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Application News

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Amino Acid Analysis / Nexera™ LC-40 X3

Microwave Digestion and On-line Pre-Column Derivatization UHPLC Method for Analysis of Total Amino Acids in Feed

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❑ Introduction

Analysis of total amino acid (AA) composition in feed is routinely performed in feed manufacture. This is because dietary amino acids in feed are crucial for lactation, growth, and health of pet and livestock. Animal diet needs amino acids as supplement, especially the essential AAs (histidine etc.) that cannot be synthesized within the animal body. Conventional HPLC analysis for determination of the total AA composition involves a tedious procedure. A feed sample is first pre-treated by acid hydrolysis (6N, HCl) to liberate AAs from proteins. Due to oxidation, tryptophan is destroyed during acid hydrolysis. Hence, alkaline hydrolysis is needed to assess tryptophan. In this study, a microwave digestion procedure was employed to carry out both acid and alkaline hydrolysis in less than 30 minutes, which is much faster in comparison to the conventional hydrolysis at 110°C for up to 24 hours.

On-line pre-column derivatization of the amino acids obtained was adopted to further speed up and simplify the analysis using the automatic pre-treatment function of the autosampler. This on-line derivatization uses o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids to produce fluorescent substances. Fluorescence (RF) detection was used for nmol/L level of high sensitivity analysis of AA quantification whereas UV detection allowed µmol/L level of AAs quantification.

❑ Experimental

Sample preparation – microwave digestion

Two types of feed samples (S1 and S2) in powder form were purchased from local supplier and used as sample matrix. 0.2 g of sample was weighed into the microwave digestion vessel and added 10 mL of 6N hydrochloric acid and 4N NaOH for acid and alkaline hydrolysis respectively. The digestion conditions were studied and optimized by varying time (15, 30, 45 minutes) and temperature (180, 195 and 210°C) in the Milestone ETHOS EASY microwave digester. The supernatant obtained was blown to dryness using a nitrogen evaporator (TurboVap®, Biotage) followed by re-constitution with aqueous solution to adjust it to a neutral pH value. Prior to pre-column derivatization and HPLC analysis, the sample was filtered by 0.22 µm hydrophilic PTFE filter.

Analytical system and conditions

A Shimadzu Nexera LC-40 X3 with photodiode array (PDA) and RF detectors were employed for the analysis. The Nexera LC-40 X3 equipped with auto pre-treatment function allows several reagents to be aspirated sequentially from multiple vials and mixed within the needle and the sample loop.

On-line pre-column derivatization with OPA (and 3-mercaptopropionic acid, MPA) followed by FMOC was performed by the auto pre-treatment function of the autosampler (SIL-40C X3). Details of a pre-set program and steps are shown in Figure 1. Addition of 0.1N HCl in step 5 was to neutralize the sample for protection of column. A Shim-pack Scepter™ C18 column (150 x 3 mm I.D., 3 µm) with a guard column was adopted, achieving good separation in 25 minutes (excluding derivatization process, which takes less than 6 minutes). The detailed UHPLC-PDA-RF conditions are compiled in Table 1.

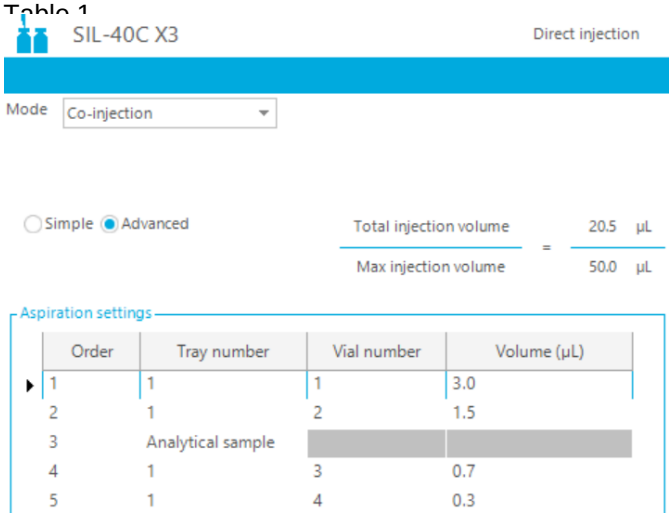

Fig 1: Preset program for auto on-line derivatization of amino acids with OPA (MPA) and FMOC in 6 minutes.

Table 1: Analytical conditions of AAs in animal feed

Column	Shim-pack Scepter 150 x 3.0 mm I.D., 3 µm with guard column
Flow Rate	0.8 mL/min
Eluent	A: 20 mM potassium phosphate buffer (pH 7.0) B: Acetonitrile/Methanol/Water = 45:40:15
Gradient Elution	7% B (0 min) ➔ 20.0% B (5.0 to 6.5 min) ➔ 27% B (8.5 min) ➔ 28.5% B (13.0 min) ➔ 65% B (18.0 min) ➔ 90% (20.0 – 20.5 min) ➔ 7%B (20.51 to 25.0 min)
Oven Temp	40°C
Inj. Vol.	0.5 µL
Det. A	Fluorescence detector, RF-20Axs Ch1: Ex: 350 nm, Em: 450 nm Ch2: Ex: 266 nm, Em: 305 nm
Det. B	Photodiode Array Detector, SPD-M40 Ch1: 338 nm Ch2: 262 nm

❑ Results and Discussion

Calibration curves of AAs by on-line derivatization

Calibration curves of 20 amino acids were established using on-line OPA/MFOC derivatization procedure (Figure 1). Figure 2 shows two representative chromatograms, 1 µmol/L mixed standard with RF detector (Top) and 100 µmol/L with PDA detector (Bottom). The sensitivity with RF detector is much higher than that of the PDA detector. The calibration curves, linearity, reproducibility, and estimated LOQ with RF detector are shown in Table 2 and Figure 3.

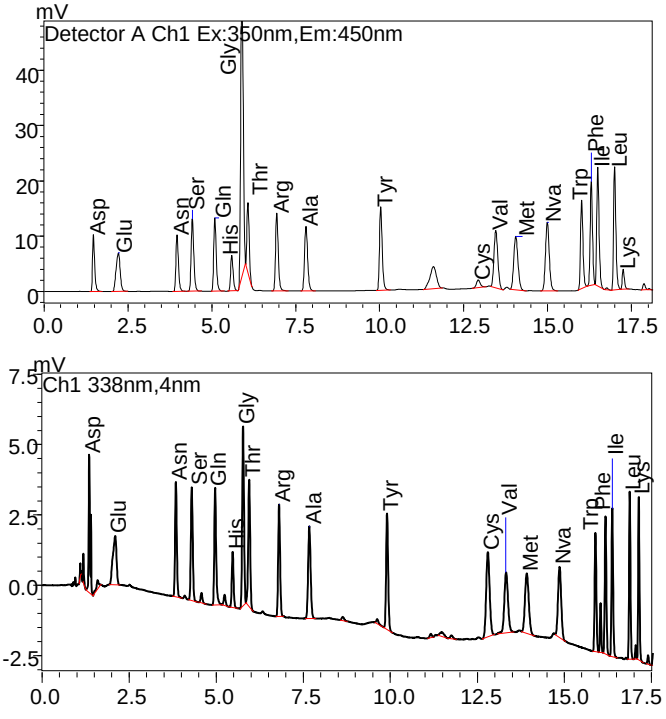

Figure 2: Chromatograms of 20 AAs in mixed standards via on-line derivatization and detection by RF detector (Top: 1 µmol/L each AA) and PDA detector (Bottom: 100 µmol/L each AA).

Table 2: Calibration curves, reproducibility and estimated LOQ for 20 AAs with on-line derivatization RF detection

No.	Amino Acids	Ret Time (min)	R^2 (*)	% RSD RT (50 µmol/L)	% RSD Peak Area (50 µmol/L)	LOQ (S/N=10), nmol/L
1	Asp	1.35	0.999	0.03	2.25	12.2
2	Glu	2.08	0.999	0.12	1.63	17.3
3	Asn	3.86	0.999	0.07	1.47	11.2
4	Ser	4.11	0.999	0.07	1.23	9.0
5	Gln	5.00	0.999	0.07	1.23	8.5
6	His	5.53	0.999	0.07	2.35	18.5
7	Gly	5.80	0.999	0.06	2.76	5.0
8	Thr	5.98	0.999	0.06	0.56	4.9
9	Arg	6.82	0.999	0.06	1.63	7.6
10	Ala	7.73	0.999	0.07	1.41	9.5
11	Tyr	9.97	0.999	0.05	1.63	7.7
12	Cys	13.10	0.999	0.06	2.11	75.0
13	Val	13.47	0.999	0.06	1.73	10.4
14	Met	14.04	0.999	0.06	1.91	10.5
15	Trp	15.94	0.999	0.02	1.58	6.8
16	Phe	16.23	0.999	0.02	1.86	7.2
17	Ile	16.43	0.999	0.02	1.59	5.2
18	Leu	16.92	0.999	0.02	1.75	5.2
19	Lys	17.20	0.999	0.02	3.9	26.5
20	Pro	19.33	0.999	0.02	4.72	12.4

** Range: 1~500 µmol/L*

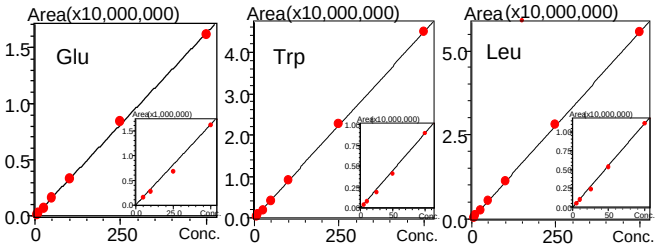

Figure 3: Three selected calibration curves of amino acids at 1~500 µmol/L with RF detector.

The reproducibility of the on-line derivatization method was evaluated with 50 µmol/L mixed standard by six consecutive injections. The %RSD (n=6) of retention time and peak area for 20 AAs were lower than 0.12% and 3.9%, respectively. The results indicate that the on-line derivatization is rather stable and reproducible. The estimated LOQs based on the lowest level (1 µmol/L) ranges 4.9~75 nmol/L as shown in Table 2.

The inbuilt PDA detector was also used to establish calibration curve for 5~500 µmol/L. The estimated LOQ with PDA detector (338 nm and 262 nm) was at 1.6~5.0 µmol/L, which was less sensitive than that of RF detector. However, based on the result, the applicable range of on-line derivatized AAs concentration is at or more than 5.0 µmol/L for UV-VIS or PDA detectors.

Optimization of microwave digestion

The animal feed samples were subjected to acid hydrolysis and alkaline hydrolysis under microwave digestion conditions. The conditions were optimized in terms of temperature and time. The results (Figure 4) indicate that the highest yields for most of the amino acids, except tryptophan and isoleucine, were obtained under microwave conditions at 195ºC for 15 minutes.

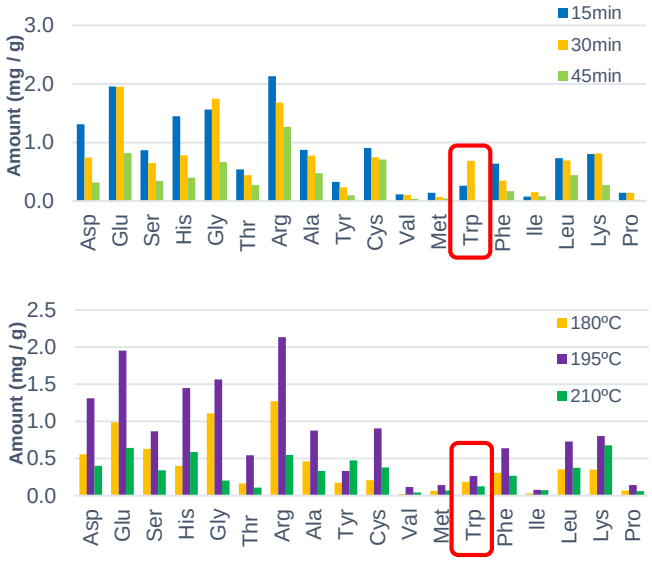

Figure 4: AA yields of feed sample (S2) by acid hydrolysis and alkaline (for Trp only) under microwave conditions. Top: at 195ºC; Bottom: at constant time of 15 minutes.

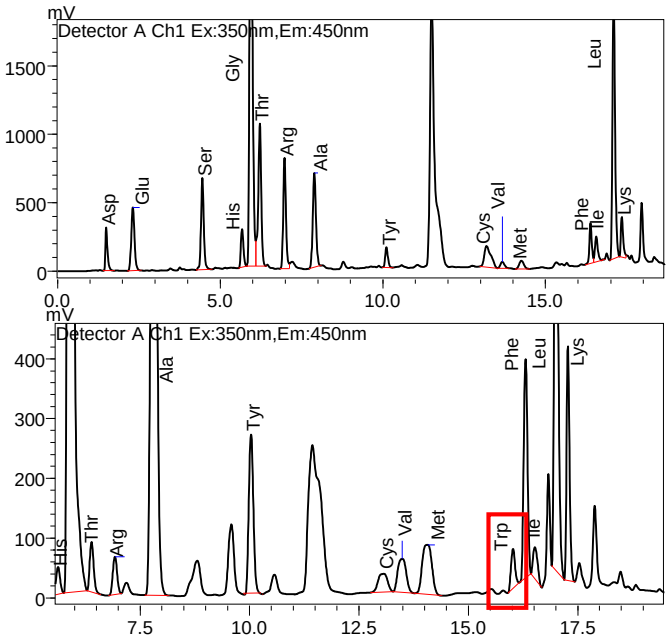

Figure 5: Chromatograms of AAs of S2 by acid hydrolysis (Top) and alkaline hydrolysis (Bottom)

Table 3: Results of 18 AAs in feed samples by microwave hydrolysis and on-line derivatization UHPLC-RF method.

Amino Acid (AA)		S1 (mg/g)	S2 (mg/g)
Essential AA	His	0.58	1.45
	Thr	0.63	0.54
	Val	0.12	0.12
	Met	0.09	0.14
	Trp*	0.11	0.26
	Phe	0.44	0.64
	Ile	0.09	0.08
	Leu	0.53	0.73
	Lys	1.08	0.8
Sum		**3.67**	**4.76**
Non-Essential AA	Asp	0.69	1.31
	Glu	1.23	1.95
	Ser	0.78	0.87
	Gly	0.58	1.56
	Arg	0.73	2.13
	Ala	0.51	0.88
	Tyr	0.12	0.33
	Cys	0.41	0.91
	Pro	0.26	0.14
Sum		**5.31**	**10.08**
Total		**8.98**	**14.84**

** Trp (tryptophan) by alkaline hydrolysis under the microwave conditions at 195ºC and 30 minutes.*

Quantitation of AAs in Feed Samples

Figure 5 shows the AA chromatograms of feed sample (S2) by acid hydrolysis (for 17 AAs) and alkaline hydrolysis (for Trp only) under the microwave conditions. Proline peak does not appear in the both chromatograms, because its Ex/Em wavelengths are different (Ex:266 and Em:305). It is well known that under the acid hydrolysis conditions, asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively. Tryptophan is totally destructed. Without pre-oxidation (like performic acid) before acid hydrolysis, methionine and cysteine are partially destructed. Eventually, eighteen amino acids in feed samples S1 and S2 were quantified, which include nine essential amino acids (see Table 3). Five essential amino acids, His, Thr, Phe, Leu, and Lys are found in relatively high contents in the feed samples.

❑ Conclusions

An automated amino acid method by on-line pre-column derivatization and UHPLC-RF analysis was set up on Nexera LC-40 X3. Combining rapid microwave acid hydrolysis and alkaline hydrolysis, a fast and simplified procedure was established for the quantitation of total AAs in animal feed. The on-line derivatization method for AAs was found to be stable, reproducible, and sensitive. The LOQs for the twenty AAs are 4.9-75.0 nmol/L and 1.6~5.0 µmol/L for RF detector and PDA detector, respectively. Eighteen AAs in two animal feed samples were quantified using the automated AA method.

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