Application Note

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Analysis of Amino Acids in Spent Media Using Automated Precolumn Derivatization with the Agilent 1290 Infinity II Bio LC System

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Abstract

Amino acids in cell culture media are important for enhancing the growth and productivity of cells. Thus, it is essential to monitor cell culture media for any changes in amino acid levels. This is to prevent depletion of essential cellular nutrients and to make informed decisions on when to change media or other inputs. In this application note, a methodology for the quantitation of amino acid changes in cell culture media, which was documented during a cell growth experiment (Hela and HepG2 cells), is provided. The analysis was performed using the Agilent 1290 Infinity II bio LC system with a serially connected diode array detector (DAD) and fluorescence detector (FLD). Automated precolumn derivatization was included using the multisampler injector program. A shorter runtime with optimized gradient was also demonstrated to showcase the possibility of employing a faster method for amino acid analysis on the Agilent 1290 Infinity II bio LC system. The automation of sample preparation and the easy setup of the LC analysis method allow for fast and efficient analysis of amino acids in spent media. This study is useful for users who need to optimize and monitor the concentration of amino acids during cell cultivation and bioprocess development.

Introduction

The impact of cell culture technology for the development of biological drugs is tremendous. From early drug discovery to the manufacturing of biopharmaceuticals, cell culture technology plays a significant role.[1] Cells in cell culture require nutrients such as amino acids, glucose, vitamins, salts, and other components to support cell growth.[2] Amino acids are essential nutrients for in vitro cell cultivation.[3] Other than being the building blocks for proteins, amino acids have multiple regulatory roles in cells.[4]

Cell culture media composition plays an instrumental role in cell growth and biotech product yields. To optimize cell culture media is to improve media composition and culture conditions to achieve the desired outcome.[5] Monitoring amino acid changes is part of the optimization process for cell culture media development. The cell culture components and their concentrations in the cell culture media will affect the important aspects of cell growth and thus, are crucial to the success of the cell culture application.[5] Agilent application note 5994-4931EN[6] shows an example of online amino acid analysis for spent media control to illustrate the importance of online amino acid concentration monitoring. In this study, the measurement of amino acid changes during cell culture is demonstrated using the 1290 Infinity II bio LC system. Some amino acids, such as histidine and glutamic acid, are known to bind to metal surfaces. The biocompatible 1290 Infinity II bio LC system was used in this analysis to reduce interactions with metal surfaces and to improve peak shape. Precolumn derivatization and method parameters were adapted from Agilent application note 5994-5950EN.[7]

Experimental

Equipment

Amino acid analysis was performed using a 1290 Infinity II bio LC system with the following components. The LC system was operated using Agilent OpenLab CDS version 2.7.

Part Number	Component
G7132A	Agilent 1290 Infinity II bio high-speed pump
G7137A	Agilent 1290 Infinity II bio multisampler
G7116B	Agilent 1290 Infinity II multicolumn thermostat
G7117B	Agilent 1290 Infinity II diode array detector (DAD) (with variable slit)
G4212-6008	Agilent Max-Light cartridge cell, 10 mm, 1.0 µL, 60 bar
G7121B	Agilent 1260 Infinity II fluorescence detector spectra
G1321-60005	Agilent flow cell, 8 µL, 20 bar

Reagents and materials

HeLa and HepG2 cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, U.S.). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, U.S.).

All solvents used were LC-grade. Acetonitrile was purchased from JT Baker (Phillipsburg, NJ, U.S.) and methanol was purchased from Merck (Darmstadt, Germany).

Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, U.S.) equipped with a 0.22 µm membrane point-of-use cartridge. Sodium phosphate dibasic, disodium tetraborate decahydrate, concentrated hydrochloric acid (37%), and phosphoric acid (85%), were purchased from Sigma Aldrich (St. Louis, MO, U.S.).

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

Part Number	Component
5061-3339	Borate buffer: 0.4 M in water, pH 10.2, 100 mL
5061-3337	9-Fluorenylmethyl chloroformate (FMOC) reagent, 2.5 mg/mL in acetonitrile, 10 x 1 mL ampules
5061-3335	o-Phthalaldehyde (OPA) reagent, 10 mg/mL in 0.4 M borate buffer, and 3-mercaptoproprionic acid, 6 x 1 mL ampules
5061-3330	Amnio acid standard (mix of 14 standards), 1,000 pmol/µL, 10 x 1 mL
5061-3331	Amnio acid standard, 250 pmol/µL, 10 x 1 mL
5061-3332	Amnio acid standard, 100 pmol/µL, 10 x 1 mL
5061-3333	Amnio acid standard, 25 pmol/µL, 10 x 1 mL
5061-3334	Amnio acid standard, 10 pmol/µL, 10 x 1 mL
5062-2478	Amino acid supplement kit (containing L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline (IS), and sarcosine (IS)), 1 g each

Preparation of solvents and reagents

- Mobile phase A contained 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$, pH 8.2
- Mobile phase B contained acetonitrile, methanol, and water (45:45:10, v:v:v)
- 0.1 N HCl was prepared by appropriate dilution of concentrated HCl using water
- Diluent: 10 mL of mobile phase A + 200 µL of phosphoric acid (85%)

Note: After opening an OPA or FMOC ampoule, the reagents were aliquoted to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024). They were stored for no longer than a week.

Note: Borate buffer and injection diluent were transferred to vials without inserts. All reagents should be stored at 4 °C and reagents in the autosampler should be exchanged daily.

Preparation of amino acid standard solutions

1. Extended amino acid (EAA) stock solution: 1,800 pmol/µL each of asparagine, glutamine, and tryptophan, in 0.1 M HCl
2. Diluted EAA stock solution:
   - 900 pmol/µL
   - 450 pmol/µL
   - 180 pmol/µL
   - 90 pmol/µL
   - 45 pmol/µL
   - 18 pmol/µL
   - 9 pmol/µL with 0.1 M HCl
3. Internal standard (IS) stock solution: 1,000 pmol/µL each of norvaline and sarcosine in 0.1 M HCl
4. EAA solutions + IS: mix EAA and IS in 1:1 ratio to obtain amino acid concentrations of 4.5 to 900 pmol/µL and IS concentrations of 500 pmol/µL
5. Final concentration of amino acid targets in calibration solutions:
   - 0.45 (L1), 0.90 (L2), 2.25 (L3), 4.5 (L4), 9.0 (L5), 22.5 (L6), 45 (L7), and 90 (L8) pmol/µL of amino acids with IS 50 pmol/µL

Spent media

DMEM spent media was collected at time points 0, 16, 24, 48, and 72 hours of cell culture of HeLa and HepG2 cells. By sampling both cell lines at corresponding time points, the study enabled a direct comparison of amino acid dynamics between HeLa and HepG2 cells during various stages of cell culture. The chosen time points provide temporal understanding of how amino acid uptake evolved during the cell culture process.

Sample preparation

Before being analyzed on the 1290 Infinity II bio LC system, 100 µL of cell culture media was diluted 10x with 850 µL of water and 50 µL of the IS stock solution.

LC analysis

Precolumn derivatization used is based on OPA and FMOC chemistry for primary and secondary amino acids. The derivatization procedures were automated using the LC autosampler injector program function. The precolumn derivatization program on the 1290 Infinity II multisampler is as shown in Table 1, and the HPLC method is as shown in Table 2.

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

Function	Parameter
Draw	Draw 5.00 µL from location 1 with the default speed using the default offset
Wash	Wash needle as defined in the method
Draw	Draw 1.00 µL from sample with the default speed using the default offset
Wash	Wash needle as defined in the method
Draw	Draw 1.00 µL from location 2 with the default speed using the default offset
Wash	Wash needle as defined in the method
Mix	Mix 7.00 µL from air with the default speed 10x
Draw	Draw 0.40 µL from location 3 with the default speed using the default offset
Wash	Wash needle as defined in the method
Mix	Mix 7.40 µL from air with the default speed 10x
Draw	Draw 32.00 µL from location 4 with the maximum speed using the default offset
Wash	Wash needle as defined in the method
Mix	Mix 20.00 µL from air with the maximum speed 5x
Inject	Inject

Table 2. HPLC method for the analysis of amino acids on the Agilent 1290 Infinity II bio LC system following the method outline in application note 5994-5950EN (Gradient 1) and a faster method (Gradient 2).

Parameter	Value	
Column	Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (p/n 695975-322) Agilent AdvanceBio AAA guard column, 3.0 × 5 mm, 2.7 µm (p/n 823750-946)	
Solvent	Mobile phase A: 10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$, pH 8.2 Mobile phase B: Acetonitrile, methanol, and water (45:45:10, v:v:v)	
Gradient	Gradient 1 Time (min) %B 0.0 2 0.4 2 13.6 57 14.0 100 Stop time: 17 min Post time: 4 min	Gradient 2 Time (min) %B 0.0 2 0.3 2 7.5 57 8.0 100 Stop time: 11 min Post time: 3 min
Flow Rate	0.6 mL/min	1.2 mL/min
Operating Pressure Range	160 to 290 bar	300 to 520 bar
Temperature	40 °C	
Detection (DAD)	338 nm, 10 nm Bandwidth, and reference wavelength 390 nm, 20 nm bandwidth	
Detection (FLD)	Excitation: 345 nm; emission: 455 nm 12.0 min: Change excitation: 265 nm; change emission: 315 nm PMT gain: 10 Peak width: > 0.025 min (18.52 Hz)	Excitation: 345 nm; emission: 455 nm 6.5 min: Change excitation: 265 nm; change emission: 315 nm PMT gain: 10 Peak width: > 0.025 min (18.52 Hz)
Injection	1 µL, Use vial/well bottom sensing Draw speed: 100 µL/min; ejection speed: 400 µL/min	
Needle Wash	Flush port, 5 seconds. Acetonitrile: 0.1 M HCl (50:50; v:v).	

Results and discussion

In this study, HeLa and HepG2 cells were chosen as their characteristics are well-documented, which made them excellent choices for experiments. HeLa cells are known for their high proliferation rate and are thus suitable for the study of rapid cell growth or metabolic activity. As for HepG2 cells, they also show good proliferation traits which are beneficial for the examination of amino acid dynamics during active cell division.

The choice of time points in a cell culture experiment is critical. The selected time points (0, 16, 24, 48, and 72 hours) correspond to key phases in the growth and proliferation of HeLa and HepG2 cells. Time 0 hour (or the initial time point) serves as a baseline before cells proliferate significantly.

Subsequent time points capture the various stages of cell growth which includes logarithmic growth (16 to 24 hours), late logarithmic growth (48 hours), and potentially reaching a growth plateau (72 hours). Cells undergo dynamic metabolic changes during the various growth phases, which can influence their nutrient requirements.

The HeLa and HepG2 cell growth at 24 and 72 hours are shown in Figure 1. The cells exhibited robust growth at 24 hours and reached near-confluence after 72 hours, adhering well to the flasks in spherical shapes. This morphology suggests that the cells remained healthy and viable when the spent media was collected from the cell culture.

Reversed-phase LC with DAD and FLD detection was employed for the separation of the 20 amino acids standard mixture together with two internal standards (Figure 2A). All amino acids showed better sensitivity in FLD than in DAD, except for cystine. The DAD chromatogram and FLD chromatogram of cystine are shown in Figures 2B and 2C. The lower sensitivity in FLD for cystine is due to the low fluorescence of the adducts, which are formed with the OPA reagent.[8] Hence, for cystine, DAD data was used for reporting result, whereas FLD data was used for the rest of the amino acids.

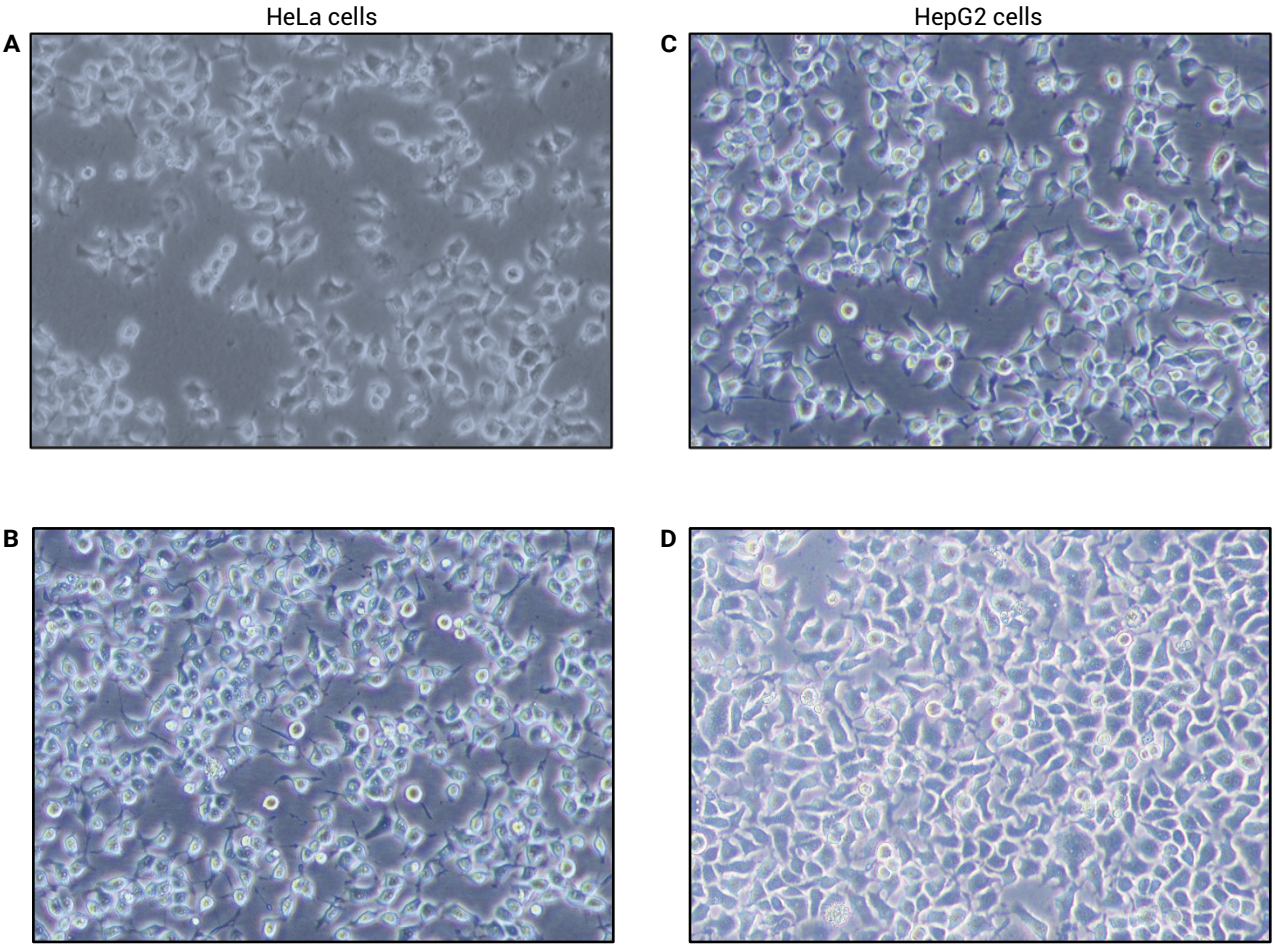

Figure 1. (A) HeLa cells after 24 hours of growth in DMEM. (B) HeLa cells after 72 hours of growth in DMEM are near confluence. (C) HepG2 cells after 24 hours of growth in DMEM. (D) HepG2 cells after 72 hours of growth in DMEM are near confluence.

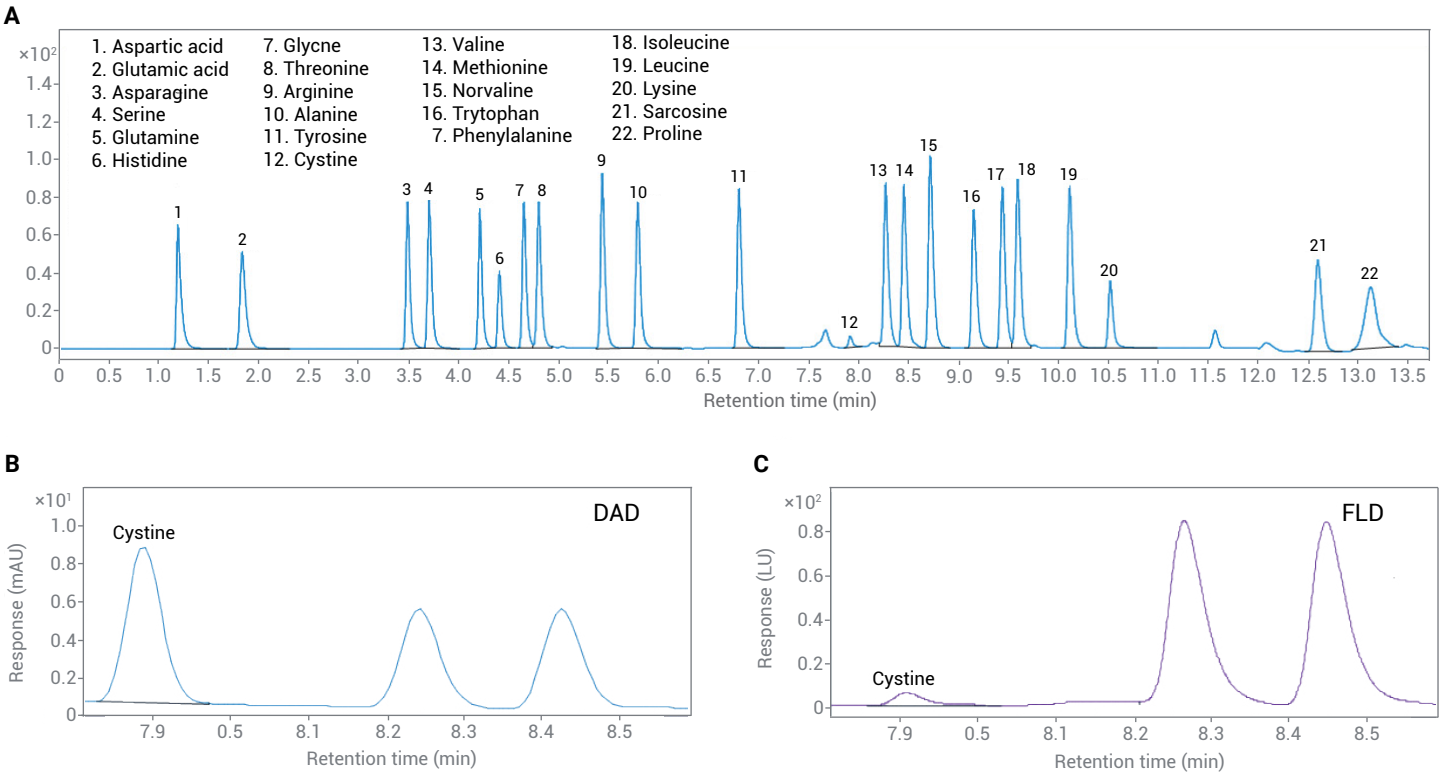

Figure 2. (A) FLD chromatogram of amino acid calibration standard solution at 45 pmol/µL. (B) DAD and (C) FLD chromatograms of cystine.

The collected spent media at the various time points of growth was analyzed on the 1290 Infinity II bio LC system. The overlay chromatogram of amino acids in DMEM spent media of HepG2 after 16, 24, 48, and 72 hours of growth is shown in Figure 3. The magnified region of the chromatogram where the amino acids change most significantly during cell culture growth is shown in Figure 4.

Close inspection of the LC data confirmed that with cell growth, amino acids such as serine, glutamine, arginine, and tyrosine show declining peak response, while amino acids such as glycine and alanine show an increasing peak response (Figure 4). Glutamine often depletes rapidly in cell culture studies.[9] This is because it produces the carbon frame for the synthesis of nonessential amino acids and tricarboxylic acid (TCA) cycle carbon.[10]

Serine is another amino acid that is quickly consumed in cell culture. Serine hydroxy methyltransferases catalyze serine to form glycine. Glycine is important for the biosynthesis of nucleotides.[11] The abundance of tyrosine and arginine in cell culture was also reported to be related to cell viability.[12] Using the prepared calibration standards (see Preparation of amino acid standard solutions), these changes can also be expressed quantitatively, as demonstrated in Figure 5. For simplicity, only a few examples are shown in Figure 5, which include some of the amino acid changes observed or mentioned in Figure 4.

The method used for the amino acid analysis in cell culture was based on the guidance described in the Amino Acid Analysis "How-To" Guide (Agilent puplication number 5991-7694EN). To showcase the ability of the 1290 Infinity II bio LC system as a UHPLC instrument, the separation of amino acids using a higher flow rate method, as mentioned in Table 2, was demonstrated. The method was within the maximum operating pressure of the column. Figure 6 shows the separation of amino acids within eight minutes, with an 11-minute runtime gradient. The separation of the amino acids at the higher flow rate shows similar performance when compared with the earlier method that was used for amino acid analysis. This method is beneficial for faster analyses of amino acids in cell culture.

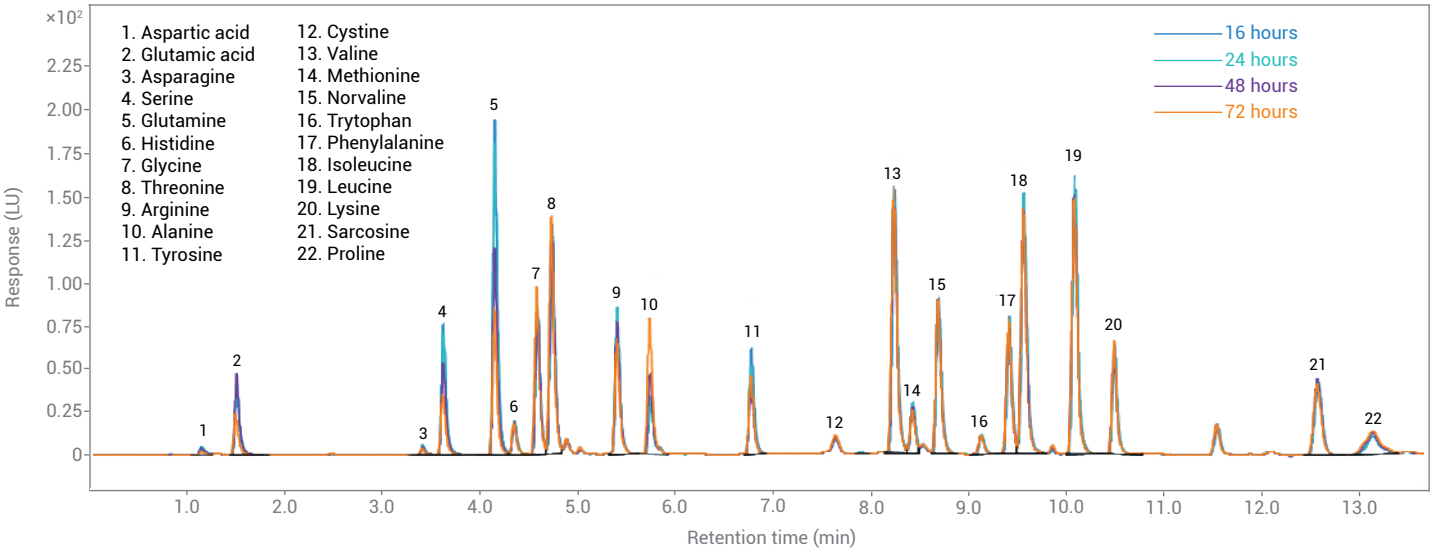

Figure 3. Analysis of amino acids in DMEM spent media of HepG2 after 16, 24, 48, and 72 hours of culture.

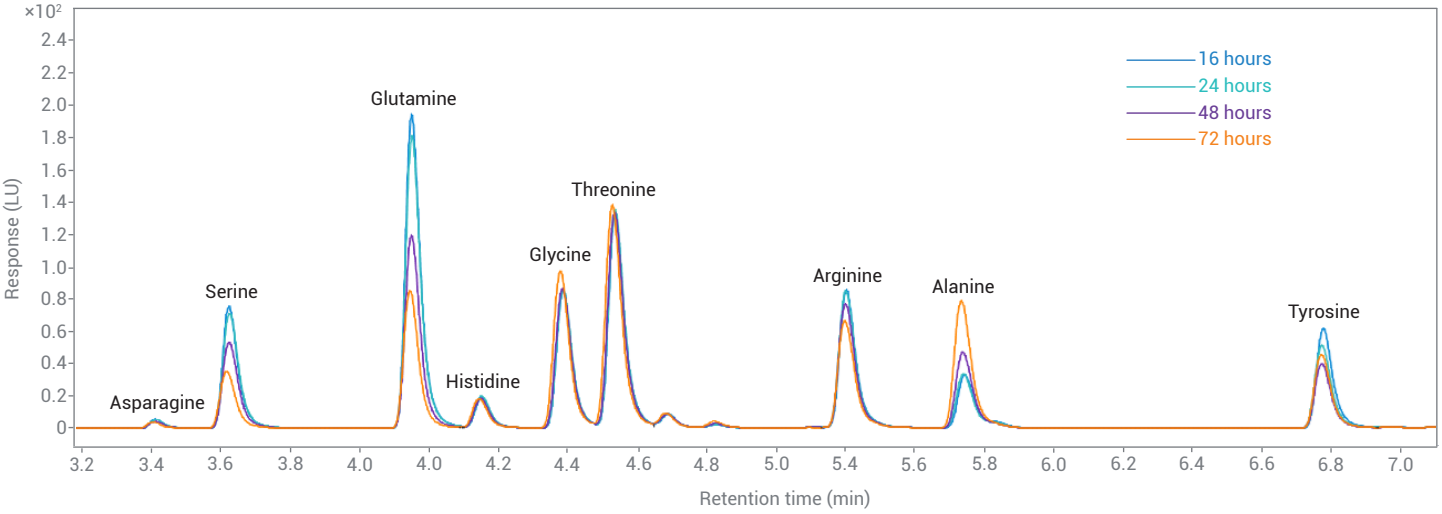

Figure 4. Analysis of critical amino acids in DMEM spent media of HepG2 after 16, 24, 48, and 72 hours of culture.

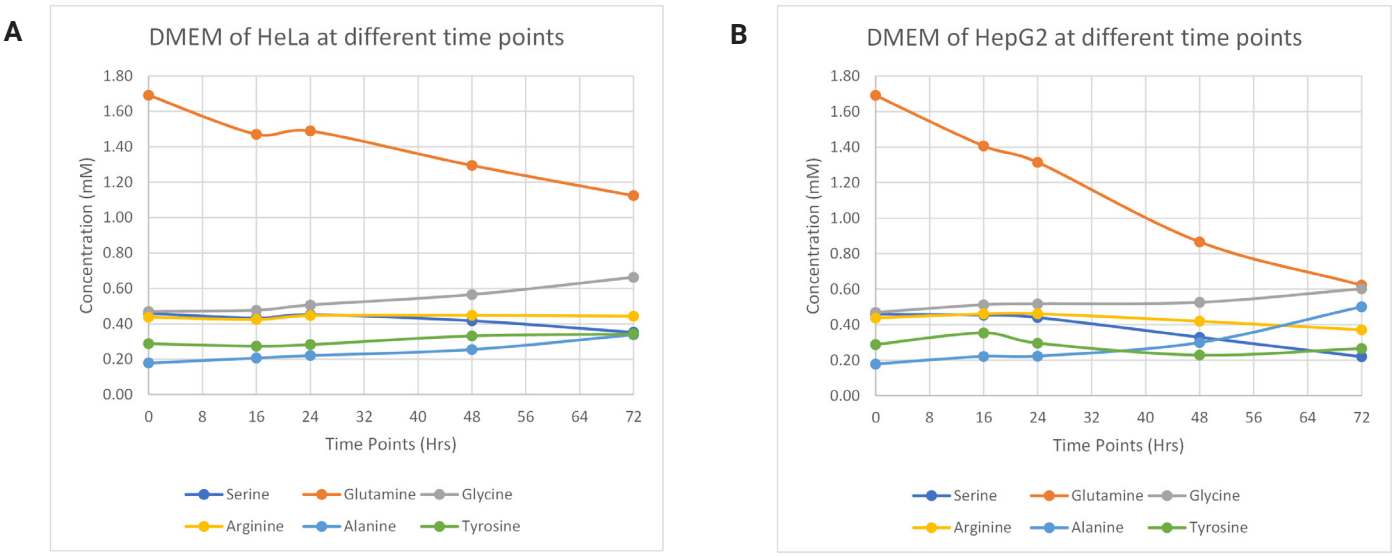

Figure 5. Quantitative trends in amino acids during cell culture of HeLa (A) and HepG2 (B) cells in DMEM.

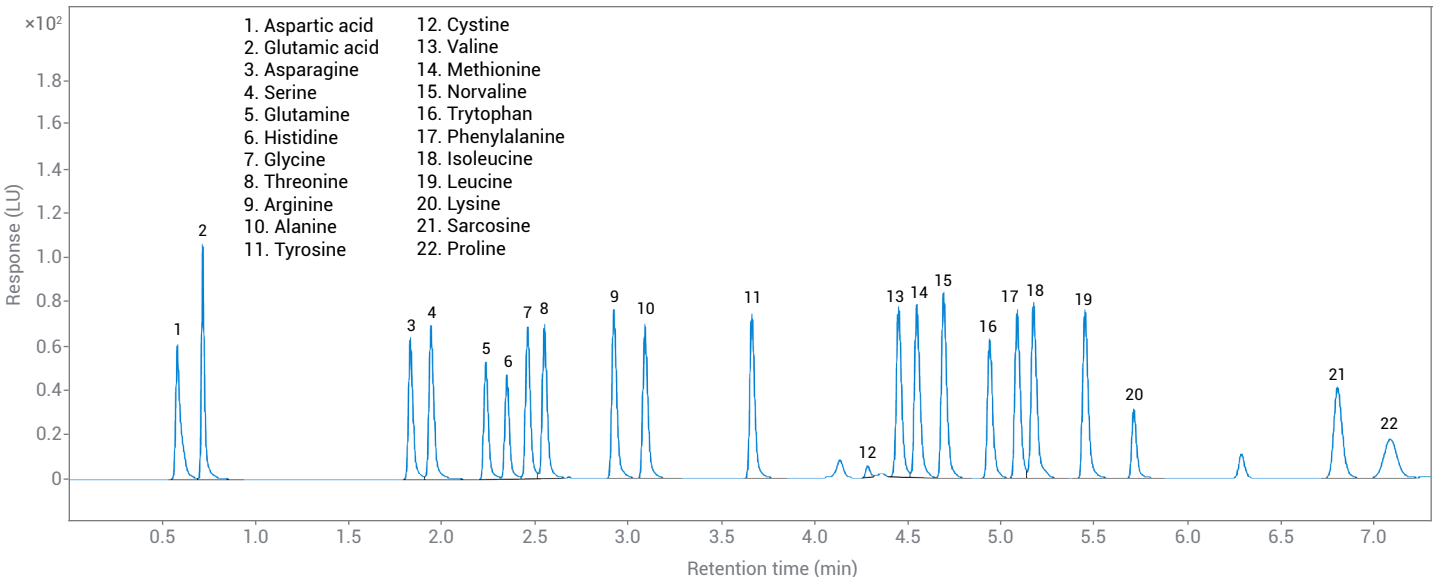

Figure 6. FLD chromatogram of amino acid calibration standard solution at 45 pmol/µL showing a shorter and faster separation of the amino acids.

Conclusion

This application note demonstrates effective amino acid profiling of spent media using the Agilent 1290 Infinity II bio LC system. No sample preparation other than simple dilution is needed for sample analysis. The automated precolumn derivatization eliminates the typical performance risks associated with manual liquid handling steps and reduces sources of operator error. Analytical results verify method applicability for precise monitoring of amino acids in DMEM media across multiple days for HeLa and HepG2 cell cultures, and the data is in excellent alignment with literature evidence. The effective use of the 1290 Infinity II bio LC system for speed-optimized amino acid analysis, without compromising chromatographic performance, is also demonstrated. This approach can be used for quality monitoring in media engineering and for exploring the optimization of cell culture conditions.

References

1. Yao, T.; Asayama, Y. Animal-Cell Culture Media: History, Characteristics, and Current Issues. RMB **2017**, 16(2), 99–117.
2. Segeritz, C. P.; Vallier, L. Cell Culture: Growing Cells as Model Systems In Vitro. *Basic Science Methods for Clinical Researchers*. Academic Press, **2017**; pp. 151–172.
3. Salazar, A.; Keusgen, M.; Von Hagen, J. Amino Acids in the Cultivation of Mammalian Cells. *J. Amino Acids*. **2016**, 48, 1161–1171.
4. Wu, G. Functional Amino Acids in Growth, Reproduction, and Health. *AN/Adv. Nutr*. **2010**, 1(1), 31–37.
5. Zhou, T.; Reji, R.; Kairon, R. S.; Chiam, K. H. A Review of Algorithmic Approaches for Cell Culture Media Optimization. *Front. Bioeng. Biotechnol*. **2023**, 11, 1195294.
6. Online Amino Acid Analysis for Spent Media Control. *Agilent Technologies application note*, publication number 5994-4931EN, **2022**.
7. Comparison of Plant-Based Meat Alternatives and Meat. *Agilent Technologies application note*, publication number 5994-5950EN, **2023**.
8. Lee, K.S.; Drescher, D.G. Derivatization of Cysteine and Cystine for Fluorescence Amino Acid Analysis With the o-Phthaldialdehyde/2-mercaptoethanol Reagent. *JBC*. **1979**, 254(14), 6248-6251.
9. Hosios, A. M.; Hecht, V. C., Danai, L. V.; Johnson, M. O.; Rathmell, J. C.; Steinhauser, M. L.; Manalis, S. R.; Vander Heiden, M. G. Amino Acids Rather Than Glucose Account for the Majority of Cell Mass in Proliferating Mammalian Cells. *Dev. Cell*. **2016**, 36(5), 540–549.
10. DeBerardinis, R. J.; Mancuso, A.; Daikhin, E.; Nissim, I.; Yudkoff, M.; Wehrli, S.; Thompson, C. Beyond Aerobic Glycolysis: Transformed Cells Can Engage in Glutamine Metabolism That Exceeds the Requirement for Protein and Nucleotide Synthesis. *PNAS*. **2007**, 104(49), 19345–19350.
11. Lukey, M. J.; Katt, W. P.; Cerione, R. A. Targeting Amino Acid Metabolism for Cancer Therapy. *Drug Discov. Today*. **2017**, 22(5), 796–804.
12. da Silva, M.R.; Zaborowska, I.; Carillo, S.; Bones, J. A Rapid, Simple and Sensitive Microfluidic Chip Electrophoresis Mass Spectrometry Method for Monitoring Amino Acids in Cell Culture Media. *J. Chromatogr. A*. **2021**, 1651, 462336.

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