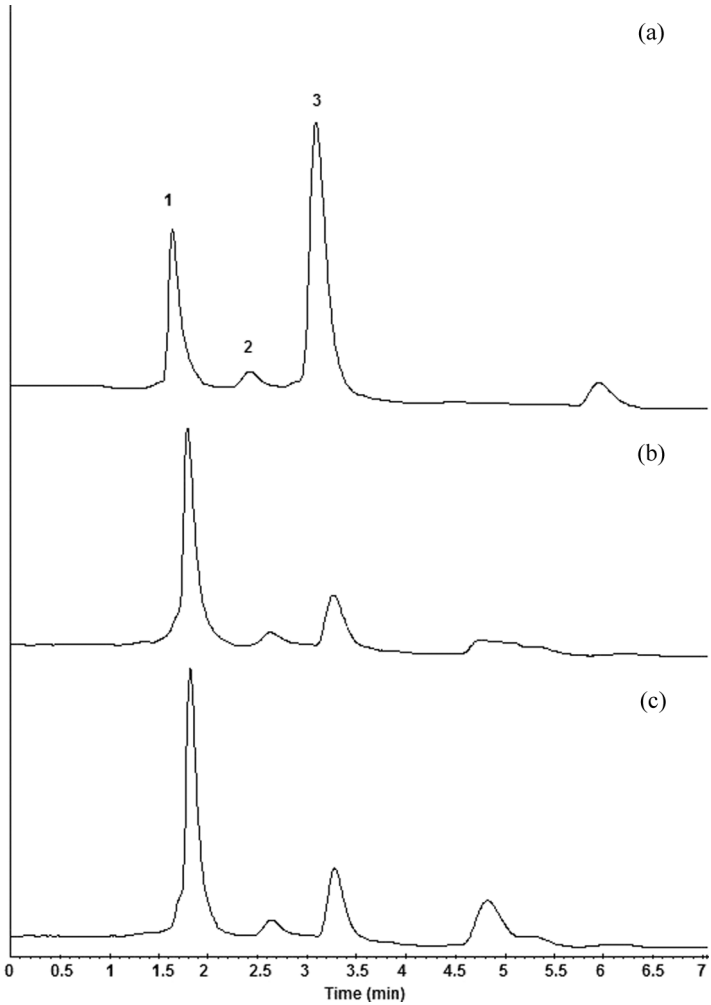

Figure 1. HPLC-FL chromatograms of samples and extracts of the liquid protein hydrolysate (a), concentrated paste (b), and dry powder (c) from fermented shrimp remnants. Peaks identified: (1) tyrosine, (2) phenylalanine, and (3) tryptophan.

hydrolysate, dry powder, and concentrated paste from fermented shrimp remnants. Figure 2 shows the HPLC-FL chromatograms of riboflavin in liquid protein hydrolysate, dry powder, and concentrated paste. Peaks were observed at 1.81 ± 0.08 min for tyrosine, 2.63 ± 0.01 min for phenylalanine, and 3.24 ± 0.06 min for tryptophan, while riboflavin peaked at 9.84 ± 0.21 min, for an average of 12 injections.

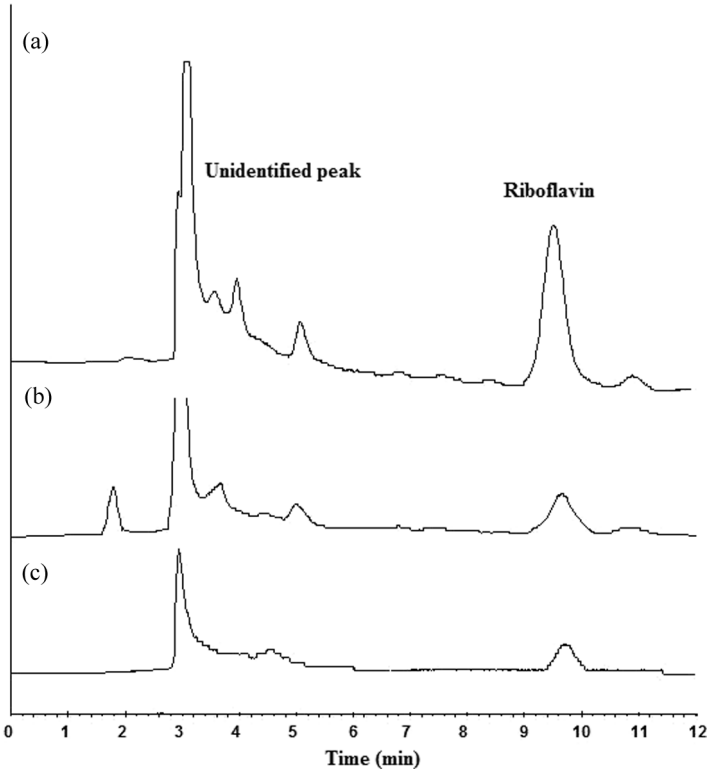

Figure 2. HPLC-FL chromatograms of riboflavin liquid protein hydrolysate (a), concentrated paste (b), and dry powder (c) from fermented shrimp remnants.

Method Validation

Linearity was calculated using stock standard solutions of tyrosine, phenylalanine, tryptophan, and riboflavin of a known concentration. The calibration curve for each aromatic amino acid and riboflavin were constructed using six concentrations ranging in concordance with the level of the compounds found in the analyzed samples (powder, concentrated paste, and liquid protein hydrolysate). In all curves, the relationships between concentrations and peak area were linear, with coefficients of determination greater than 0.999. Table 1 shows regression equations for the calibration plots for tyrosine, phenylalanine, tryptophan, and riboflavin.

The precision study was comprised of repeatability and reproducibility studies. A total of eight procedures were performed in replicate on a sample, which were performed under optimum conditions to determine

Table 1. Linearity for tyrosine, phenylalanine, tryptophan and riboflavin analyzed

Compound	Range ($\mu g\,mL^{-1}$)	Equation	r^2
Tyrosine	1.62–16.24	$y = 70.48x + 1.621$	0.9994
Phenylalanine	9.79–97.92	$y = 2.298x + 11.145$	0.9992
Tryptophan	1.73–17.36	$y = 100.04x + 9.391$	0.9991
Riboflavin	0.012–0.96	$y = 2393.2x + 9.123$	0.9992

x, amount ($\mu g\,ml^{-1}$); y, peak area; r^2, determination coefficient.

repeatability. And three replicate analyses of the same sample were made on different days to determine reproducibility. Table 2 shows precision results. The relative standard deviations (RSD) for the determination of tyrosine, phenylalanine, tryptophan, and riboflavin were less than 7% for all compounds. Considering these RSD values, the reproducibility and repeatability are satisfactory. These results indicate that the present method can be used for quantitative analyses of free aromatic amino acids and riboflavin in a protein hydrolysate from the fermentation of shrimp remnants.

Table 2. Precision of the proposed method for determination of free aromatic amino acids and riboflavin

	Repeatability ($n = 8$)		Reproducibility ($n = 3$)	
Compound	Mean ± SD[a]	RSD (%)	Mean ± SD[a]	RSD (%)
Liquid protein hydrolysate				
Tyrosine ($mg\,g^{-1}$)	1.19 ± 0.02	1.59	1.17 ± 0.05	4.40
Phenylalanine ($mg\,g^{-1}$)	15.66 ± 0.58	3.71	15.21 ± 0.71	4.71
Tryptophan ($mg\,g^{-1}$)	2.52 ± 0.03	1.21	2.47 ± 0.08	3.59
Riboflavin ($\mu g\,g^{-1}$)	3.36 ± 0.06	1.87	3.22 ± 0.11	3.63
Dry powder				
Tyrosine ($mg\,g^{-1}$)	6.60 ± 0.05	0.82	6.07 ± 0.40	6.56
Phenylalanine ($mg\,g^{-1}$)	17.37 ± 0.29	1.72	16.32 ± 0.68	4.20
Tryptophan ($mg\,g^{-1}$)	1.42 ± 0.01	1.06	1.30 ± 0.05	4.34
Riboflavin ($\mu g\,g^{-1}$)	0.63 ± 0.00	0.95	0.64 ± 0.02	2.69
Concentrated paste				
Tyrosine ($mg\,g^{-1}$)	7.44 ± 0.27	3.65	7.55 ± 0.26	3.42
Phenylalanine ($mg\,g^{-1}$)	7.04 ± 0.13	1.97	7.05 ± 0.13	1.92
Tryptophan ($mg\,g^{-1}$)	0.68 ± 0.01	2.47	0.70 ± 0.01	2.71
Riboflavin ($\mu g\,g^{-1}$)	1.11 ± 0.05	4.72	1.16 ± 0.00	0.84

[a]Results expressed as dry mass.

Table 3. Accuracy of the assay for free aromatic amino acids and riboflavin

Compound	Mean value[a]	Amount addition[a]	Amount found[a]	Recovery (%)[b]	RSD
Liquid protein hydrolysate					
Tyrosine ($mg\,g^{-1}$)	1.20	0.46	1.63	93.39 ± 1.75	1.87
Phenylalanine ($mg\,g^{-1}$)	15.39	2.30	17.64	97.77 ± 2.89	2.96
Tryptophan ($mg\,g^{-1}$)	2.50	0.49	2.98	96.12 ± 0.78	0.81
Riboflavin ($\mu g\,g^{-1}$)	3.37	2.17	5.46	96.51 ± 1.26	1.31
Dry powder					
Tyrosine ($mg\,g^{-1}$)	6.46	3.42	9.50	88.81 ± 0.01	1.14
Phenylalanine ($mg\,g^{-1}$)	17.66	4.23	21.89	99.87 ± 5.73	5.74
Tryptophan ($mg\,g^{-1}$)	1.50	0.90	2.39	99.11 ± 2.00	2.02
Riboflavin ($\mu g\,g^{-1}$)	0.63	0.63	1.25	97.76 ± 3.21	3.29
Concentrated paste					
Tyrosine ($mg\,g^{-1}$)	7.06	2.33	9.31	96.48 ± 1.98	2.05
Phenylalanine ($mg\,g^{-1}$)	7.26	1.17	8.41	98.59 ± 6.98	7.08
Tryptophan ($mg\,g^{-1}$)	0.70	0.25	0.94	98.69 ± 1.76	1.79
Riboflavin ($\mu g\,g^{-1}$)	1.12	1.44	2.51	98.26 ± 2.13	2.17

[a]Results expressed as dry mass.
[b]Percent recovery = (amount found − mean value)/(amount added) × 100.

The detection limits (three times the basis of signal-to-noise ratio, as per American Chemical Society guidelines, 1980)[31] for tyrosine, phenylalanine, tryptophan, and riboflavin were 10, 90, 10, and $20\,ng\,mL^{-1}$, respectively, which are smaller than those presented in other works.[15,17]

Accuracy was estimated by means of recovery assays. For the recovery, six samples of dry powder, concentrated paste, and liquid protein hydrolysate were spiked with known concentrations of the three aromatic amino acids and riboflavin prior to extraction, and quantitation. Table 3 shows the recovery of free tyrosine, phenylalanine, tryptophan, and riboflavin. These results are similar to those reported in previous works of free amino acids and riboflavin.[18,32–34]

Free Aromatic Amino Acids and Riboflavin Contents in Three Forms of Shrimp Protein Hydrolysate

The free tyrosine, phenylalanine, tryptophan, and riboflavin in liquid protein hydrolysate, dry powder, and concentrated paste were determined in separate preparations and analysis of 5 different batches of each. The free amino acid of higher concentration in the three samples was phenylalanine (Table 4). The mean contents of free phenylalanine were $16.28 \pm 1.02\,mg\,g^{-1}$ dry mass, $16.00 \pm 1.58\,mg\,g^{-1}$ dry mass, and

Table 4. Free aromatic amino acids and riboflavin content in three forms of shrimp protein hydrolysate

Sample (batch number)	Tyrosine ($mg\,g^{-1}$)[a]	Phenylalanine ($mg\,g^{-1}$)[a]	Tryptophan ($mg\,g^{-1}$)[a]	Riboflavin ($\mu g\,g^{-1}$)[a]
Liquid protein hydrolysate				
1	1.08	15.20	2.80	3.10
2	1.23	15.82	2.44	1.14
3	1.21	15.83	2.67	2.95
4	1.22	16.74	2.50	2.74
5	1.24	17.82	2.45	1.95
Dry powder				
1	5.59	18.03	1.48	0.67
2	5.71	13.95	1.26	0.63
3	6.37	15.25	1.47	0.59
4	5.24	15.75	1.46	0.77
5	5.58	17.03	1.46	0.88
Concentrated paste				
1	7.06	7.59	0.68	0.84
2	7.02	7.41	0.70	1.05
3	8.06	8.41	0.69	0.81
4	6.55	6.01	0.48	0.70
5	6.47	6.78	0.49	0.84

[a]Results expressed as dry mass.

$7.24 \pm 0.90\,mg\,g^{-1}$ dry mass, for liquid protein hydrolysate, dry powder, and concentrate paste, respectively. These values are higher than those reported in other studies.[35,36] The sample that presented the higher content of free tyrosine was the concentrated paste and the one with the smaller concentration was liquid protein hydrolysate. The contents of free tyrosine in all cases were superior to those reported in other researches.[33,37] In the case of free tryptophan, the liquid protein hydrolysate presents the highest content and the concentrated paste the lowest. The mean content of tryptophan in liquid protein hydrolysate and dry powder are similar to those reported in previous studies.[26] Valdez-Martinez, (2006)[38] mentions that a reductor sugar and an amine group (from amino acid or protein), such as lysine, arginine, and tryptophan are involved in the initial stage of the Milliard reaction. In consequence, the loss of tryptophan caused by the Milliard reaction can be more than 50%, as is noted in the low content of free tryptophan in the concentrated paste.

In other comparisons, the mean contents of riboflavin were 2.38 ± 0.82, 0.71 ± 0.12, and $0.85 \pm 0.13\,\mu g\,g^{-1}$ dry mass for liquid

protein hydrolysate, dry powder, and concentrate paste, respectively. These values are similar to reports of other seafood.[39,40] The amount of water soluble vitamins in marine foods is less dependent upon the specie; however, generally, a ration of seafood may significantly contribute to satisfying the human daily need for B vitamins.[41]

The observed differences in the content of the aromatic amino acids and riboflavin in the three forms of shrimp protein hydrolysate may be a result of the fermentation procedure, the kind of shrimp, and the type of production process.

CONCLUSIONS

The method of HPLC developed in this work results in a rapid, sensitive, precise, and accurate procedure for determination of riboflavin and aromatic amino acid content in protein hydrolysate from fermented shrimp remnants. With this high performance liquid chromatography method (HPLC), the quantification of the four compounds is possible with the same conditions, only varying the wavelengths specific to each compound.

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