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Amino Acid Analysis / LCMS-8045

Non-Derivatization LC/MS/MS Method for Determination of Amino Acids in Infant and Adult Nutritional Formulas Following AOAC Requirements

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❑ Introduction

Quantitative analysis of proteinogenic L-α-amino acids and taurine in infant and adult formulas are performed routinely in manufacturer and nutritional laboratories. Milk powder, a source of high quality proteins, contains the essential amino acids (EAA) that must be obtained from nutritional intake. Taurine is commonly found in infant formula milk, which can protect human from exposure to toxic substances. Quantitative analysis of amino acids is conventionally carried out by HPLC-RF methods with pre- or post-column derivatization, which involves a tedious procedure. Normally, three hydrolysis procedures are needed to determine all the AA listed in the AOAC requirements [1-2]. Recently, non-derivatization LC/MS/MS method was reported, which is simple and fast, and it was applied to quantitation of free amino acids [3] and hydrolyzed proteins in various food or biological samples. This study focuses on validation of the non-derivatization LC/MS/MS method for determination of total proteinogenic amino acids and taurine using the standard reference materials (SRM) NIST® 1849a infant/adult nutritional formula.

❑ Experimental

Analytical conditions and sample preparation

A stock solution of 20 amino acids (AA) and taurine (Tau) were prepared from a commercial amino acid standard (Sigma-Aldrich). The NIST® 1849a Infant/adult nutritional formula (Sigma-Aldrich) was used for the purpose of method validation. Six nutritional formulas for adults and infants were purchased from the local markets and used for the amino acid and taurine analysis in this study. Different hydrolysis approaches were used in sample handling. (1) Acid hydrolysis: 0.5mL 37% hydrochloric acid (HCl) and 0.5mL propionic acid were added to a 50mg sample and heated at 160ºC for 1 hour, followed by drying with N_2 gas. (2) Alkaline hydrolysis: 1mL of 4N NaOH was added to a 50mg sample and heated at 195ºC for 1 hour. The pH was then adjusted to pH 4 using 1N HCl solution and the salts formed were removed by centrifuge. (3) Pre-oxidation followed by acid hydrolysis: 2mL of freshly prepared performic acid (9:1 formic acid: 30% H_2O_2) was added to a 50mg sample and heated at 50ºC in water bath for 1 hour. Then, the sample was hydrolyzed by acid hydrolysis method as described above.

A dedicated amino acid column was adopted for separation of the compounds with a gradient elution program. The detailed LC and MS conditions are compiled in Table 1. The hydrolyzed samples were injected to LCMS-8045 triple quadrupole for analysis.

Table 1. LCMS acquisition parameters on LCMS-8045

Column	Intrada Amino Acid Column (100 mmL x 3 mmI.D, 3 µm)
Flow Rate	0.6 mL/min
Mobile Phase	A: ACN/THF/25 mM $HCOONH_4$/HCOOH =9/75/16/0.3 B: ACN/100mM $HCOONH_4$
Elution Mode	Gradient elution, 0% B (0 – 3 min) -> 17%B (9 min) ->100%B (16-18 min) 0%B (18.10 min) -> stop (21 min)
Oven Temp.	35ºC
Injection Vol	3.0µL
Interface & Temp.	Heated ESI, 300ºC
MS Mode	MRM (positive and negative)
Block Temp.	400ºC
DL Temp	250ºC
CID Gas	Ar (230 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

A. Quantitative analysis of 20 amino acids and taurine on LC/MS/MS

The 20 proteinogenic amino acids and taurine are the target analytes in infant/adult nutritional formula used in this study. An MRM method for quantitative analysis of the 20 amino acids and taurine was established using the mixed standards. For construction of the calibration curve, a calibrant series of six concentration levels (0.1, 0.5, 1, 5, 20 and 50 µM) were prepared in 0.1N HCl and used for analysis of the SRM and real samples. The MRM chromatograms of the standards of the 20 amino acids and taurine obtained on LCMS-8045 are shown in Figure 1 (top), and a few selected calibration curves are shown in Figure 2. The accuracy and repeatability test (based on area) of the MRM method was done by a repeat injection of the 20 µM neat standard. The results obtained are satisfying for calibration establishment (data not shown).

** Student from Nanyang Technological University, Singapore for ITS training*

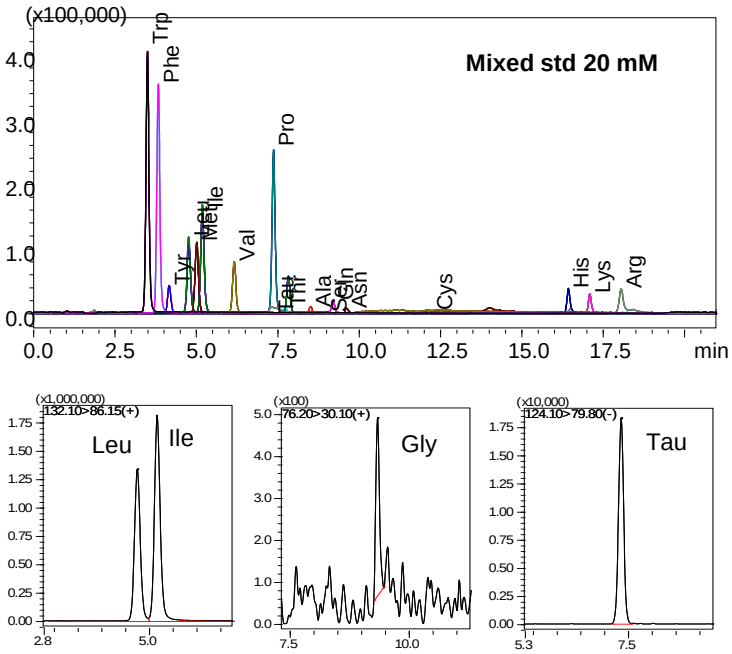

Figure 1. MRM chromatograms of mixed standard of 20 AA and taurine prepared in 0.1N HCl solution. (top) mixed std. conc. of 20 mM; (bottom): MRM peaks of selected AA, 20 mM

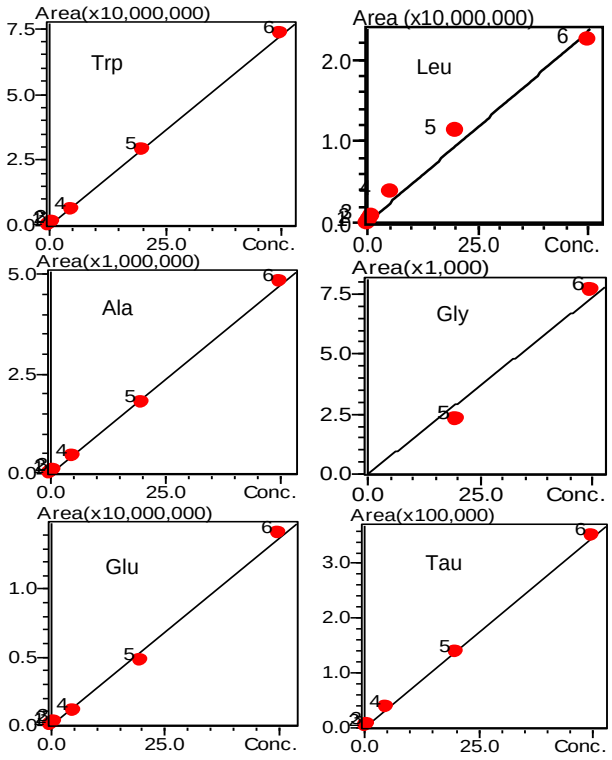

Figure 2. Representative calibration curves of six concentration levels of neat standards from 0.1 µM to 50 µM established on LC/MS/MS.

B. Evaluation of sample hydrolysis methods with SRM

Standard reference materials (SRM) NIST 1849a Infant/adult nutritional formula was used for evaluation of sample pre-treatment (hydrolysis) approach and LC/MS/MS analysis. Proteins in the nutritional formula were hydrolysed by acid hydrolysis (method 1) at 160ºC. The duration time of hydrolysis was tested and the results (Figure 3) indicate clearly that highest amounts of amino acids were produced with 1 hour hydrolysis. Due to degradation of tryptophan under acidic hydrolysis condition, alkaline hydrolysis (method 2) was used for analysis of tryptophan. While, pre-oxidation followed by acid hydrolysis (method 3) was carried out for cysteine/cystine analysis. In alkaline hydrolysis, the sample was hydrolyzed with 4N NaOH solution for 30 minutes at different temperatures. The results shown in Figure 3 indicate that hydrolysis at 195ºC gave the best results for tryptophan and most amino acids except proline, aspartic acid and taurine, which results are better with hydrolysis at 180ºC. Thus, a final heating time of 1 hour at 195ºC was used for alkaline hydrolysis. In the pre-oxidation hydrolysis method with performic acid, cysteine and cystine are converted into cysteic acid, which is then quantified using a separate calibration curve. The analysis results of the hydrolyzed SRM are summarized in Table 2, which are compared with the NIST reference values. The quantitative results of 6 out of the 18 amino acids match the official reference values (Ile, Val, Glu, Cys, His and Tau, recovery 86%~109%). The results of 11 amino acids are slightly out of the reference values (8 lower recovery at 71%~91%, 3 higher recovery at 123%~138%), but the measured result of Ala is far below the reference value (recovery: 26.4%).

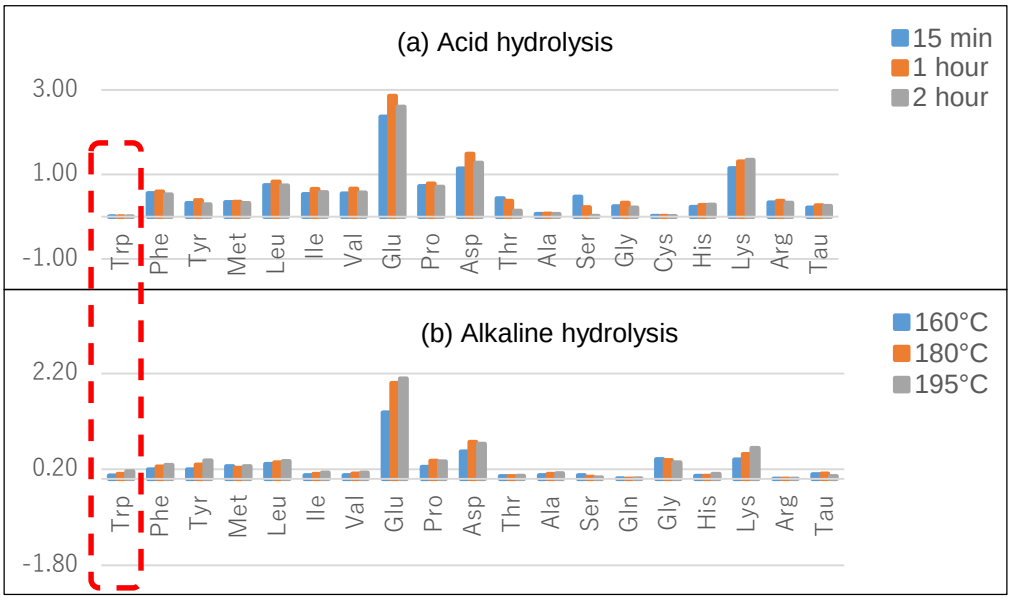

Figure 3. Profiles of total amino acids in SRM under the different hydrolysis conditions. (a) acid hydrolysis at 160ºC with different times. (b) alkaline hydrolysis at different temperatures with a constant time of 0.5h. Unit: g/100g

Table 2. Summary of quantification results of amino acids and taurine (g/100g) in SRM (NIST 1849a)

Amino Acid	MRM Transition	RT (min)	Measured Cont. (g/100g)	NIST 1849a Value	Extended Uncertainty	Recovery (%)	Matrix Effect (%) [3]
Tryptophan[1]	205.1>188.1	3.49	0.161	0.184	0.01	87.5	- [3]
Phenylalanine	166.1>120.1	3.83	0.711	0.58	0.021	122.6	80.8
Tyrosine	182.1>136.0	4.16	0.435	0.51	0.043	85.2	82.6
Leucine	132.1>86.2	4.76	0.947	1.261	0.05	75.1	86.9
Isoleucine	132.2>86.2	5.18	0.666	0.66	0.071	100.9	85.4
Valine	118.1>72.1	6.16	0.654	0.76	0.11	86.1	90.5
Glutamic acid	148.1>84.1	7.21	2.832	2.59	0.27	109.3	104.2
Proline	116.1>70.1	7.37	0.842	1.195	0.086	70.5	90.4
Aspartic acid	134.2>74.1	8.24	1.228	1.07	0.057	114.8	118.1
Threonine	120.1>74.0	7.73	0.524	0.64	0.022	81.9	104.5
Alanine[1]	90.1>44.1	8.51	0.120	0.455	0.021	26.4	-[3]
Serine	106.1>60.2	9.00	0.526	0.72	0.03	73.1	112.3
Glycine	76.2>30.1	9.37	0.180	0.241	0.019	74.7	133.5
Cystine[2]	241.0>152.0	12.24	0.125	0.1286	0.0071	97.2	-
Histidine	156.1>110.1	16.44	0.305	0.315	0.036	96.9	123.6
Lysine	147.0>84.1	17.10	1.392	1.01	0.071	137.9	115.0
Arginine	175.1>70.1	18.07	0.366	0.4	0.029	91.4	114.5
Taurine[1]	(-)124.1>80.0	7.36	0.040	0.0366	0.0018	109.3	-[3]

Note: [1] Results of Trp, Ala, Tau are based on alkaline hydrolysis method. [2] Result of cystine/cysteine is calculated based on the data of cysteic acid by pre-oxidation hydrolysis method. [3] ME was determined only for acid hydrolysis sample.

C. Determination of amino acids in infant & adult formulas

The above optimized sample preparation procedure and LC/MS/MS method were applied to actual infant and adult nutritional formulas. The results of the six milk powder samples are shown in Table 3 & Figures 4 and 5.

It can be seen that the total amino acid amounts in adult formulas are more than double the amounts of the amino acids in infant formulas, with the exception of taurine. In addition, lysine, aspartic acid and glutamic acid are typically present in high amounts compared to other amino acids.

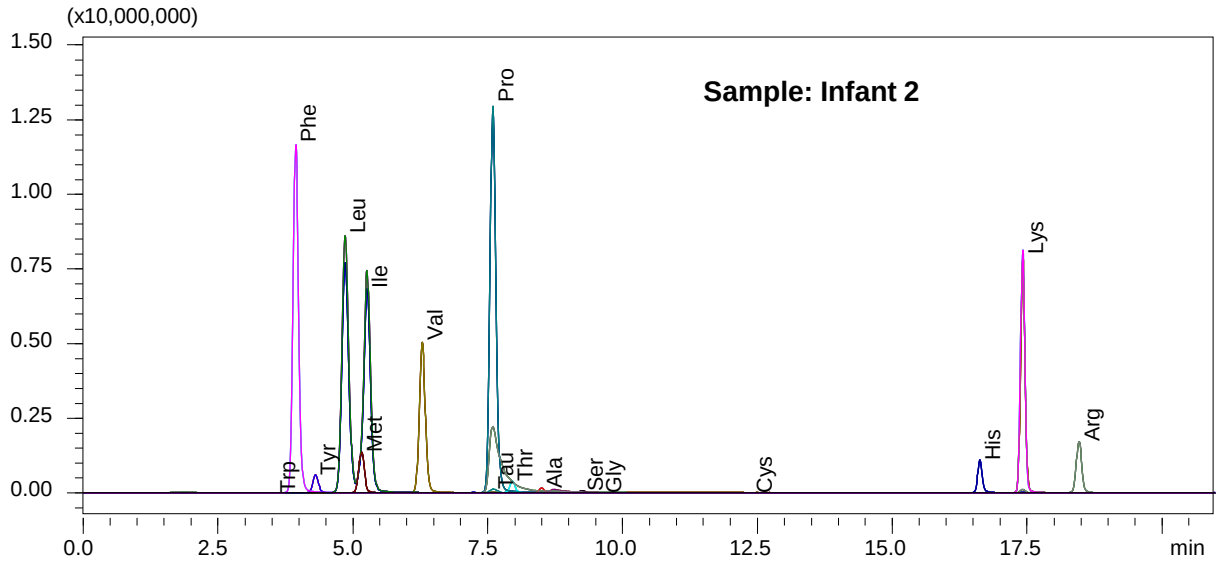

Figure 4. MRM chromatograms of 18 amino acids in milk powder samples: infant 2 and adult 3.

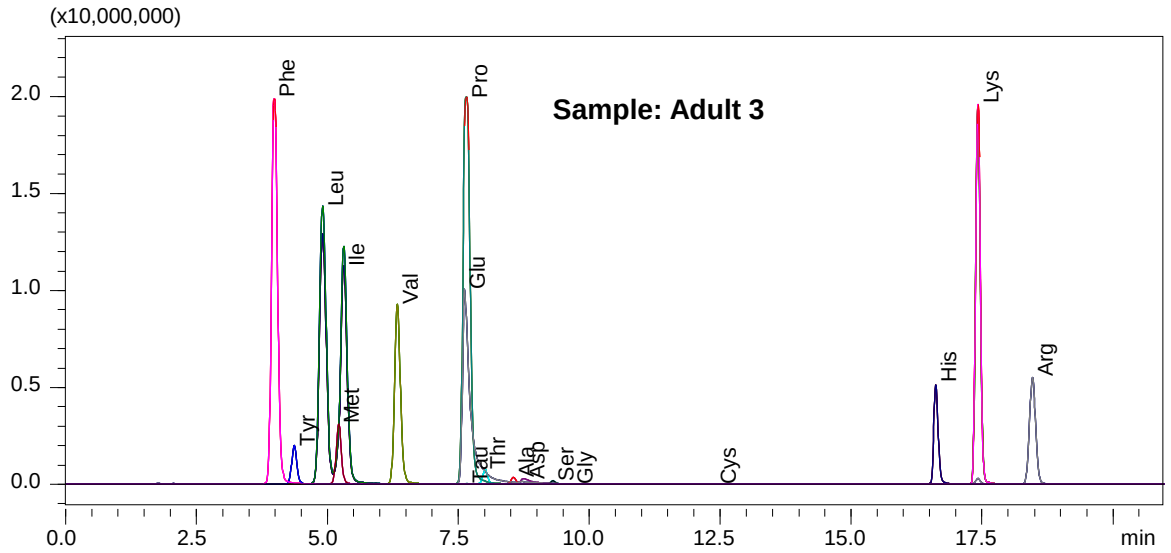

Figure 5. MRM chromatograms of 18 amino acids in milk powder samples: infant 2 and adult 3.

Table 3. Quantitative results of 17 AA and taurine (g/100g) in infant and adult formulas samples by LC/MS/MS method.

Milk Powder	Infant 1	Infant 2	Ave (Infant)	Adult 1	Adult 2	Adult 3	Adult 4	Ave (Adult)
Tryptophan[1]	0.09	0.16	0.12	0.22	0.23	0.20	0.19	0.21
Phenylalanine	0.40	0.40	0.40	0.73	0.70	0.76	0.83	0.76
Tyrosine	0.17	0.16	0.16	0.44	0.36	0.50	0.57	0.47
Leucine	0.69	0.67	0.68	1.02	1.06	1.13	1.21	1.10
Isoleucine	0.58	0.56	0.57	0.82	0.57	0.89	0.92	0.80
Valine	0.48	0.46	0.47	0.77	0.79	0.86	1.07	0.87
Glutamic acid	1.39	1.52	1.46	3.98	3.64	4.36	5.61	4.40
Proline	0.57	0.58	0.58	0.96	1.03	1.06	1.16	1.05
Aspartic acid	0.70	0.70	0.70	1.37	1.29	1.43	1.66	1.43
Threonine	0.23	0.21	0.22	0.33	0.39	0.40	0.59	0.43
Alanine[1]	0.11	0.10	0.11	0.21	0.21	0.18	0.17	0.19
Serine	0.09	0.08	0.09	0.19	0.25	0.24	0.30	0.24
Glycine	0.12	0.12	0.12	0.30	0.65	0.34	0.37	0.42
Cystine[2]	0.20	0.21	0.21	0.26	0.17	0.29	0.29	0.25
Histidine	0.13	0.14	0.13	0.46	0.44	0.63	0.61	0.53
Lysine	0.75	0.76	0.76	1.66	1.78	2.00	1.97	1.85
Arginine	0.16	0.20	0.18	0.55	0.66	0.72	0.73	0.67
Taurine[1]	0.048	0.037	0.04	0.0010	0.010	0.050	0.030	0.02
Total	**6.91**	**7.07**	**6.99**	**14.27**	**14.21**	**16.04**	**18.28**	**15.70**

Notes: [1] Results of Trp, Ala, Tau are based on alkaline hydrolysis method. [2] Result of cystine/cysteine is calculated based on the data of cysteic acid by pre-oxidation hydrolysis method.

❑ Conclusion

A SRM NIST® 1849a infant/adult nutritional formula was used for method validation of hydrolysis procedure and LC/MS/MS analysis of 18 amino acids (AA) without derivatization. Three hydrolysis procedures are needed to determine all the AA listed in the AOAC requirements. The measured results of the 18 AA are matched or closed to the reference values, except alanine, which recovery is only 26.4%. Further study on this low recovery of alanine is necessary.

❑ References

1. Standard Method Performance Requirements for Determination of Amino Acids in Infant Formula and Adult/Pediatric Nutritional Formula, AOAC SMPR 2014.013
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