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

The Agilent AdvanceBio Amino Acid Solution

Application Note

Biopharmaceuticals

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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.

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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 AdvanceBioAAA 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), Na_2HPO_4, and $Na_2B_4O_7 \cdot 10H_2O$, 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.

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

Each of the components in the table can be ordered separately.

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.

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. Standard analytical sensitivity calibration standards.

	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 G1376C 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.

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

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.

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.

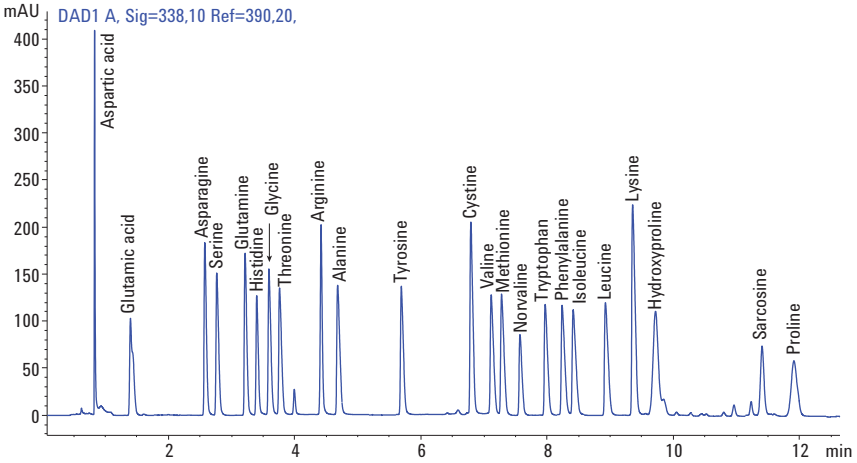


Figure 1. Separation of amino acid standard (1 nmol) on an Agilent AdvanceBio AAA 4.6 × 100 mm, 2.7 µm column, using the amino acid method.

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).

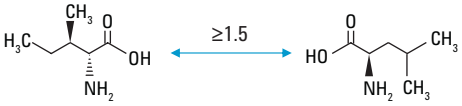
Amino acid	RT RSD (%)	Area RSD (%)	Amino acid	RT RSD (%)	Area RSD (%)
1. Aspartic acid	1.270	1.066	13. Valine	0.084	2.47
2. Glutamic acid	0.973	1.85	14. Methionine	0.073	1.82
3. Asparagine	0.605	1.79	15. Norvaline	0.073	1.72
4. Serine	0.629	1.82	16. Tryptophan	0.054	1.57
5. Glutamine	0.470	1.56	17. Phenylalanine	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			

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].



Isoleucine Leucine

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

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 (R^2) 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.

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

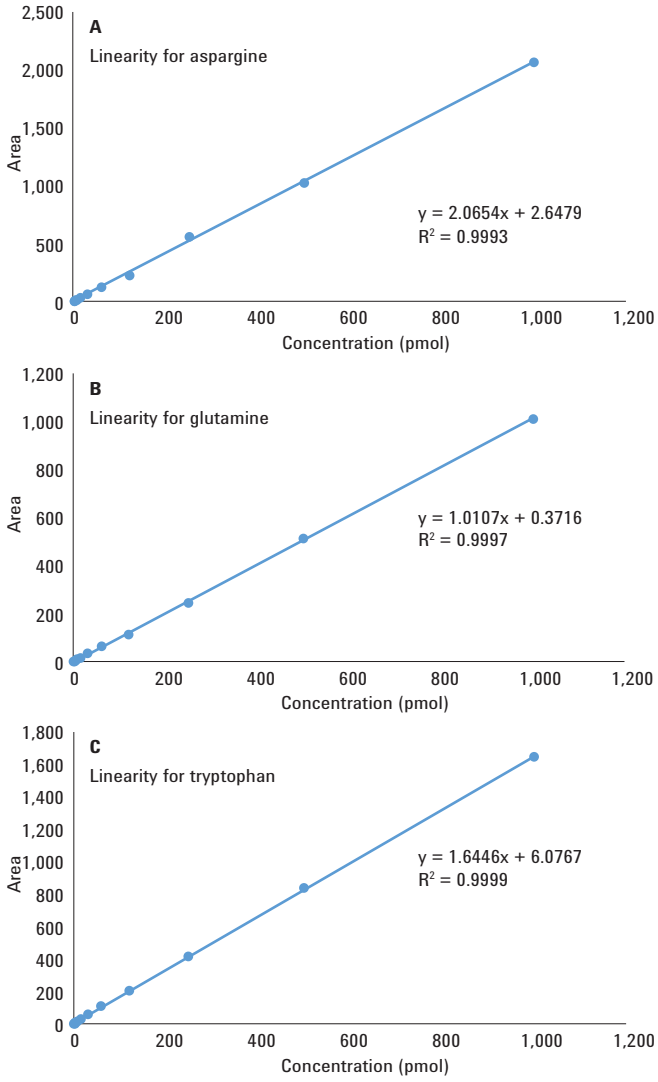


Figure 3. Linearity curve with 10 standard concentrations of asparagine, glutamine, and tryptophan ranging from 0.9 to 1,000 pmol, showing excellent coefficient values.

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.

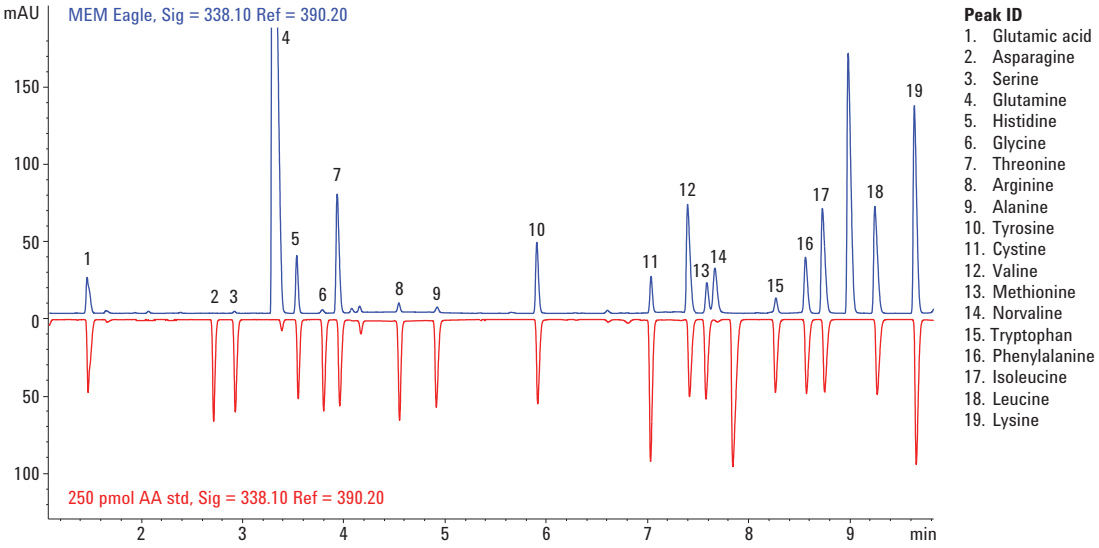


Figure 4. Amino acid analysis of MEM media (blue trace) mirrored with AA standards (red trace).

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

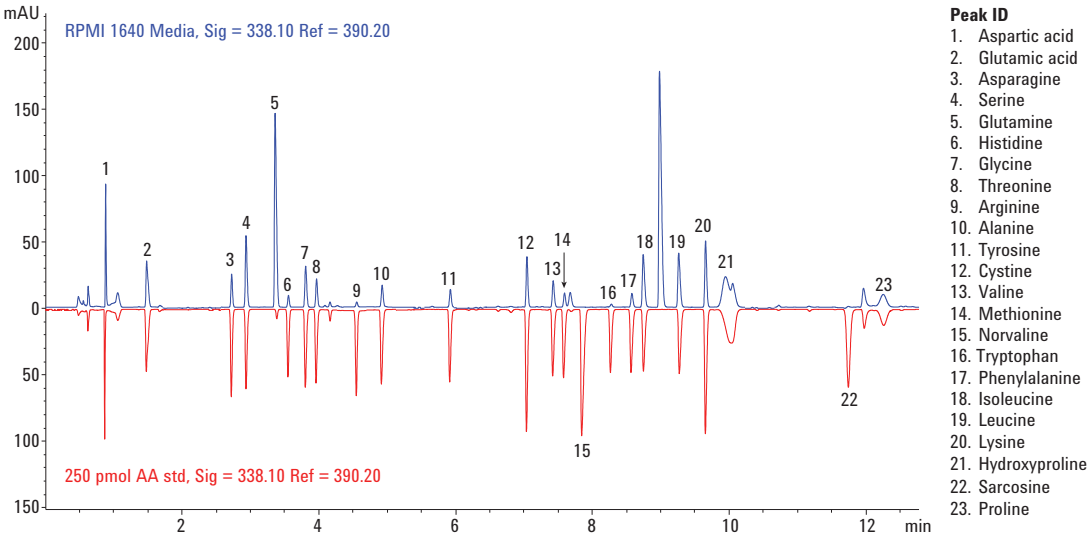


Figure 6. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with AA standards (red trace).

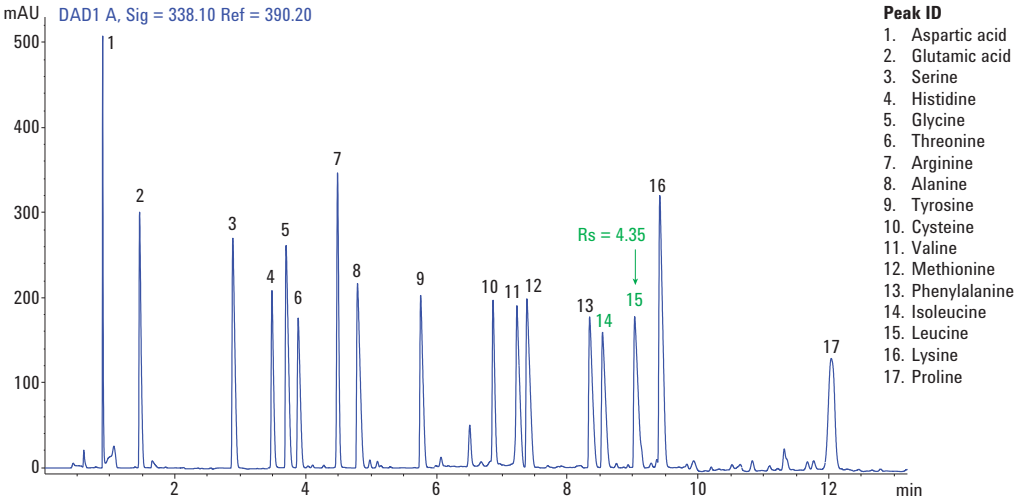


Figure 7. Amino acid analysis of protein hydrolysate standard. The resolution between leucine and isoleucine with the Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement for this pair.

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.

For More Information

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