Application Note

Pharma & Biopharma

Online Amino Acid Analysis for Spend Media Control

Long-time reaction control by the Agilent InfinityLab Online LC Solution

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Abstract

This application note shows the capability of the Agilent InfinityLab Online LC Solution to measure the amino acid concentration in cell growth media over the course of a production process. This process typically takes days and samples can be drawn in intervals of hours. The Agilent InfinityLab Online LC Solution uses different analytical methods and sample preparation methods for sample measurements, blanks, and quality controls (QCs). The InfinityLab Online LC Solution can be kept in standby mode between the measurements. The complete experiment is orchestrated by the Agilent Online LC Monitoring Software. The data, acquired over several days, can be analyzed within the Online LC Monitoring Software nearly in real time.

Introduction

In the modern biopharmaceutical industry, active pharmaceutical ingredients (APIs) such as monoclonal antibodies (mAbs) are produced from genetically modified living cells kept in nutrition media. These media contain amino acids, vitamins, minerals, and sugars. Typically, an essential amino acid such as glutamine is present in large excess as a source of external nitrogen for the living cells. The content of this essential amino acid must be monitored quantitatively. The monitoring must occur over the time needed to grow the cells for production of the desired biological compounds. If the essential amino acid is consumed by the cells in the bioreactor, more amino acid must be added to maintain a level necessary to keep the cells alive. The concentration of the amino acid can be determined by their derivatization, making them measurable by UV or fluorescent detection after a chromatographic separation.

This application note demonstrates the analysis of the essential amino acid glutamine added to a Dulbecco's Modified Eagle Medium (DMEM) formulation. This analysis was done by a precolumn derivatization and quantitative UV detection. To simulate a biological experiment, samples were drawn and measured every 5 hours over 2 days. Sample preparation in the Agilent 1260 Infinity II Online Sample Manager enables fully automated sample handling in a time and cost saving manner. The Online LC Monitoring Software controls the complete experiment in an unattended, safe, and economic method.

Experimental

Instrument

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1260 Infinity II Online Sample Manager Set (G3167AA): Agilent 1260 Infinity II Online Sample Manager (G3167A) clustered with external valve (part number 5067-6680) located at the Agilent 1290 Infinity Valve Drive (G1170A) and Agilent Online LC Monitoring Software
- Thermostat for 1260 Infinity II Online Sample Manager (G7167-60005)
- Agilent 1260 Infinity High Performance Degasser (G4225A)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Agilent InfinityLab Max-Light Cartridge Cell (10 mm, G4212-60008)
- Agilent 1260 Infinity II Isocratic Pump (G7110B)

Software

- Agilent OpenLab CDS, version 2.6 or later
- Agilent Online LC Monitoring Software, version 1.0

Columns

- Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322)
- Agilent AdvanceBio AAA guard columns, 3.0 × 5 mm, 2.7 µm, 3/pk (part number 823750-946)

Sample preparation (Injector program)

1. Draw 2.5 µL from location 1 (borate buffer) with the default speed using the default offset.
2. Wash the needle as defined in the method.
3. Draw 1.00 µL from the sample with 100 µL/min draw speed and offset 0.0 mm.
4. Wash the needle as defined in the method.

Analytical method*

Parameter	Value
Solvents	A) See "Solvents and chemicals" B) See "Solvents and chemicals"
Analytical Flow Rate	0.6 mL/min
Gradient	Time (min) %B 0 2 0.35 2 0.35 to 13.4 2 to 57 13.4 to 13.5 100 Stop time: 15.0 min Post time: 5.0 min
Column Temperature	40 °C
Flowthrough Injection	Draw speed: 100 µL/min Eject speed: 400 µL/min Wait time after draw: 1.2 s
Sample Volume	1 µL
Needle Wash	3 s, water (S1)
Sample Temperature	10 °C
Sampling	see sampling methods for sampling to vial
Diode Array Detector	A) 238 ±10 nm, Ref.: 390 ±20 B) 262 ±16 nm, Ref.: 324 ±20 nm, data rate 20 Hz

* This method is used for blanks and with the sample preparation method for the determination of amino acids in media samples and QCs. The solvents S1 and S2 were degassed by an extra degasser.

5. Mix 3.5 µL from air with the default speed five times.
6. Wait 0.2 minutes.
7. Draw 0.50 µL from location 2 (OPA reagent) with the default speed using the default offset.
8. Wash the needle as defined in the method.
9. Mix 4.00 µL from air with the default speed 10 times.
10. Draw 0.40 µL from location 3 (FMOC reagent) with the default speed using the default offset.*
11. Wash the needle as defined in the method.
12. Mix 4.40 µL from air with the default speed 10 times.
13. Draw 32.00 µL from location 4 (injection diluent) with the maximum speed using the default offset.
14. Mix 20.00 µL from air with the maximum speed 8 times.
15. Inject.

* If the medium does not contain secondary amino acids, such as proline, steps 10 to 12 can be skipped.

The sleep method was applied after the measurement of the QC sample and the wake-up method started 10 minutes before the measurement of the blank sample. After the complete measurement of 10 blank, sample, and QC combinations, the stop method was applied.

Sample delivery pump

Pump: Agilent 1260 Infinity II Isocratic Pump (G7110B)

Flow rate: 0.3 mL/min

Sampling to vial

Parameter	Value
Sampling	Sampling from reactor to 2 mL vials with preslit caps
Target Volume	700 µL
Dilution Factor	20
Dilution Speed	10.000 µL/min (ejection of dilution solvent, S2)
Dilution Solvent	Water (S2)
Draw Speed	Setting 3 (draw speed: 70 µL/min, wait time: 4.8 s, dispense speed: 90 µL/min (ejection of sample into well before dilution)

Schedule

Parameter	Value
Blank	Start time: 00d:00h:00m, interval: 5 hrs, count: 10
Samples	Start time: 00d:00h:30m, interval: 5 hrs, count: 10
QCs	Start time: 00d:01h:00m, interval: 5 hrs, count: 10
Sleep Method	Flow rate: 0.1 mL/min Solvent: 100% B Gradient: Time (min) %B Flow (mL/min) 0.0 2 0.1 0.1 2 0.6 0.2 100 0.6 4.9 100 0.6 5.0 100 0.1
Wake-Up Method	Flow rate: 0.6 mL/min Solvent: 2% B Stop time: 5 min
Stop Method	Flow rate: 0.0 mL/min Solvent: 100% B Gradient: Time (min) %B Flow (mL/min) 0.0 2 0.0 0.1 2 0.6 0.2 100 0.6 4.9 100 0.6 5.0 100 0.0 Column temperature: not controlled

The pump was controlled by the OpenLab CDS and switched on before the sampling. This flow flushes out the delay volume of the pump and the tubing to the sampling interface of the 1260 Infinity II Online Sample Manager. After injecting the sample, the sampling pump was switched off. To maintain stable operating conditions of the pump, a restriction capillary (part number 5022-2159) was used. The restriction capillary was placed after the sampling interface valve of the 1260 Infinity II Online Sample Manger before going to waste.

Reagents

- **Borate buffer:**
  0.4 M in water, pH 10.2, 100 mL (part number 50613339)
- **FMOC reagent:**
  2.5 mg/mL in ACN, 10 × 1 mL ampoules (part number 50613337)
- **OPA reagent:**
  10 mg/mL in 0.4 M borate buffer and 3-mercaptopropionic acid, 6 × 1 mL ampoules (part number 5061-3335)
- **Injection diluent:**
  100 mL mobile phase A + 400 µL orthophosphoric acid (85%)

Standards and calibrants

- Amino acid supplement (part number 50622478)
- AA standard, 1 nmol/µL (part number 50613330)
- AA standard, 250 pmol/µL (part number 5061-3331)
- AA standard, 100 pmol/µL (part number 5061-3332)
- To make the extended amino acid (EAA) stock solution, weigh 59.45 mg of asparagine, 59.0 mg of hydroxyproline, 65.77 mg of glutamine, and 91.95 mg of tryptophan and add all amino acids to a 25 mL volumetric flask; dissolve with 0.1 N HCl (18 nmol/µL each amino acid).
- For primary amino acid ISTD stock solutions, weigh 58.58 mg of norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg of sarcosine into the same 50 mL flask. Fill with 0.1 N HCl for final concentration of 10 nmol for each amino acid/µL
- For the preparation of the calibration solutions, the EAA stock solution was used undiluted and diluted 1:4 and 1:10. These dilutions are mixed 1:1 with the ISTD stock solution used. This solution was mixed 1:10 with the AA standards to get final concentrations of 900, 225, and 90 pmol/µL of each amino acid and 500 pmol/µL ISTD

Solvents and chemicals

- **Mobile phase A:** 2.8 g of sodium phosphate dibasic (Na_2HPO_4) and 7.6 g of disodium tetraborate decahydrate ($Na_2B_4O_7$•10 H_2O), added to 2.0 L of water. The pH was adjusted to 8.2 by adding fuming hydrochloric acid (37%)
- **Mobile phase B:** acetonitrile/methanol/water 45/45/10 (v/v/v)
- Dulbecco's Modified Eagle Medium (DMEM), 0.1% NaN_3 was added to prevent undesired growth of algae and bacteria
- **Chemicals:** Sodium phosphate dibasic (Na_2HPO_4), disodium tetraborate decahydrate ($Na_2B_4O_7$•10 H_2O), sodium azide (NaN_3), H_3PO_4 85%, HCl concentrated 37%, glutamine, acetonitrile, and methanol
- All solvents were purchased from Merck, Germany
- Chemicals were purchased from VWR, Germany
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point of use cartridge (Millipak)

Additional material

- Agilent amber wide opening vial (part number 5182-0716)
- Agilent preslit screw caps (part number 5185-5824)

Results and discussion

For the quantitative measurement of the amino acids in the growth medium, a calibration was done before the experiment. For this calibration, amino acid solutions with 900, 225, and 90 pmol/µL were used. The calibration solutions contained 19 primary and two secondary amino acids. The solution also contained two quality control standards with 500 pmol/µL (primary amino acid norvaline and secondary amino acid sarcosine). The derivatization was done with OPA for the primary and Fmoc for the secondary amino acids (Figure 1). Typical performance values, such as retention time and area precision, can be found in the Amino Acid Analysis guide.[1]

For the following Online LC experiment, Dulbecco's Modified Eagle Medium (DMEM) was used. This growth medium contains a selection of primary amino acids and, in addition, buffer salts, vitamins, and sugars. This medium does not contain all amino acids necessary for the growth of cells (Figure 2). Therefore, an amino acid such as glutamine is typically added in excess as an energy and nitrogen source. The consumption of this amino acid can be monitored

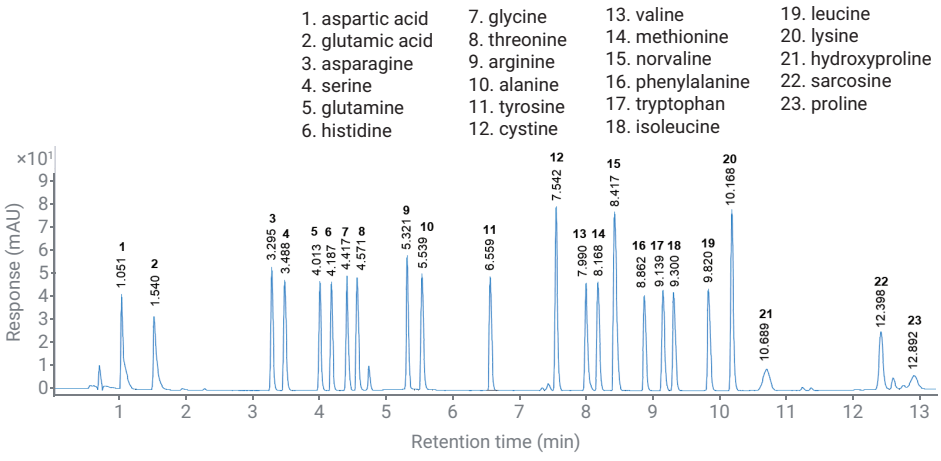

Figure 1. Amino acid calibration, 225 pmol/µL (µmol/L), including secondary amino acid proline and hydroxy proline and quality controls norvaline and sarcosine (500 pmol/µL).

by online LC during the experiment to ensure that the amino acid is maintained in predefined upper and lower limits.

The online LC experiment was run for 2 days, and a medium sample was drawn every 5 hours for analysis. To minimize solvent consumption, the online LC was switched automatically to a sleep mode method that flushed the column with solvent B and maintained a flow rate of 0.1 mL/min. A wake up method was used to ramp up the flow again and equilibrate the column with starting conditions automatically before the next analytical cycle. During the cycle, the amino acid method was accompanied by a blank run before, and a quality control after, the amino acid analysis of the medium. The DMEM medium was enriched with 4.5 mM glutamine as a potential source of nitrogen, which was monitored during the experiment (Figure 3).

To simulate a typical biological experiment, the sample was pumped out of the reactor starting before the sample was drawn and the reactor was switched off after the sampling. An extra filtration step as well as aseptic sampling must be implemented to prevent cells from being flushed into the LC instrument. The obtained results can be analyzed and displayed during the running experiment by the Online LC Monitoring Software. This enables dosing of additional glutamine by the user, maintaining a defined concentration window in the growth medium.

To demonstrate the performance of the method, the measured concentrations for the added amino acid glutamine were monitored continuously during the experiment and were displayed in a trending plot (Figure 4A). The measured average glutamine concentration was 4.635 mM/L with a relative standard deviation (RSD) of 2.3%. The glutamine concentrations were automatically corrected for the applied 1:20 dilution.

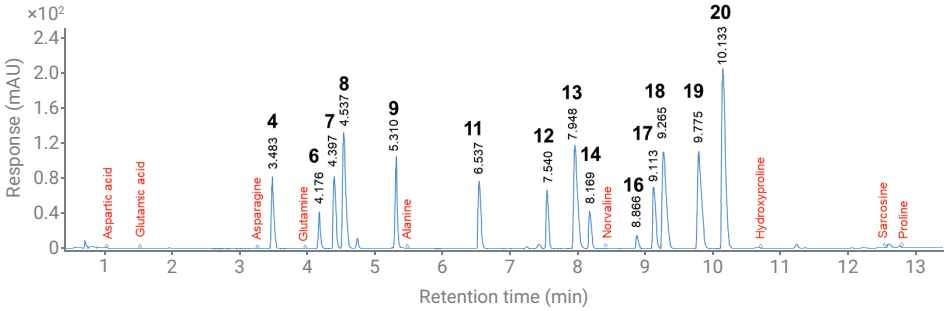

Figure 2. Amino acids analysis in Dulbecco's Modified Eagle Medium (DMEM) showing primary amino acids according to the listed compounds (Figure 1).

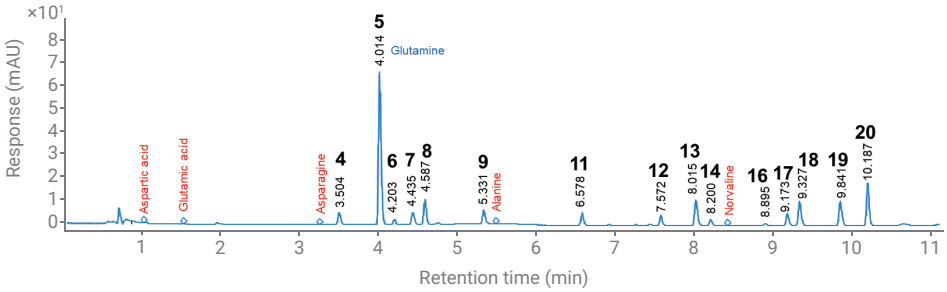

Figure 3. DMEM enriched with 4.5 mM glutamine drawn from the bioreactor followed by a 1/20 dilution.

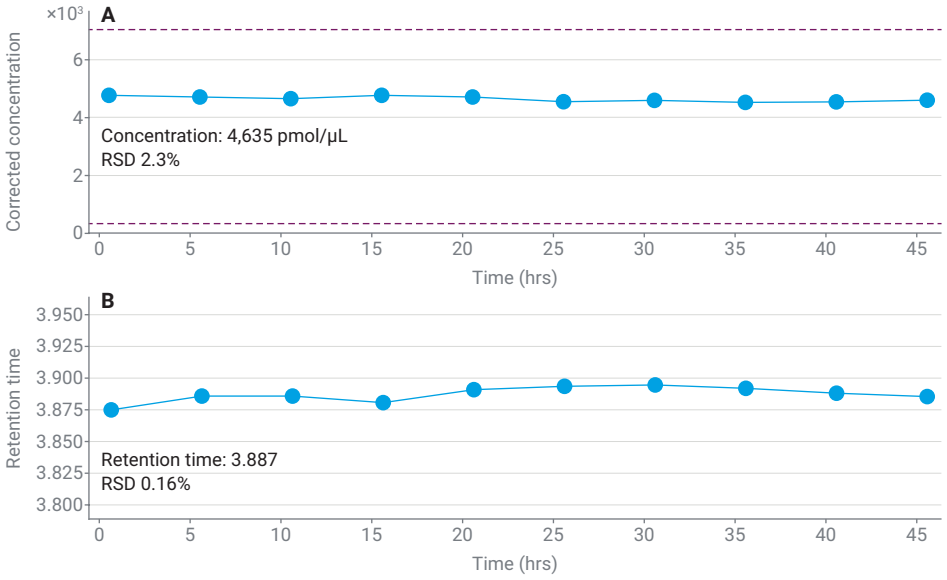

Figure 4. (A) Glutamine concentrations measured during the experiment, in average 4,635 pmol/µL. The chosen concentration limits of 7 mM/L and 0.25 mM/L, which will trigger a notification to the user are displayed as dashed lines. (B) A trending plot of the retention times of glutamine in all samples, average retention time 3.887 minutes with an RSD of 0.16%. (To prevent consumption of glutamine due to undesired growth of algae and bacteria in the medium NaN_3 was added. And to prevent glutamine degradation, the experiment was done at room temperature.)

As with the concentration, the retention time of the amino acid glutamine can be displayed in a trending plot (Figure 4B). The average retention time of glutamine was 3.887 minutes with an RSD of 0.16%.

Since all amino acids in the mixture were initially calibrated, each can be monitored individually as described for glutamine. This approach of switching between analytical methods and LC sleep phase enables unattended runs over several days with high performance and reduced solvent consumption.

To control the applied sample preparation and chromatographic separation method, a quality control sample was measured after each sample measurement. As this quality control, a 500 pmol/µL solution of the primary amino acid norvaline was used (Figure 5). The concentration measure in average was 510 pmol/µL with an RSD of 0.56%. The average retention time was 8.433 minutes with an RSD of 0.06%.

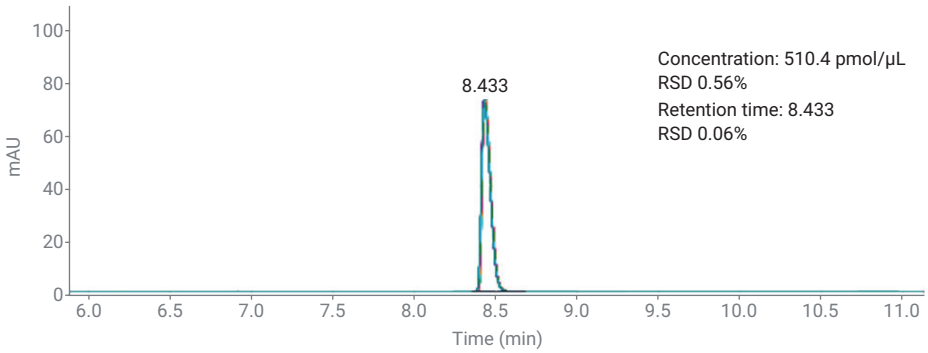

Figure 5. Overlay of 10 measured quality control samples. The RSDs of concentration and retention time were 0.56 and 0.06%, respectively.

Conclusion

This application note demonstrates the use of the Agilent InfinityLab Online LC Solution for the determination of amino acids in spend media. As an example, dosed glutamine was determined quantitatively with high performance in stabilized Dulbecco's Modified Eagle Medium (DMEM). To simulate a real application, the experiment was run for two days including sleep mode and wake-up phases. The experiment used blank samples, quality control samples, and a dilution step for the drawn media sample. The amino acids were derivatized in a fully automated manner using the Agilent 1260 Infinity II Online Sample Manager robotics as part of a sample preparation method.

References

1. Amino Acid Analysis "How-To" Guide, *Agilent Technologies*, publication number 5991-7694EN, **2021**.

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