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VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE QUANTIFICATION OF AMINO ACIDS IN THE FISH *Anisotremus scapularis* "Chita"

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ABSTRACT

The *Anisotremus scapularis* "Chita" is a species that lives on the coast of Peru and is considered potential for fish farming. Studies on the amino acid profile have not yet been reported. The aim of this investigation was to developed an analytical method for the quantification of amino acids in lyophilized muscle from the Chita by HPLC-FL. To achieve this objective, the hydrolysis conditions was optimized, as a result, the HCl concentration, hydrolysis time and temperature were 6 M, 24 h and 100 °C respectively. The results of the most relevant performance parameters were: the linearity had a coefficient of determination (R^2>0,999), the accuracy showed that all amino acids did not exceed the bias of 15%. The recovery ranged from 97,08% to 102,44%. The coefficients of variation for repeatability and intermediate precision were ranging from 1,93% to 5,43% and 2,08% to 10,69% respectively. The LOD and LOQ were ranging from 0,002 to 0,014 g/100 g and 0,005 to 0,043 g/100 g respectively. The robustness showed that the method is sensible to changes to the studied factors. Finally, the method met the majority of acceptance criteria, and it is suitable for the analysis of amino acids in Chita.

Key words: Optimization, Derivatization, Reference material, Box-Behnken, *Anisotremus scapularis*.

VALIDACIÓN DE UNA METODOLOGÍA ANALÍTICA PARA LA CUANTIFICACIÓN DE AMINOÁCIDOS EN EL PEZ *Anisotremus scapularis* "Chita"

RESUMEN

El pez *Anisotremus scapularis* "Chita" es una especie que habita en la costa de Perú y se considera potencial para la acuicultura. Los estudios sobre el perfil de aminoácidos aún no han sido reportados. El objetivo de esta investigación fue desarrollar un método analítico para la cuantificación de aminoácidos en músculo liofilizado de Chita mediante HPLC-FL. Para lograr este objetivo, se optimizaron las condiciones de hidrólisis, resultando una concentración de HCl de 6 M, un tiempo de hidrólisis de 24 horas y una temperatura de 100 °C. Los resultados de los parámetros de rendimiento más relevantes

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fueron los siguientes: la linealidad presentó un coeficiente de determinación (R^2>0,999), la veracidad mostró que todos los aminoácidos no superaron un sesgo del 15%. La recuperación varió del 97,08% al 102,44%. Los coeficientes de variación para la repetibilidad y la precisión intermedia variaron del 1,93% al 5,43% y del 2,08% al 10,69%, respectivamente. El LOD y LOQ oscilaron entre 0,002 y 0,014 g/100 g y entre 0,005 y 0,043 g/100 g, respectivamente. La robustez mostró que el método es sensible a cambios en los factores estudiados. Finalmente, el método cumplió con la mayoría de los criterios de aceptación y es adecuado para el análisis de aminoácidos en Chita.

Palabras clave: Optimización, Derivatización, Material de referencia, Box-Behnken, *Anisotremus scapularis*.

INTRODUCTION

Since the 1970s, the most trustful analytical technique for the quantification of amino acids is the high-performance liquid chromatography (HPLC) in reverse phase coupled with a detector of fluorescence, ultraviolet or mass spectrometry, due to his great precision and high detection sensitivity[1].

Over the years, the analytical laboratories have developed different analytical methods for the amino acids analysis in food and feed products by HPLC. These methods generally include the derivatization pre-column or post-column. Some of the best known derivatizing reagents are the o-phtaldialdehyde (OPA), 9-fluorentylmethyl chloroformate (FMOC), phenyl isothiocyanate (PITC), ninhydrin, etc[2]. However, some of these reagents present disadvantages such as the lack of reactivity with secondary amino acids and the instability of the formed fluorescent products. In the early 1990s, Cohen and Michaud[3] developed a pre-column derivatization method, and they used a novel derivatizing reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) for amino acids. The advantages of AQC are that it reacts with primary and secondary amines, its derivatized products are stable, they are highly fluorescent, they have a fast kinetic reaction and present low matrix interference[4,5].

Additionally, according to current international standards, the developed methodologies must be validated to meet specifications regarding the intended use of analytical results[6,7]. Thus, the International Organization for Standardization (ISO), Asociación Española de Farmacéuticos de la Industria (AEFI), Eurachem, Association of Official Analytical Collaboration (AOAC International), the Codex Alimentarius, the US Pharmacopeia (USP) and others, recommend the validation of the analytical method by evaluating the performance parameters, such as the accuracy, precision, specificity, linearity, detection limit, quantification limit, sensibility and robustness[8-11].

Worldwide, the fishing and aquaculture industry have incremented their production levels due to the high demand for food, reaching a historical maximum of 179 million tons in the year 2018, where the aquaculture represented a total production of 46%, and 88% of that was used for direct human consumption, registering a per capita consumption of 20,5 kg, promoting the global efforts directed to eradicate hunger and malnutrition[12].

The Plan Nacional de Desarrollo Acuícola – (DS N°30-2001-PE)[13] and the Programa Nacional de Ciencia, Desarrollo Tecnológico e Innovación en Acuicultura 2013-2021 (C+DT+i)[14] indicate that, in Peru, the aquaculture has experimented a sustained growth, letting the supply of aquatic species such as shrimp (*Penaeus vannamei*), Peruvian scallop

(*Argopecten purpuratus*), rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis niloticus*). These represent around 95% of the total market production, having an important contribution towards the country's economy and the human nutrition. Additionally, the law N° 27460, "Ley de promoción y desarrollo de la acuicultura"[15], establishes the need to support research for the technological development of aquaculture based on species with potential for cultivation and commercialization. For this reason, IMARPE has developed research projects in the fish aquaculture of "chita" (*Anisotremus scapularis*), "lenguado" (*Paralichthys adspersus*) and "cabrilla" (*Paralabrax humeralis*) since 2013[16].

Particularly, the "Chita" is considered a specie with high aquaculture potential and is one of the principal specie that sustain the traditional coastal fishery in Peru. The "Chita" has a high commercial value and great demand for the direct human consumption due to its delicious taste. Moreover, it has a good nutritional quality, due to its high concentration of proteins and polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA)[17]. A critical point to achieve the cultivation of this specie is the evaluation of the quality of the protein, and therefore, the objective of this work was to develop and validate an analytical method, with the goal of obtaining a method for quantification of amino acids in the fish Anisotremus scapularis "Chita" by HPLC-FL according to the Peruvian guideline for validation INACAL DA-acr-20D:2017.

EXPERIMENTAL PART

Chemicals and reagents

The 17 amino acids (AAs) mixture standard (code AAS18) was purchased from Sigma Aldrich (St. Louis, USA). It contained 2,5 µmol/mL of each amino acid: L-alanine (Ala), amonium chloride, L-arginine (Arg), L-aspartic acid (Asp), L-glutamic acid (Glu), L-glycine (Gly), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-tyrosine (Tyr), L-valine (Val), except L-cystine, which contained a concentration of 1,25 µmol/mL. The internal standard L-2-aminobutyric acid (Aab) (code A1879) was purchased from Sigma Aldrich (St. Louis, USA). The derivatizing reagent AQC, AccQ-Fluor™ reagent kit (code WAT052880) was purchased from Waters Corporation (Milford, MA, USA). The type 1 water was provided by a purification system Barnstead™ EASYpure™ II (Dubuque, USA). The sodium acetate anhydrous ACS® grade and the phosphoric acid 85% ACS® grade were obtained by Merck® (Darmstadt, Alemania). The acetonitrile HPLC grade was purchased by J.T.Baker (California, USA) and the hydrochloric acid fuming 37% ACS® grade was obtained by Supelco (Darmstadt, Germany).

Biological sample and pretreatment

The fish *Anisotremus scapularis* "Chita" was provided by the Laboratorio de cultivo de peces of IMARPE. The samples were processed according to the procedure of the Department for Environmental Protection of Kentucky-USA[18]. The fish fillet of 2 cm was lyophilized in a freeze-dryer Labconco (Kansas, USA) at a vacuum pressure between 0,022-0,070 mbar. The collector temperature was -56 °C. The temperature ramp was -15

°C, for 8 h; then 0,5 °C/min, until 5 °C, for 15 h; and finally 0,5 °C/min until 25 °C, for 7 h. The sample was homogenized, placed in a sample bag and stored at -20 °C in a freezer Egiasac (Lima, Peru) until analysis.

Hydrolysis

An amount of 10,00 ± 0,50 mg of lyophilized sample was weighted in a 13x100 mm glass tube and 2 mL of HCl 6 M was added. The sample was hydrolyzed at 100 °C for 24 h in a thermoblock Thermoscientific (China). An aliquot of 50 µL of the hydrolyzed sample was transferred to a 16 x 100 mm glass tube, then 100 µL of internal standard Aab 2,5 mM and 4850 µL of water were added. The final solution was filtered in a polytetrafluoroethylene (PTFE) syringe filter of 0,45 µm.

Derivatization

An amount of 10 µL of hydrolyzed sample was derivatized according to the instructions of the reagents kit AccQ.Fluor™ [19,20]. The concentration of the amino acids was calculated using the following equation:

$$C_{AA}\ (g/100\ g) = \frac{C_m x V_e x V_f\ x\ PM}{W_S x V_a x\ 10000000} \qquad (1)$$

Where C_{AA} is the amino acid concentration from the lyophilized sample, C_m is the amino acid concentration from the calibration curve (pmol/µL), PM is the amino acid molecular weight, V_e is the HCl 6 M volume (mL), V_a is the aliquot volume (mL), V_f is the final dilution volume from the aliquot (mL) and W_s is the sample weight (g).

Chromatography method

The separation of amino acids was performed according to the AccQ-Tag method[21] with certain modifications. The HPLC system LaChrom Elite® (Tokyo, Japan) had a fluorescence detector Hitachi L-2485. The analytical column was Thermoscientific™ Hypersil GOLD C18, 5 µm, 150 mm x 4,6 mm (Lithuania) with a guard column Thermoscientific™, Hypersil GOLD, 5 µm, 10 x 4 mm (Lithuania). The mobile phase consisted in water (A), a solution of sodium acetate 19 g/L at pH of 5,10 ± 0,01 adjusted with phosphoric acid 85% (B) and acetonitrile (C), the gradient elution is shown in table 1. The flow was 1 mL/min, the column temperature was set at 37 °C, the injection volume was 5 µL, the excitation and emission wavelength were 250 nm and 395 nm respectively. The data processing from these experiments was performed with the software EZChrom Elite 3.2.1 (Agilent). The amino acids identified were Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr and Val. The applied analytical technique was not able to identify the amino acids asparagine, glutamine, cysteine, methionine and tryptophan (Asn, Gln, Cys, Met and Trp).

Optimization of the hydrolysis

Box-Behnken response surface methodology was applied to maximize the amino acid concentration in the fish sample. The factors in the experimental design were the Temperature (100, 110 and 120 °C), Hydrolysis time (8, 16 and 24 hours) and HCl

concentration (3, 6 and 9 M). The experimental design consisted in 15 completely randomized runs with two replicates.

Method Validation

The developed method was validated according to Peruvian guideline for validation INACAL DA-acr-20D:2017[7], which are based on the study of the following performance parameters such as accuracy, recovery, precision, selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ) and robustness.

Accuracy

Twenty replicates of the certified reference material of soy flour code SRM 3234 NIST (Gaithersburg, USA) was analyzed to compare the amino acid concentration. The %bias was calculated according to the equation 2, and a percentage lower than 15% was proposed as acceptance criteria[22]. The certified reference material of soy flour was chosen, since there is no reference material of the fish muscle matrix to quantify the concentration of amino acids.

$$\%\text{Bias} = \frac{\bar{x}-\mu}{\mu} \times 100\% \qquad (2)$$

Where $\bar{x}$ is the average of all the measurements and μ is the accepted reference value.

Recovery

The hydrolyzed sample of 50 µL was fortified by triplicate in 3 concentration levels corresponding to the 25, 50 and 75% of the estimated amino acid concentration in the sample. The percentage recovery was calculated following the equation 3. It was proposed as acceptance criteria the theoretical range of 97-103% according to FAO/WHO[11] and NMKL[23].

$$R\ (\%) = \frac{C_f - C_m}{C_a} \times 100\% \qquad (3)$$

Where C_f is the fortified amino acid concentration (pmol/µL), C_m is the original amino acid concentration (pmol/µL) and C_a is the added amino acid concentration (pmol/µL).

Precision

The instrumental precision was evaluated by injecting the same sample four times. The repeatability was evaluated analyzing the sample twenty times, and the result was expressed as a percentage of the relative standard deviation (%RSDr). It was proposed as acceptance criteria a maximum %RSDr of 5%[24,25].

The within-laboratory reproducibility was evaluated analyzing the sample by triplicate around 5 different days with 2 analysts and the relative standard ($\%RSD_{Rw}$) was calculated according to the guidelines NTP-ISO 5727-2:2021[26]. It was proposed as acceptance criteria a maximum $\%RSD_{RW}$ of 15%[27].

Selectivity

100 µL of the specific interference Aab 2,5 mM was added to the sample, the influence of Aab in the separation of the analytes was determined by calculating the differences in the retention time of the peaks before and after the addition of Aab. The resolution for each analyte was calculated according to the following equation:

$$R_s = \frac{2 \times \triangle t}{w_1 + w_2} \qquad (4)$$

Where Δt is the difference of the retention time of both near peaks and w is the width of both peaks. AOAC International recommended that a good value of Rs between both peaks would have to be at least 1,5, and 1 is the minimal usable separation[10].

Linearity

The calibration curve was prepared in six levels of amino acid standard solutions with 4 replicates for each level. The concentrations of the calibration standards were 5, 12,5, 25, 37,5, 50 and 100 µM. The acceptance criteria for the linearity was a coefficient of determination (R^2) of at least 0,99[28].

Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the equations 5 and 6[29]: LOD = $3{,}3s_b/m$ (5) and LOQ = $10s_b/m$ (6). Where m is the slope of the calibration curve and sb is the intercept error. According to the laboratory requirements, the acceptance criteria for LOQ was established at 0,1 g/100g.

Robustness

The fractional factorial design 2^{7-4} of resolution III was performed with seven factors, two center points and two levels. The factors were: column temperature (35, 37 and 39 °C), pH of the mobile phase B (5,0, 5,1 and 5,2), excitation wavelength (245, 250 and 255 nm), emission wavelength (390, 395 and 400 nm), flow (0,9, 1,0 and 1,1 mL/min), injection volume (4, 5 and 6 µL) and sample weight (9, 10 and 11 mg).

Statistical analysis

The data analysis was performed in the software Minitab 19. The analysis of variance (ANOVA), t–student was used at a significance level of α=0,05. The outliers were identified using the Grubbs, Cochran or Mandel's k and h consistency according the guidelines NTP-ISO 5727-2:2021[26].

RESULTS AND DISCUSSION

Optimization of the hydrolysis

The Box-Behnken response surface design was used to optimize hydrolysis factors such as Temperature, Hydrolysis time and HCl concentration, in order to maximize the concentration of amino acids in fish sample. The thirty experimental runs were conducted in random order, with six center points, and the results are shown in Table 1. The results

presented in Table 1 indicate that the concentration for the case of the amino acid Ile ranged from 2.56 to 4.80 g/100 g respect to the experimental runs used.

Table 1. Box-Behnken design (BBD) and responses for the optimization of hydrolysis.

Run	Coded level			Uncoded level			Amino acids (g/100 g)[a]														
	X_1	X_2	X_3	X_1	X_2	X_3	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Lys	Ile	Leu	Phe
1	-1	0	1	100	16	9	9,25	13,68	3,69	5,07	1,75	6,06	3,76	5,33	2,85	2,77	3,56	8,18	3,54	6,63	3,57
2	1	0	-1	120	16	3	9,24	13,77	3,19	4,58	1,88	6,07	3,61	5,19	2,61	2,88	4,41	8,33	4,39	6,94	3,67
3	-1	1	0	100	24	6	9,06	13,63	3,52	4,82	1,85	6,02	3,71	5,17	2,77	2,66	4,19	8,20	4,14	6,74	3,60
4	0	1	1	110	24	9	9,94	14,85	3,56	4,91	2,04	6,39	3,96	5,54	2,77	3,08	4,73	9,04	4,66	7,40	3,91
5	1	0	1	120	16	9	9,25	13,81	3,26	4,79	1,91	6,25	3,63	5,20	2,59	2,86	4,40	8,47	4,43	6,97	3,71
6	0	0	0	110	16	6	9,21	13,73	3,50	4,59	1,84	6,10	3,74	5,15	2,61	2,88	4,07	8,52	4,11	6,84	3,63
7	-1	-1	0	100	8	6	8,04	9,74	2,81	4,18	1,04	4,26	2,31	4,25	2,15	2,15	1,59	7,23	2,13	4,41	2,44
8	0	1	-1	110	24	3	9,19	13,78	3,16	4,56	1,89	5,84	3,61	5,16	2,61	2,71	4,49	8,68	4,51	6,96	3,70
9	0	-1	1	110	8	9	9,46	12,88	3,59	4,52	1,44	5,17	3,39	5,13	2,42	2,79	2,59	8,08	2,63	6,07	3,26
10	0	-1	-1	110	8	3	9,21	12,59	3,43	4,40	1,42	5,09	3,29	5,00	2,42	2,69	2,53	7,87	2,56	5,83	3,12
11	1	1	0	120	24	6	9,72	14,54	3,15	4,78	2,01	6,17	3,71	5,40	2,72	3,00	4,68	8,99	4,80	7,26	3,91
12	1	-1	0	120	8	6	10,29	14,82	3,93	4,89	1,84	5,97	3,93	5,64	2,87	2,98	3,52	8,78	3,50	7,09	3,83
13	-1	0	-1	100	16	3	9,40	13,91	3,70	4,65	1,81	6,10	3,83	5,24	2,67	2,82	3,66	8,51	3,75	6,79	3,68

[a]The results were presented as the average of two replicates (n=2), except the center point (n=6).
X_1: Temperature (°C), X_2: Hydrolysis time (min), X_3: HCl concentration (M).

The analysis of variance (ANOVA) of the Box-Behnken design for the amino acid Ile is shown in Table 2. The factors, temperature, hydrolysis time, $(\text{HCl concentration})^2$, $(\text{Hydrolysis time})^2$ and the interaction (Temperature*Hydrolysis time) presented significant differences (p-value<0,05). The quadratic polynomial model is the one that adjusts better (R^2=0,9883). Additionally, the lack of fit is not statistically significant (p-value= 0,442>0,05), which indicates that the specified model adjusts properly to the data. Therefore, the quadratic model from the response surface design for the amino acid Ile establishes a relationship between the concentration of Ile (dependent variable) and the factors (independent variables): Temperature (X_1), Hydrolysis time (X_2), and HCl concentration (X_3), as represented by the following equation:

$$\text{Ile (g/100 g)} = 4.1391 + 0.4117*X_1 + 0.8943*X_2 - 0.0365*X_3 - 0.4715\,X_2^2 - 0.1358*X_3^2 - 0.2495*X_1*X_2 + 0.0624*X_1*X_3 - 0.0653*X_2*X_3 \quad (7)$$

Table 2. ANOVA of Box-Behnken design for amino acid Isoleucine.

Source	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Model	8	16,2464	2,03080	189,48	0,000
X_1	1	12,0035	4,00117	210,83	0,000
X_2	1	2,2596	2,25957	889,26	0,000
X_3	1	9,5307	9,53072	1,81	0,195
X_2^2	1	0,0194	0,01944	133,60	0,000
X_3^2	1	1,5293	0,76463	11,34	0,003
X_1*X_2	1	1,4319	1,43191	33,18	0,000
X_1*X_3	1	0,1216	0,12156	2,91	0,105
X_2*X_3	1	0,4153	0,13842	2,66	0,120
Error	18	0,3556	0,35563		
Lack of Fit	4	0,0311	0,03114	1,00	**0,442**
Pure Error	14	0,0285	0,02849		
Total	26	0,1929			
R^2 = 0,9883					

Additionally, the Table 2 shows that the pure error sum of squares (0,0285) is less than the total sum of squares (0,1929), indicating the correct reproducibility of the evaluated central point[30]. On the other hand, the coefficient of determination (R^2) introduced a value of 0.9883, establishing that 98.83% of the variability of the results is explained by the model proposed that a good R^2 should be greater than 0.8[31], demonstrating that the quadratic model is optimal for predicting the concentration of the amino acid Ile in fish samples.

Based on the quadratic model from equation 7, the 3D surface plots (Figure 1) were developed to represent the relationship between the independent variables and the response variable (Isoleucine concentration).

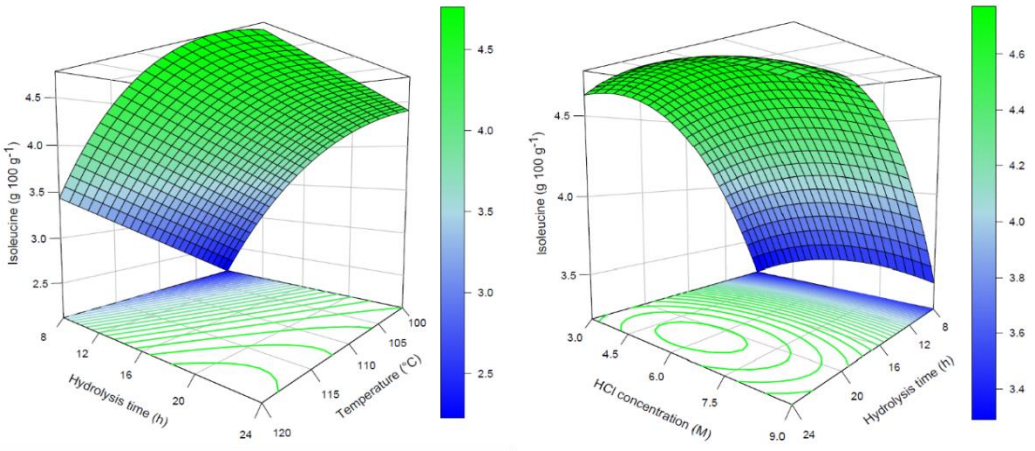

Figure 1. Left: Surface plot of isoleucine concentration vs temperature, hydrolysis time, HCl concentration=6 M. Right: Surface plot of isoleucine concentration vs HCl concentration, hydrolysis time, Temperature= 120 ºC.

On the left side of Figure 1, the presence of a quadratic model due to the curvature in the levels of the Hydrolysis time factor can be observed. In contrast, the temperature levels presented a linear model with a maximum concentration of Ile at its highest level (120 °C). However, for the rest of the amino acids, the maximum concentration occurred at the lowest temperature level (100 °C) except for Val (Table 3).

On the other hand, on the right side of Figure 1, the presence of a quadratic model is also shown due to the presence of a curvature in the concentration levels of the HCl concentration factor, where the highest concentration of Ile is found at the center level (~ 6 M).

According to the experimental results, the HCl concentration factor did not present significant differences (p value > 0.05) for the rest of the amino acids: Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Lys and Leu as shown in Table 3. Consequently, it was obtained that the maximum concentration in majority of AAs was obtained through the following hydrolysis conditions: HCl concentration 6 M, hydrolysis time 24 h and temperature 100 °C except for Ile, Arg and Val. The HCl concentration 6 M is reported by many authors in different matrix of fishes such as, grass carp (*Ctenopharyngodon idella*), pacu (*Piaractus mesopotamicus*) and catfish (*Ictalurus punctatus*)[32,33] and food[34,35,36]. Besides, the hydrolysis time of 24 h is reported by different studies for the quantification of amino acids in muscle of the snakehead fish (*Channa striatus*)[37], catfish (*Sperata seenghala*)[38] and feed[34]. Additionally, the temperature depends on the ratio HCl volume/sample weight, obtaining values from 100 to 120 °C for different matrix of food[39]. The temperature of 100 °C is in the range of 105 ± 5 ºC reported in the article about the quantification of amino acids in tissue`s fish of Tilapia (*Oreochromis Mossambicus*)[40].

Table 3. Optimum values of hydrolysis.

Amino acid	**X_1**	**X_2**	**X_3**
Asp	100	24	N.S
Glu	100	24	N.S
Ser	100	24	N.S
Gly	100	24	N.S
His	100	24	N.S
Arg	100	17	N.S
Thr	100	24	N.S
Ala	100	24	N.S
Pro	100	24	N.S
Tyr	100	24	N.S
Val	120	12	N.S
Lys	100	24	N.S
Ile	120	17	6
Leu	100	24	N.S
Phe	100	24	N.S

X_1: Temperature (°C), X_2: Hydrolysis time (min), X_3: HCl concentration (M), N.S: No significance

Method Validation

The elution profile of 15 AAs derivatized with AQC and separated on the Hypersil GOLD C-18 column with fluorescence detection is shown in Figure 2. The chromatographic separation was optimal for all the AAs analyzed.

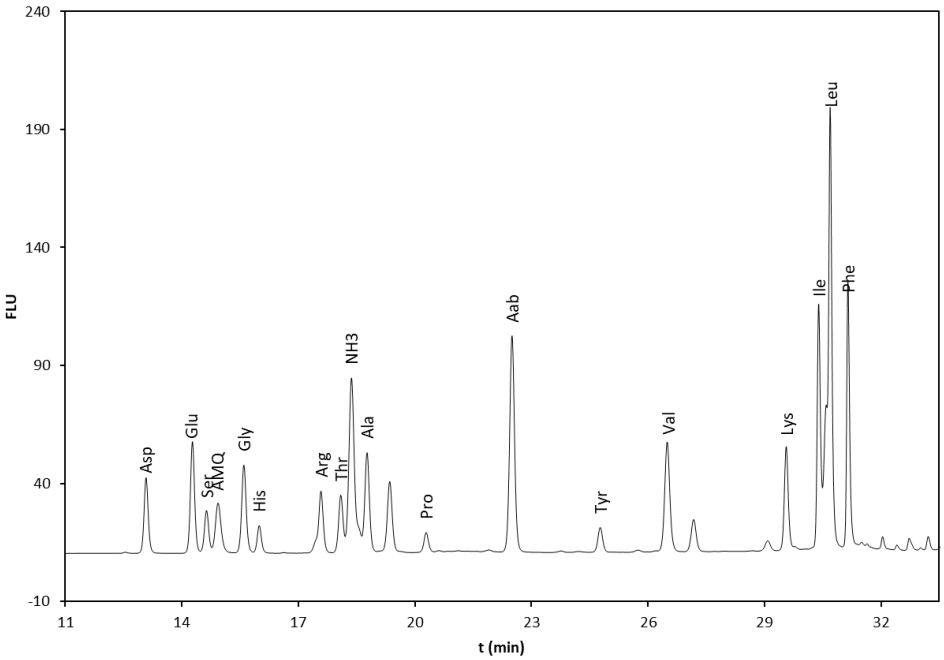

Figure 2. Typical amino acid chromatogram of tissue fish *Anisotremus Scapularis* "Chita".

The results of method validation are shown below. First, the type of distribution of the data obtained from 30 repetitions was evaluated using the Anderson-Darling test. Our results indicated that the data obtained from all AAs studied presented a normal distribution (p value > 0.05)[41].

Accuracy was assessed by the bias percentage of each amino acid as shown in Table 4. A certified reference material (CRM) for the studied matrix was not available on the market, for this reason, the soy flour was chosen as a reference sample for the accuracy. According to our results, the % bias for all AAs were less than 15%. These results comply with the acceptance criteria proposed[22]. Similar values of bias% were reported for other authors ranging from (-12.84% to 12.37%)[42], (2.3% to 11%)[2] and (1.86% to 12.85%)[43], respectively.

On the other hand, accuracy was evaluated using spiked samples. The table 4 shows the recovery values of each amino acid. Satisfactory recovery rates were obtained for all AAs, ranging from 97.08% to 102,44%. These recoveries are within the acceptance range (97-103%), according to Codex Alimentarius[11], AEFI[44] and AOAC International[45] fulfilling the veracity of the method. Our results had less dispersion than that described by other authors, introducing recoveries between (87 to 104%)[2] and (90 to 110%)[46], respectively. In addition, the Student's t-test indicated that there is no significant difference between the mean recovery and 100% for all AAs.

Table 4. Percentage recovery, bias percentage, precision expressed in relative standard deviation percentage and selectivity of each amino acid.

Amino acid	Recovery		Accuracy	Precision			Selectivity		
	R (%) (n=3)	t-exp[a]	%Bias (n=20)	Instrumental (n=4)	RSD_r (%) (n=20)	RSD_{RW} (%) (n=3)	Δt_R	ΔA	R_s
Asp	97,08 ± 3,75	1,31	-3,58	0,68	2,51	2,79	0,01	0,02	3,01
Glu	97,20 ± 4,27	1,10	-8,37	0,58	2,26	2,44	0,00	0,01	1,07
Ser	100,08 ± 2,27	0,06	-0,61	0,37	2,64	10,69	0,00	0,02	0,69
Gly	102,44 ± 3,61	1,20	-4,41	0,46	2,60	5,39	0,00	0,01	1,02
His	100,42 ± 0,74	0,99	2,03	0,59	2,24	2,34	0,00	0,00	4,42
Arg	100,33 ± 1,45	0,40	9,61	0,51	2,98	6,26	0,00	0,06	4,97
Thr	101,80 ± 1,33	2,39	-0,98	0,22	2,33	3,19	0,00	0,04	0,83
Ala	100,48 ± 2,27	0,37	-3,65	0,27	2,47	9,34	0,00	0,00	1,26
Pro	99,01 ± 1,48	1,15	-6,12	1,76	2,13	5,85	0,00	0,03	4,92
Tyr	99,23 ± 1,49	0,89	-13,26	1,47	5,43	7,42	0,02	0,02	3,92
Val	100,44 ± 1,97	0,39	-11,15	0,60	1,93	2,08	0,02	0,02	1,50
Lys	101,95 ± 3,70	0,93	1,63	1,70	3,03	3,30	0,01	0,28	1,85
Ile	101,34 ± 1,98	1,19	-1,96	1,79	3,40	4,41	0,01	0,18	1,06
Leu	101,16 ± 0,76	2,67	-6,47	1,49	3,24	4,88	0,01	0,09	1,38
Phe	100,63 ± 1,24	0,89	-2,33	1,88	2,92	4,15	0,01	0,12	1,56

[a] $t\text{-}_{exp} < t_{crit}$ (α=0,05) = 4,30

Δt_R: Difference in the amino acid retention time (min) before and after the addition of Aab.
ΔA: Difference in the amino acid percentage area before and after the addition of Aab.
R_s: Resolution.

The precision was measured as repeatability and intermediate presicion. The RSD (%) of repeatability and intermediate precision is summarized in Table 4. The instrumental precision was within 1,88%, similar values were reported in the literature with RSD ranging from 0.04 to 1.28%[47]. The repeatability for all AAs was within 5,43%, these similar values are reported in other validation studies[24,46,48]. The within-laboratory reproducibility for all AAs was within 10,69%, complying with the acceptance criteria (%RSD < 15%)[27] and similar to the values reported by other authors[2,49,50,48]. The precision values reported in the literature are similar to obtained here, using the same derivatizing reagent, fullfiling with the proposed acceptance criteria.

The selectivity, acoording to EURACHEM, is the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behavior[9]. Selectivity was determined by comparing the sample chromatograms respect to the sample chromatogram with the addition of the internal standard Aab. As shown in Figure 3, the amino acid chromatogram was not affected by the addition of the internal standard Aab. Hence, the validated method is selective for the amino acids analyzed in presence of the internal standard Aab.

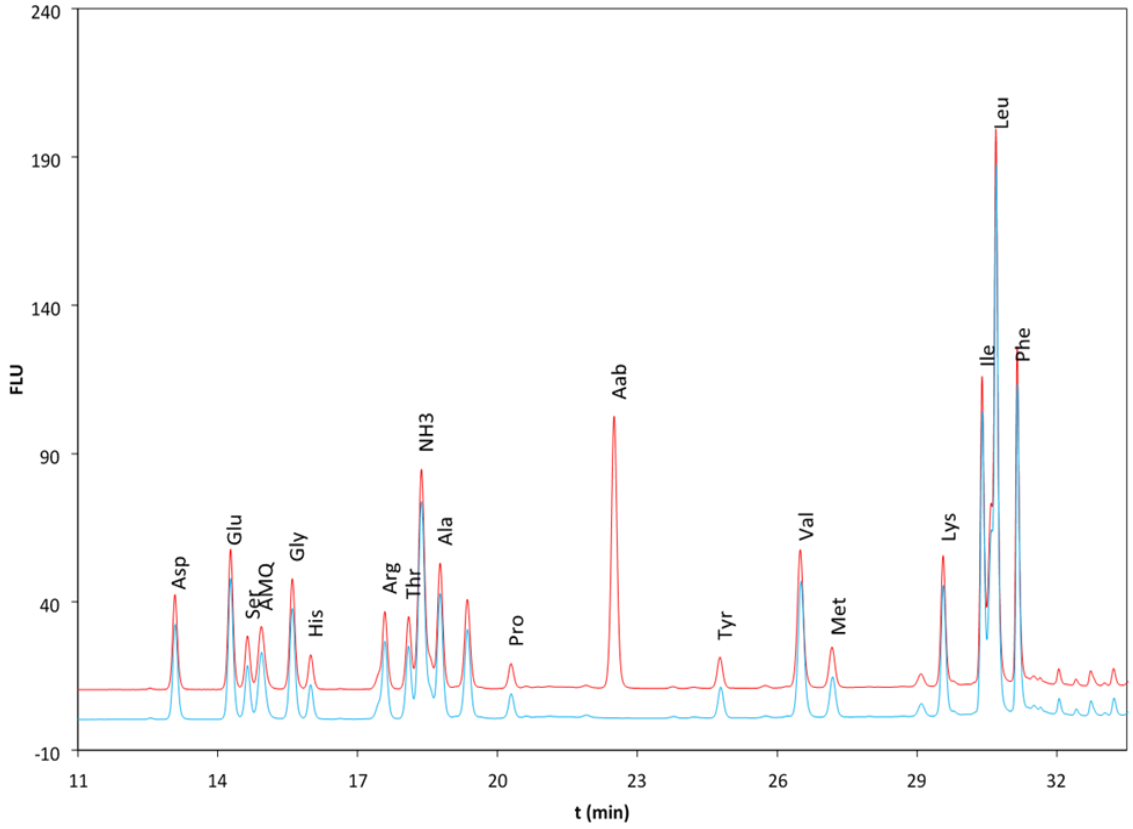

Figure 3. Chromatogram of amino acid analyses in fish *Anisotremus Scapularis* "Chita". Red line: sample with internal standard and blue line: sample without internal standard.

Furthermore, as can be seen in Table 4, it shows a minimal difference of retention time (0,02 min) for Tyr and Val, and (0,00 min) for the rest of AAs. Moreover, it was found that the difference in the percentage area of the chromatogram peak is lower than 0,28%. These results are similar to other validation study using AQC derivatizing reagent in gelatin. The authors obtained $\triangle t_R$ between 0.06 to 0.13 min higher than our results (0,00-0,02 min). Similarly, they obtained ΔA between 0,00 to 0,54%, higher than our results of 0,28%[51].

Good chromatographic separation was achieved with Rs > 1.5 for most of the AAs. However, acceptable Rs (1.0-1.5) were obtained for Glu, Gly, Ala, Ile and Leu. Figure 2 shows that most AAs had well-defined peaks, except for Ser and Thr with Rs < 1, due to overlapping chromatographic peaks by the presence of secondary products in the reaction such as AMQ (6-Aminoquinolone) and NH_3(ammonia)[5,52]. Other authors reported values of Rs > 0.8 for the separation of AAs by HPLC using AQC derivatizing reagent[53,54].

The linearity was tested on concentration range of 5–100 µM. Each concentration was prepared with four replicates. Linearity was evaluated by the correlation coefficient (R^2) of the regression line. The Table 5 show that the coefficient of determination (R^2) for all AAs is greater than 0,990 and the intercepts were close to zero, indicating excellent linearity and fulfilling the proposed aceptance criteria (R^2> 0,99)[28,55]. Similarly, in validation studies, other authors reported R^2 greater than 0,990[53,56]. Moreover, the F test for each amino acid presented values greater than F_{crit}= 7,71, showing that the slope is different from zero. As estimated from the calibration curve, the LOD and LOQ values

were in the range of 0,002 - 0,014 g/100 g and 0,005 - 0,043 g/100 g, respectively (Table 5). The highest LOD and LOQ were registered for Phe (0,014 - 0,043 g/100 g), while the lowest values were registered for Gly (0,002 - 0,005 g/100 g). These values of LOD and LOQ obtained in this work are similar to those previously reported by other authors using AQC derivatizing reagent with fluorescence detection, ranging from 0,0002 to 0,1570 g/100 g and 0,006 to 0,5230 g/100 g, respectively[2,46,57]. Hence, the present method is sensitive to detect low concentrations of these AAs.

Table 5. Linearity parameters, limits of detection (LOD) and limit of quantification (LOQ).

Amino acid	Regression equation[a]	Range (µM)	Coefficient of determination (R^2)	Slope (m)	Intercept	LOD (g/100 g)	LOQ (g/100 g)	F-value[b]
Asp	y = 0,4375x - 0,00003	5-100	0,9999	0,437	0,0000	0,008	0,023	31159,9
Glu	y = 0,4815x - 0,0002	5-100	0,9999	0,482	-0,0002	0,008	0,024	36801,0
Ser	y = 0,6054x + 0,0005	5-100	0,9999	0,605	0,0005	0,004	0,013	58044,5
Gly	y = 0,583x + 0,0098	5-100	1,0000	0,583	0,0098	0,002	0,005	220327,6
His	y = 0,8517x + 0,00003	5-100	0,9999	0,852	0,0000	0,007	0,023	44498,4
Arg	y = 0,8419x - 0,0029	5-100	0,9999	0,842	-0,0029	0,010	0,031	30659,5
Thr	y = 0,8049x + 0,0015	5-100	1,0000	0,805	0,0015	0,004	0,011	118907,0
Ala	y = 0,7614x + 0,00009	5-100	0,9999	0,761	0,0001	0,005	0,017	27070,6
Pro	y = 0,3621x + 0,0015	5-100	0,9999	0,362	0,0015	0,005	0,017	45119,1
Tyr	y = 0,7358x - 0,002	5-100	0,9999	0,736	-0,0020	0,010	0,029	35677,6
Val	y = 1,3021x + 0,0009	5-100	0,9999	1,302	0,0009	0,007	0,020	31321,0
Lys	y = 0,6985x + 0,0044	5-100	0,9999	0,698	0,0044	0,009	0,027	28541,7
Ile	y = 2,1208x + 0,011	5-100	0,9997	2,121	0,0110	0,012	0,036	12748,0
Leu	y = 2,2313x + 0,0034	5-100	0,9997	2,231	0,0034	0,011	0,033	14656,1
Phe	y = 3,1108x + 0,0115	5-100	0,9997	3,111	0,0115	0,014	0,043	14007,4

The ruggedness (robustness) of an analytical procedure, according to EURACHEM is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Ruggedness provides an indication of the method's reliability during normal usage[9].

Previous studies have revealed that a good chromatography resolution requires stringent control in different factors such as, pH of mobil phase, mobile phase composition and organic solvent concentration[58,59,60]. For this reason, small changes in seven factors were studied as shown in Table 6. The amino acids Asp and Glu were only sensitive to changes in the excitation and emission wavelengths, so they were more robust compared to others. In contrast, the amino acids Ser, Gly, His, Thr, Ala, Ile and Leu were sensitive to changes all the factors, due to the overlapping of some amino acid with the secondary products in the reaction, such as, 6-aminoquinoline (AMQ) and NH_3[5,52,61]. Also, Cohen & Michaud[3] reported that the separation of polar amino acids and AMQ depended completely on the pH, with AMQ eluting in the middle of the chromatogram under alkaline conditions of mobile phase. In this method, the peak of AMQ elute between Ser and Gly as shown in Figure 2. Other studies mentioned that the factors such as pH and temperature of column play an important role in the resolution and separation of the AAs[62].

Table 6. Summary of the effects for each parameter.

Amino acid	Effects						
	Temperature of column	**pH of mobile phase B**	**Excitation wavelength**	**Emission wavelength**	**Flow rate**	**Injection volume**	**Sample weight**
Asp	0,15*	0,22*	-0,26	0,23*	0,20*	-0,19*	0,23*
Glu	0,14*	0,09*	-0,24*	0,35	0,34*	-0,32*	0,25*
Ser	-2,33	-2,28	-2,51	2,44	2,49	2,27	-2,37
Gly	4,08	3,88	-0,79	4,60	0,81	-0,71	0,99
His	-0,62	-0,33	0,47	-0,54	0,50	0,54	0,58
Arg	0,11*	0,61	-0,30	0,20	-0,14*	-0,29	0,41
Thr	-0,71	1,12	-0,92	1,07	-0,77	0,89	1,08
Ala	1,22	1,19	1,73	0,55	0,6	0,44	2,02
Pro	0,02*	-0,19	0,29	0,04*	0,17	-0,04*	-0,01*
Tyr	0,02*	-0,21	0,35	0,09*	0,17*	0,04*	-0,06*
Val	-0,06*	-0,12*	0,13	0,16	-0,06*	-0,15	0,10*
Lys	0,06*	1,85	0,19	0,12*	-2,48	-0,58	0,48
Ile	-1,47	-1,62	-1,00	1,40	1,22	1,07	-0,91
Leu	1,36	-2,03	-1,54	-1,95	-1,99	1,34	1,63
Phe	-0,28	-0,16	-0,15	-0,42	-0,18	-0,06*	0,26

*No statistically significant difference (α=0,05).

CONCLUSION

The analytical method was validated according to the Peruvian guideline for validation INACAL DA-acr-20D:2017. According to our results, the optimal hydrolysis conditions for the amino acids analysis in the lyophilized muscle were the concentration of HCl 6 M, time of 24 h and temperature of 100 °C. The performance parameters evaluated were the accuracy that showed that all amino acids don't exceed the limit %bias of 15%. The recovery ranged from 97,08% to 102,44%. The repeatability and intermediate precision were ranging from 1,93% to 5,43% and 2,08% to 10,69% respectively. The linearity had a working range from 5 to 100 µM with coefficients of determination (R^2) higher than 0,999. The limits of detection and quantification were in the range from 0,002 to 0,014 g/100 g and 0,005 to 0,043 g/100 g respectively. The selectivity showed that the amino acid chromatogram was not affected by the presence of the internal standard. The robustness showed that the method is sensible against changes in the column temperature, pH of the mobile phase, excitation wavelength, emission wavelength, flow, injection volume and sample weight. Finally, the analytical methodology met the majority of defined acceptance criteria and is considered suitable for use in the laboratory.

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