Application Note

Pharmaceutical and Food Testing

Analysis of Amino Acids Derived Online Using an Agilent AdvanceBio AAA Column

Author

Lu Yufei
Agilent Technologies, Inc.

Abstract

A liquid chromatographic (LC) method capable of quick, high analytical sensitivity quantitative analysis of amino acids has been developed. This method uses automated online precolumn amino acid derivatization performed using an Agilent autosampler combined with an Agilent AdvanceBio AAA column packed with superficially porous particles. This method was successfully applied in LC separation and precise determination of 23 amino acids in three different sample matrices.

Introduction

Amino acid analysis, with a wide range of applications in food, pharmaceutical, and biological industries, has always been a challenging area in chromatography. How to analyze different types of amino acids with high efficiency is an issue for many analytical chemists. Many commercially available methods are flawed due, for example, to short column lifetime at high pH, low analytical efficiency, and rare reagents that are not easily available.

An LC method was developed to enable high speed and high analytical sensitivity for the quantitative analysis of amino acids. This method used reliable online precolumn amino acid derivatization, combined with an Agilent AdvanceBio AAA C18 column packed with superficially porous particles. The automated online derivatization can easily be achieved using an Agilent autosampler. The primary amino acids were derived from o-phthalaldehyde (OPA), and the secondary amino acids were derived from 9-fluorenylmethyl chloroformate (FMOC). All reagents and amino acid standards needed for this analysis are available from the AdvanceBio Amino Acid Analysis reagents kit.

Experimental

Apparatus and reagents

Agilent 1260 Infinity LC equipped with the following Agilent components:

- G1312B binary pump
- G1367D liquid autosampler
- G1316A thermostatted column compartment
- G4212B diode array detector

The Agilent AdvanceBio AAA C18, 4.6 × 100 mm, 2.7 µm (p/n 655950-802) was the column selected for use in this experiment.

Methanol and acetonitrile were chromatographic grade, and were purchased from Dikma Technologies Inc. Hydrochloric acid, dibasic sodium phosphate, and sodium borate were AR grade, and were purchased from J&K Scientific Ltd. Ultrapure deionized water was prepared using a Millipore Milli-Q ultrapure water system. Twenty-three types of amino acid reference standard (Table 1), OPA, FMOC, and borate buffer were obtained from the AdvanceBio Amino Acid kit from Agilent Technologies. Samples including compound amino acid injection, compound amino acid tablets, and goat milk, were obtained from local pharmacies and supermarkets.

Preparation of amino acid standard solutions

Divide 250 pmol/µL amino acid standard, contained in a 1 mL ampule, into 10 aliquots (100 µL each). Put each aliquot into a vial with a vial insert. Cover the vials and store at 4 °C.

Accurately weigh 59.45 mg of asparagine, 59.0 mg of hydroxyproline, 65.77 mg of glutamine, and 91.95 mg of tryptophan from the amino acid supplement kit. Then add these amino acids to a 25 mL volumetric flask, before adding 12.5 mL of 0.1 M hydrochloric acid solution, and ultrasonicating until the amino acids are dissolved. Next, dilute to the mark with water, shake well, and store at 4 °C as amino acid supplement stock solution 1.

Accurately weigh 58.58 mg of norvaline and 44.54 mg of sarcosine from the amino acid supplement kit, and add to a 50 mL volumetric flask. Then add 25 mL of 0.1 M hydrochloric acid solution to the flask, and ultrasonicate until the amino acids are dissolved. Next, dilute to the mark with water, shake well, and store at 4 °C as amino acid supplement stock solution 2. This solution can serve as internal standard solution.

Take a suitable amount of amino acid supplement stock solutions 1 and 2, add them to each aliquoted amino acid standard, and mix well using a vortex mixer; label as amino acid standard solution for later use.

Preparation of derivatization reagents

FMOC Reagent: Divide FMOC, contained in a 1 mL ampule, into 10 aliquots (100 µL each). Put each aliquot into a vial with a vial insert. Cover the vials, and store at 4 °C. Under these storage conditions, the aliquoted solutions are stable for 7–10 days.

OPA Reagent: Divide OPA, contained in a 1 mL ampule, into 10 aliquots (100 µL each). Put each aliquot into a vial with a vial insert. Cap the vials, and store at 4 °C. Under these storage conditions, the aliquoted solutions are stable for 7–10 days.

Diluent: 100 mL mobile phase A (containing 1.2 mL phosphoric acid), stored at 4 °C.

Sample preparation

Compound amino acid injection: Take 1 mL of sample solution, and put it into a 50-mL volumetric flask. Then, add 25 mL of 0.1 M hydrochloric acid solution, shake well, dilute with water to the mark, and shake well again. Pass the mixture through a 0.2 µm filtration membrane, and save the subsequent filtrate for later use.

Compound amino acid tablets: Take five tablets of sample, grind well, then precisely weigh the fine powder for an amount equivalent to one tablet. Put the powder into a 15 mL centrifuge tube, add 0.1 M hydrochloric acid solution, and ultrasonicate until the powder is dissolved. Then, pass the solution through a 0.2 µm filtration membrane. Take 1 mL of the subsequent filtrate, add it to a 50 mL volumetric flask, dilute with water to the mark, and shake well for later use.

Goat milk: Prepare as per GB/T 5009.124–2003 for dairy products. Briefly, proteins in the goat milk are denatured with HCl, hydrolyzed to amino acids at high heat, filtered, dried, then resuspended in sodium citrate buffer.

Autosampler procedure

- Draw 2.5 µL from a borate vial.
- Draw 1.0 µL from a sample vial.
- Mix 3.5 µL in wash port, five times.
- Wait 0.2 minutes, then draw 0.5 µL of OPA
- Mix 4 µL at wash port, 10 times.
- Draw 0.4 µL of FMOC.
- Mix 4.4 µL at wash port, 10 times.
- Draw 32 µL from a diluent vial.
- Mix 20 µL in wash port, eight times.
- Inject the sample.
- Wait 0.1 minutes.
- Valve bypass

LC Conditions

Parameter	Value
Column	AdvanceBio AAA C18, 4.6 × 100 mm, 2.7 µm
Flow rate	1.5 mL/min
Column temperature	40 °C
Mobile phases	A) 10 mM Dibasic sodium phosphate and 10 mM sodium borate, pH adjusted to 8.2 with hydrochloric acid. B) Methanol:acetonitrile:water, 45:45:10 (v:v:v)
Gradient program	Time (min) %B 0.0 2 0.35 2 13.4 57 13.5 100 15.7 100 15.8 2 18.0 2
Detector	338 nm, 10 nm bandwidth; reference 390 nm, 20 nm bandwidth (primary amino acids) 262 nm, 16 nm bandwidth; reference 324 nm, 8 nm bandwidth (secondary amino acids) Wavelength is switched after lysine peak appears. Experimental setting: 338 nm & 262 nm at 10.0 minutes

Results and Discussion

Online amino acid derivatization samples were injected as per the sample injection program described in this report. An Agilent 1260 Infinity LC system equipped with an AdvanceBio AAA column was used to perform baseline separation for 23 types of amino acids in 18 minutes. Figure 1 and Table 1 show the chromatogram of amino acid standard solutions and a list of 23 amino acids involved. The analytical method described in this report is a method developed using an AdvanceBio AAA C18 column based on the previous method[1,2]. As column selectivity is unchanged, the order of peaks for amino acids is not changed. Since there is an interval of 0.1 minutes between lysine and hydroxyproline peaks, compound analysis is unaffected at the time of wavelength switching.

The presence of amino acids in real samples was determined using the method described in this report, as shown in Figures 2–4. Complete baseline separation was achieved for amino acids in the real samples. This enabled the successful determination of 18 amino acids in the compound amino acid injection, 17 amino acids in the compound amino acid tablets, and 16 amino acids in the dairy products.

Table 1. List of 23 amino acid standards.

Peak number	Amino acid
1.	Aspartic acid
2.	Glutamic acid
3.	Asparagine
4.	Serine
5.	Glutamine
6.	Histidine
7.	Glycine
8.	Threonine
9.	Arginine
10.	Alanine
11.	Tyrosine
12.	Cystine

Peak number	Amino acid
13.	Valine
14.	Methionine
15.	Norvaline
16.	Tryptophan
17.	Phenylalanine
18.	Isoleucine
19.	Leucine
20.	Lysine
21.	Hydroxyproline
22.	Sarcosine
23.	Proline

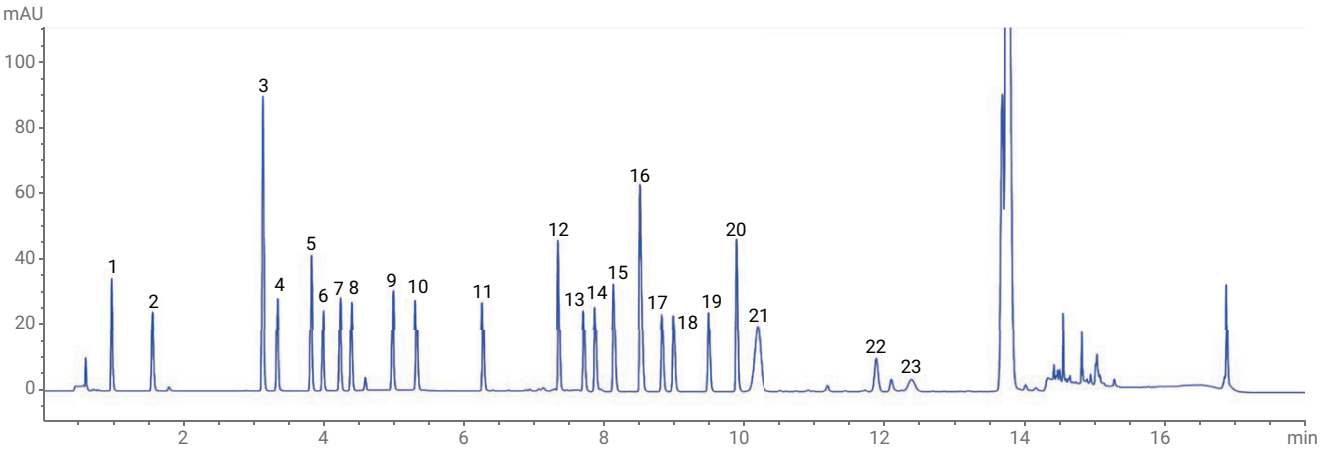

Figure 1. Separation of 23 amino acid standards.

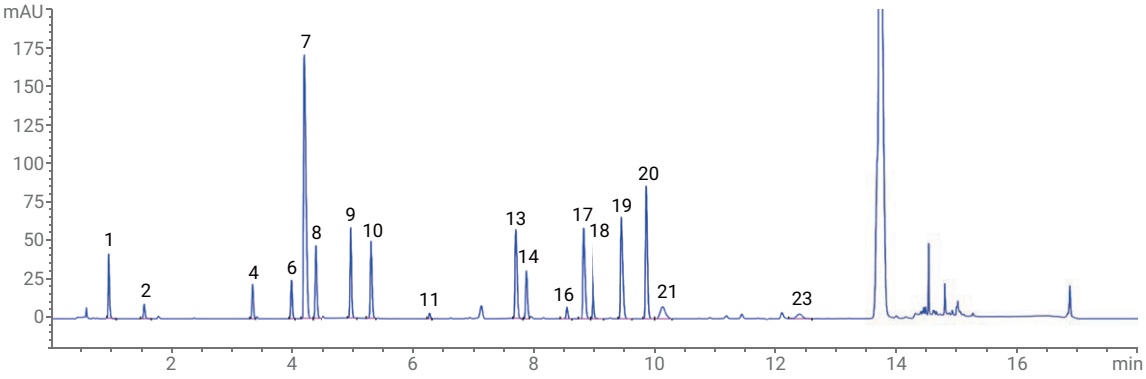

Figure 2. Separation of compound amino acid injection.

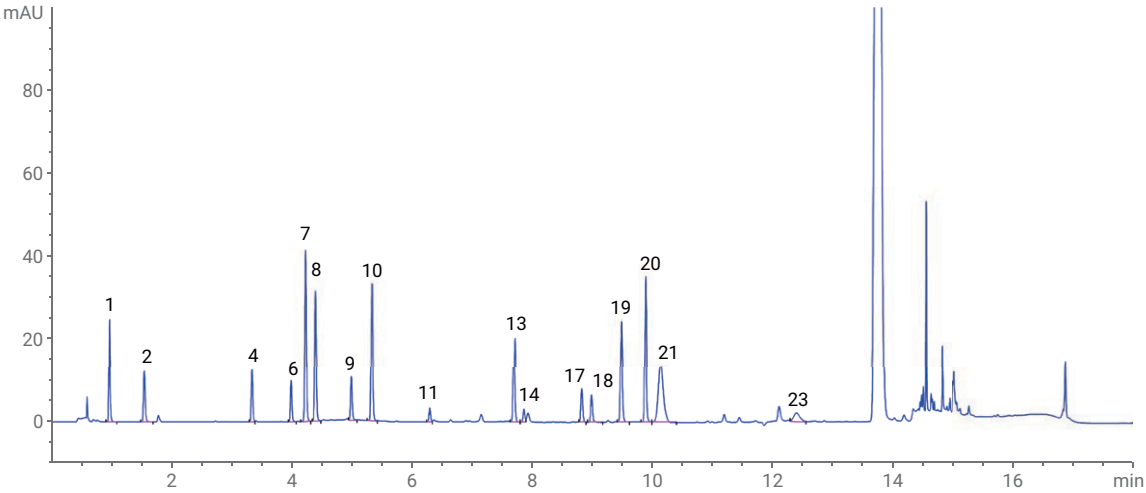

Figure 3. Separation of compound amino acid tablet sample.

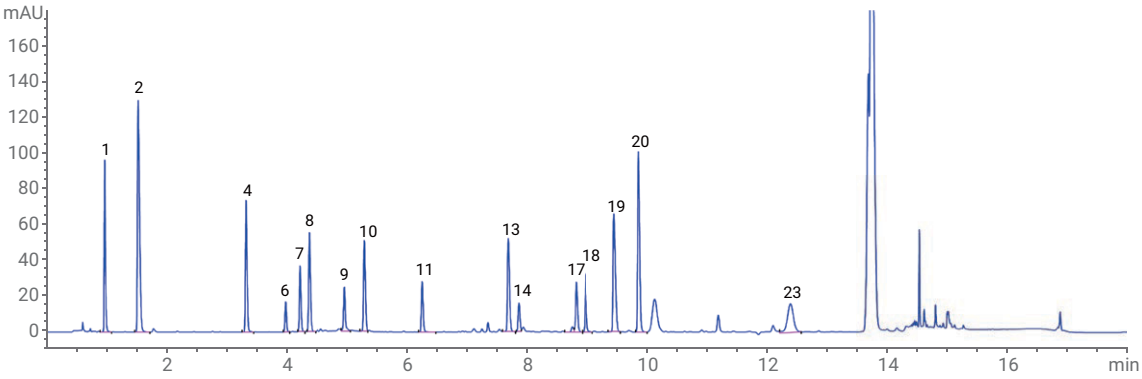

Figure 4. Separation of amino acids in a dairy sample, goat milk.

Conclusion

In this experiment, an accurate, quick, and reproducible amino acid analytical method was established using an Agilent AdvanceBio AAA C18 column. This method was based on the previous amino acid analytical method, enabling qualitative and quantitative analysis of 23 amino acids. In this experiment, amino acids in three different matrices for chemical drugs and food were analyzed with good resolution, providing accurate quantitation. Online derivatization reduced the time needed for sample preparation during the analysis process. Therefore, this method is suitable for amino acid analysis of mass samples in pharmaceutical, food, and other industries. Packed with superficially porous particles, the AdvanceBio AAA C18 offers efficiency comparable to columns packed with sub-2 µm particles, and its column pressure is approximately 50 % of those with sub-2 µm particles. The highest gradient pressure in the experiment was 220 bar, fully compatible with conventional LC systems. As the mobile phase system used in this amino acid analytical method is alkaline, the robustness of the silica-based column is important. After pH tolerance tests, the AdvanceBio AAA C18 column proved to be stable under alkaline conditions, effectively guaranteeing reliable repeatability of amino acid analysis[3,4]. For the analysis of real samples, it is recommended to use an AdvanceBio AAA C18 guard column.

References

1. Henderson, J. W.; *et al.* Rapid, Accurate, Sensitive and Reproducible HPLC Analysis of Amino Acids. *Agilent Technologies Application Note*, publication number 5980-1193E, **2000**.
2. Henderson Jr., J. W.; Brooks, A. Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals. *Agilent Technologies Application Note*, publication number 5990-4547EN, **2010**.
3. Extending Column Lifetime in Pharmaceutical Methods with High pH-Stable Poroshell HPH Chemistries. *Agilent Technologies Technical Overview*, publication number 5991-5022EN, **2014**. For Research Use Only. Not for use in diagnostic procedures.
4. Long, W. Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column. *Agilent Technologies Application Note*, publication number 5991-5571EN, **2015**.

Consumables Ordering Information

Part number	Component
655950-802	AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm column
820750-931	AdvanceBio AAA guard column, 4.6 × 5 mm
5190-9426	Agilent AdvanceBio AAA standard and reagents kit, which includes:
5061-3339	Borate buffer, 0.4 M in water, pH 10.2, 100 mL
5061-3337	FMOC reagent, 2.5 mg/mL in ACN, 10 × 1 mL
5061-3335	OPA reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampules
5062-2479	Dithiodipropionic acid (DTDPA) reagent, 5 g
5061-3330	AA standard, 1 nmol/µL, 10 × 1 mL
5061-3331	AA standard, 250 pmol, 10/pk
5061-3332	AA standard, 100 pmol/µL, 10 × 1 mL
5061-3333	AA standard, 25 pmol/µL, 10 × 1 mL
5061-3334	AA standard, 10 pmol/µL, 10 × 1 mL
5062-2478	Amino acids supplement kit, 1 g each of norvaline, sarcosine, asparagine, glutamine, tryptophan, and 4-hydroxyproline
Each component can be ordered separately.	

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Printed in the USA, September 21, 2017
5991-6572EN

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