Application Note

Food Testing and Agriculture

# Amino Acid Profiling of Fish Feeds Using Agilent 1260 Infinity II LC with DAD and FLD

## An optimized method covering an extended list of 24 amino acids

**Author**

Aveline Neo
Agilent Technologies, Inc.

## Abstract

This application note shows the quantitation of the amino acid composition of two different fish feeds following acidic hydrolysis. The existing Agilent amino acid chromatographic method (publication number 5991-7694EN) was further optimized to accommodate another four targets (taurine, theanine, glucosamine, and cysteine). The analysis was performed using an Agilent 1260 Infinity II LC with a serially connected Agilent 1260 Infinity II Diode Array Detector (DAD) and Agilent 1260 Infinity II Fluorescence Detector (FLD). Automated precolumn derivatization was included using the 1260 Infinity II Multisampler injector program. The superior DAD sensitivity for targets cysteine and cystine confirmed the benefit of adding UV detection together with FLD.

## Introduction

Fish feed nutrition is critical in fish farming. Understanding the amino acid profile in fish feed is essential to ensure the growth and health of fish. The amino acid composition of fish feed is an important factor that contributes to the growth performance of fish.[1] Taurine[2], cysteine[3], theanine[4], and glucosamine[5] are the additional amino acids that have been reported as growth and immunity enhancers in fish. Hence, a chromatographic method capable of separating all 24 amino acids is essential for screening amino acids in fish feeds. Precolumn derivatization and method parameters were adopted from the Agilent amino acid analysis "how to" guide (publication number 5991-7694EN).

## Experimental

### Equipment

Amino acid analysis was performed using an Agilent 1260 Infinity II LC system with the following components. The LC system was operated using Agilent OpenLab CDS version 2.7.

| Part Number | Component |
|---|---|
| G7112B | Agilent 1260 Infinity II Binary Pump |
| G7167A | Agilent 1260 Infinity II Multisampler |
| G7116A | Agilent 1260 Infinity II Multicolumn Thermostat |
| G7117C | Agilent 1260 Infinity II Diode Array Detector HS (with fixed slit) |
| G7121B | Agilent 1260 Infinity II Fluorescence Detector Spectra |
| G1321-60005 | Flow cell, 8 µL, 20 bar |

### Chemicals

All solvents used were LC grade. Acetonitrile was purchased from JT Baker (Phillipsburg, NJ, USA) and methanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, USA) equipped with a 0.22 µm membrane point-of-use cartridge. Sodium phosphate dibasic, disodium tetraborate decahydrate, concentrated hydrochloric acid (37%), and phosphoric acid (85%), were purchased from Sigma Aldrich (St. Louis, MO, USA).

The Agilent AdvanceBio AAA standards and reagents kit (part number 5190-9426) includes:

| Part Number | Component |
|---|---|
| 5061-3339 | Borate buffer: 0.4 M in water, pH 10.2, 100 mL |
| 5061-3337 | FMOC reagent, 2.5 mg/ml in acetonitrile, 10 × 1 mL ampules |
| 5061-3335 | OPA reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampules |
| 5061-3330 | Amnio acid standard (mix of 14 standards), 1 nmol/µL, 10 × 1 mL |
| 5061-3331 | Amnio acid standard, 250 pmol/µL, 10 × 1 mL |
| 5061-3332 | Amnio acid standard, 100 pmol/µL, 10 × 1 mL |
| 5061-3333 | Amnio acid standard, 25 pmol/µL, 10 × 1 mL |
| 5061-3334 | Amnio acid standard, 10 pmol/µL, 10 × 1 mL |
| 5062-2478 | Amino acids supplement kit (containing L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline (IS), and sarcosine (IS)), 1 g each |

The four other amino acids that were added to the amino acid analysis method were purchased from Sigma-Aldrich.

| Part Number | Amino acids |
|---|---|
| SMB00395 | L-Theanine |
| PHR1109 | Taurine |
| PHR1199 | Glucosamine hydrochloride |
| 168149 | L-Cysteine |

### Preparation of solvents and reagents

- Mobile phase A contained 10 mM $Na_2HPO_4$, and 10 mM $Na_2B_4O_7$, pH 8.2.
- Mobile phase B contained acetonitrile, methanol, and water (45/45/10, v/v/v).
- 0.1 N HCl was prepared by appropriate dilution of concentrated HCl using water
- Diluent: 10 mL of mobile phase A + 200 µL of phosphoric acid (85%)
- **Note**: After opening an OPA or FMOC ampoule, the reagents are aliquoted to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024). They were stored for no longer than a week.
- **Note**: Borate buffer and injection diluent were transferred to vials without inserts. All reagents should be stored at 4 °C and reagents in the autosampler should be exchanged daily.

### Preparation of amino acid standard solutions

1. Extended amino acid (EAA) stock solution: 1.8 nmol/µL each of asparagine, glutamine, and tryptophan, theanine, taurine, glucosamine, and 18 nmol/µL of cysteine in 0.1 M HCl.
2. Diluted EAA stock solution:
   - 0.9 nmol/µL,
   - 0.45 nmol/µL,
   - 0.18 nmol/µL,
   - 90 pmol/µL,
   - 45 pmol/µL,
   - 18 pmol/µL, and
   - 9 pmol/µL with 0.1 M HCl.

   Cysteine concentration in diluted EAA:
   - 9 nmol/µL,
   - 4.5 nmol/µL,
   - 1.8 nmol/µL,
   - 900 pmol/µL,
   - 450 pmol/µL,
   - 180 pmol/µL, and
   - 90 pmol/µL with 0.1 M HCl.
3. Internal standard (IS) stock solution: 1.0 nmol/µL each of norvaline and sarcosine in 0.1 M HCl.
4. EAA solutions + IS: mix EAA and IS in 1:1 ratio to obtain amino acid concentrations of 4.5 to 900 pmol/µL (cysteine was at 45 to 9,000 pmol/µL) and IS concentrations of 500 pmol/µL.
5. Final concentration of amino acid targets in calibration solutions:
   - 0.45 (L1), 0.90 (L2), 2.25 (L3), 4.5 (L4), 9.0 (L5), 22.5 (L6), 45 (L7), and 90 (L8) pmol/µL of amino acids (for all except cysteine) with IS 50 pmol/µL
   - For cysteine, the calibration level concentrations were 4.5 (L1), 9.0 (L2), 22.5 (L3), 45 (L4), 90 (L5), 225 (L6), 450 (L7), and 900 (L8) pmol/µL with IS 50 pmol/µL

### Fish feed sample source

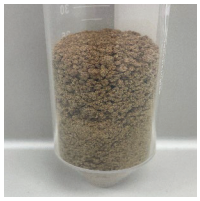
Fish feed 1 and fish feed 2 were generously provided by a customer.

### Sample preparation

For the extraction of amino acids in the fish feeds, the samples were hydrolyzed using 6 N HCl following a procedure described by Dai *et al*[6] as shown in Figure 1. Approximately 10 g of fish feed was weighed and homogenized using a mechanical shaker. A 500 mg sample of the homogenized fish feed was weighed into a 15 mL glass tube for extraction. Water and concentrated HCl (37%) were added to the sample to result in 10 mL of 6 N HCl. The tubes were capped and kept at a temperature of 110 °C for 24 hours for digestion. The tubes were gently shaken after 2 hours to facilitate efficient digestion.

After digestion, the tubes were cooled to room temperature and centrifuged at 3,000 rpm for 5 minutes. Agilent Captiva premium syringe filters (part number 5190-5108) were used to filter 1 mL of the supernatant. Then, 10 µL of the filtered supernatant was diluted with 990 µL water to get a 100x dilution. Later, 950 µL of the diluted sample homogenate was added to 50 µL of the IS stock solution and analyzed on the 1260 Infinity II LC system.

Fish feed 1



Fish feed 2

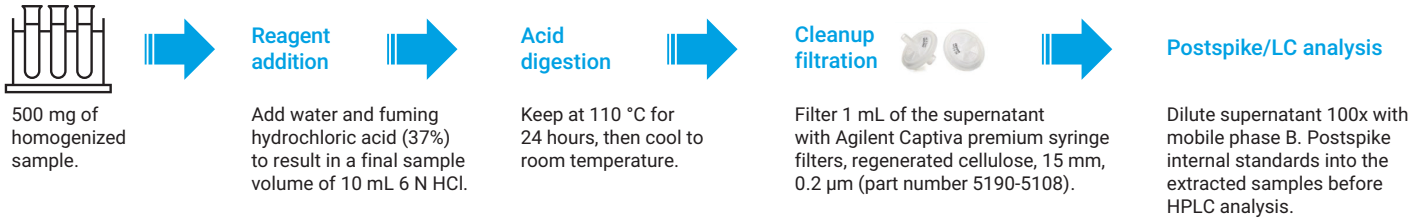



**Figure 1.** Sample preparation workflow.

### LC analysis

Precolumn derivatization is employed based on o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) chemistry for primary and secondary amino acids. The derivatization procedures were automated using the LC autosampler injector program function.The precolumn derivatization program on the 1260 Infinity II Multisampler is shown in Table 1 and the HPLC method is shown in Table 2.

**Table 1.** Injector program for precolumn derivatization of amino acids. Where location 1 is borate buffer, 2 is OPA reagent, 3 is FMOC reagent and 4 is injection diluent.

| Function | Parameter |
|---|---|
| Draw | Draw 5.00 µL from location "1" with default speed using default offset |
| Wash | Wash needle as defined in method |
| Draw | Draw 1.00 µL from sample with default speed using default offset |
| Wash | Wash needle as defined in method |
| Draw | Draw 1.00 µL from location "2" with default speed using default offset |
| Wash | Wash needle as defined in method |
| Mix | Mix 7.00 µL from air with default speed 10 times |
| Draw | Draw 0.40 µL from location "3" with default speed using default offset |
| Wash | Wash needle as defined in method |
| Mix | Mix 7.40 µL from air with default speed 10 times |
| Draw | Draw 32.00 µL from location "4" with maximum speed using default offset |
| Wash | Wash needle as defined in method |
| Mix | Mix 20.00 µL from air with maximum speed five times |
| Inject | Inject |

**Table 2.** HPLC method for analysis of amino acids.

| Parameter | Value |
|---|---|
| Column | Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322)<br>Agilent AdvanceBio AAA guard column, 3.0 × 5 mm, 2.7 µm (part number 823750-946) |
| Solvent | Mobile phase A: 10 mM $Na_2HPO_4$, and 10 mM $Na_2B_4O_7$, pH 8.2<br>Mobile phase B: acetonitrile, methanol, and water (45/45/10, v/v/v) |
| Gradient | Time (min) %B<br>0.0 2<br>0.4 2<br>2.0 15<br>5.0 15<br>12.0 38<br>14.0 57<br>16.0 57<br>16.5 100<br>20.0 100<br>20.5 2<br>24.0 2<br>Stop time: 24 minutes<br>Post time: 2 minutes |

**Table 2.** HPLC method for analysis of amino acids. (continued)

| Parameter | Value |
|---|---|
| Flow Rate | 0.6 mL/min |
| Temperature | 40 °C |
| Detection (DAD) | 338 nm, 10 nm Bandwidth, and reference wavelength 390 nm, 20 nm bandwidth |
| Detection (FLD) | Excitation: 345 nm; emission: 455 nm<br>15.20 min: Change excitation: 265 nm; change emission: 315 nm<br>PMT gain: 10<br>Peak width: >0.025 min (18.52 Hz) |
| Injection | 1 µL, Use vial/well bottom sensing<br>Draw speed 100 µL/min; ejection speed 400 µL/min |
| Needle Wash | Flush port, 20 seconds; acetonitrile: 0.1 M HCl (50:50; v:v) |

## Results and discussion

### Neat standard mix

The existing Agilent AdvanceBio AAA HPLC method was improvised for the analysis of the extended list of 24 amino acids that includes taurine, theanine, cysteine, and glucosamine. Reversed-phase LC with DAD, and FLD, detection was employed for the analysis. Figure 2 shows the separation of the 24 amino acids standard mixture together with two internal standards. All the amino acids showed better sensitivity in FLD than in DAD except for cysteine and cystine (Figures 3 and 4). The lower sensitivity in FLD for cysteine and cystine is due to the low fluorescence of the adducts, which are formed with the OPA reagent.[7] Hence, for cysteine and cystine, DAD data was used for result reporting whereas FLD data was used for the rest of the amino acids.

Limit of detection (LOD) and signal-to-noise (s/n) were ≥ 3 and limit of quantification (LOQ) and s/n were ≥ 10 for the amino acid analyzed, where these values were 0.45 pmol/µL and 0.90 pmol/µL, respectively, for all the amino acids except cysteine, which was at 4.5 pmol/µL and 9.00 pmol/µL. The calibration range was from 0.90 to 90 pmol/µL for all the amino acids except for cysteine, which was in the range of 9 to 900 pmol/µL. Excellent coefficient of determination ($R^2$) values above 0.999 were achieved for all the amino acids.

Retention time and area precision values were below 0.4 and 1.0% respectively (n = 3). At 22.5 pmol/µL, the average resolutions of all amino acid peaks were above 1.5 except for arginine. All amino acid targets exhibited a good accuracy value (back calculated using linearity equations) between 91 to 101%. Precision (retention time (RT) and area % relative standard deviation (RSD)), accuracy, resolution, and calibration results obtained from the analysis of the amino acid calibration standards are presented in Table 3.

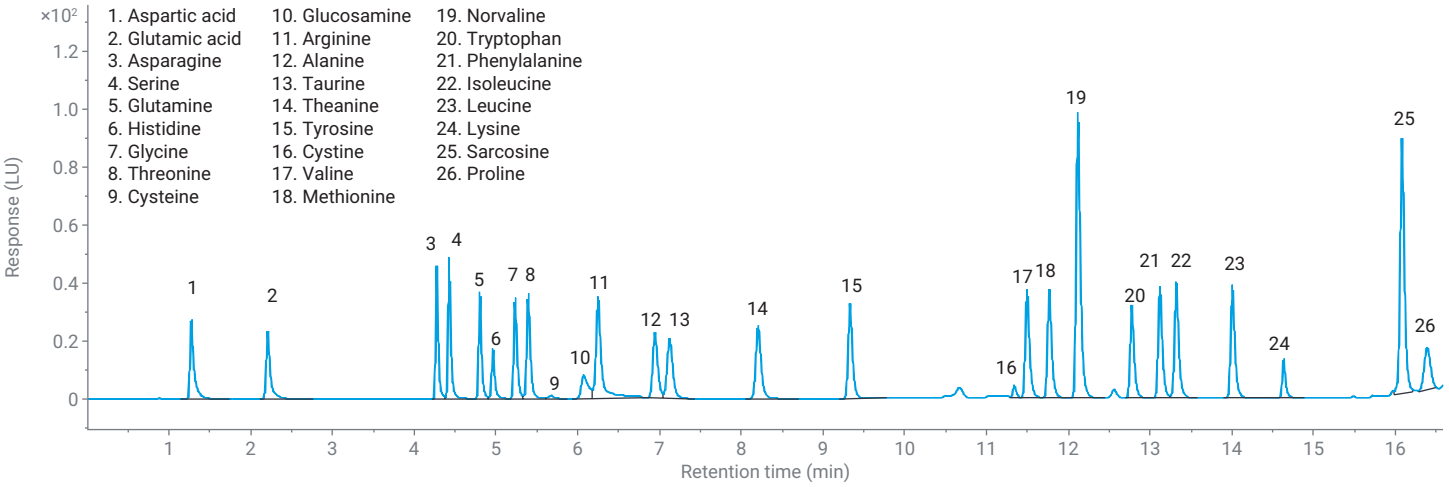



**Figure 2.** FLD chromatogram of amino acid calibration standard solution level 6.

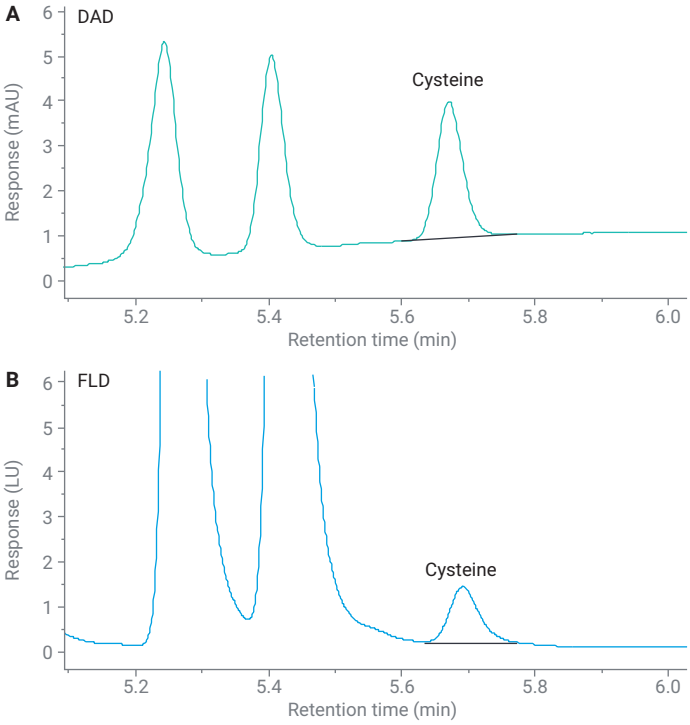



**Figure 3.** DAD (A) and FLD (B) chromatograms of cysteine at 225 pmol/µL.

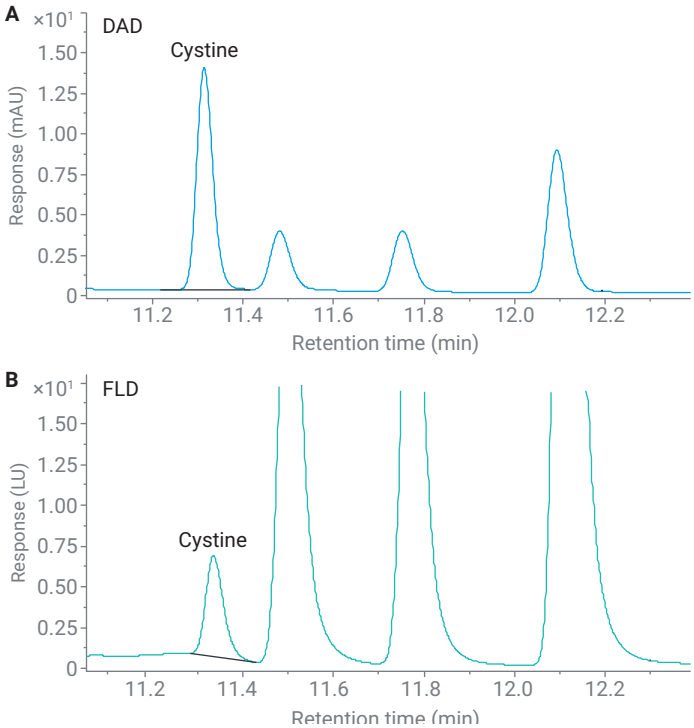



**Figure 4.** DAD (A) and FLD (B) chromatograms of cystine at 22.5 pmol/µL.

**Table 3.** Method performance summary using neat standard mix. Retention time, precision, accuracy, and resolution results are calculated at calibration level 6 concentration.

| Peak Number | Compound | Retention Time (min) | RT RSD (%) | Area RSD (%) | Accuracy (%) | Resolution | Calibration Range (pmol/µL) | Calibration Type | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Aspartic acid | 1.30 | 0.3 | 0.3 | 95 | NA | 0.90 to 90 | Linear | 1.000 |
| 2 | Glutamic acid | 2.34 | 0.8 | 0.3 | 91 | 12.1 | 0.90 to 90 | Linear | 0.999 |
| 3 | Asparagine | 4.36 | 0.4 | 0.3 | 98 | 30.1 | 0.90 to 90 | Linear | 1.000 |
| 4 | Serine | 4.51 | 0.4 | 0.3 | 96 | 2.9 | 0.90 to 90 | Linear | 1.000 |
| 5 | Glutamine | 4.88 | 0.3 | 0.6 | 97 | 6.7 | 0.90 to 90 | Linear | 0.999 |
| 6 | Histidine | 5.05 | 0.3 | 1.2 | 101 | 2.7 | 0.90 to 90 | Quadratic | 1.000 |
| 7 | Glycine | 5.33 | 0.3 | 0.2 | 96 | 4.1 | 0.90 to 90 | Linear | 1.000 |
| 8 | Threonine | 5.49 | 0.3 | 0.4 | 97 | 2.2 | 0.90 to 90 | Linear | 1.000 |
| 9 | Cysteine* | 5.77 | 0.3 | 0.2 | 97 | 3.3 | 9 to 900 | Quadratic | 0.999 |
| 10 | Glucosamine | 6.21 | 0.3 | 0.3 | 98 | 3.8 | 0.90 to 90 | Linear | 1.000 |
| 11 | Arginine | 6.36 | 0.3 | 0.6 | 97 | 1.3 | 0.90 to 90 | Linear | 1.000 |
| 12 | Alanine | 7.06 | 0.3 | 0.3 | 94 | 6.1 | 0.90 to 90 | Linear | 0.999 |
| 13 | Taurine | 7.25 | 0.3 | 0.2 | 93 | 1.5 | 0.90 to 90 | Linear | 0.999 |
| 14 | Theanine | 8.30 | 0.3 | 0.3 | 96 | 8.7 | 0.90 to 90 | Linear | 0.999 |
| 15 | Tyrosine | 9.38 | 0.4 | 0.3 | 96 | 10.3 | 0.90 to 90 | Linear | 1.000 |
| 16 | Cystine* | 11.28 | 0.3 | 1.8 | 95 | 25.5 | 0.90 to 90 | Linear | 0.999 |
| 17 | Valine | 11.46 | 0.3 | 0.2 | 96 | 2.1 | 0.90 to 90 | Linear | 1.000 |
| 18 | Methionine | 11.73 | 0.3 | 0.2 | 96 | 3.0 | 0.90 to 90 | Linear | 1.000 |
| 19 | Norvaline | 12.06 | 0.3 | 0.2 | NA | 3.7 | NA | NA | NA |
| 20 | Tryptophan | 12.70 | 0.2 | 0.5 | 97 | 7.2 | 0.90 to 90 | Linear | 1.000 |
| 21 | Phenylalanine | 13.03 | 0.2 | 0.5 | 97 | 3.7 | 0.90 to 90 | Linear | 1.000 |
| 22 | Isoleucine | 13.22 | 0.2 | 0.6 | 97 | 2.1 | 0.90 to 90 | Linear | 1.000 |
| 23 | Leucine | 13.89 | 0.1 | 0.7 | 97 | 7.3 | 0.90 to 90 | Linear | 1.000 |
| 24 | Lysine | 14.50 | 0.0 | 1.9 | 101 | 7.6 | 0.90 to 90 | Linear | 0.999 |
| 25 | Sarcosine | 16.02 | 0.1 | 1.7 | NA | 18.4 | NA | NA | NA |
| 26 | Proline | 16.38 | 0.1 | 1.7 | 94 | 2.6 | 0.90 to 90 | Linear | 0.999 |

*Cysteine and cystine results were based on DAD.

## Method applicability for fish feed analysis

The FLD chromatogram of endogenous amino acids present in fish feed 1 is shown as Figure 5A. Cysteine and cystine were only detectable in DAD (Figures 5B and C). Using DAD and FLD in series leads to unambiguous identification of amino acids. The amino acid profile from fish feed samples 1 and 2 are similar and comparison results are shown in Figure 6.

Acidic hydrolysis can lead to the conversion of asparagine, glutamine, and cysteine to aspartic acid, glutamic acid, and cystine, respectively, and tryptophan is decomposed.[6] As a result, in both fish matrices, these amino acids are either absent or present in low concentrations.

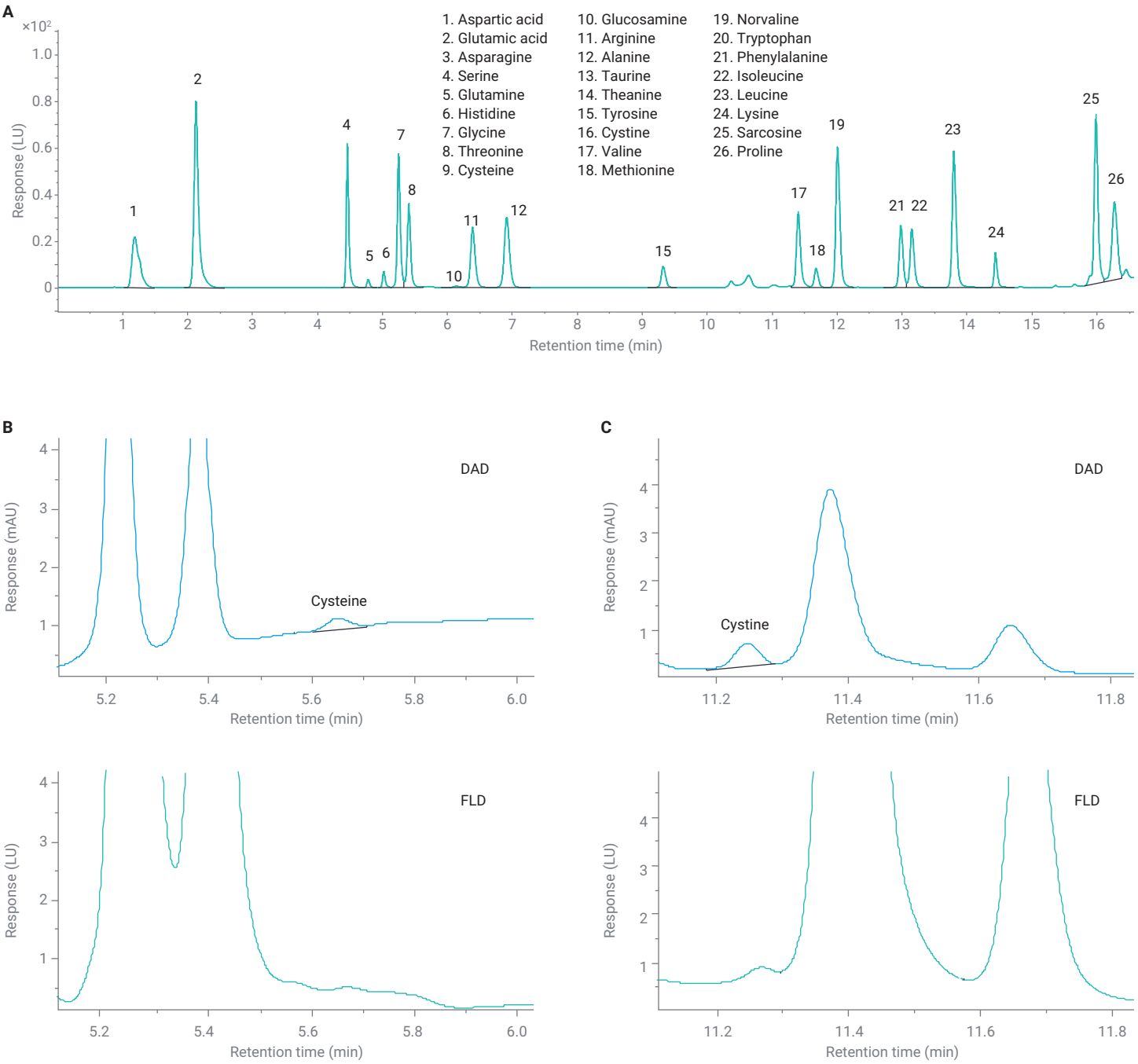



**Figure 5.** FLD chromatogram of amino acid analysis of fish feed 1 (A). DAD and FLD chromatograms of endogenous cysteine (B) and cystine (C) in fish feed 1.

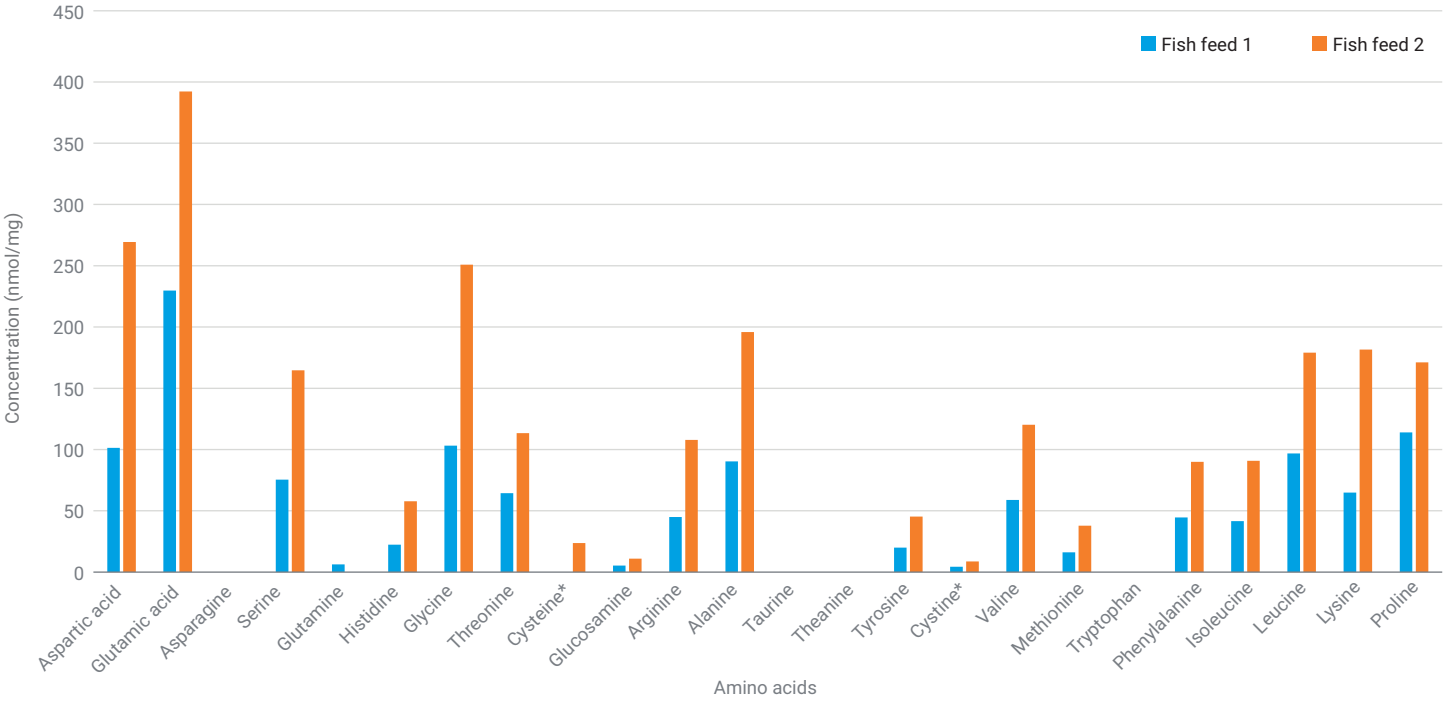



**Figure 6.** Comparison of amino acid profiles between fish feed 1 and 2. *Cysteine and cystine results were based on DAD.

For amino acid recovery analysis, the matrix blank was spiked at level 6 concentration. The endogenous concentration of targets was subtracted from the postspiked matrix and compared with calibration level 6 and expressed as percentage (Table 4). The recovery of individual amino acids ranged between 70 to 130% for all compounds in both fish feed matrices except for glutamic acid in fish feed 1. These results demonstrated the suitability of the method for the analysis of the amino acid profile for the fish feeds.

**Table 4.** Spike recovery results at level 6 calibration concentration.

| Peak Number | Name | % Recovery | |
|---|---|---|---|
| | | Fish Feed 1 | Fish Feed 2 |
| 1 | Aspartic acid | 88 | 109 |
| 2 | Glutamic acid | 68 | 106 |
| 3 | Asparagine | 103 | 101 |
| 4 | Serine | 99 | 109 |
| 5 | Glutamine | 80 | 93 |
| 6 | Histidine | 71 | 95 |
| 7 | Glycine | 93 | 103 |
| 8 | Threonine | 91 | 98 |
| 9 | Cysteine* | 128 | 130 |
| 10 | Glucosamine | 113 | 83 |
| 11 | Arginine | 96 | 106 |
| 12 | Alanine | 91 | 101 |
| 13 | Taurine | 102 | 116 |
| 14 | Theanine | 104 | 101 |
| 15 | Tyrosine | 103 | 106 |
| 16 | Cystine* | 123 | 78 |
| 17 | Valine | 94 | 129 |
| 18 | Methionine | 101 | 109 |
| 19 | Tryptophan | 102 | 100 |
| 20 | Phenylalanine | 100 | 107 |
| 21 | Isoleucine | 102 | 109 |
| 22 | Leucine | 93 | 107 |
| 23 | Lysine | 107 | 123 |
| 24 | Proline | 90 | 117 |

*Cysteine and cystine results were based on DAD.

## Conclusion

This application note demonstrates the effective amino acid profiling of fish feed samples using the Agilent 1260 Infinity II LC system with the Agilent 1260 Infinity II Diode Array Detector (DAD) and Agilent 1260 Infinity II Fluorescence Detector (FLD). The method achieved good sensitivity for the detection of 24 amino acids using the lowest calibration standard mix. The excellent area and RT precision confirmed the reproducibility of the method. The peak resolution together with DAD/FLD detection offers specificity and selectivity for confident identification of amino acids. The automated precolumn derivatization removes the manual liquid handling steps and reduces sources of human error, with the added benefit of time saved. The efficiency of the newly developed method for the precise quantitation of amino acids from complex fish feed sample is demonstrated. The sample cleanup using Agilent Captiva premium syringe filters minimizes the matrix interferences and offers good target recovery. The method is suitable for profiling amino acids from various fish feed samples.

## References


1. Deng, J.; Zhang, X.; Bi, B.; Kong, L.; and Kang, B. Dietary Protein Requirement of Juvenile Asian Red-Tailed Catfish *Hemibagrus wyckioides*. *Animal Feed Science and Technology* **2011**, *170(3)*, 231–238.
2. Sampath, W. W. H. A.; Rathnayake, R. M. D. S.; Yang, M.; Zhang, W.; and Mai, K. Roles of Dietary Taurine in Fish Nutrition. *Marine Life Science & Technology* **2020**, *2(4)*, 360–375.
3. Liu, J. X.; Zhu, K. C.; Guo, H. Y.; Liu, B. S.; Zhang, N.; Zhang, D. C. Effects of Cysteine Addition to Low-Fishmeal Diets on the Growth, Anti-Oxidative Stress, Intestine Immunity, and Streptococcus Agalactiae Resistance in Juvenile Golden Pompano (*Trachinotus ovatus*). *Frontiers in Immunology* **2022**, 13, 1066936.
4. Alagawany, M.; Abd El-Hack, M. E.; Saeed, M.; Naveed, M.; Arain, M. A.; Arif, M.; Tiwari, R.; Khandia, R.; Khurana, S.K.; Karthik, K.; *et al.* Nutritional Applications and Beneficial Health Applications of Green Tea and L-Theanine In Some Animal Species: A Review. *Journal of Animal Physiology and Animal Nutrition* **2020**, *104(1)*, 245-256.
5. Chowdhary, S.; Srivastava, P. P.; Mishra, S.; Yadav, A. K.; Dayal, R.; Raizada, S.; Jena, J. K. Partial Replacement of Dietary Animal Protein with Vegetable Protein Blend With Different Proportions of Glucosamine on Growth, Feed Efficiency, Body Composition and Survival of Fingerlings of Asian Catfish (*Clarias batrachus*). *National Academy Science Letters* **2012**, 35, 291-297.
6. Dai, Z.; Wu, Z.; Jia, S.; Wu, G. Analysis of Amino Acid Composition in Proteins of Animal Tissues and Foods as Pre-Column O-Phthaldialdehyde Derivatives by HPLC with Fluorescence Detection. *Journal of Chromatography B*, 964, 116-27, **2014**.
7. Lee, K. S.; Drescher, D. G. Derivatization of Cysteine and Cystine for Fluorescence Amino Acid Analysis with the O-Phthaldialdehyde/2-Mercaptoethanol Reagent. *Journal of Biological Chemistry* **1979**, *254(14)*, 6248-6251.



**www.agilent.com**

DE03297942

This information is subject to change without notice.





Printed in the USA, January 23, 2024
5994-7063EN


Agilent
Trusted Answers