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Application News

No. AD-0185

Amino acids in fish/ LCMS-8060

Development and Validation of Non-derivatization LC/MS/MS Method for Fast Determination of Proteinogenic Amino Acids in Fish

Wan Tung Liw[1], Zhe Sun[1], Chan Nicole[2]*, Jie Xing[1], and Zhaoqi Zhan[1]
[1] Application Development & Support Centre, Shimadzu (Asia Pacific), Singapore, [2] School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore, *ITP Student

❑ Introduction

Amino acids (AA) play a central role as building blocks of proteins and intermediates in metabolism that helps to maintain health and vitality. Fish is a source of proteins rich in essential amino acids which cannot be synthesized in human. Conventional methods for analysis of AA are based on HPLC-RF using pre- or post-column derivatization which runtime is normally from 30 mins to 2 hours. Thereby, there are demands in simpler and faster analysis without derivatization method for AA determination in various food matrixes. This study focuses on development and validation of a novel non-derivatization MRM-based method for fast quantitation of total amino acids in hydrolyzed fish using an Imtakt column. The method performance was validated and compared with UHPLC-RF method.

❑ Experimental

Analytical conditions and sample preparation

A commercial amino acids standard mixture was acquired from Sigma Aldrich. Two amino acids standards, asparagine and glutamine were added to the commercial standard to prepare a total of 21 amino acids working standards. Red snapper, seabass and Spanish mackerel from fresh water were purchased from local fish market and used as sample matrix. Blended fish meat of 50 mg was hydrolyzed with 1 mL of mixed concentrated HCl/Propionic acid (1:1; v/v) in a Chemglass vacuum hydrolysis tube at 160°C for varied hydrolysis time, 15, 30, 60 and 120 minutes. Sample was blown to dryness by N_2 followed by reconstitution with ammonia solution to pH 5. Hexane was added to sample for defatting (hexane to sample ratio 2:1). After vortex and centrifuge for 15 minutes respectively, the supernatant was diluted 400 times and filtered with 0.2 µm nylon filter. A LCMS-8060 triple quadrupole and a Nexera UHPLC with fluorescence detector were employed for LC/MS/MS and UHPLC-RF analysis, respectively. An Imtakt mixed-mode column (100x3mm, 3µm) was adopted for separation of the 21 amino acids without derivatization with a fast gradient elution of 15 minutes. On the other hand, on-line pre-column derivatization with OPA followed by FMOC performed in autosampler (SIL-30AC) at room temperature was carried out before injection to a C18 column (Shim-pack HR-ODS, 150x3mm, 3µm). The total runtime of UHPLC-RF method is 25 minutes excluding derivatization process. The detailed LC and MS/MS conditions are compiled in Table 1.

Table 1. Analytical conditions of 21 amino acids on LCMS-8060 without derivatization

Column	Intrada Amino Acid column (100 x 3 mm I.D, 3 µm)
Flow rate	0.6 mL/min
Mobile phase	A: ACN/ THF/ 25 mM HCOONH4/ FA = 9/ 75/ 16/ 0.3 (v/v/v/v) B: ACN/ 100 mM HCOONH4 = 20/ 80
Elution mode	Gradient elution, 0 %B (0 – 3 min) → 17 %B (6.5 min) → 100 %B (10 - 12 min) → 0 %B (12.01 min) → 0 %B (15 min) → Stop
Oven temp.	40 °C
Injection vol.	2.0 µL

Interface & temp.	Heated ESI, 300°C
MS mode	MRM (+ and -)
Block temp.	400°C
DL temp.	250°C
CID gas	Ar (270 kPa)
Nebulizing gas flow	N_2, 2 L/min
Drying gas flow	N_2, 10 L/min
Heating gas flow	Zero air, 10 L/min

❑ Results and Discussion

Quantitative analysis of 20 amino acids and taurine (Tau) on LC-MS/MS

The 20 proteinogenic amino acids (AA) as well as taurine (Tau) are the targeted analytes in fish in this study. A MRM method for quantitative analysis of the 20 AA and taurine was established as summarized in Table 2. It was observed that glycine exhibited low peak intensity and sensitivity in MRM mode (76.1>30.1). Thus, SIM mode (m/z 76.2) was selected for glycine. Figure 1 shows the MRM chromatograms of the 20 AA and Tau mixed standards obtained on LCMS-8060. For calibration curve construction, a calibrant series of seven levels (0.01, 0.05, 0.1, 1, 5, 10 and 20 µM) were prepared and analysed, except for glycine which calibration standard concentration range is 5-20 µM. A few selected calibration curves are shown in Figure 2,

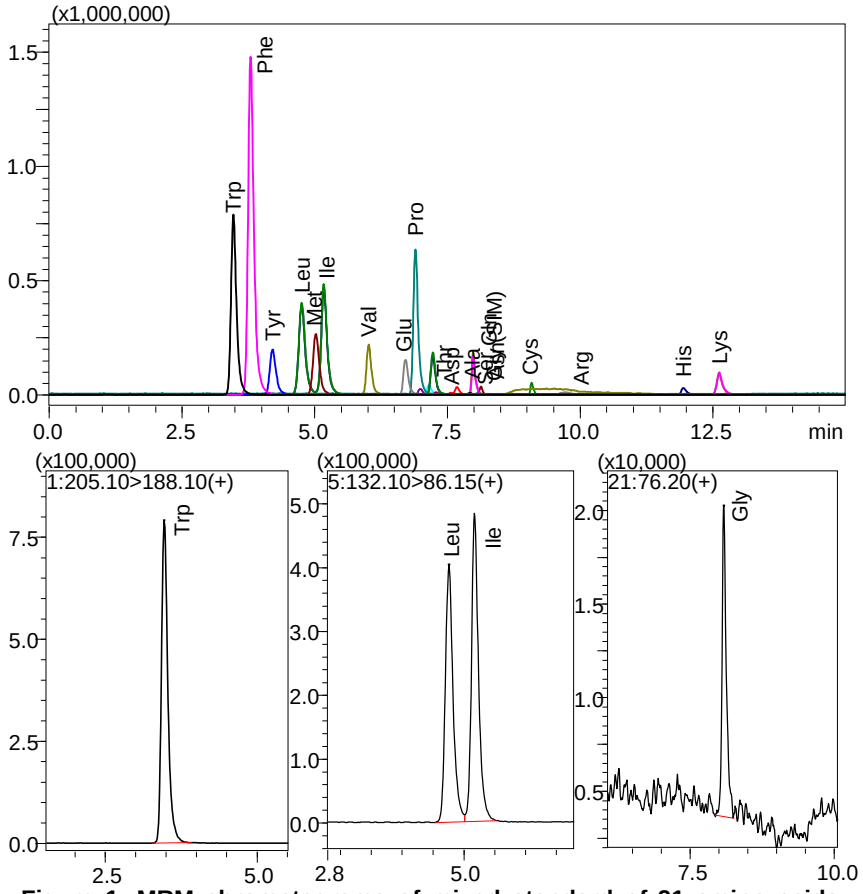

Figure 1. MRM chromatograms of mixed standard of 21 amino acids with 5 µM each except Gly (10 µM) on LCMS-8060.

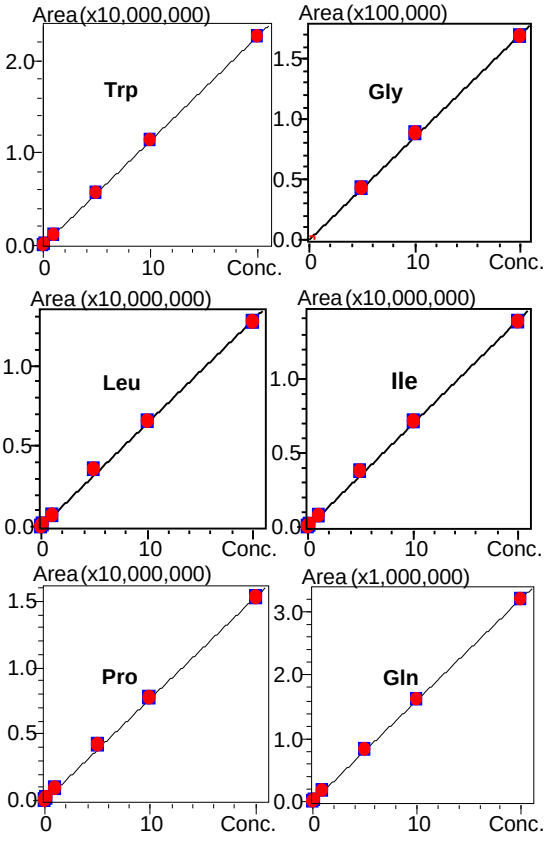

Figure 2. Representative calibration curves from 0.01 to 20 µM (Gly 5~20 µM) on LCMS-8060

with linearity (R^2) of 0.997 for all AA except for arginine which R2 is 0.995. The LOQ of the amino acids standards obtained range at 0.004~0.10 µM, except glycine 5 µM. Repeatability evaluation (based on area) for the amino acids was performed (n=6, injection volume 2 µL) with 0.5 µM mixed standard (except for glycine, 5 µM), giving results of % RSD of peak areas are at 0.8 – 7.8% for all amino acids except for glycine (11.9%). The performance and repeatability of the method are also shown in Table 2.

Validation of acid hydrolysis procedure and LC/MS/MS method

Acid hydrolysis of proteins in fish is normally carried out with 6N HCl at 110°C for 24 hours. In this study, a fast acid hydrolysis procedure was used with

Table 2. Summary of MRM quantitation method and performance of 20 AA and Tau based on 0.5 µM standard

No.	Name	RT (min)	MRM transition (m/z)	Calibration Curves and Quantitation Performance				
				Range (µM)	R^2	LOQ (µM)	LOD (µM)	%RSD (n=6)
1	Trp	3.52	205.1>188.1	0.01-20	0.999	0.008	0.003	0.9
2	Phe	3.82	166.1>120.1	0.01-10	0.998	0.005	0.002	0.8
3	Tyr	4.22	182.1>136.0	0.05-20	0.999	0.050	0.020	1.8
4	Met	5.03	150.1>56.1	0.01-20	0.999	0.005	0.002	1.1
5	Leu	4.79	132.1>86.2	0.01-20	0.999	0.010	0.003	1.3
6	Ile	5.18	132.0>86.2	0.01-20	0.999	0.010	0.003	2.3
7	Val	5.99	118.1>72.1	0.05-20	0.999	0.020	0.006	2.5
8	Glu	6.68	148.1>84.1	0.01-20	0.998	0.010	0.003	1.7
9	Pro	6.91	116.1>70.1	0.01-20	0.999	0.010	0.003	3.0
10	Asp	7.24	134.2>74.1	0.10-20	0.997	0.100	0.030	7.8
11	Thr	7.12	120.1>74.0	0.05-20	0.999	0.050	0.015	3.9
12	Ala	7.61	90.1>44.1	0.05-20	0.998	0.014	0.004	2.4
13	Ser	7.89	106.1>60.2	0.10-20	0.999	0.100	0.030	3.9
14	Gln	7.96	147.1>84.1	0.05-20	0.998	0.050	0.015	4.4
15	Asn	8.11	133.1>74.1	0.05-20	0.997	0.050	0.015	4.6
16	Cys	9.07	241.0>152.0	0.05-20	0.998	0.050	0.015	3.6
17	His	11.98	156.1>110.0	0.05-20	0.999	0.050	0.015	4.4
18	Lys	12.60	147.0>84.1	0.05-20	0.998	0.050	0.015	3.9
19	Arg	9.66	175.1>70.1	0.01-20	0.995	0.010	0.003	6.3
20	Gly (SIM)	8.07	76.2 (SIM)	5.0-20	0.999	5.00	1.500	11.9
21	Tau	6.99	(-) 124.1>80.0	0.10-20	0.998	0.010	0.003	1.8

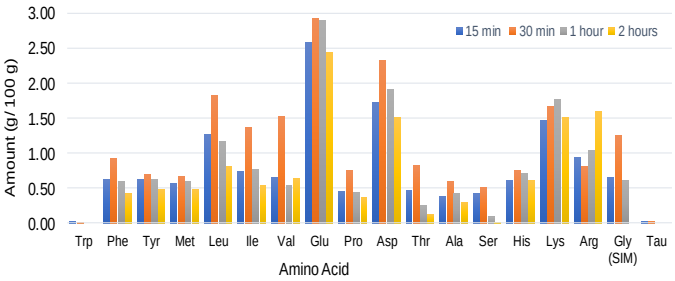

Figure 3. Profiles of amino acids of fish (Spanish Mackerel) produced by acid hydrolysis with different time

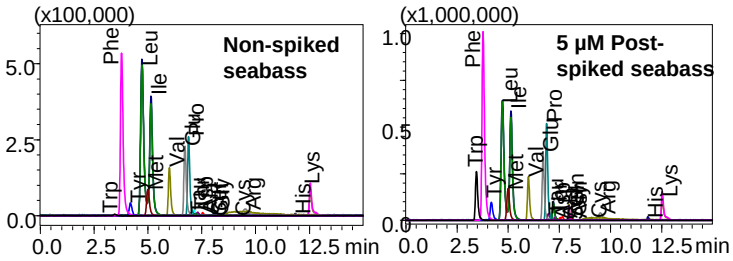

Figure 4. MRM chromatograms of seabass sample non-spiked (top) and 5 µM post-spiked analysed on LCMS-8060. The peak area differences of spiked and non-spiked were used to calculate matrix effect of each amino acid (see Table 3).

concentrated HCl and propionic acid at 160°C. The hydrolysis time is optimized between 15 mins and 2 hours. The amounts of amino acid (g/100g) produced are displayed in Figure 3. All the amino acids except for lysine exhibit the highest yields with 30 mins of hydrolysis. As thus, this acid hydrolysis condition with 30 mins was applied to all validation experiments with three species of fish including red snapper, seabass and Spanish mackerel. This fast acid hydrolysis was conducted in duplicate (S1 and S2) for each type of fish respectively and validated by acquiring inter-day and intraday reproducibility results with the established LC/MS/MS method. Matrix effect of the method was evaluated first with seabass by post-spiking 5 µM of mixed AA in the hydrolysed sample (Figure 4). The results of the matrix effects for the 21 AA are shown in Table 3, which were used to correct the quantitative results of the AA obtained with neat standard calibration curves. The AA results of three types of fish of intra-day and inter-day are shown in Table 3. The reproducibility are well accepted for inter-day and intraday results. In addition, the quantitative results obtained by LC/MS/MS is essentially in good agreement with that of the HPLC-Fluorescence for Tilapia fish. The predominant amino acids amongst the essential amino acids were lysine and leucine. The sum of total essential amino acids (EAA) in all the fish samples were at 8.5~11.9 g/100g. The relative higher amounts of glutamic acid and aspartic acid are due to conversion from glutamine and asparagine respectively in acid hydrolysis process. The extremely low level of tryptophan obtained is due to its degradation under acid hydrolysis condition. It is known

Table 3. LC/MS/MS results of amino acids (g/100g) in fish, matrix effect, reproducibility and comparison with HPLC results

No.	Amino Acid	Matrix effect (n=4) [4]	Intra-day (g/ 100g), n=2						Inter-day (g/ 100g), n=2						HPLC results of Tilapia fish HPLC (g/100g)
			Red Snapper		Sea Bass		Spanish Mackerel		Red Snapper		Sea Bass		Spanish Mackerel		
			S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	
1	Trp [3]	96.5	0.025	0.029	0.025	0.022	0.036	0.032	0.019	0.037	0.021	0.010	0.039	0.039	0.050
2	Phe	86.5	1.17	1.16	1.04	1.08	1.06	1.05	1.15	1.13	0.91	0.88	1.06	1.07	1.11
3	Tyr	94.6	1.05	1.25	1.06	1.05	1.07	1.16	1.26	1.21	0.64	0.60	1.19	1.15	0.77
4	Met	79.8	0.75	0.77	0.63	0.64	0.65	0.63	0.74	0.73	0.70	0.66	0.66	0.63	0.48
5	Leu	70.1	2.52	2.55	2.17	2.27	2.51	2.50	2.21	2.23	2.08	2.04	2.53	2.50	1.78
6	Ile	73.0	1.42	1.53	1.36	1.43	1.55	1.57	1.58	1.46	1.33	1.30	1.55	1.52	1.16
7	Val	73.9	1.30	1.49	1.23	1.23	1.40	1.40	1.39	1.38	1.19	1.14	1.37	1.38	1.12
8	Glu	103.7	2.02	2.47	2.18	2.15	2.03	2.09	2.13	2.46	2.64	2.36	2.11	2.10	4.44
9	Pro	90.7	0.76	0.92	0.75	0.72	0.88	0.87	0.81	0.85	0.69	0.66	0.95	0.93	2.93
10	Asp	81.3	2.62	2.85	2.66	2.53	2.46	2.52	2.27	2.25	2.27	1.99	2.47	2.22	3.95
11	Thr	99.5	0.79	0.81	0.63	0.57	0.73	0.67	0.85	0.86	0.66	0.57	0.67	0.69	0.94
12	Ala	90.0	0.61	0.71	0.54	0.55	0.69	0.65	0.62	0.63	3.12	2.83	0.61	0.67	1.65
13	Ser	107.8	0.35	0.31	0.32	0.36	0.34	0.33	0.38	0.39	0.40	0.33	0.46	0.40	0.85
14 [1]	Gln	106.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15 [1]	Asn	120.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	Cys	84.1	0.063	0.047	0.038	0.040	0.046	0.033	0.468	0.477	0.059	0.071	0.039	0.036	0.100
17	His	86.3	0.40	0.42	0.42	0.43	0.64	0.57	0.46	0.46	0.36	0.32	0.62	0.60	0.78
18	Lys	86.7	2.95	3.15	2.89	2.86	2.70	2.68	1.60	1.59	1.64	1.57	2.65	2.69	3.41
19	Arg	119.9	2.46	2.89	3.69	1.92	1.94	1.85	1.41	1.44	0.86	0.78	1.99	2.13	1.67
20	Gly (SIM)	86.4	1.81	2.26	2.43	2.14	2.39	2.25	2.14	2.14	1.06	1.00	2.44	2.65	1.12
21	Tau	107.5	0.12	0.12	0.12	0.12	0.02	0.02	0.25	0.21	0.24	0.21	0.02	0.02	N.A.
Total EAA (9) [2]			11.6		10.5		11.2		9.9		8.7		11.1		10.8
Other AA			12.9		12.7		11.8		11.9		11.4		12.3		17.5

[1] Conversion under acid hydrolysis condition: Asn --> Asp; Gln --> Glu; [2] EAA = essential amino acids (red colour remark); [3] value is low due to degradation in acid hydrolysis. Alkaline hydrolysis for Trp is needed. [4] ME = [post-spiked – non-spiked] / Neat std.

that alkaline hydrolysis process must be adopted for determination of tryptophan. The AA results of three types of fish of intra-day and inter-day are shown in Table 3. The reproducibility are well accepted for inter-day and intraday results. In addition, the quantitative results obtained by LC/MS/MS is essentially in good agreement with that of the HPLC-Fluorescence for Tilapia fish. The predominant amino acids amongst the essential amino acids were lysine and leucine. The sum of total essential amino acids (EAA) in all the fish samples were at 8.5~11.9 g/100g. The relative higher amounts of glutamic acid and aspartic acid are due to conversion from glutamine and asparagine respectively in acid hydrolysis process. The extremely low level of tryptophan obtained is due to its degradation under acid hydrolysis condition. It is known that alkaline hydrolysis process must be adopted for determination of tryptophan.

❑ Conclusion

A non-derivatization LC/MS/MS method with fast acid hydrolysis at 160°C was developed and partially validated for the determination of proteinogenic amino acids and taurine in fish. The results of EAA in different types of fish are in consistence with each other and comparable with the results obtained by UHPLC-fluorescence method. The main advantage of the current method is its much faster speed in both sample pre-treatment and LC/MS/MS analysis due to the fast acid hydrolysis at higher temperature and direct analysis of amino acids without the need for derivatization.

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SHIMADZU (Asia Pacific) Pte. Ltd
79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264,
www.shimadzu.com.sg; Tel: +65-6778 6280 Fax: +65-6778 2050