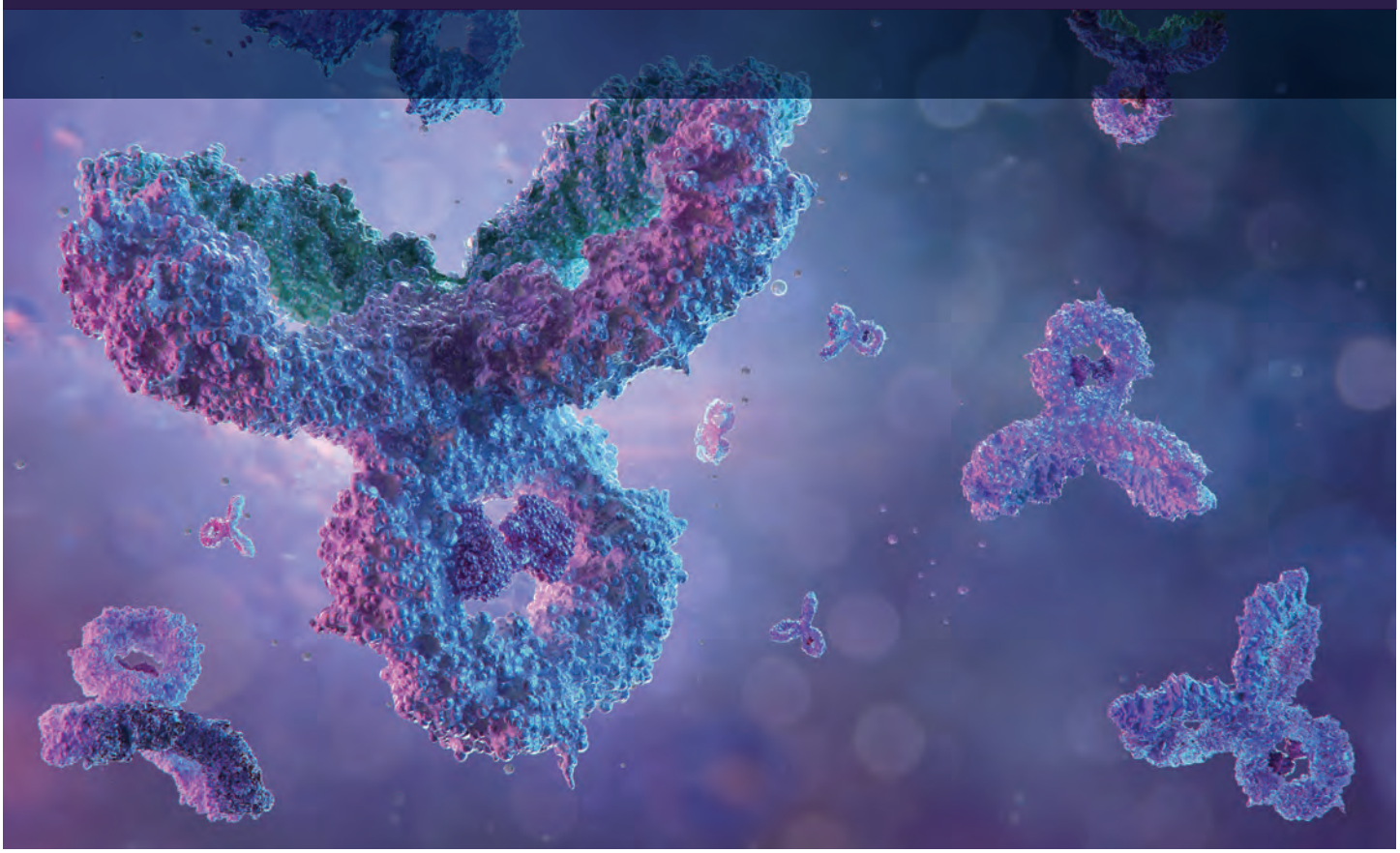
Agilent
Trusted Answers

Agilent Biocolumns

Amino Acid Analysis

Application Compendium



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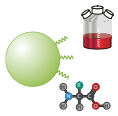
Amino Acid Analysis

Background

Determining the amino acid composition of a protein is a well-established technique that is used with other techniques to confirm the correct structure. Acid hydrolysis is typically used to hydrolyze the protein into its constituent amino acids before analysis. Amino acids are also key ingredients in the cell culture medium used to prepare recombinant proteins. It is often desirable to monitor the consumption of individual amino acids during the fermentation reaction and so the same chromatographic approach may be used.

Amino acids are inherently zwitterionic and possess a variety of side chains, including neutral, hydrophobic, hydrophilic, acid, and basic groups. They also lack a suitable UV chromophore, making the separation and detection of the twenty or so naturally occurring amino acids challenging. Agilent introduced a unique method of amino acid analysis combining precolumn derivatization, employing the liquid handling capabilities of the multisampler, together with reversed-phase separation to baseline resolution of all the common amino acids.

To perform precolumn derivatization the sample is first neutralized in borate buffer at high pH to ensure that the amino terminus of each amino acid is neutralized. Primary amines are then reacted with ortho-phthaldehyde (OPA) and secondary amines (proline, hydroxyproline etc.) are reacted with 9-fluorenylmethylchloroformate (FMOC-Cl). This enables the subsequent separation by reversed-phase to be performed, but the high pH conditions required to obtain baseline resolution means that the latest pH stabilized columns provide the longest column lifetime.



Amino Acid and Cell Culture Analysis

Small molecule chromatography (<150 Å)

Delivers robust, high-resolution separations

AdvanceBio Amino Acid Analysis (AAA)

LC/UV or LC/FLD with sample derivatization

Attribute	Advantage
Exceptional resolution	More reliable results
High pH–resistant C18 stationary phase	Longer column lifetimes
HPLC and UHPLC compatible	Increased flexibility

AdvanceBio MS Spent Media

LC/MS without sample derivatization

Attribute	Advantage
HILIC LC separation/ MS detection	One method for multiple metabolite classes
No sample derivatization needed	Use any LC/MS system
PEEK-lined stainless steel column hardware	Excellent peak shape and recovery

Getting Started

The AdvanceBio Amino Acid Analysis kit combines all the reagents and calibration standards necessary for the analysis into a single part number, 5190-9426. Each component can also be ordered separately if needed. The 'How-To" Guide on the following pages contains detailed instructions for mobile phase preparation, setting up the automated, online amino acid derivatization in the autosampler, and the LC method.

The AdvanceBio Amino Acid Analysis column is packed with C18 silica particles that have been chemically modified for high pH stability. Amino acid separations are most efficient at high pH, and this improvement over previous amino acid analysis solutions significantly extends the lifetime of the column under these mobile phase conditions. To maximize the benefit of this advancement, take care to never store the column in mobile phase A. Short term storage should be in mobile phase B, while long-term storage should be in 50 % acetonitrile. The column is meant to withstand short-term exposure to basic pH during a gradient, but prolonged exposure to high pH will still lead to shortened column lifetimes.

Amino Acid Analysis: “How-To” Guide

Accurate results with AdvanceBio end-to-end solution

The Agilent AdvanceBio Amino Acid Analysis (AAA) end-to-end solution optimizes workflow efficiency by combining the advantages of the Agilent InfinityLab LC Series instrumentation and column technology with proven precolumn derivatization chemistry. It is part of the AdvanceBio family that delivers consistent, exceptional performance for the complete characterization of proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals.

This complete, single vendor solution (including chemicals/standards, columns, and application support) provides fast, sensitive, and automated amino acid analysis. It is based on the latest InfinityLab LC Series instrument and column technology. The automated online derivatization in the Agilent 1290/1260 Infinity II vialsampler eliminates tedious manual procedures and delivers reproducible reaction results. AdvanceBio AAA columns provide the speed and resolution of sub-2 µm columns, but with 50 % less backpressure and reduced risk of column clogging.

The AdvanceBio AAA solution has evolved from proven Agilent ortho-phthalaldehyde/ 9-fluorenyl-methyl chloroformate (OPA/FMOC) reagents for amino acid derivatization. Together with AdvanceBio AAA columns and standards, these reagents provide an ideal, quantitative and qualitative amino acid analysis that combines speed and sensitivity. When used according to the protocol described in this document, the AdvanceBio AAA solution enables the user to separate the amino acids commonly found in protein/peptide hydrolysates.

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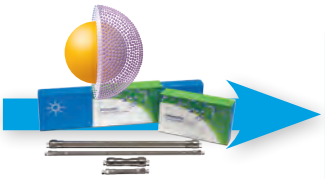
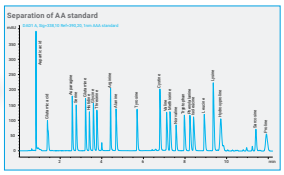
Expert applications and technical support

AdvanceBio AAA reagents and standards
Ready to use

Agilent analytical LC systems
Efficient LC solutions



AdvanceBio AAA columns
Fast, rugged amino acid separation



Fast, reliable data

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AdvanceBio AAA columns: Superficially porous particle (SPP) technology

AdvanceBio AAA columns are based on Agilent's innovative 2.7 µm superficially porous particle (SPP) Poroshell technology—particles consist of a 1.7 µm solid core with a 0.5 µm porous shell.

The 2.7 µm SPPs provide 40-50 % lower backpressure with 80-90 % of the efficiency of sub-2 µm totally porous particles. The SPPs have a narrower particle size distribution than totally porous particles, which results in a more homogeneous column bed, and reduces dispersion in the column. At the same time, the thin porous shell provides lower resistance to mass transfer. What's more, since the columns incorporate a 2 µm frit, they are as resistant to clogging as 3.5 and 5 µm columns.

Until recently, all silica-based SPP materials possessed limited lifetime in higher pH buffers, including phosphate buffers. To achieve longer lifetimes, it is necessary to protect the base particle by either surface modification or special bonding modification. The surface is chemically modified using a proprietary process to form an organic layer that is resistant to silica dissolution at high pH conditions.

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AdvanceBio AAA columns: Superficially porous particle (SPP) technology

The AdvanceBio AAA columns ensure excellent selectivity for amino acid analysis.

Fast and rugged amino acid separation

- The speed and resolution of a sub-2 µm column with up to 50 % less backpressure
- More forgiving for dirty samples, due to 2 µm frits
- Unique chemical modification for high pH stability and column lifetime
- Guard column options reduce your operating costs by extending the life of the columns

Everyday efficiency with confidence

- Higher speed and higher resolution thanks to the operating power up to 600 bar and 5 mL/min
- Injector programming for automated online derivatization
- High-sensitivity UV detection based on diode array technology for uncompromised sensitivity for simultaneous multiwavelength detection
- Optional full spectral detection for identification and peak purity analysis
- Wide flexibility for other LC or UHPLC applications with 100 % HPLC compatibility

Agilent detectors – flexibility in detection

Multiple Wavelength Detector:

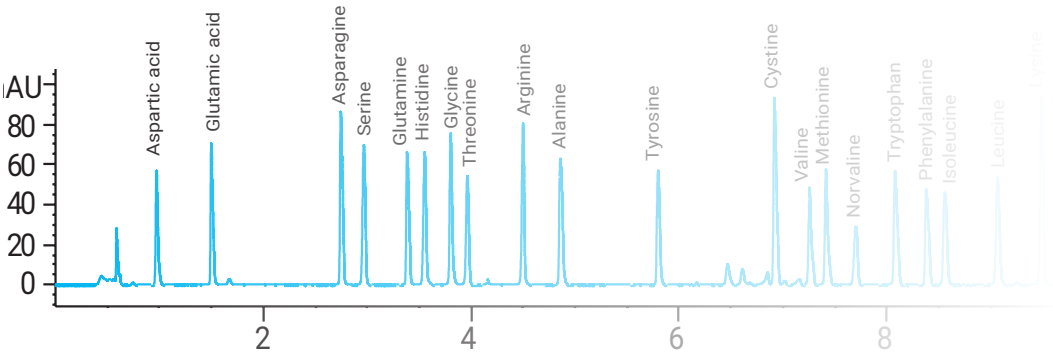
Uncompromised sensitivity for simultaneous multiwavelength detection.

Diode Array Detector with spectral data:

Identification and peak purity analysis with more selectivity and fewer matrix effects.

Fluorescence Detector:

Superior sensitivity in the multi-signal mode in the femto-mole range.



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Advance your confidence: Agilent AdvanceBio Amino Acid Analysis (AAA)

Achieve fast, sensitive, and reproducible separation of amino acids in biological samples

Steps for AAA analysis

1. Prepare HPLC mobile phases
2. Prepare amino acid standards
3. Prepare Internal Standard (ISTD) stock solution
4. Perform online derivatization
5. Set parameters for detection
6. Run high throughput routine analysis
7. Ensure system suitability per European Pharmacacopoeia (Ph. Eur.)
8. Optimize cell culture media and protein hydrolysate standard

Learn more about analyzing amino acids with utmost confidence, visit

www.agilent.com/chem/advancebioaaa

Step 1:

Prepare HPLC mobile phases

Mobile phase A:

10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$ pH 8.2

To prepare 1 L, weigh out 1.4 g anhydrous Na_2HPO_4 and 3.8 g $Na_2B_4O_7 \cdot {}_{10}H_2O$ in 1 L water. Adjust to approximately pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid and adjust to a final pH of 8.2. Allow stirring time for complete dissolution of borate crystals before adjusting pH. Filter through 0.45 µm regenerated cellulose membranes (p/n3150-0576).

Mobile phase B:

Acetonitrile:methanol:water (45:45:10, v:v:v)

All mobile-phase solvents are HPLC grade.

Mobile phase A is consumed at a faster rate than mobile phase B. Therefore, we recommend preparing 2 L of mobile phase A for every 1 L of mobile phase B.

Injection diluent

The injection diluent is 100 mL of mobile phase A and 0.4 mL concentrated H_3PO_4. This solution is prepared in a 100 mL bottle that should be stored at 4 °C.

0.1 N HCl

Extended amino acid and internal standard stock solutions are prepared in 0.1 N HCl solution. To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) to a 500 mL volumetric flask that is partially filled with water. Mix, and fill to the mark with water. Store at 4 °C.

Derivatization reagents

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent. Simply transfer these reagents from their container into an autosampler vial. Recommended precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7 to 10 days. We recommend transferring 100 µL aliquots of OPA to microvial inserts and storing in a refrigerator. Replace the OPA autosampler microvial daily.
- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred to microvial inserts in 100 µL aliquots, and stored in a refrigerator. Like the OPA, an open FMOC ampoule transferred to 10 microvial inserts is potent for about 7 to 10 days.
- Borate buffer can be transferred to a 1.5 mL autosampler vial without a vial insert. Replace every three days.

Step 2:

Prepare amino acid standards

Solutions of 17 amino acids (AA) in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Store solutions at 4 °C.

To make the extended amino acid (EAA) stock solution, weigh:

- 59.45 mg asparagine
- 59.00 mg hydroxyproline
- 65.77 mg glutamine
- 91.95 mg tryptophan

Add the weighed out amino acids to a 25 mL volumetric flask, fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Then fill to mark with water for a total concentration of 18 nmol/µL of each amino acid.

For the high-sensitivity EAA stock solution, take 5 mL of this standard-sensitivity solution and dilute with 45 mL water (1.8 nmol/µL). Solutions containing extended standards are unstable at room temperature. Keep them frozen and discard at first signs of reduced intensity.

Step 3:

Prepare Internal Standard (ISTD) stock solution

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill halfway with 0.1 N HCl and shake or sonicate until dissolved. Finally fill to mark with water for a final concentration of 10 nmol for each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, take 5 mL of standard-sensitivity solution and dilute with 45 mL of water. The final concentration of the high-sensitivity ISTD is 1 nmol for each amino acid/µL. Store at 4 °C.

Calibration curves may be made using two to five standards depending on experimental need. Typically 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for "standard sensitivity" analysis.

The following tables should be followed if an internal standard or other amino acid (for example, the extended amino acids) is added. Table 1 describes "standard sensitivity" concentrations typically used in UV analysis. Table 2 is typically used for "high sensitivity" fluorescence analysis.

Table 1. Standard sensitivity calibration standards

	Concentration of Final AA Solution (pmol/µL)		
	900	225	90
Take 5 mL of 18 nmol EAA Dilute with water	5 mL -	5 mL 15 mL	5 mL 45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA-ISTD mix	100 µL	100 µL	100 µL
For 1 nmol AA, add	900 µL	-	-
For 250 pmol AA, add		900 µL	-
For 100 pmol AA, add	-	-	900 µL
Final AA solution with EAA and 500 pmol/µL ISTD	1 mL	1 mL	1 mL

Table 2. High sensitivity calibration standards

	Concentration of Final AA Solution (pmol/µL)		
	90	22.5	9
Take 5 mL of 1.8 nmol EAA Dilute with water	5 mL -	5 mL 15 mL	5 mL 45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 1 nmol ISTD solution	5 mL	5 mL	5 mL
High-sensitivity EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA-ISTD mix	100 µL	100 µL	100 µL
For 100 nmol AA, add	900 µL	-	-
For 25 pmol AA, add		900 µL	-
For 10 pmol AA, add	-	-	900 µL
Final AA solution with EAA and 50 pmol/µL ISTD	1 mL	1 mL	1 mL

Step 4:

Perform online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G7129A well plate automatic liquid sampler with 100 µL capillary* (WPALS), with injection program is as follows:

1. Draw 2.5 µL from borate vial (p/n5061-3339)
2. Draw 1.0 µL from sample vial
3. Mix 3.5 µL in wash port five times
4. Wait 0.2 minutes
5. Draw 0.5 µL from OPA vial (p/n 5061-3335)
6. Mix 4.0 µL in wash port 10 times default speed
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337)
8. Mix 4.4 µL in wash port 10 times default speed
9. Draw 32 µL from injection diluent vial
10. Mix 20 µL in wash port eight times
11. Inject
12. Wait 0.1 minutes
13. Valve bypass

* Note: other autosampler models may have a different volume capillary installed, which will require adjustment of volumes

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G7129A with a 2 × 56 well plate tray (p/n G2258-44502), the locations are:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection diluent
- P1-A-1: Sample

Note: Use the correct vials, closures, and pumps parameters

Conical vial inserts with polymer feet (Figure 1A) are required to hold the OPA and FMOC reagents because of the limited volumes involved. The inserts are compatible with wideopening screw-top (Figures 1B and 1C) or crimp-top vials. For this procedure, an airtight seal is needed for both FMOC, which is highly volatile, and OPA, as it slowly degrades in the presence of oxygen. Snap-cap vials should therefore not be used in this procedure. Be careful not to use vials or caps designed for other instruments, to prevent damage to the auto injector.

Pump parameters for all methods include compressibility (×10-6 bar) A: 40, B: 80, with minimal stroke A, B of 20 µL.

Figure 1. Insert, vial, and cap for amino acid analysis using the Agilent 4226A autosampler: A) Conical insert (Agilent p/n 5181-1270), B) amber wide opening vial (Agilent p/n 5182-0716), and C) screw cap (Agilent p/n 5182-0721).

Increase precision with Autosampler automation

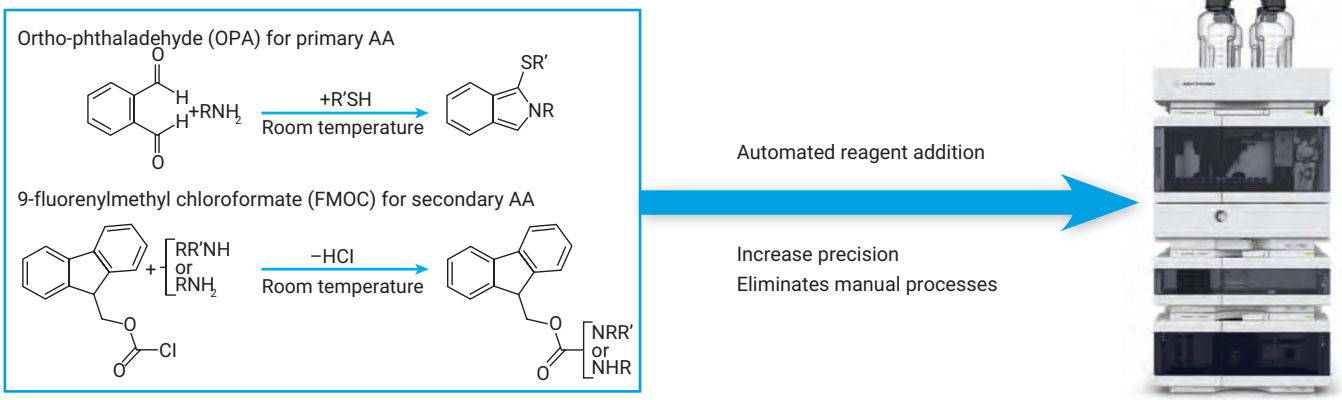

Figure 2. Online derivatization of OPA and FMOC: Separation of polar amino acids on RP-phase and detection by UV and Fluorescence

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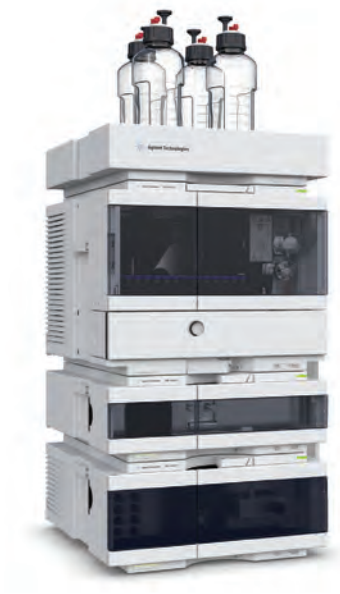
Step 5:



Set parameters for detection

Thermostatted column compartment (TCC)

Left and right temperatures should be set to 40 °C. Enable analysis when the temperature is within ± 0.8 °C.

Diode array detector (DAD)

Signal A: 338 nm	10 nm bandwidth	Reference wavelength 390 nm	20 nm bandwidth
Signal B: 262 nm	16 nm band-width	Reference wavelength 324 nm	8 nm bandwidth
Signal C*: 338 nmix	10 nm band-width	Reference wavelength 390 nm	20 nm bandwidth

*Signal C is not required if the instructions below are followed.

To detect both OPA and FMOC derivitized amino acids in a single chromatogram it is necessary to switch detector wavelength between the last eluting OPA derivitized amino acid, lysine (peak 20 in the standard), and before the first eluting FMOC derivitized amino acid, hydroxyproline (peak 21 in the standard).

Determining the appropriate transition point using the DAD is possible by initially collecting two channels (Signal A 338 nm, to detect OPA derivitized amino acids and Signal B 262 nm, to detect FMOC derivitized amino acids). This will determine the ideal point at which to switch the wavelength during the run. Subsequent runs can be made using a single channel with the detector timetable function used to program a wavelength switch from 338 to 262 nm at the appropriate time. Between the elution of OPA-lysine and FMOC-hydroxyproline, allow time for both OPA and FMOC derivitized amino acids to be detected in a single chromatogram.

Peak width settings of > 0.01 minutes are used for all columns.

Fluorescence detection

FLD should always be the last detector module in the flow stream to avoid damage to the pressure sensitive flow cell (max 20 bar).

Peak width 0.01 min, stop time 18 min (adjust as needed)

Excitation 340 nm; Emission 450 nm; Filter 390 nm (Default filter)

Timetable Signal:

0.00 min Excitation 340 nm, Emission 450 nm; Gain (as needed)

5.53 min Excitation 260 nm, Emission 325 nm;

PMT Gain 10 (as needed; transition between lysine and hydroxyproline)

To determine the transition point needed with fluorescence detection (FLD), it is necessary to perform two separate runs: the first using Excitation 340 nm, Emission 450 nm to detect the OPA derivitized amino acids and the second using Excitation 260 nm, Emission 325 nm to detect the FMOC derivitized amino acids. Both OPA and FMOC derivitized amino acids can be detected in a single chromatogram, using the detector timetable function. This function programs a wavelength switch at the appropriate point after the last eluting OPA derivitized amino acid, lysine (peak 20 in the standard) and before the first eluting FMOC derivitized amino acid, hydroxyproline (peak 21 in the standard).

Note: If the concentration of amino acids is below 100 pmol, we recommend the use of fluorescence detection.

Gradient program

Time (min)	% B
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	end

Flow rate: 1.5 mL/min for 4.6 mm id columns and 0.62 mL/min for 3 mm id columns.

Injection volume: 1 µL with needle wash at the port for 7 s.

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Typical Separations

A separation of 20 amino acids using an AdvanceBio AAA column is shown in Figure 3.
The following parameters are noted:

- No change in elution profile of amino acids with and without NaN_3 in mobile phase.
- NaN_3 is used only as a preservative to contain bacterial/fungal growth.
- Filtering the mobile phase using 0.45 µm filter is highly recommended. Note: If the concentration of amino acids is below 100 pmol, we recommend the use of fluorescence detection.

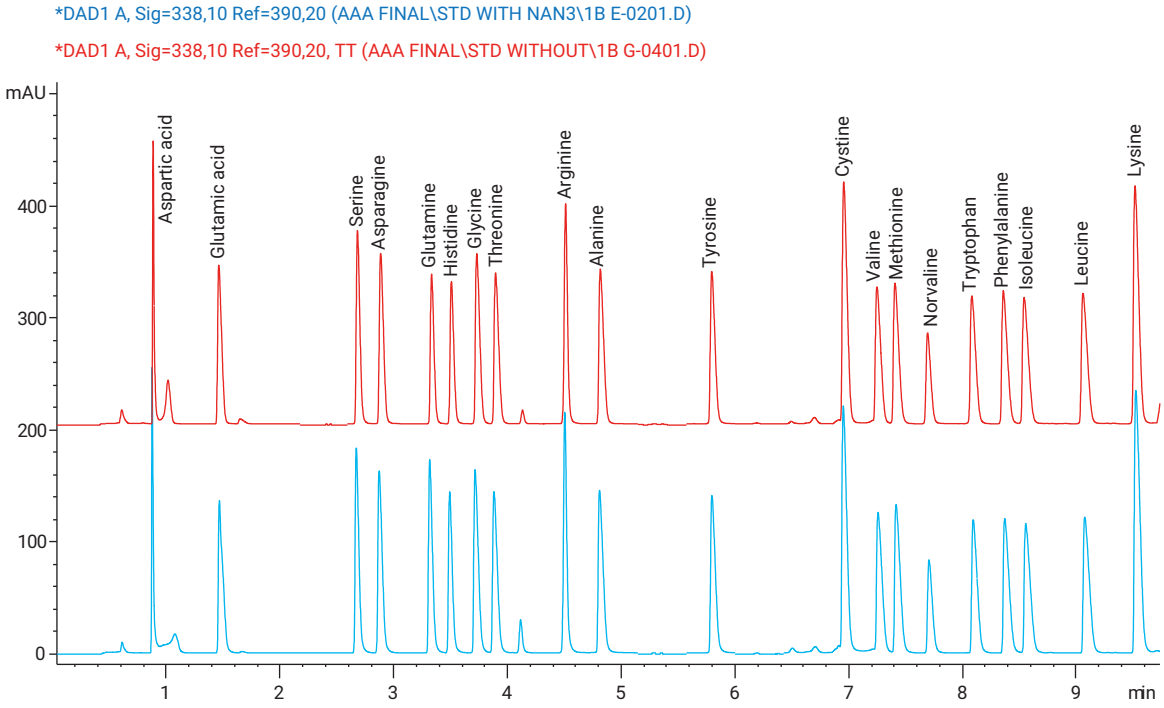

Figure 3. Separation of 20 amino acid standards using an Agilent AdvanceBio AAA 4.6 x 100 mm column with and without 5 mM sodium azide in mobile phase.

Note: Addition of 5 mM sodium azide (NaN_3) to mobile phase A is optional to prevent microbiological growth and extend shelf life of buffers.

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Step 6:

Run high-throughput amino acid analysis

The chromatogram in Figure 4 illustrates typical routine standard sensitivity in high-throughput applications that can be obtained using Agilent AdvanceBio AAA columns. These separations were produced using the Agilent 1260 Infinity II HPLC binary system with AdvanceBio AAA, 100 mm, 2.7 µm columns of different internal diameters, and DAD detection. A single run can be completed in under 20 minutes (including re-equilibration) with adequate resolution. The primary amino acids (1-20, OPA-derivatized), were monitored at 338 nm, while the secondary amino acids (21-23, FMOC-derivatized), were monitored at 262 nm.

The first 20 amino acids in Figure 4, the primary amino acids, are derivatized with OPA. The last three, hydroxyproline, sarcosine, and proline, are derivatized with FMOC. A programmable wavelength switch from 338 to 265 nm takes place after lysine (peak 20) elutes and before hydroxyproline (peak 21) elutes.

- The method can easily be scaled to different column dimensions.
- In this case, the only changes to the method were made by altering the flow rate, changed geometrically with the diameter of the column.
- The low-volume heat exchanger was used with short red tubing to minimize extra column volume.

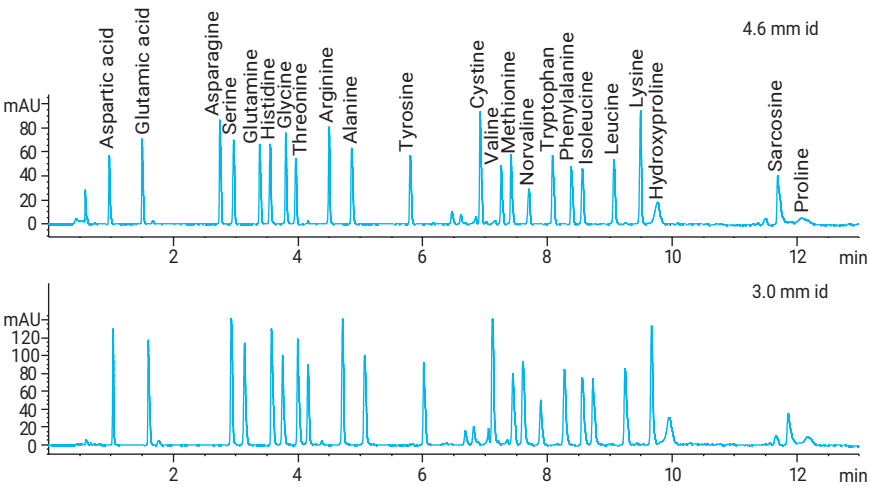

Figure 4. Separation of AA standards using Agilent AdvanceBio AAA columns of different internal diameters using the amino acid method.

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Retention time and area precision for 100 pmol and 1000 pmol analysis (n=6)

Table 3. Retention time and area RSD precision for amino acids (100 pmol) separated using an AdvanceBio AAA, 4.6 x 100 mm column (six replicates.)

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.851	1.270	1.066
2. Glutamic acid	1.428	0.973	1.850
3. Asparagine	2.639	0.605	1.790
4. Serine	2.835	0.629	1.820
5. Glutamine	3.285	0.470	1.560
6. Histidine	3.465	0.430	1.220
7. Glycine	3.681	0.477	1.920
8. Threonine	3.837	0.440	1.950
9. Arginine	4.458	0.251	2.150
10. Alanine	4.764	0.280	3.060
11. Tyrosine	5.762	0.128	1.650
12. Cysteine	6.870	0.067	1.900

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
13. Valine	7.201	0.084	2.47
14. Methionine	7.363	0.073	1.82
15. Norvaline	7.602	0.073	1.72
16. Tryptophan	8.055	0.054	1.57
17. Phenylalanine	8.341	0.051	1.66
18. Isoleucine	8.503	0.047	1.72
19. Leucine	9.000	0.030	1.70
20. Lysine	9.428	0.028	1.66
21. Hydroxyproline	9.747	0.021	4.13
22. Sarcosine	10.980	0.026	1.15
23. Proline	11.620	0.021	4.36

Table 4. Retention time and area RSD precision for amino acids (1000 pmol) separated using an AdvanceBio AAA, 4.6 x 100 mm column (six replicates).

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.837	0.151	2.60
2. Glutamic acid	1.400	0.512	2.19
3. Asparagine	2.583	0.124	2.13
4. Serine	2.772	0.114	1.74
5. Glutamine	3.220	0.092	1.80
6. Histidine	3.405	0.077	1.39
7. Glycine	3.598	0.068	1.48
8. Threonine	3.766	0.059	2.26
9. Arginine	4.422	0.027	1.66
10. Alanine	4.685	0.031	1.87
11. Tyrosine	5.695	0.034	2.04
12. Cysteine	6.794	0.030	2.22

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
13. Valine	7.118	0.025	2.40
14. Methionine	7.281	0.025	1.78
15. Norvaline	7.573	0.019	1.77
16. Tryptophan	7.970	0.024	2.03
17. Phenylalanine	8.238	0.027	1.98
18. Isoleucine	8.413	0.025	2.17
19. Leucine	8.925	0.020	1.81
20. Lysine	9.357	0.022	2.00
21. Hydroxyproline	9.718	0.014	3.14
22. Sarcosine	10.961	0.015	5.91
23. Proline	11.911	0.011	2.58

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Step 7:

Ensure system suitability as per European Pharmacopoeia

The The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid, produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of not less than 1.5 [1]

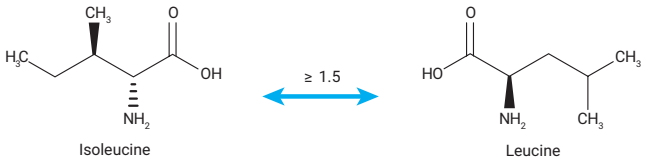

Table 5. System suitability testing using Agilent AdvanceBio AAA columns and AA standards

System Suitability	AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm	AdvanceBio AAA, C18, 3.0 × 100 mm, 2.7 µm
Resolution between Leucine and Isoleucine (≥1.5)	4.5	4.6

Reference:

1. European pharmacopoeia 9.0 (2.2.56)
Amino Acid Analysis

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Step 8:

Optimize cell culture media and protein hydrolysate standard

Cell cultures are widely used to produce biopharmaceuticals and other biologically active compounds. The composition of the cell culture media affects the yield and structure of the desired products and must be carefully optimized. Cell culture media is typically composed of mixtures of amino acids, vitamins, carbohydrates, inorganic salts, as well as different peptides, proteins, and other compounds. As the cells grow, they consume nutrients and release target biopharmaceuticals as well as waste products. Amino acids serve as the building blocks of proteins, as well as intermediates in many metabolic pathways. Therefore, amino acids are typically added to cell culture media to provide nutritional requirements for the cells.

Determination of amino acid flux in cultured cells is an important indicator of the metabolic rate and health of those cells. It can also be used as an indicator of the remaining carbon and nitrogenous fuel available. This is especially true in hepatocyte and hepatoma cell lines, where extensive gluconeogenesis, urea production, and protein synthesis may consume larger quantities of amino acids than other cell types.

HPLC with precolumn derivatization is a standard technique in the analysis of amino acids. Precolumn derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is at times done manually offline. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique; extra sample manipulation; extra time required; and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. A rugged high-resolution HPLC method including online derivatization, therefore, can increase productivity compared to offline methods.

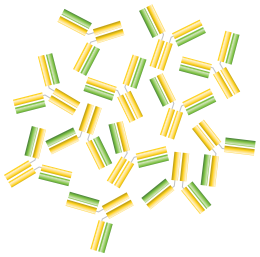
Amino acid compositional analyses of commonly used cell culture media and protein hydrolysate are shown in Figures 5-8. This analysis confirms that the amino acid composition of cell culture media accurately matches with their theoretical composition. Such applications are useful for monitoring and adjusting amino acid composition. This analysis is an essential part of optimizing the manufacturing process to ensure high quality and optimum yield of the final biopharmaceutical product.

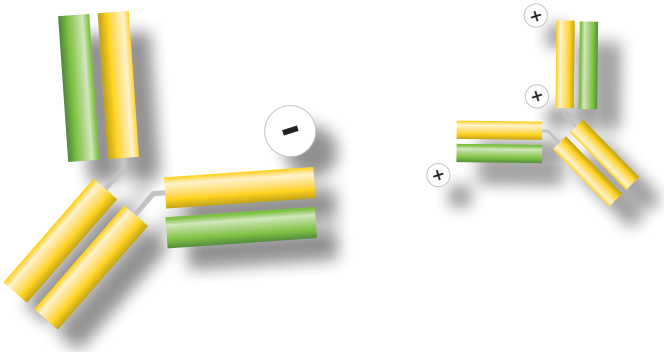
The following cell culture media are used for compositional analysis using amino acid method with an AdvanceBio AAA 4.6 x 100 mm column (Figures 5-8).

1. Minimum Essential Medium Eagle (MEM) M4655: L-arginine, L-Cystine, L-Glutamine, L-Histidine, L-Isoleucine, L- Leucine, L-Lysine, L-Methionine, L- Phenylalanine, L-Threonine, L-Tryptophan, L- Tyrosine, and L-Valine.

2. Non-Essential Amino Acid (NEAA) Cell Culture Supplement M7145: L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, Glycine, L-Proline, and L-Serine.

3. RPMI 1640 R0083: L-arginine, L-Asparagine, L-Cystine, Glycine, L-Histidine, Hydroxy-L-Proline, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, and L-Valine.





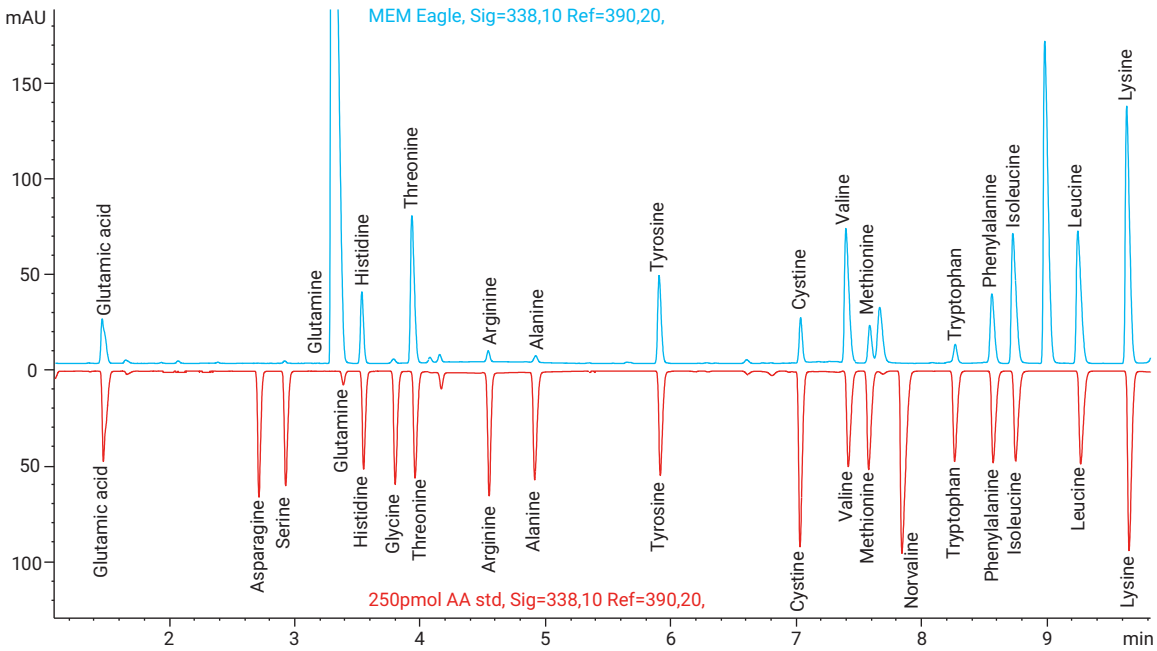


Figure 5. Amino acid analysis of Eagles MEM media (blue trace) and comparison with amino acid standards using the Agilent AdvanceBio AAA solution.

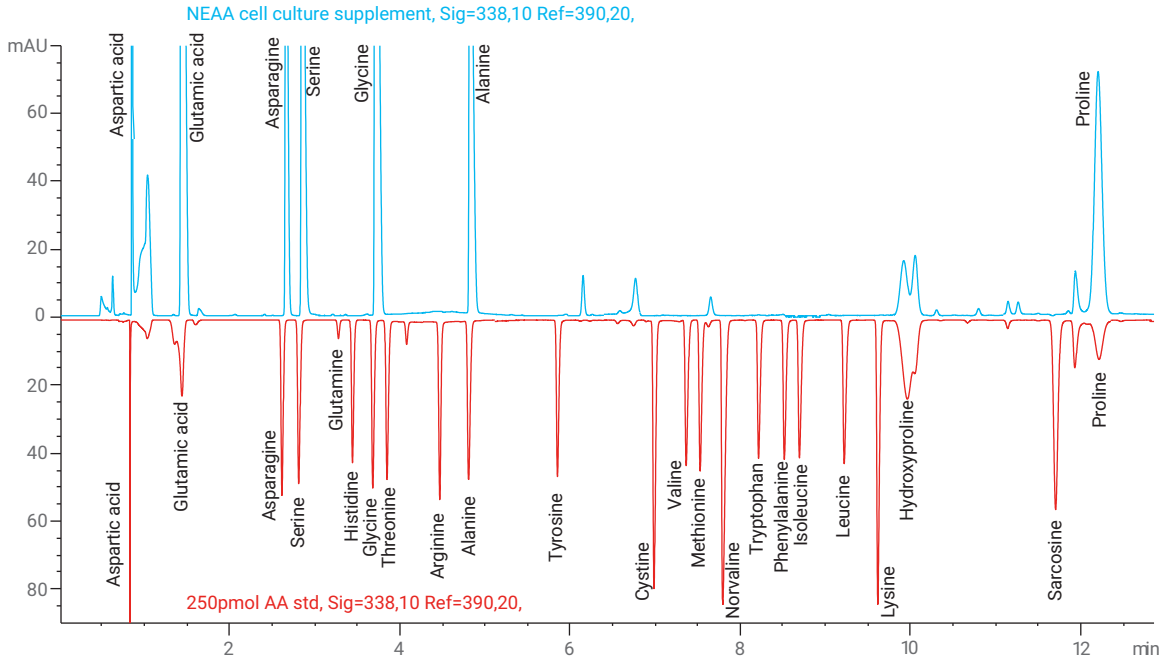


Figure 6. Amino acid analysis of Non-Essential Amino Acid (NEAA) media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

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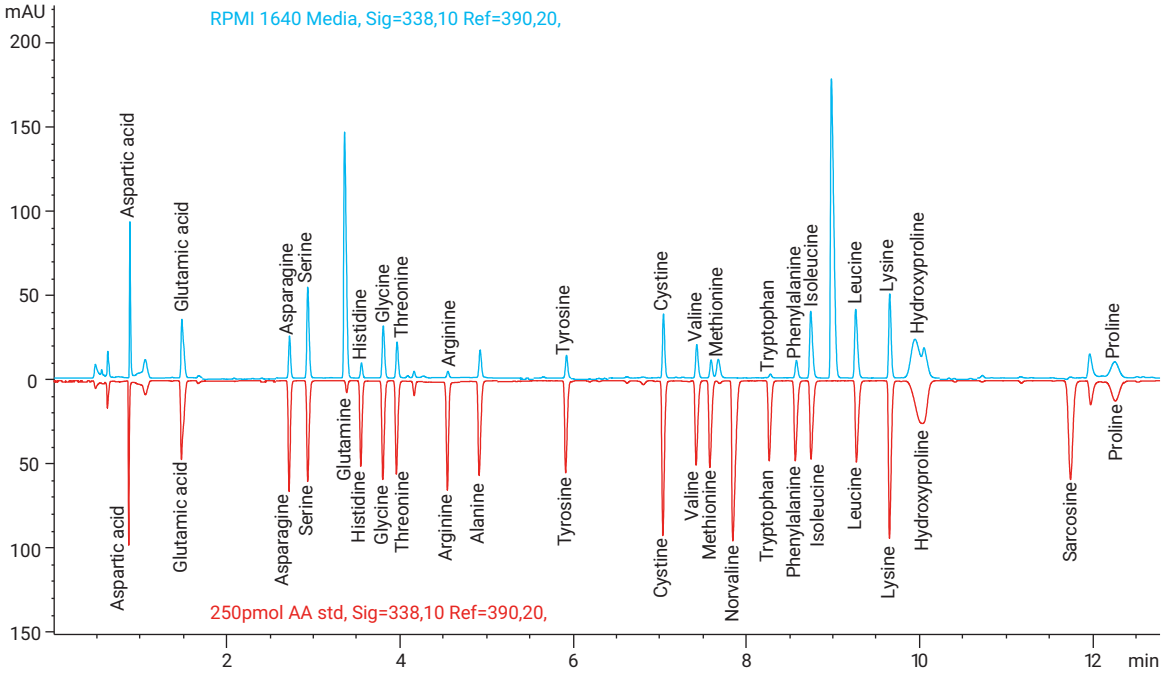

Figure 7. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

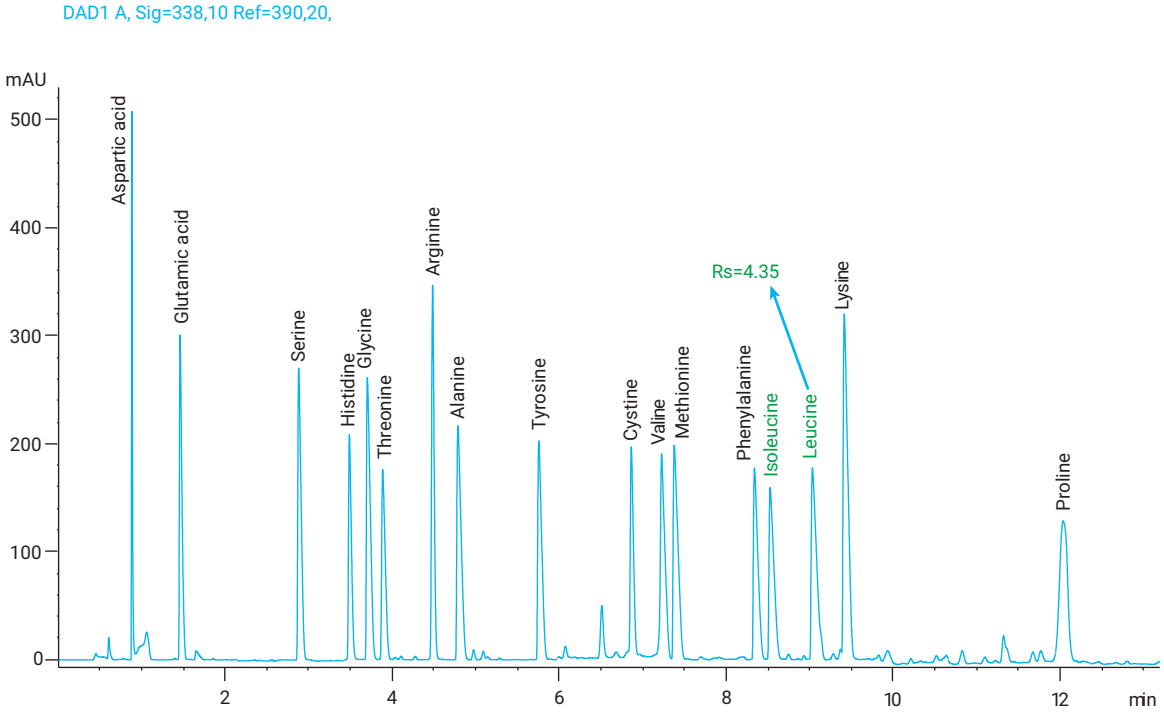

Figure 8. Amino acid analysis of protein hydrolysate. The resolution between leucine and Isoleucine with the AdvanceBio AAA, 4.6 x 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement.

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Maintenance and troubleshooting

The Agilent AdvanceBio AAA solution includes technical and application support. The following maintenance and troubleshooting tips are recommended to keep your InfinityLab LC Series instrument systems running smoothly.

Daily Maintenance:

- Replace derivatization reagent, borate buffer, amino acid standards, and wash water, which are placed in autosampler tray.
- Recalibration of retention times and response factors.
- Check column and guard column performance using system suitability report.
- Every two days replace mobile phase A and B with freshly made solvents

Troubleshooting:

Poor chromatographic resolution

- Exhausted guard column
- Damaged analytical column
- Post column band broadening due to too long connections.
- Always use short red tubing with the low-volume heat exchanger be to minimize extracolumn volume

Low Intensity Chromatogram

- OPA reagent has deteriorated
- FMOC reagent has deteriorated
- Glycine contamination

Ordering Information

Columns, supplies and chemicals	Size	Part No.
AdvanceBio AAA LC column	4.6 x 100 mm, 2.7 µm	655950-802
AdvanceBio AAA guard columns	4.6 x 5 mm, 2.7 µm, 3/pk	820750-931
AdvanceBio AAA LC column	3.0 x 100 mm, 2.7 µm	695975-322
AdvanceBio AAA guard columns	3.0 x 5 mm, 2.7 µm, 3/pk	823750-946
Borate Buffer	0.4 M in water, pH 10.2, 100 mL	5061-3339
FMOC Reagent	2.5 mg/mL in ACN, 10 x 1 mL ampoules	5061-3337
OPA Reagent	10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 x 1 mL ampoules	5061-3335
Dithiodipropionic Acid (DTDPA) reagent	5 g	5062-2479
Inserts, with polymer feet	250 µL, 100/pk	5181-1270
Vial, screw top, amber with write-on spot	2 mL, certified, 100/pk	5182-0716
Cap, screw, green, PTFE/white silicone septum	100/pk	5182-0721
Vial, screw top, clear, flat bottom	for LC, 6 mL, certified, 100/pk	9301-1377
Cap, screw	for 6 mL vials, 100/pk	9301-1379
Septum	for 6 mL vials, 100/pk	9301-1378
AA standard	1 nmol/µL, 10 x 1 mL	5061-3330
AA standard	250 pmol, 10/pk	5061-3331
AA standard	100 pmol/µL, 10 x 1 mL	5061-3332
AA standard	25 pmol/µL, 10 x 1 mL	5061-3333
AA standard	10 pmol/µL, 10 x 1 mL	5061-3334
Amino acids supplement kit		5062-2478

Learn more about the Agilent AdvanceBio family of innovations, designed specifically for biomolecule characterization, visit www.agilent.com/chem/advancebio

Application Note

Amino Acid Analysis

Agilent
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Determination of Amino Acid Composition of Cell Culture Media and Protein Hydrosylate Standard

The Agilent AdvanceBio Amino Acid Solution

Authors

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Abstract

This study presents a method for analyzing primary amino acids in cell culture media using the Agilent AdvanceBio Amino Acid Analysis (AAA) solution with absorbance detection. Derivitization using an online injector program with OPA and FMOC decreases sample preparation time, and increases reproducibility over traditional offline methods. The effectiveness of this solution for routine analysis was confirmed using a system suitability test and retention time and area precision studies. The AdvanceBio AAA solution provides sensitive and high-resolution separation of all amino acids in cell culture media. The limit of detection (LOD), limit of quantification (LOQ), and linearity for selected amino acids for qualitative assays are also reported.

Introduction

Amino acids are the basic building blocks of proteins. They constitute all proteinaceous material of the cell including the cytoskeleton and the protein component of enzymes, receptors, and signaling molecules. In addition, amino acids are used for the growth and maintenance of cells. Cell culture media plays a key role in the biopharma industry. A large proportion of the amino acids supplied from cell culture media are diverted to pathways that could influence the fate of the cells in a culture. The identification of the optimal concentration of amino acids is important in fed batch and perfusion culture. Therefore, the design of an amino acid supplementation strategy might be streamlined by identifying the amino acid demands of a cell culture due to host cell growth and product production.

HPLC with precolumn derivatization is commonly used for the analysis of amino acids. Precolumn derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is sometimes done manually, offline. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique. Other drawbacks include extra sample manipulation, extra time required, and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. Thus, a rugged high-resolution HPLC method including online derivatization, can increase productivity compared to offline methods. Consistent automated OPA derivatization, using the injector programming of the HPLC's autosampler and highly efficient Agilent AdvanceBio AAA columns, generate a rapid-reproducible amino acid method ideal for cell culture media. This method is convenient because the cell media samples are simply transferred to autosampler vials and analyzed. The selectivity of the AdvanceBio AAA column and the mobile phase gradient provides high resolution of 23 amino acids.

Materials and Methods

Instrumentation

Analyses were performed using an Agilent 1290 Infinity LC, which was equipped with an Agilent 1290 Infinity binary pump delivery system (G4220A), Agilent 1290 Infinity autosampler (G4226A), Agilent 1290 Infinity thermostatted column compartment (G1316C), and Agilent 1290 Infinity DAD (G4212A).

Reagents, samples, and materials

Cell culture media for compositional analysis, Minimum Essential Medium Eagle (M4655), Non-Essential Amino Acid (M7145), RPMI 1640 (R0083), Na2HPO4, and Na2B4O7•10H2O, were bought from Sigma-Aldrich. Protein hydrolysate was obtained from Fisher Scientific. Acetonitrile and methanol used were bought from Lab-Scan (Bangkok, Thailand). HPLC grade and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Column

Agilent AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm (p/n 655950-802)

Preparation of HPLC mobile phase

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). Since mobile phase A is consumed at a faster rate than B, it is convenient to make 2 L of A for every 1 L of B produced.

Injection diluent

The injection diluent was 100 mL mobile phase A, plus 0.4 mL concentrated H_3PO_4 in a 100 mL bottle, stored at 4 °C. To prepare 0.1 N HCl, 4.2 mL concentrated HCl (36%) was added to a 500 mL volumetric flask that was partially filled with water, mixed, then filled to the mark with water. This solution was then used for making extended amino acid and internal standard stock solutions. It was stored at 4 °C.

Agilent AdvanceBio AAA standards and reagents kit, p/n 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 ACN, 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
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

Derivatization reagents

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent, and were transferred from their containers to autosampler vials. Precautions included:

- OPA is shipped in ampules under inert gas to prevent oxidation. Once opened, the OPA is potent for approximately 7 to 10 days. Therefore, 100 µL aliquots of OPA were transferred in microvial inserts and refrigerated. The OPA autosampler microvial was then replaced daily. Each ampule lasted 10 days (one vial/day).
- FMOC is stable in dry air, but deteriorates in moisture. Therefore, FMOC was transferred in 100 µL aliquots to microvial inserts and refrigerated. An open FMOC ampule transferred to 10 microvial inserts should last 10 days.
- Borate buffer was transferred to a 1.5 mL autosampler vial without a vial insert, and replaced every 3 days.

Preparation of amino acid standards

- Solutions of 17 amino acids in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Each 1 mL ampule of standards was divided into 100 µL portions in conical vial inserts, and stored at 4 °C.
- The extended amino acid (EAA) stock solution was produced by weighing 59.45 mg asparagine, 59.00 mg hydroxyproline, 65.77 mg glutamine, and 91.95 mg tryptophan into a 25 mL volumetric flask. This flask was filled halfway with 0.1 N HCL, and shaken or sonicated until the amino acids were dissolved. It was then filled to mark with water to produce a total concentration of 18 nmol/µL of each amino acid.
- For the high-sensitivity EAA stock solution, 5 mL of this standard-sensitivity solution was diluted with 45 mL water (1.8 nmol/µL). Solutions containing extended standards were unstable at room temperature, and were kept frozen, and discarded at the first signs of reduced intensity.

Internal Standard (ISTD) stock solution

For primary amino acid ISTD stock solutions, 58.58 mg norvaline was weighed into a 50 mL volumetric flask. For secondary amino acids, 44.54 mg sarcosine was weighed into the same 50 mL flask. This flask was filled halfway with 0.1 N HCl, and shaken or sonicated until dissolved, then filled to mark with water for a final concentration of 10 nmol each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, 5 mL of standard-sensitivity solution was diluted with 45 mL of water, and stored at 4 °C.

Calibration curves may be made using two to five standards, depending on experimental need. Typically, 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for standard analytical sensitivity analysis. The following tables should be followed if an internal standard or other amino acids (for example, the extended amino acids) are added. Table 1 describes standard analytical sensitivity concentrations typically used in UV analysis.

Table 1. Chromatographic parameters used for intact and reduced analysis.

	Concentration of Final AA solution (pmol/µL)		
	900	**225**	**90**
Take 5 mL of 18 nmol EAA	5 mL	5 mL	5 mL
Dilute with water	–	15 mL	45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nmol ISTD solution	5 mL	5 mL	5 mL
EAA–ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA–ISTD mix	100 µL	100 µL	100 µL
For 1 nmol AA, add:	900 µL	–	–
For 250 pmol AA, add:	900 µL	–	
For 100 pmol AA, add:	–	–	900 µL
Final AA solution with EAA and 500 pmol/µL ISTD	1 mL	1 mL	1 mL

Online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G4226A well plate automatic liquid sampler (WPALS), the injection program was:

1. Draw 2.5 µL from borate vial (p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in wash port five times.
4. Wait 0.2 minutes.
5. Draw 0.5 µL from OPA vial (p/n 5061-3335).
6. Mix 4.0 µL in wash port 10 times default speed.
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337).
8. Mix 4.4 µL in wash port 10 times default speed.
9. Draw 32 µL from injection diluent vial.
10. Mix 20 µL in wash port eight times.
11. Inject.
12. Wait 0.1 minutes.
13. Valve bypass.

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G1367C with a 2 × 56-well plate tray (p/n G2258-44502), the locations were:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection diluent
- P1-A-1: Sample

Thermostatted column compartment (TCC)

Left and right temperatures were set to 40 °C. Analysis was enabled when the temperature was within ±0.8 °C.

Diode array detector (DAD)

Signal A: 338 nm, 10 nm bandwidth, reference wavelength 390 nm, 20 nm bandwidth.

Signal B: 262 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth.

Signal C: 338 nm, 10 nm bandwidth, reference wavelength 390 nm, 20 nm bandwidth.

To detect both OPA and FMOC derivatized amino acids in a single chromatogram, it was necessary to switch detector wavelengths. This switch took place between the last eluting OPA derivatized amino acid, lysine (peak 20 in the standard), and the first eluting FMOC derivatized amino acid, hydroxyproline (peak 21 in the standard).

With the DAD, determining the appropriate transition point was possible by initially collecting two channels. Signal A, 338 nm, detected OPA derivatized amino acids, and signal B, 262 nm, detected FMOC derivatized amino acids. From this analysis, the ideal point at which to switch wavelength during the run was determined. Subsequent runs were then made using a single channel, with the detector timetable function used to program a wavelength switch from 338 to 262 nm at the appropriate time between the elution of OPA-lysine and FMOC-hydroxyproline. This switch allowed both OPA and FMOC-derivatized amino acids to be detected in a single chromatogram. Peak width settings of >0.01 minutes were used for all columns.:

Linearity, limit of detection (LOD), and limit of quantification (LOQ) determination

As an example extended amino acid (EAA) stock solution, asparagine (59.45 mg), glutamine (65.77 mg), and tryptophan (91.95 mg) were used for linearity, LOD, and LOQ determination. These standards were weighed into a 25 mL volumetric flask, which was filled halfway with 0.1 N HCl, and mixed or sonicated until they dissolved. The flask was then filled to mark with water for a total concentration of 18 nmol/µL of each amino acid.

Linearity was studied in the range of 0.9–1,000 pmol/µL of these standard amino acids. Appropriate AA standard solutions were prepared in triplicate and injected into the chromatograph. The LOD and LOQ were estimated from the calibration function. LOD and LOQ were calculated as 3 (SD(a)/b) and 10 (SD(a)/b), respectively, where SD(a) is the standard deviation of the intercept, and b is the slope of the calibration function.

Gradient program	
Time (min)	**%B**
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	end
Flow rate: 1.5 mL/min for 4.6 mm id	

Table 3. Retention time and area RSD precision for amino acids (1,000 pmol) separated on an Agilent AdvanceBio AAA, 4.6 × 100 mm, column (n = 6).

Amino acid	RT RSD (%)	Area RSD (%)	Amino acid	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.151	2.60	13. Valine	0.025	2.4
2. Glutamic acid	0.512	2.19	14. Methionine	0.025	1.78
3. Asparagine	0.124	2.13	15. Norvaline	0.019	1.77
4. Serine	0.114	1.74	16. Tryptophan	0.024	2.03
5. Glutamine	0.092	1.8	17. Phenylalanine	0.027	1.98
6. Histidine	0.077	1.39	18. Isoleucine	0.025	2.17
7. Glycine	0.068	1.48	19. Leucine	0.020	1.81
8. Threonine	0.059	2.26	20. Lysine	0.022	2
9. Arginine	0.027	1.66	21. Hydroxyproline	0.014	3.14
10. Alanine	0.031	1.87	22. Sarcosine	0.015	5.01
11. Tyrosine	0.034	2.04	23. Proline	0.011	2.58
12. Cysteine	0.030	2.22			

System suitability as per the European Pharmacopoeia (Ph. Eur.)

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe. Leucine (Leu) is a branched-chain α-amino acid that is produced by a fermentation process (Figure 2). During this process, isoleucine can be produced as a by-product. The Ph. Eur. states that leucine and isoleucine should have a resolution of not less than 1.5 [1].

Ten concentration points for three amino acids were assayed in triplicate. The three standard amino acids showed good linearity in the tested range. The area response obeyed the equation y = mx + C, where the intercept C was zero within 95 % confidence limits, and the square correlation coefficient (R2) was always greater than 0.99. Figure 3 shows the linearity curve for asparagine, glutamine, and tryptophan in the concentration range evaluated.

The LOD and LOQ were approximately 0.9 pmol and 3.8 pmol, respectively, using UV detection, indicating that the method was sensitive. Table 5 shows the observed LOD and LOQ values of asparagine, glutamine, and tryptophan.

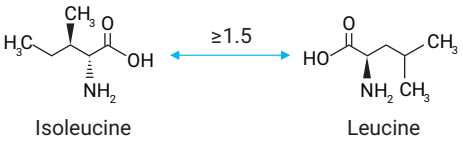

Figure 2. Isoleucine and leucine chemical relationship.

Table 4. System suitability testing using AdvanceBio AAA columns and AA standards.

System suitability	Agilent AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm	Agilent AdvanceBio AAA, C18, 3 × 100 mm, 2.7 µm
Resolution between leucine and isoleucine (≥1.5)	4.5	4.6

Table 5. LODs and LOQs for three amino acids.

Asparagine		Glutamine		Tryptophan	
Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio
0.9 (LOD)	5.3	0.9 (LOD)	3.0	0.9 (LOD)	4.5
1.9 (LOQ)	10.8	3.8 (LOQ)	13.8	3.8 (LOQ)	20.5

Results and Discussion

High-throughput routine analysis

The chromatogram in Figure 1 illustrates the standard analytical sensitivity achieved in high-throughput separations of amino acids. This chromatogram was obtained using an Agilent 1290 Infinity LC with an Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column using the amino acid method with DAD detection. A single run was completed in 18 minutes (including re-equilibration) with adequate resolution. The primary amino acids (1-20, OPA-derivatized), shown in Figure 1, were monitored at 338 nm, while the secondary amino acids (21-23, FMOC-derivatized) were monitored at 262 nm.

Precision of retention time and area (n = 6)

Tables 2 and 3 summarize the average retention times and area RSDs for all the amino acids for the 100 and 1,000 pmol from six replicates of an amino acid method. The retention time RSDs for all amino acid peaks, including the early eluting peak 1 were less than 1.2%, demonstrating excellent gradient reproducibility. Peak area RSDs were less than 5 %, indicating precise sample injection. The RSD values demonstrate the robustness and precision of the amino acid method.

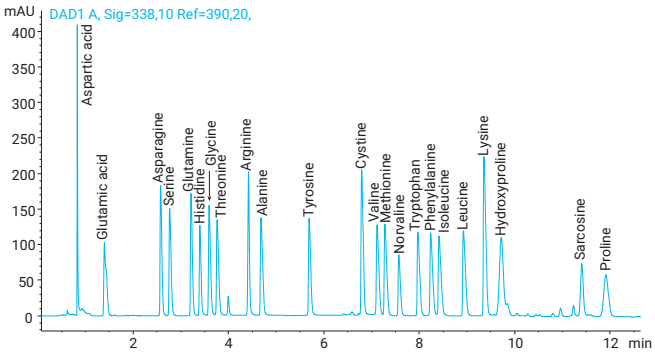

Figure 1. Separation of amino acid standard (1 nmol) on an Agilent AdvanceBio AAA 4.6 × 100 mm, 2.7 µm column, using the amino acid method.

Table 2. Retention time and area RSD precision for amino acids (100 pmol) separated on an Agilent AdvanceBio AAA, 4.6 × 100 mm, column (n = 6).

Amino acid	RT RSD (%)	Area RSD (%)	Amino acid	RT RSD (%)	Area RSD (%)
1. Aspartic acid	1.270	1.066	13. Valine	0.084	2.47
2. Glutamic acid	0.973	1.85	14. Methionine	0.073	1.82
3. Asparagine	0.605	1.79	15. Norvaline	0.073	1.72
4. Serine	0.629	1.82	16. Tryptophan	0.054	1.57
5. Glutamine	0.470	1.56	17. Phenylalanine	0.051	1.66
6. Histidine	0.430	1.22	18. Isoleucine	0.047	1.72
7. Glycine	0.477	1.92	19. Leucine	0.03	1.7
8. Threonine	0.440	1.95	20. Lysine	0.028	1.66
9. Arginine	0.251	2.15	21. Hydroxyproline	0.021	4.13
10. Alanine	0.280	3.06	22. Sarcosine	0.026	1.15
11. Tyrosine	0.128	1.65	23. Proline	0.021	4.36
12. Cysteine	0.067	1.9			

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Amino acid analysis of cell culture media and protein hydrolysate standard

We analyzed the amino acid composition of commonly used cell culture supplements. These standards included: Minimum Essential Medium Eagle (MEM), Non-Essential Amino Acid (NEAA), RPMI 1640 R0083, and protein hydrolysate standard. The results were then compared with the amino acid standards. Figures 4 to 7 show the overlays of amino acid composition of the media and the amino acid standards.

It is evident that the amino acid composition of cell culture supplements, as determined by this method, matches accurately with their theoretical composition. In addition, baseline resolution of isoleucine and leucine was observed with a resolution factor of 4.35 for the protein hydrolysate standard, meeting the regulatory requirements for these components significantly better than competitive columns. Such applications are useful in monitoring and adjusting amino acid composition, which is an essential part of optimizing the manufacturing process to ensure high quality and optimum yield of the final biopharmaceutical product.

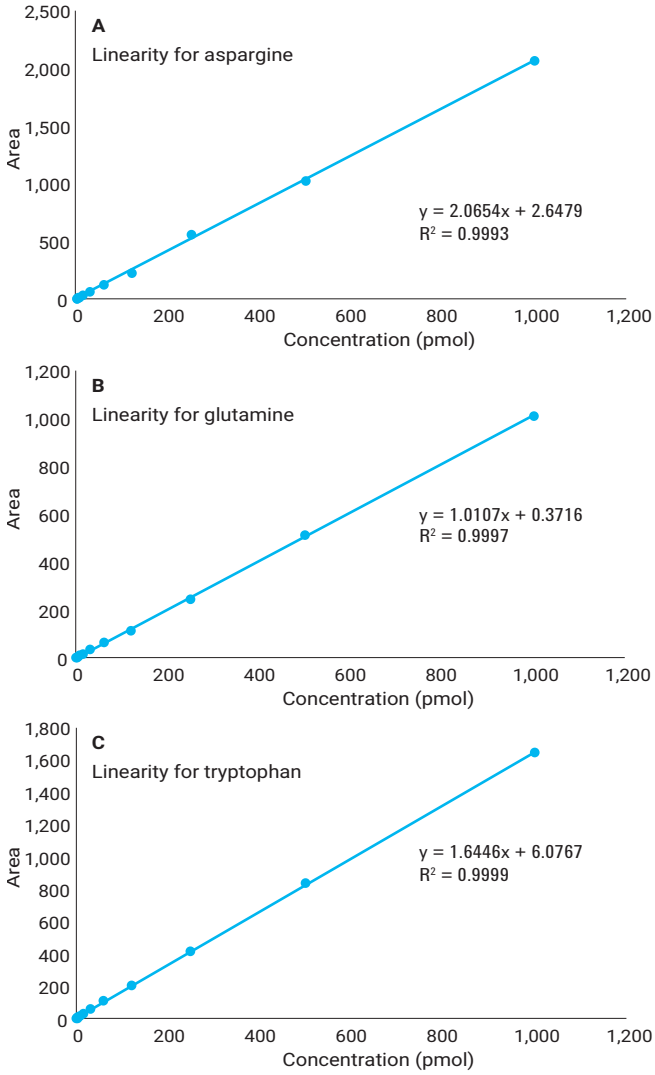

Figure 3. Linearity curve with 10 standard concentrations of asparagine, glutamine, and tryptophan ranging from 0.9 to 1,000 pmol, showing excellent coefficient values.

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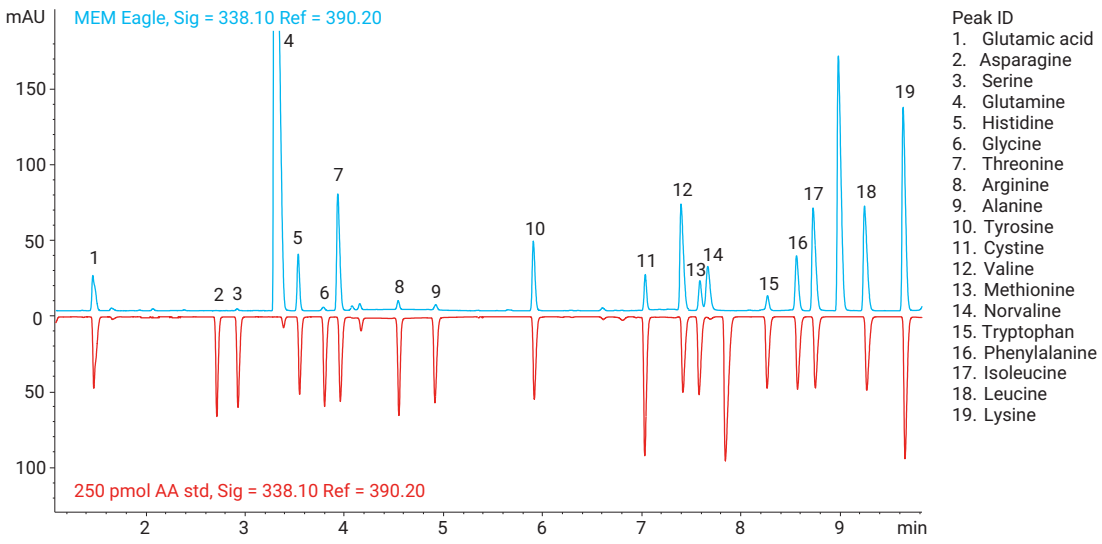

Figure 4. Amino acid analysis of MEM media (blue trace) mirrored with AA standards (red trace).

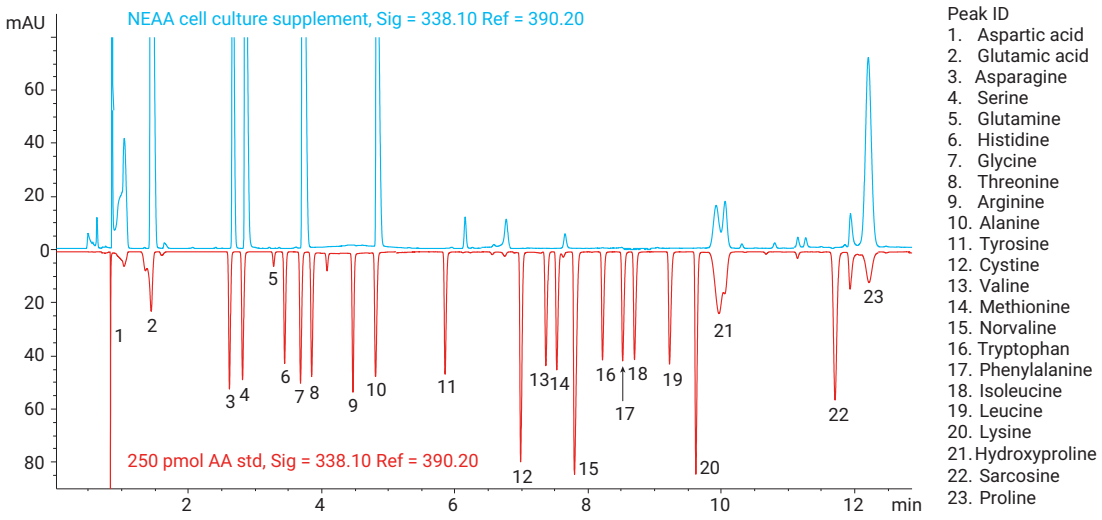

Figure 5. Amino acid analysis of NEAA media (blue trace) and comparison with AA standards (red trace).

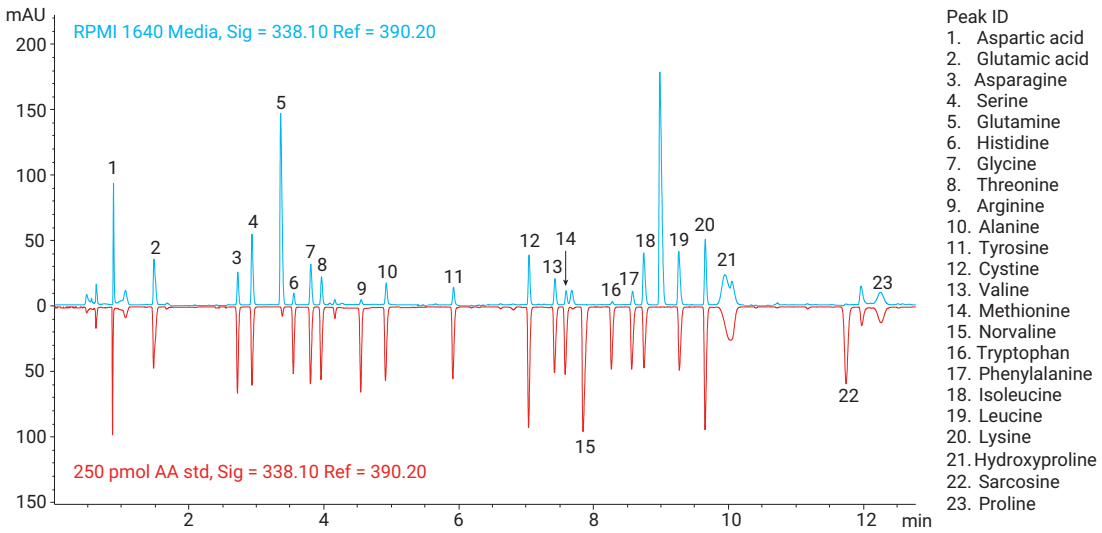

Figure 6. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with AA standards (red trace).

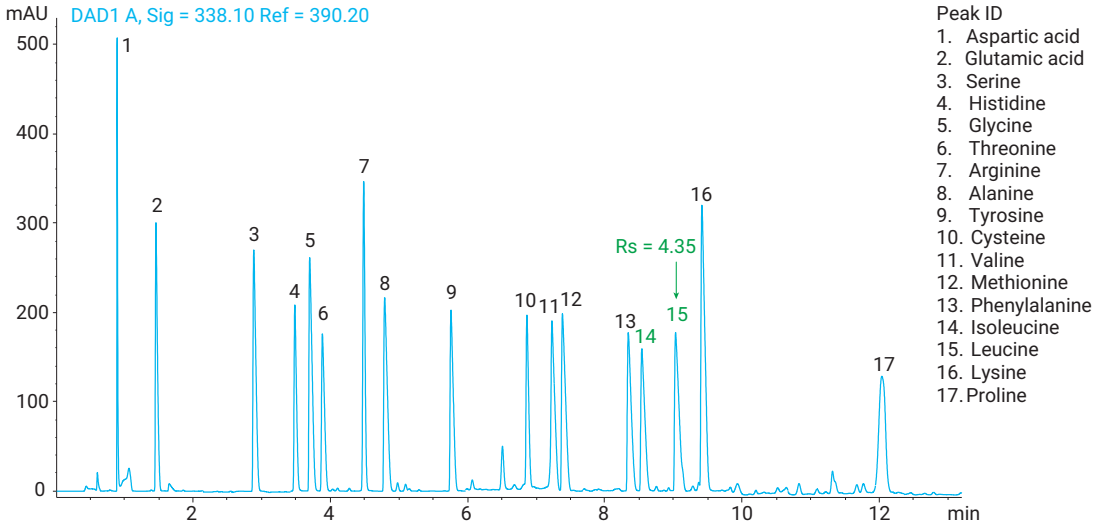

Figure 7. Amino acid analysis of protein hydrolysate standard. The resolution between leucine and isoleucine with the Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement for this pair.

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Conclusion

Amino acid analysis is an important approach for the characterization of protein and peptide-based products. Studying the roles of amino acids during bioprocesses leads to better understanding the feeding strategy, and to improving the yield and quality of the product. In addition, the determined amino acid composition can confirm sample identity, and give a measure of sample purity. This Application Note demonstrates several Agilent tools for the analysis of amino acids. We first used the Agilent 1290 Infinity LC and the Agilent AdvanceBio AAA kit for the automated online derivatization of amino acids using OPA/FMOC chemistries. The derivitized amino acids were then separated on an AdvanceBio AAA LC column to achieve a fast, sensitive, and reproducible separation of amino acids. Area and RT precision of the method were excellent, and met the system suitability requirement. Linearity curves with 10 standard concentrations of three amino acids, ranging from 0.9 pmol to 1 nmol, had excellent coefficient of linearity values, indicating that the method was quantitative and accurate. The LOD and LOQ for the amino acids were 0.9 pmol and 3.8 pmol, respectively, indicating that the method was sensitive. In addition, this method was able to separate and detect amino acids from cell culture media and protein hydrolysate standard. The amino acid composition determined using this method correlated well with their theoretical compositions..

Reference

1. European Pharmacopoeia 9.0 (2.2.56) *Amino Acid Analysis*

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Application Note

Amino Acid Analysis

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Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column

Authors

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Agilent Technologies, Inc

Abstract

In this application note, an automated precolumn OPA/FMOC amino acid method, previously developed on 3.5 and 1.8 µm Agilent ZORBAX Eclipse Plus C18 columns, is expanded to include 2.7 µm Agilent Poroshell HPH-C18 superficially porous columns. This column exhibits good lifetime and transferability to different column dimensions, both of which are shown in this work. Applications of the column to fermentation products are also shown.

Introduction

Superficially porous particle (SPP) technology is based on particles with a solid core and a superficially porous shell. These particles consist of a 1.7 µm solid core with a 0.5 µm porous shell. In total, the particle size is about 2.7 µm. The 2.7 µm superficially porous particles provide 40 to 50 % lower backpressure and 80 to 90 % of the efficiency of sub-2 µm totally porous particles. The superficially porous particles have a narrower particle size distribution than totally porous particles. This results in a more homogeneous column bed, and reduces dispersion in the column. At the same time, the thin porous shell provides lower resistance to mass transfer. The result is minimal loss of efficiency at higher flow rates [1]. Additionally, since the columns incorporate a 2 µm frit, they are as resistant to clogging as 3.5 and 5 µm columns. Until recently, all silica-based SPP materials possessed limited lifetime in higher pH buffers, including phosphate buffers. To achieve these longer lifetimes, it is necessary to protect the base particle by either surface modification or special bonding modification. The surface of Agilent Poroshell HPH-C18 particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process. The continuous improvement in HPLC columns and instrumentation presents an opportunity to improve HPLC methods. A proven ortho-phthalaldehyde/9-fluorenylmethyl chloroformate (OPA/FMOC)-derivatized amino acid method developed on HP 1090 Series HPLC systems, and later updated for the Agilent 1100 Series, has now evolved further taking advantage of the Agilent 1260 Infinity Binary LC and superficially porous Agilent Poroshell HPH-C18 columns [2-8].

Experimental

Preparation of HPLC mobile phase

Mobile phase A contained 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 5 mM NaN_3. For 1 L, weigh 1.4 g anhydrous Na_2HPO_4 plus 3.8 g $Na_2B_4O_7{\cdot}_{10}H_2O$ in 1 L water plus 32 mg NaN_3. Adjust to about pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid to pH 8.2. Allow stirring time for complete dissolution of borate crystals before adjusting pH. Filter through 0.45 µm regenerated cellulose membranes (p/n 3150-0576). Mobile phase B contains acetonitrile:methanol:water (45:45:10, v:v:v). All mobile-phase solvents were HPLC grade. Since mobile phase A is consumed at a faster rate than B, it is convenient to make 2 L of A for every 1 L of B.

The injection diluent was 100 mL mobile phase A, plus 0.4 mL concentrated H_3PO_4 in a 100 mL bottle, stored at 4 °C.

To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) to a 500 mL volumetric flask that is partially filled with water. Mix, and fill to the mark with water. This solution is for making extended amino acid and internal standard stock solutions. Store at 4 °C

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent. They simply need to be transferred from their container into an autosampler vial. Some precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7 to 10 days. We recommend transferring 100 µL aliquots of OPA to microvial inserts. Label with name and date, cap, and refrigerate. Replace the OPA autosampler microvial daily. Each ampoule lasts 10 days.
- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred in 100 µL aliquots to microvial inserts. Label with name and date, cap tightly, and refrigerate. Like the OPA, an open FMOC ampoule transferred to 10 microvial inserts should last 10 days (one vial/day).
- Borate buffer can be transferred to a 1.5 mL autosampler vial without a vial insert. Replace every three days.

Preparation of amino acid standards

Solutions of 17 amino acids in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Divide each 1 mL ampoule of standards (p/n 5061-3330 through 5061-3334) into 100 µL portions in conical vial inserts. Cap and refrigerate aliquots at 4 °C. To make the extended amino acid (EAA) stock solution, weigh 59.45 mg asparagine, 59.00 mg hydroxyproline, 65.77 mg glutamine, and 91.95 mg tryptophan into a 25 mL volumetric flask. Fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Fill to mark with water for a total concentration of 18 nmol/µL of each amino acid. For the high-sensitivity EAA stock solution, take 5 mL of this standard-sensitivity solution and dilute with 45 mL water (1.8 nmol/µL). Solutions containing extended standards are unstable at room temperature. Keep them frozen and discard at first signs of reduced intensity.

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill half way with 0.1 N HCl and shake or sonicate until dissolved, then fill to mark with water for a final concentration of 10 nmol each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, take 5 mL of standard-sensitivity solution and dilute with 45 mL of water. Store at 4 °C. Calibration curves are made using two to five standards depending on experimental need. Typically, 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for standard-sensitivity analysis.

Pump parameters

Pump parameters for all methods include compressibility (×10–6 bar) A: 35, B: 80, with minimal stroke A, B of 20 µL.

Online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G1376C well plate automatic liquid sampler (WPALS), with injection program:

1. Draw 2.5 µL from borate vial (p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in wash port five times.
4. Wait 0.2 minutes.
5. Draw 0.5 µL from OPA vial (p/n 5061-3335).
6. Mix 4.0 µL in wash port 10 times default speed.
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337).
8. Mix 4.4 µL in wash port 10 times default speed.
9. Draw 32 µL from injection diluent vial.
10. Mix 20 µL in wash port eight times.
11. Inject.
12. Wait 0.1 minutes.
13. Valve bypass

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G1367C with a 2 × 56 well plate tray (p/n G2258-44502), the locations were:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection diluent
- P1-A-1: Sample

Thermostatted column compartment (TCC)

Left and right temperatures were set to 40 °C. Enable analysis when the temperature is within ± 0.8 °C. See Table 5 for which heat sink to use.

Diode array detector (DAD)

Signal A: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth.

Signal B: 262 nm, 16 nm bandwidth, and reference wavelength 324 nm, 8 nm bandwidth.

Signal C: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth.

The DAD was programmed to switch to 262 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth, after lysine elutes, and before hydroxyproline elutes. Signal C was determined by examining signal A and B timeframes between peaks 20 and 21, then choosing a suitable point to switch wavelengths. Once the switch time was established and programmed into the method, signals A and B were optional.

Peak width settings of > 0.01 minutes were used for all columns.

Results and Discussion

As can be seen in Figure 1, using the same chromatographic conditions, the separation was very similar. The elution order of the mixture on both columns was the same, and as shown in Figure 2, the relationship of retention times of the amino acid samples was highly correlated between an Eclipse Plus C18 and a Poroshell HPH-C18, with a correlation co-efficient of 0.997. As can be seen in the chromatograms, the retention times were slightly less on the Poroshell HPH-C18 column. Some chromatographic differences are notable. Thus, separation of leucine and lysine looks better on Poroshell HPH-C18, while the separation between lysine and hydroxyproline and the sarcosine/proline pair looks worse. As suggested in previous application notes, the chromatography can be altered to enhance resolution of desired peak pairs.

Conditions for Figure 1.

Parameter	Value	
Column:	Agilent Poroshell HPH C18, 4.6 × 100 mm, 2.7 µm (p/n695975-702) or Agilent Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (p/n959961-902)	
Flow rate:	1.5 mL/min	
Gradient:	Time (min)	% B
	0	2
	0.35	2
	13.4	57
	13.5	100
	15.7	100
	15.	8 2
	18	end

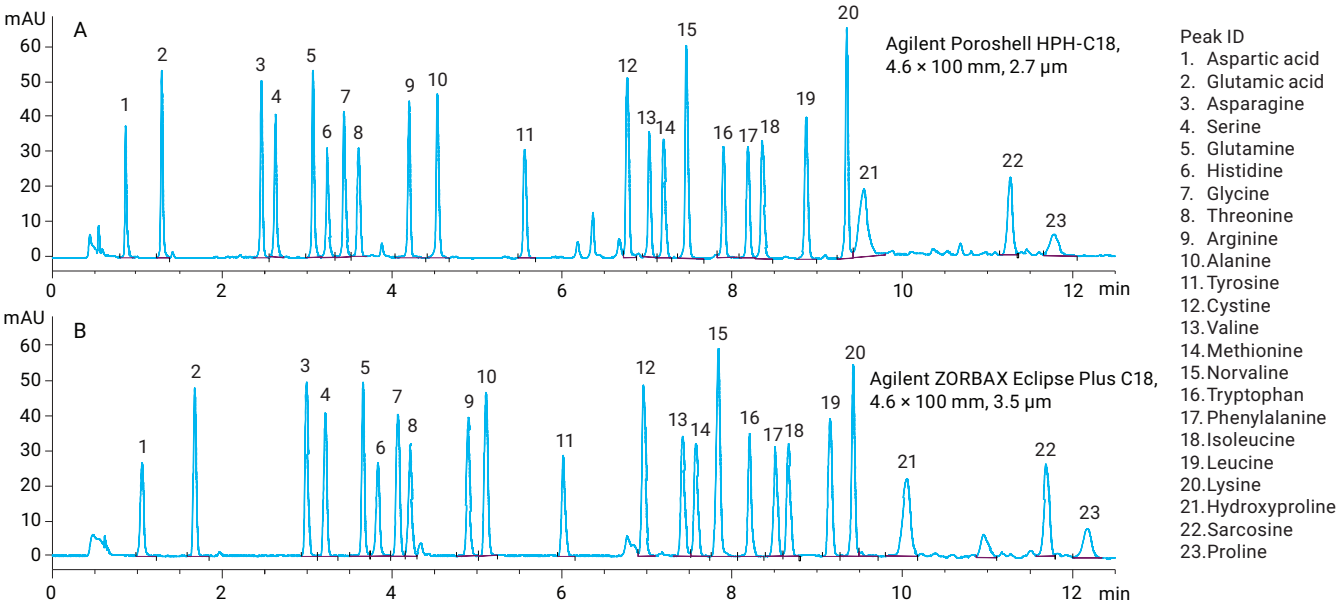

Figure 1. Comparison of an Agilent Poroshell HPH C18 to an Agilent ZORBAX Eclipse Plus C18 column using the Amino Acid Method.

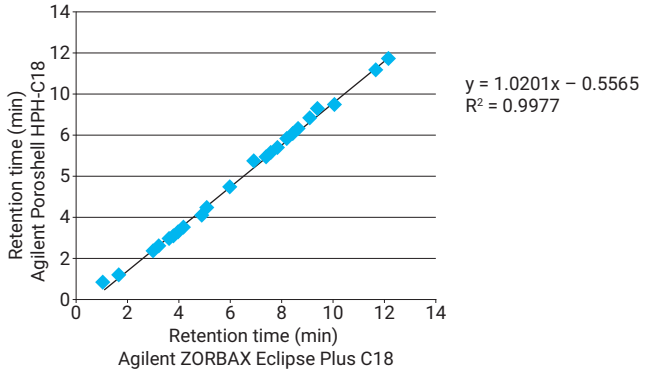

Figure 2. Correlation of retention times using Agilent Poroshell HPH-C18 and Agilent ZORBAX Eclipse Plus C18 columns.

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Column dimensions

The method can easily be scaled to different column dimensions. In this work, three column dimensions were studied. All columns were 100 mm in length with 4.6, 3.0, or 2.1 mm internal diameter, as shown in Figure 3. In this case, the only changes to the method were made by altering the flow rate. Table 1 lists the gradient program used throughout. Flow rates are changed geometrically with the diameter of the column. The flow rate used with the 4.6 × 100 mm column was 1.5 mL/minute. The flow rates for the 3 and 2.1 mm columns were 0.62 and 0.21 mL/min, respectively. In all cases, the low-volume heat exchanger was used with short red tubing to minimize extra column volume. Using the Agilent 1260 Infinity Binary LC with low dispersion heating and tubing, the column pressure was approximately 175 bar. We observed that retention time of all analytes increased slightly (without changing selectivity) as columns were changed from larger to smaller internal diameter. This is due to the increase in gradient delay time. As the flow rates are scaled and consequently reduced from larger to smaller column ids, the gradient delay volume remains constant, thereby increasing the time it takes for the gradient to reach the column. The difference in retention between various column ids could potentially be reduced or eliminated by scaling the gradient delay volume on the LC system (adding or removing capillary length/diameter/volume between the pump and column inlet).

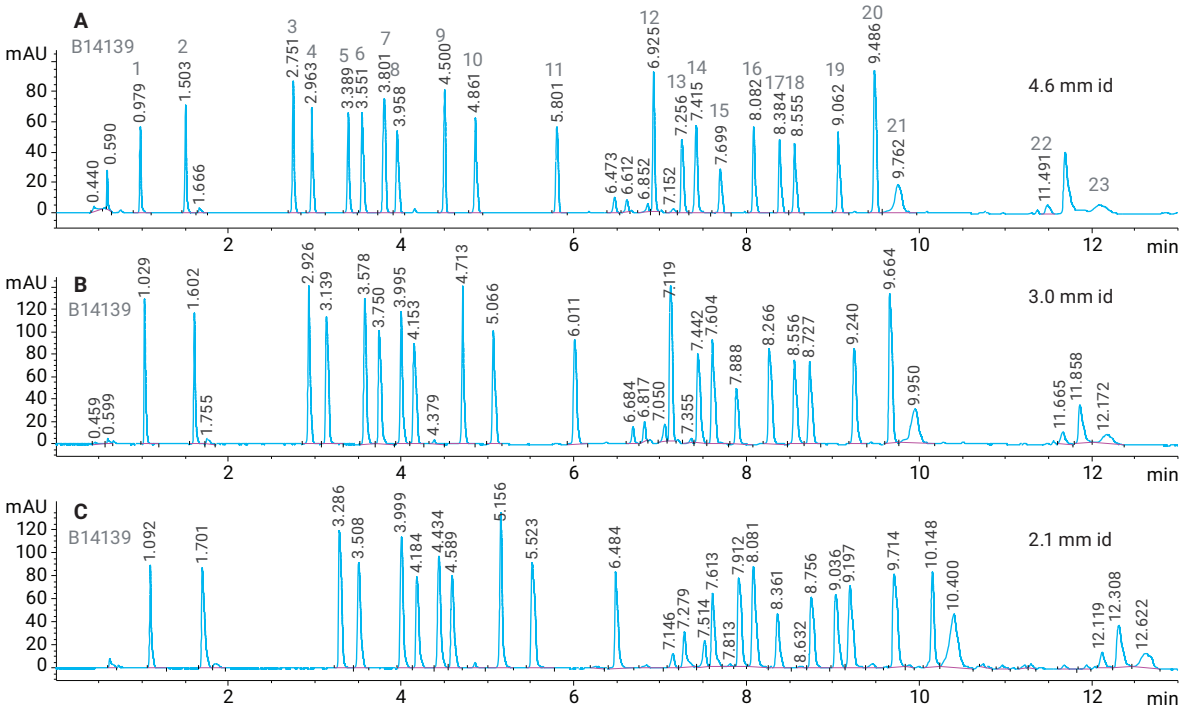

Figure 3. Agilent Poroshell HPH-C18 100 mm columns of different inside dimension using the amino acid method.

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Lot-to-lot variability

Batch-to-batch or lot-to lot reproducibility is also an import factor in method development. It is recommended that, before a method is adopted, one of the earliest validation steps is to examine the method performance on at least three columns made from different lots.

Following good validation practice, three columns loaded with particles from different production batches were examined for 4.6, 3.0, and 2.1 × 100 mm columns. The overlays of these three sets are shown in Figures 4A-C. As can be seen in Figure 4A, the amino acid separation on the 4.6 × 100 mm column achieved good peak as well as baseline separation shape for all compounds. No change in elution order was noted, and lot-to-lot reproducibility looked good. A slight change in retention time can be seen in Figure 4A though the k' remained constant. However, a slight change in the wavelength switch time is required as it is tied to the elution times of lucine and hydroxyproline. Similar reproducibility is evident in Figures 4B and 4C for the smaller id columns.

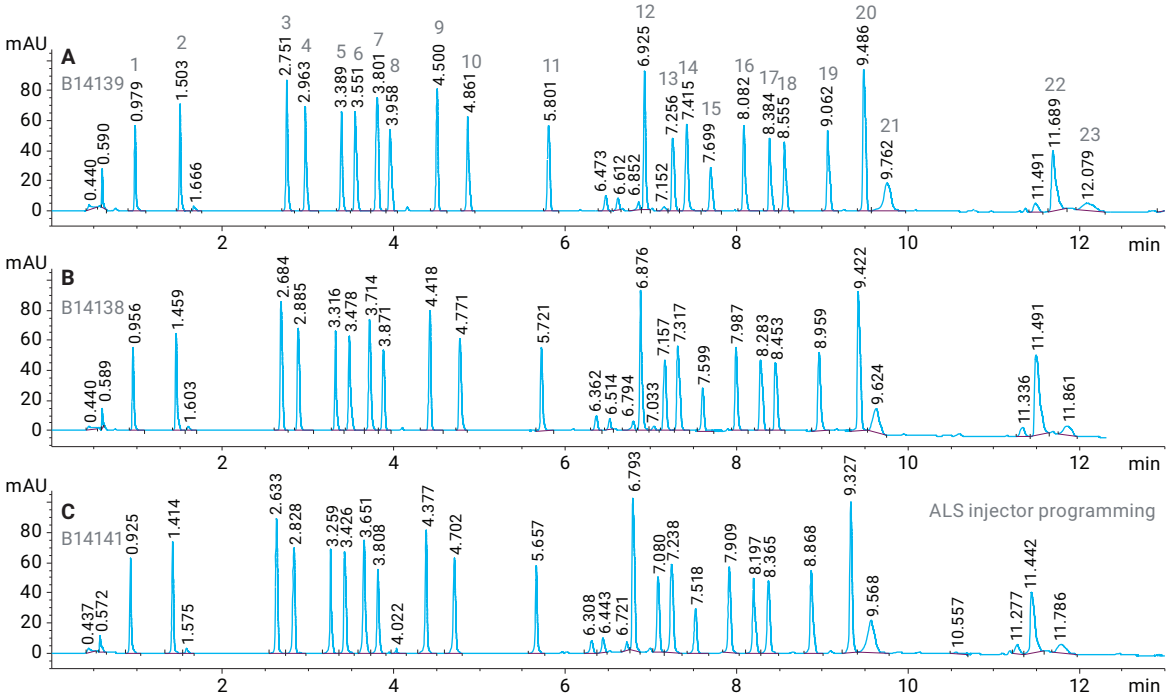

Figure 4A. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-702).

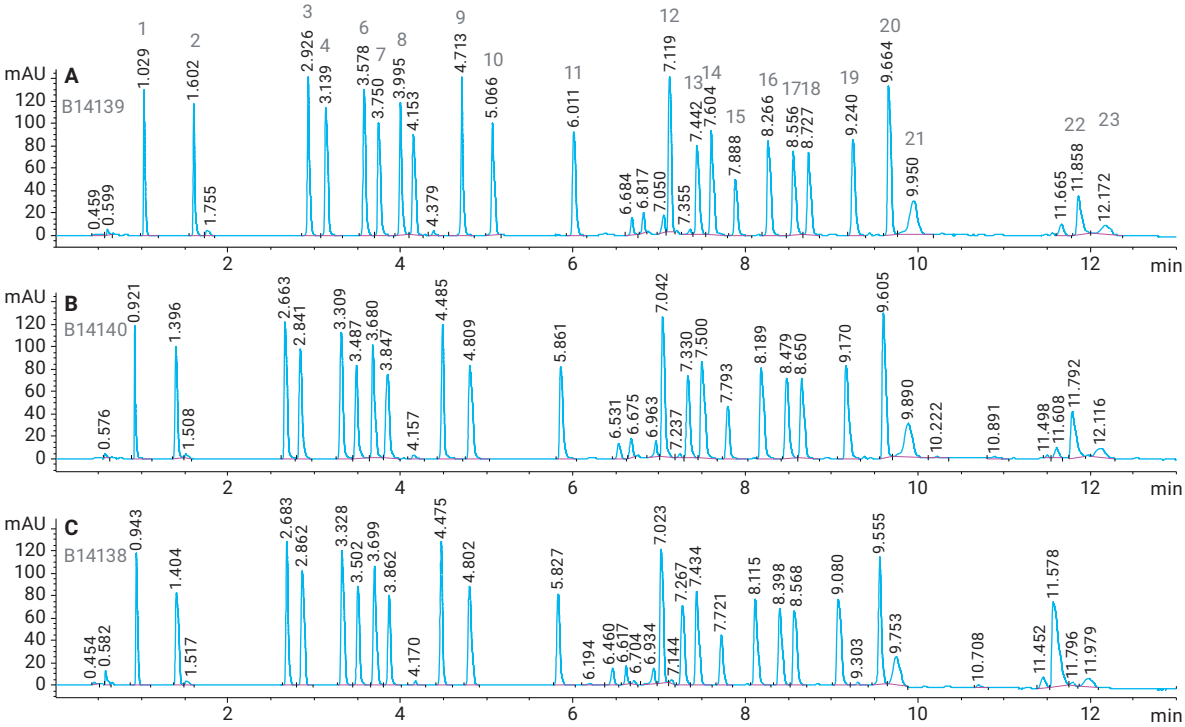

Figure 4B. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (p/n 695975-502).

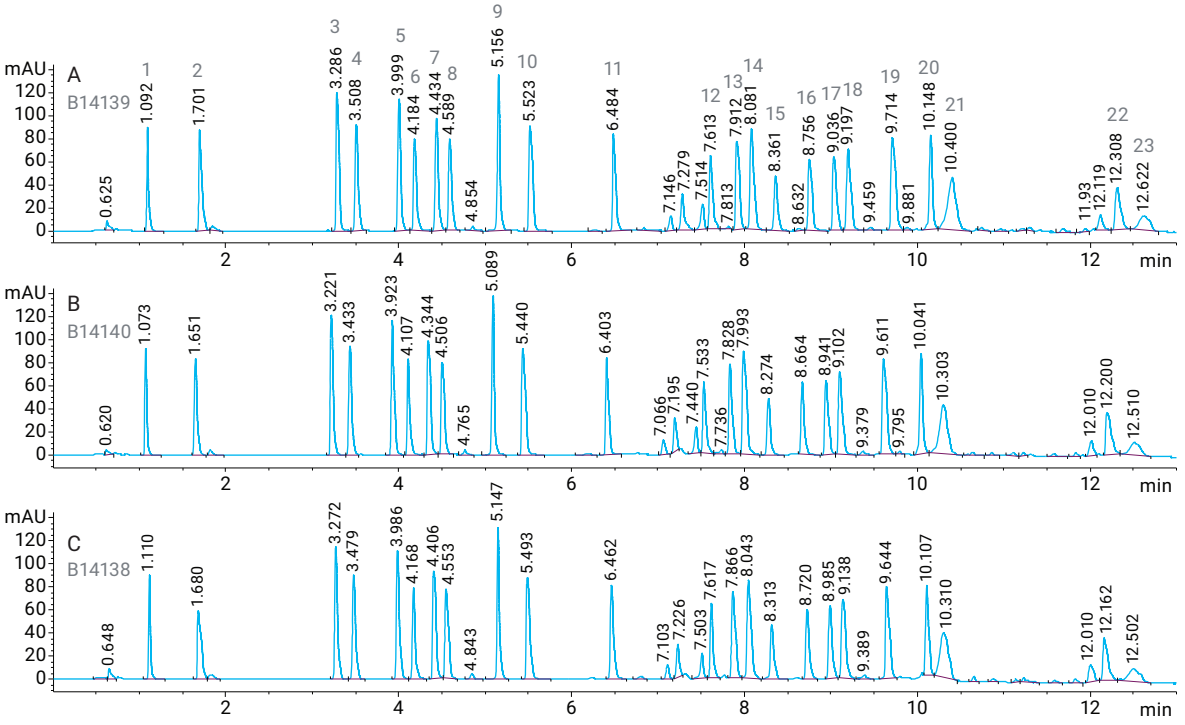

Figure 4C. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702).

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Lifetime

Column lifetime is an important consideration for chromatographers analyzing amino acid samples. Most silica columns lose efficiency after prolonged exposure to these conditions. Kirkland et al. [9] and Tindall and Perry [10] discussed possible reasons for the reduced lifetime of silica columns in phosphate buffer, but both agree that columns do not last as long.

There are two approaches to achieving high pH stability in silica HPLC columns. One way is to employ special bonding chemistry, as in the Agilent ZORBAX Extend C18 column. This column uses bidentate bonding to protect the silica from dissolution at high pH. Another way to achieve high pH stability is to modify the silica itself, making it less soluble. The surface of Poroshell HPH particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process [11].

Figure 5 is an overlay of four chromatograms. Single 4 L bottles of mobile phase A and B were prepared. A single 2.1 × 100 mm column was used for lifetime testing from a series of 500 analyses over a period of four weeks. In this series, approximately 102 injections were made each week using freshly opened amino acid standard mix and reagents. At the end of the sequence, the column was flushed with 100 % B mobile phase for 40 minutes and the instrument was shut down. In this manner, the method was run for 3.5 days and the column was stored with no analysis for 3.5 days. This simulated typical practice in a lab where samples are run for an extended time, and then a column is washed and stored. Storing a column in 100 % mobile phase B was recommended in the original amino quant methods, and is common practice in many successful laboratories that frequently run amino acids. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence. As can be seen in Figure 5, the 17 amino acid sample lost no resolution and only a slight retention time shift was seen.

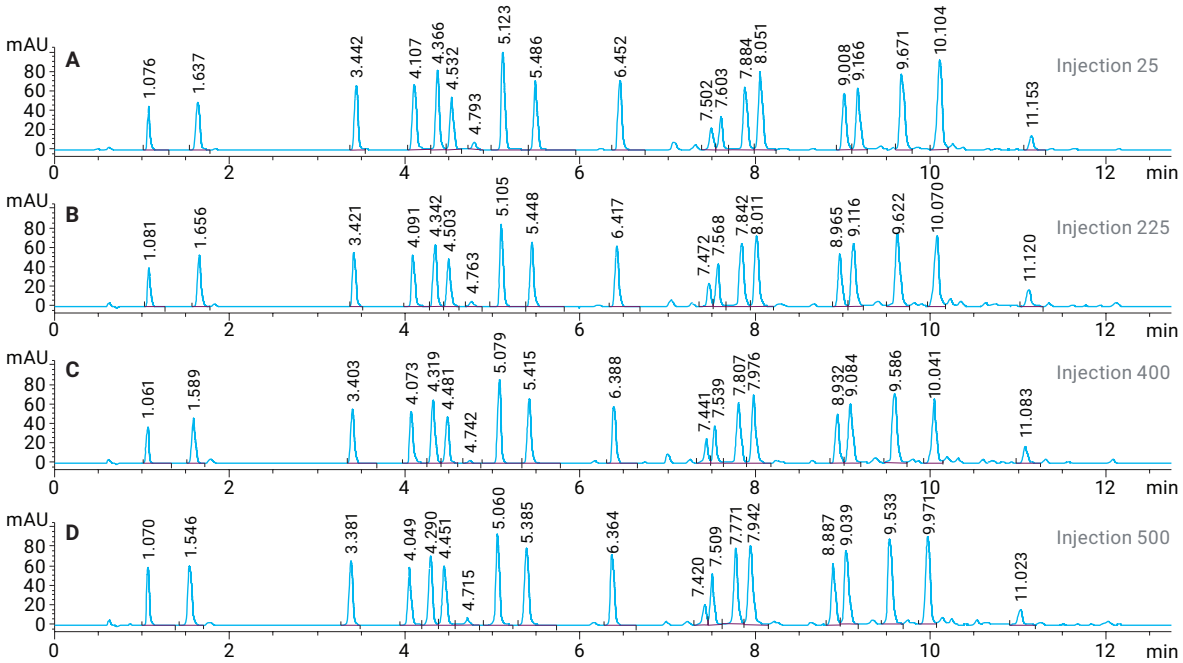

Figure 5. Column lifetime test using an Agilent Poroshell HPH-C18, 2.1 × 100 mm column running an amino acid method.

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Conclusions

Agilent Poroshell HPH-C18 has selectivity similar to totally porous Agilent ZORBAX Eclipse Plus C18. This allows easy transfer of existing methods such as the amino acid method. In this work, no changes to the chromatographic conditions were made although changes in the gradient could be done to improve resolution on selected amino acids. In most cases, Poroshell HPH-C18 was slightly less retentive than totally porous Eclipse Plus C18. The method was investigated with 4.6, 3.0, and 2.1 mm × 100 mm columns. Use of the low volume column heater is recommended. In total, four particle lots were investigated, requiring only slight changes to the wavelength switch time. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence.

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