Validation of microwave-assisted sample extraction-high performance liquid chromatography (HPLC) method for quantitative analysis of hydrolysed amino acids in aquatic biological samples

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Abstract: A sensitive and widely applicable High-Performance Liquid Chromatography (HPLC) with fluorescence detector (FLD) method for determination hydrolysed amino acids in fish and aquatic products was developed and validated through study. The method involved a microwave-assisted sample extraction and protein hydrolysed with the hydrochloric acid and propionic acid. The separation was performed by Shim-pack HR-ODS column with a gradient mobile phase. The total separation time was 25 min per run. The method showed satisfactory linearity, repeatability, accuracy, and limit of detection for 21 amino acids with the 1-100 mg/L calibration range based on 10 µL injection. The fish, fish feed, canned fish and seaweeds samples were analysed successfully by using this developed method.

Keywords: Amino acids, fish, HPLC-FLD, method validation

INTRODUCTION

Amino acids play a major role in cellular metabolism, as a structural unit for protein, energy source, regulating gene expression act as a neurotransmitter, participate in the modulation of protein synthesis, biosynthesis of trace amines, lipid transport (Lorenzo et al. 2013; Yanting et al. 2018; Min et al. 2017) and biosynthesis of vitamins (Mudiam and Ratnasekhar 2013). Some amino acids such as aspartic acid (Asp), glutamic acid (Glu), γ-aminobutyric acid (GABA), and taurine (Tau) act as a neurotransmitter (Yanting et al. 2018) while some of them such as glutamine (Gln) and glycine (Gly) inhibitory signals (Lorenzo et al. 2013).

Determination of amino acids in biological samples such as fish and the aquatic product is very important for modern fisheries, aquaculture, and the human health sector. Today aquaculture achieved this development through proper farm management, genetics, disease control and a better understanding of nutrient requirements of farmed animals, ingredient processing and feed manufacturing (Nunes et al. 2014). Due to inadequate consumption of quality protein through human diets leads to protein-calorie malnutrition, Kwashiorkor, Marasmus (Bimal et al. 2014), type II diabetics, renal disease and carcinogenesis (Yanting et al. 2018).

Quantitative analysis of amino acid profile in fish and fisheries products is an important as well as the challenge, in food analysis laboratory, because it do not have a chromophore, high hydrophilic and polarity property and some of these amino acids can be lost during the sample treatment (Choi et al. 2017). Most of the amino acids show poor solubility near the Iso-electric point when separation. The combination of the above factors is critical to the separation and quantification of amino acids in different food matrices.

There are several methods for amino acids analysis in fish and aquatic samples. The classical amino acids analysis method is consisting two steps, (i) hydrolysis, which helps to liberate analyte from the substrate and (ii) chromatographic analysis for the qualitatively and quantitatively analysis of the analyte. Several factors affect to hydrolysis step such as temperature, time, hydrolysis agent, additives (Fountoulakis and Lahm 1998), sample matrices and reagents (Dai et al. 2014). There are several types of hydrolysis process involve, known as acid hydrolysis, alkyl hydrolysis, enzyme hydrolysis, polyacrylamide gel hydrolysis and

cleavage hydrolysis (Fountoulakis and Lahm 1998).

The common hydrolysis type expresses the many disadvantages; when using 6M HCl, the Tryptophan (Trp) destroyed completely. On the other hand, there is little loss of Trp when protein hydrolysed with alkaline such as NaOH. But Arginine (Arg), Asparagine (Asn), Cysteine (Cys), Glutamine (Gln), Methionine (Met), Serine (Ser) and Threonine (Thr) are significantly decomposed in such processes (Dai et al. 2014). The traditional acid hydrolyses method generally performed with 6N HCl at 110°C for 24 hrs (Damm et al. 2010; Afiuni-Zadeh et al. 2011). The microwave assisted hydrolysed method to develop recently as a useful tool for a range of protein sample preparation for proteome analysis (Chen et al. 2014; Wang and Li 2010). This method helps to reduce hydrolysing time significantly as a major advantage. Nevertheless, this technique helps to recover higher yield of residues and reproducibility (Yu et al. 1988).

The High-Performance Liquid Chromatography (HPLC) combine with the pre-column derivatizing technique provides advantages of amino acids analysis such as wide acceptance and ability to use different derivatization reagents (Jajić et al. 2013). o-phthaldialdehyde (OPA) is one of the common derivatizing agent for the primary amino acids at room temperature (Li et al. 2011). 9-fluorenylmethylchloroformate (FMOC) used as a derivatizing agent for secondary amino acids such as hydroxyproline, sarcosine, and proline (Einarsson 1985). Through the chromatographic analysis part involving ultraviolet, fluorescent, electrochemical and mass spectrometry detection are used (Yanting et al. 2018).

There are few references available for microwave assisted sample hydrolysing method for determination of amino acids in fish and aquatic products. The objective of this work is to optimize and validate a robust and easy pre-column derivatizing, HPLC method with a microwave-assisted sample extraction procedure for the analysis of amino acids in fish, fishery products, and aquatic resources as well as its application to real sample analysis.

MATERIALS AND METHODS

Chemicals and reagents

The number of 21, L-Amino Acids (1 g each, HPLC), L-Glycine, L-Alanine, L-Arginine Hydrochloride, L-Asparagine, L-Aspartic Acid, L-Cysteine hydrochloride, L-Glutamic Acid, L-Glutamine, L-Histidine Hydrochloride, L-Isoleucine, L-Leucine, L-Lysine Hydrochloride, L-Methionine, L-Phenyl alanine, L-Proline, L-Hydroxyproline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine were from Sigma Aldrich, Germany. Boric acid, Mercapto-propionic acid (MPA), o-phthalalaldehyde (OPA), 9- fluorenylmethyl-chloroformate (FMOC), Propionic acid, Hydrochloric acid (HCl), Sodium Hydroxide (NaOH), Methanol and Acetonitrile were purchased from Sigma (USA). All chemicals and reagents were in AR or HPLC grade (High purity, ≥99%).

Sample collection and preparation

The fresh fish (*Lutjanus* sp.), canned fish (yellowfin tuna), fish feed and seaweed (*Sargassum* sp.) were used for the method validation. Samples were homogenised using a domestic blender to obtain an equal portion. Then, weight 50.0 mg of sample into microwave vial and added 0.50 mL of hydrochloric acid and 0.50 mL of propionic acid and freeze (-20°C) into the domestic freezer for 1 hour. Then the frozen vials were connected to the microwave accelerated unit (CEM, MARS 6, USA) and heated 160°C (ramping time 10 min), and holding time was 15 min. The vials were allowed to reach the room temperature and then hydrolysed solutions were completely sparged under nitrogen gas and reconstitute it around pH 8 using, 983 µL 0.1N NaOH and 17 µL of 7.5 N NaOH. Then followed the below mentioned optimized derivatisation procedure.

In HPLC vial, 67.5 µL of MPA, 33 µL of OPA and 11.25 µL of sample (or standard) mixed together and waited 1 min prior to adding 15 µL of FMOC. Again, the vial was mixed and waited for 2 min and then added 0.1 N HCl 7.5 µL and injected to the HPLC. Each sample was run in triplicate. Limit of Detection (LoD) and Limit of Quantification (LoQ) for each amino acid was calculated based on the EURACHEM/CITAC method by injecting 6 replicates.

HPLC Analysis

Totally 10 µL of samples were injected to Shimadzu-Nexera UHPLC (Shimadzu, Japan), equipped with a DGU-20As degausser unit, RF-10AXL, SPD-M20A detectors, SIL-10A auto-injector and Shim-pack HR-ODS column (150 x 3.0 mm ID, 3 µm) eluted with 0.8 mL/min binary gradient containing mobile phase (A) 20 mmol/L potassium phosphate buffer (pH 6.5) in ultra-pure water and (B) 45:40:15 Acetonitrile: Methanol: H_2O respectively. The gradient program and summarized are given in Table 1. The column oven temperature was at 40 °C and the excitation-emission wavelength condition was set as for FLD (Shimadzu, FLD-20 Axs) channel 1: 350-450 nm and channel 2: 266-305 nm

Table 1 HPLC program and summary

Column	Shim-pack HR-ODS column (150 x 3.0 mm ID, 3 µm)		
Injection volume	10 µL		
Flow rate	800 µL/min		
Mobile phase composition	20 mmol/L potassium phosphate buffer (pH 6.5) in ultra-pure water, (A) 45:40:15 Acetonitrile: Methanol: H_2O (B)		
	Total time (min)	A%	B%
	0.1	85	15
	4.0	85	15
	5.5	80	20
	7.5	65	35
	11.5	64.5	34.5
	13	100	0
	18	85	15

Validation Study

Individual 1, 10, 50 and 100 mg/L of amino acids were prepared as the serial dilution with 0.1 N HCl. On the day of analysis, the above concentration was prepared. The calibration range and linearity were studied by injection of 5 concentrations of each amino acids standard of above and below the level of the range. The repeatability of standard and samples were checked using 100 mg/L standards injected in 6 times. The recovery was calculated by comparing in the triplicate value of each sample matrices and spiked sample preparing to the linearity range and treated as the same manner of samples.

RESULTS AND DISCUSSION

The microwave power and time optimized during the study, and maximum recovery for the spiked sample was received on 800 W power and 160 °C temperature reach on 10 min ramping time with 15 min holding time. In the harsh condition like the temperature >160 °C, observed the low recovery of some more labile amino acids such as Thr and Sys. Lorenzo et al. (2013), reported the almost similar condition (160-180 °C) for the bovine serum albumin samples, but they used a small sample volume than our study. Moreover, microwave assisted extraction technique used for the determination of free amino acids in plant and animal origin foods and found 10% higher extraction yield than traditional conventional techniques (Kovács et al. 1998). Liman and others (2016) reported, the microwave assisted extraction technique used for amino acids analysis in Tunisian animal feedstuff and they compared this method with the traditional hydrolysed method. The method performance showed very good separation to 18 amino acid with the low detection limits (Limam et al. 2016).

The chromatographic separation of 21 amino acid standards on FLD detector is shown in Figure 1. (Upper, Chanel 1: Ex:350 nm, Em:450 nm, Lower: Chanel 2: Ex:266 nm, Em:305 nm).

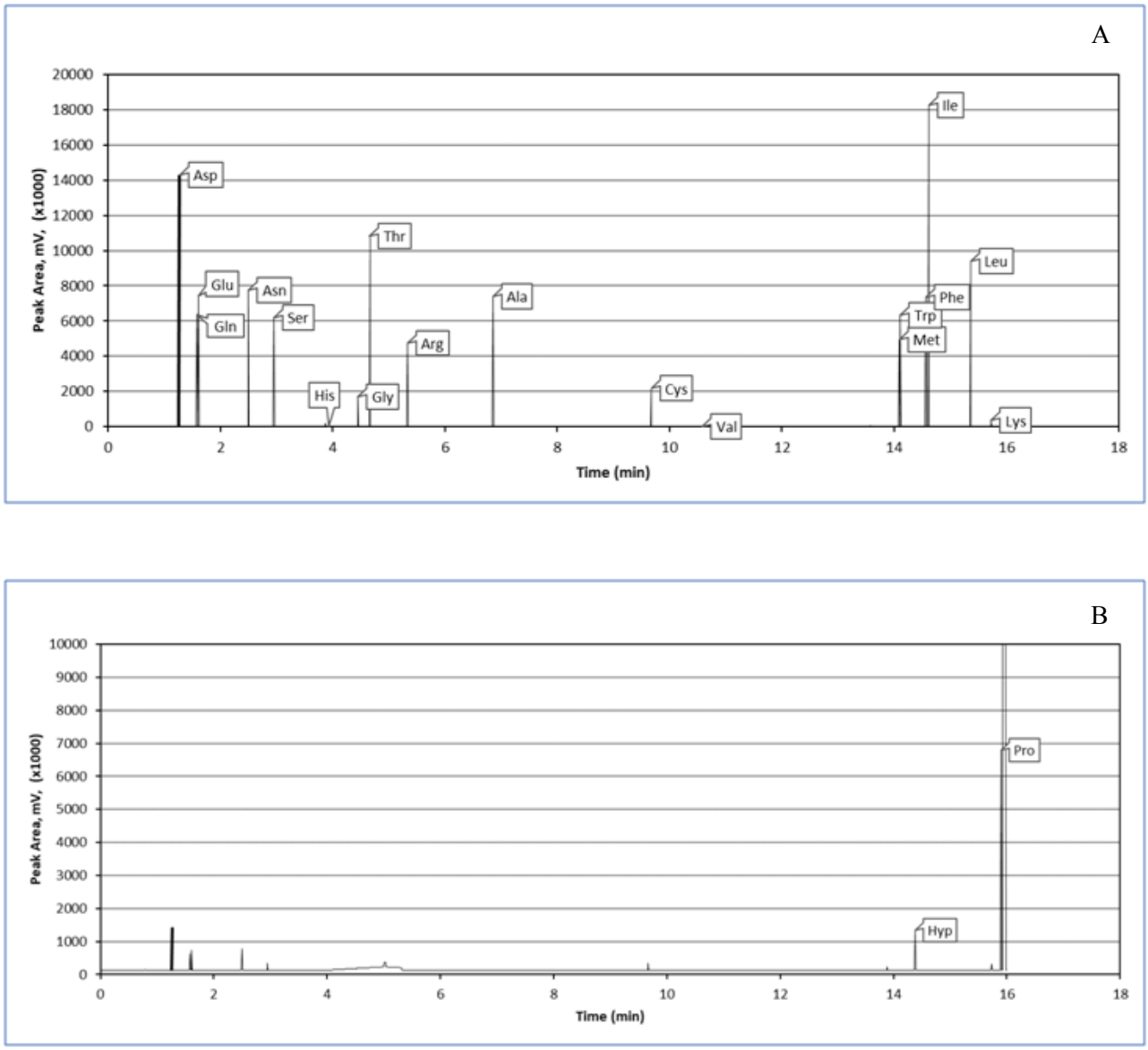

Fig 1 Amino acid chromatogram, HPLC-FLD, 100 mg/L, 10 µL injection (upper: detector channel A, ex.350 nm, em. 450 nm, lower: detector channel B, ex. 266 nm, em. 305 nm)

The repeatability (use as a precision) of the method was observed and the retention time (Rt, min), standard deviation of retention time (Rt-SD, %), average peak area (x1000, mv), standard deviation of area (RSD, %), concentration after injection of 100 mg/L (Con-mg/L) and standard deviation of concentration (Con.-SD, %) are listed in Table 2. The best FLD detector response and linearity of each amino acids were given more than 0.99 (except proline and hydroxyproline, - 0.99) correlation coefficient in between 1-100 mg/L (Figure 2). As Table 2, the method of recovery was very good for all 21 amino acids and ranges were complying with international standards recommended value (75-125%). The LoD and LoQ were calculated based on the standard deviation of the response and the slope of the linearity plot. The lowest LoQ was observed form Glu (0.03 g/kg) while the highest LoQ was observed from Gln (2.13 g/kg).

Table 2 Validation parameters of amino acids analysis

	Compound	Rt (min)	Rt-SD, %	Area	Area, SD, %	Con. (mg/L)	Con.-SD, %	LoD (g/kg)	LoQ (g/kg)
1	Asp	1.27	0.01	14300661	2.08	97	1.82	0.36	1.08
2	Gln	1.58	0.01	6373522	3.58	82	2.05	0.71	2.13
3	Glu	1.61	0.01	7414772	1.98	97	1.68	0.01	0.03
4	Asn	2.50	0.01	7752380	1.57	96	1.29	0.02	0.06
5	Ser	2.95	0.06	6205232	2.00	96	1.70	0.38	1.15
6	His	3.87	0.02	219216	2.27	95	2.12	0.18	0.54
7	Gly	4.45	0.03	1705205	1.90	89	1.57	0.27	0.81
8	Thr	4.66	0.01	10827093	1.98	89	1.74	0.26	0.78
9	Arg	5.33	0.01	4746499	1.96	96	1.64	0.11	0.33
10	Ala	6.85	0.01	7415506	1.84	95	1.74	0.13	0.39
11	Cys	9.67	0.02	2163069	2.48	88	2.50	0.15	0.45
12	Tyr	10.59	0.01	70016	2.76	98	2.35	0.11	0.33
13	Val	13.58	0.01	85147	2.21	77	1.88	0.21	0.64
14	Met	14.09	0.02	303475	3.68	78	3.13	0.04	0.12
15	Trp	14.11	0.01	6343431	3.94	99	3.35	0.15	0.45
16	Hyp	14.38	0.05	134181	1.21	101	3.22	0.17	0.51
17	Phe	14.56	0.01	7348101	2.64	99	2.04	0.56	1.69
18	Ile	14.61	0.01	18272117	1.21	101	2.49	0.08	0.25
19	Leu	15.36	0.01	9417973	1.16	97	2.93	0.09	0.27
20	Lys	15.73	0.03	335681	3.43	90	2.90	0.09	0.28
21	Pro	15.90	0.04	67990	5.52	86	6.59	0.31	0.93

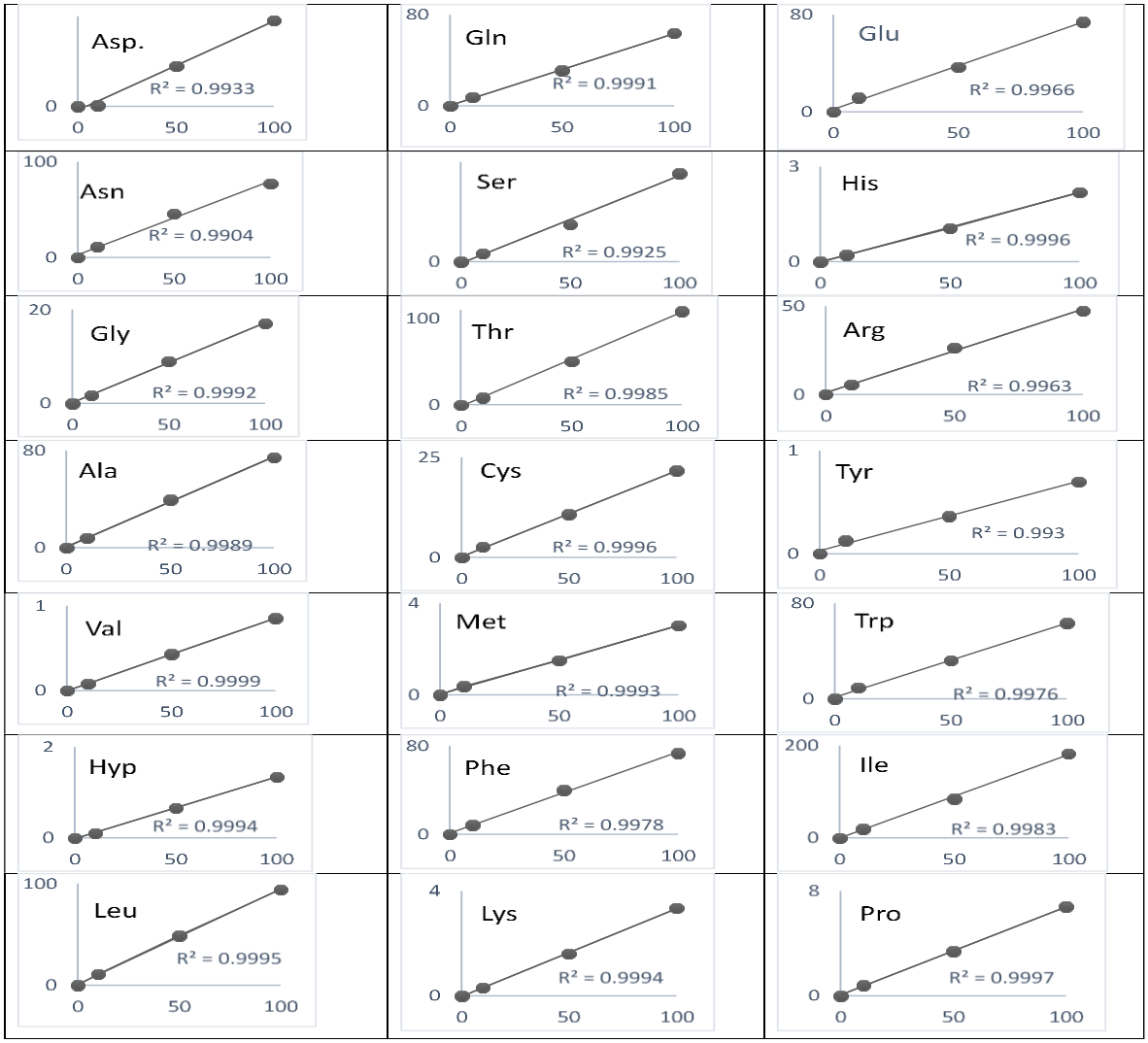

Fig 2 Calibration curve of pre-column derivatized 21 amino acids (Peak area x100,000)

Quantitative results of sample

The quantitative results of the studied samples are given in Table 3. To confirmation of the results, Norvalin (Nva) and Sarcosine (Sar) used as an internal standard (IS) with all samples. The recovery of the IS was in-between 80-110%. When quantification, we considered that the values below the LoQ as a not detected (ND).

Table 3 The hydrolysed amino acid concentration of fish (*Lutjanus* sp.), canned fish, fish feed & seaweed (*Sargassum* sp.), the value (mg/kg)

	Compound	Lutjanus sp.	Canned fish	Fish feed	Seaweed
1	Asp	4420	3018	20753	1987
2	Gln	22454	20586	21270	2337
3	Glu	ND	ND	37	ND
4	Asn	65	72	2794	ND
5	Ser	2548	1314	5508	1349
6	His	1312	1476	1344	ND
7	Gly	2662	3575	1433	6614
8	Thr	1903	1678	2128	4265
9	Arg	5128	2283	70977	368
10	Ala	6316	6981	2804	3617
11	Cys	3106	15042	5088	ND
12	Tyr	495	2727	4615	ND
13	Val	2095	4355	2320	4361
14	Met	1595	1370	2166	ND
15	Trp	3711	558	3936	ND
16	Hyp	2852	2627	3077	ND
17	Phe	3575	3350	2011	ND
18	Ile	4525	1434	2087	ND
19	Leu	9535	2193	76550	340
20	Lys	1282	318	805	329
21	Pro	9789	13806	26576	8801

CONCLUSION

New pre-column derivatizing method supported with microwave-assisted sample hydrolysing procedure was developed to quantify the amino acid profile of fish, fisheries products (canned fish, fish feed), and seaweed samples. The high sensitivity was achieved for 21 amino acids with FLD detector with the calibration range 1-100 mg/L based on 10 µL injections. The validated method can be used for hydrolysed amino acids detection of the fish, dried fish, fish feed and seaweed samples respectively.

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