Application Note

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Automated Bioprocess Monitoring with Agilent 1290 Infinity III Bio Online LC Solutions

Process analytics of L-alanine production using genetically modified *Vibrio natriegens*

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Abstract

This application note illustrates the implementation of an Agilent 1290 Infinity III Bio Online LC solution in combination with Flownamics Seg-Flow S3 and Sample-Mod S3 for the purpose of sampling from a bioreactor. This configuration facilitates automatic sampling and sample preparation from a bioreactor that can be left unattended, thereby enabling continuous monitoring of bioprocesses. This was demonstrated through the analysis and online monitoring of a bioprocess that used genetically modified *Vibrio natriegens* as the production host to produce L-alanine.

Introduction

Bioprocess engineering is a challenging field because of the constantly changing nature of bioprocess models which make them difficult to validate.[1] Consequently, process control needs to be developed at various levels using strategies tailored to these specific requirements.[1] The performance and effectiveness of the bioreactor are determined by key performance indicators such as product yield, titer, production rate, and quality of the product.[1] These factors can be quickly influenced by changes in process variables and the raw materials used.[1,2] The ability to react quickly to problems is therefore crucial for preserving ongoing production, a factor that can be particularly significant in bioprocesses due to the substantial cost and time investmented.[1,2] Such challenges can be tackled by constantly monitoring the bioprocess, which requires the ability to analyze and collect data quickly. Therefore, monitoring is of the utmost importance.

This application note describes the advantages of using an Agilent 1290 Infinity III Bio Online LC solution with Agilent Online LC Monitoring software. A bioreactor was interfaced with a Flownamics Seg-Flow S_3 sampling system for aseptic withdrawal and an Agilent 1290 Infinity III Bio Online LC to make an hourly analysis.

This is a fully automated approach for taking sterile samples from a bioreactor and preparing or diluting them. In addition, the 1290 Infinity III Bio Online LC solution displays data in near real time, which is a crucial element for effective bioprocess monitoring.

Experimental

Equipment

The 1290 Infinity III Bio Online LC comprised the following modules:

- Agilent 1290 Infinity III Bio Flexible Pump (G7131A) with an InfinityLab Quick Change Bio inline filter assembly (part number 5067-1607), 2.1 mm id, 0.2 µm pore size
- Agilent 1290 Infinity III Bio Online Sample Manager (G3167B) with Sample Thermostat (option #101) clustered with Agilent 1290 Infinity Valve Drive (G1170A), featuring a reactor valve pod (part number 5067-6680) and Online LC Monitoring Software
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) equipped with an Agilent Quick Connect Bio Heat Exchanger Std. (G7116-60071)
- Agilent 1290 Infinity III Diode Array Detector (DAD) (G7117B), equipped with a Bio Max-Light Cartridge Cell (G4212-6008), 10 mm, 1 µL
- Agilent 1260 Infinity High-Performance Degasser (G4225A) is connected between S1 (transport solvent) solvent line and Agilent 1290 Infinity III Bio Online Sample Manager (G3167B)

Software

- Agilent OpenLab CDS version 2.8 or later versions
- Agilent Online LC Monitoring Software version 1.3 or later versions
- Flownamics FlowWeb control software

Instrumental configuration

The 1290 Infinity III Bio Online LC solution was used with a Seg-Flow interface for automatic sampling and online analysis (see Figure 1). The Seg-Flow interface was used to withdraw samples from a bioreactor through a Flownamics F-Series 310 FISP probe with a ceramic membrane filter (0.2 µm pore size) from a 5 L bioreactor. The drawn samples were sent through the built-in sample collection cup on the Seg-Flow to the LC and the samples were retained into vials. The collected samples were then automatically derivatized by using an injector program and subsequently analyzed. A more detailed description of the instrumental configuration has been published.[3]

Columns

Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322)

Chemicals, solvents, and samples

Agilent InfinityLab gradient grade acetonitrile for LC (part number 5191-5100*) and Agilent InfinityLab gradient grade methanol for LC (part number 5191-5110*) were used for all experiments. Fresh ultrapure water was obtained from a Milli-Q integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate dibasic (Na_2HPO_4) and disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (HCl) (37%) and orthophosphoric acid (85%) were obtained from Merck (Darmstadt, Germany). The genetically modified strain Vibrio natriegens Δdldh Δlldh Δpfl Δmdh[2] was provided by Bastian Blombach at the Technical University of Munich.

Culture media and reagents for *V. natriegens* cultivation were obtained from Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), VWR Chemicals (VWR, Darmstadt, Germany), and Gibco (Thermo Fischer Scientific, Grand Island, New York, USA).

* Only available in select countries.

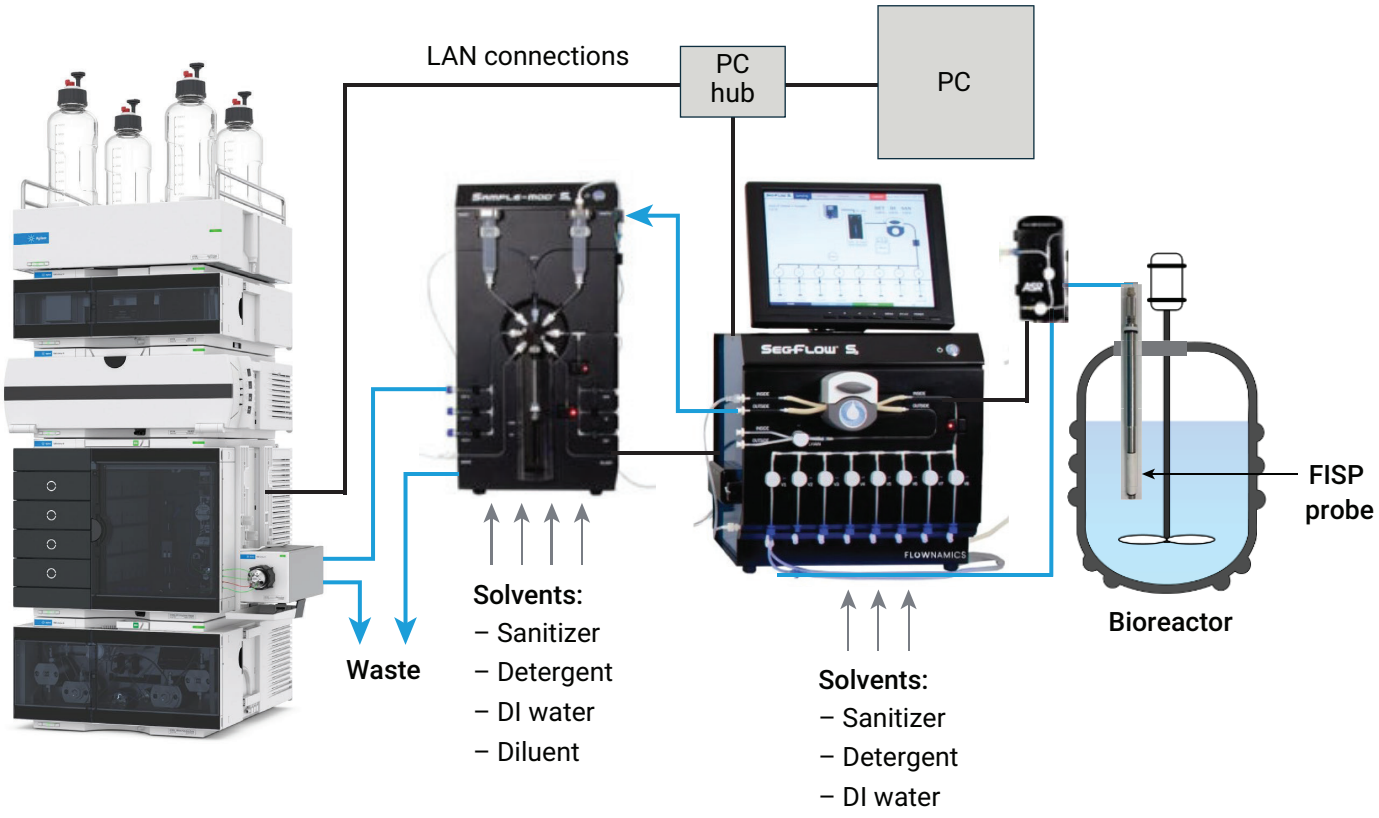

Figure 1. Agilent 1290 Infinity III Bio Online LC combined with the Flownamics Seg-Flow S3 and Sample-Mod S3 (black: electrical connections, blue: flow connections).

Standards and derivatization reagents were obtained from Agilent, including:

- Amino acids supplement kit (part number 50622478) containing: L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline, and sarcosine (1 g each)
- Amino acid standard, 100 pmol/µL (part number 5061-3332)
- Borate buffer, 0.4 N in water, pH 10.2, 100 mL (part number 50613339)
- FMOC reagent, 2.5 mg/mL 9-fluorenylmethylchloroformate in acetonitrile, 10 × 1 mL (part number 50613337)
- OPA reagent, 10 mg/mL each of o-phthalaldehyde and 3-mercaptopropionic acid in 0.4 M borate buffer, 6 × 1 mL (part number 5061-3335)

Media for cultivation

Brain heart infusion (BHIN) agar plates: The BHIN agar was prepared by dissolving 37 g of BHIN, 15 g of NaCl, and 15 g of agar in one liter of water. The medium was then sterilized in an autoclave at 121 °C and 1 bar pressure for 20 minutes.

The agar plates were prepared by subjecting the BHIN agar to microwave until it was liquid again. The agar was then transferred into Petri dishes under sterile conditions and allowed to cool.

Yeast-trypton (2x YTN) medium: The 2x YTN medium was prepared by first dissolving 16 g of tryptone, 10 g of yeast extract, and 15 g of NaCl in 1 liter of water. The solution was then sterilized in an autoclave.

Prior to use, $MgCl_2$ was added by filtration with a sterile filter with a pore size of 0.2 µm. The final concentration of $MgCl_2$ was 50 mM.

***Vibrio natriegens* (VN) medium:** To prepare 4 L of VN medium[2] (5 L bioreactor), the following reagents were dissolved in water: 20 g $(NH_4)_2SO_4$, 60 g NaCl, 4 g K_2HPO_4, and 4 g KH_2PO_4. The pH was adjusted to 7.5 using 5 N KOH. The medium was sterilized within the bioreactor via autoclaving.

After the autoclaving process, the following substances were introduced into the bioreactor by filtration through a sterile filter with a pore size of 0.2 µm: 144 g of glucose, 80 g of $NaHCO_3$, 1 g of $MgSO_4$, and 0.04 g of $CaCl_2$, dissolved in 200 mL of water. Then, the following reagents, dissolved in 4 mL of 0.1 M HCl, were added:

- 65.6 mg of $FeSO_4 \cdot 7\ H_2O$
- 40 mg of $MnSO_4 \cdot H_2O$
- 1.2 mg of $CuSO_4 \cdot 5\ H_2O$
- 4 mg of $ZnSO_4 \cdot 7\ H_2O$
- 0.08 mg of $NiCl_2 \cdot 6\ H_2O$.

Solvents for amino acid analysis

Mobile phase A: Dissolve 2.8 g of Na_2HPO_4 and 7.6 g of $Na_2B_4O_7 \cdot 10\ H_2O$ in 1.9 L of water and add 1.5 mL of HCl (37%). Mix until homogeneous, adjust the pH to 8.2 with HCl, and fill up to 2 L with water. It is recommended to use an amber 2 L solvent bottle (part number 93016341) to avoid algae growth.

Mobile phase B: Acetonitrile:methanol:water 45:45:10 (v:v:v)

Injection diluent: 10 mL of mobile phase A + 200 µL of orthophosphoric acid (85%)

After opening an OPA or FMOC ampoule, the reagents were distributed to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024) and stored for no longer than a week. Borate buffer and injection diluent were transferred to vials without inserts. All reagents should be stored at 4 to 8 °C, and reagents in the autosampler should be exchanged daily.

Amino acid standard solutions

To prepare the extended amino acid (EAA) stock solution containing 18 nmol/µL of asparagine, glutamine, and tryptophan, weigh 59.45 mg of asparagine, 65.77 mg of glutamine, and 91.95 mg of tryptophan into a 25 mL volumetric flask and fill it to the volume with 0.1 M HCl.

For the internal standard (ISTD) stock solution containing 10 nmol/µL of norvaline and sarcosine, weigh 58.58 mg of norvaline and 44.54 mg of sarcosine into a 50 mL volumetric flask and fill it to the volume with 0.1 M HCl.

It is recommended to aliquot the stock solutions before freezing them at –20 °C to avoid freeze-thaw cycles.

For the preparation of the calibration standards, the EAA stock solution was diluted to 9, 4.5, 2.25, 0.90, and 0.45 nmol/µL and combined 1:1 with ISTD stock solution. This was later mixed at a ratio of 1:10 with respective concentrations of amino acid standard to obtain final concentrations of 900, 450, 225, 90, 45, and 22.5 pmol/µL of each amino acid and 500 pmol/µL of ISTD.

V. natriegens precultivation

To inoculate the 4 L of fermentation medium, it was necessary to create a pre-culture. For this, a small amount of a deep-frozen glycerol stock with *V. natriegens* ΔdldhΔlldh Δpfl Δmdh was extracted using an inoculation loop and dispersed onto a BHIN agar plate. The plate was then incubated at 37 °C for a duration of 6 to 8 hours or, alternatively, left to incubate overnight.

A single colony was then transferred into 100 mL of 2x YTN medium, in a shaking flask. The flask was incubated overnight at 37 °C with 300 rpm and an amplitude of 50 mm in a shaking incubator.

V. natriegens bioreactor cultivation and L-alanine production

The bioprocess was performed with the following parameters: a temperature of 37 °C, a pH of 7.5, anaerobic conditions, and a stirrer operating at 400 rpm. The pH was adjusted using 1 N H_3PO_4 as the acid and 1 N NaOH as the base.

The bioreactor was inoculated with 100 mL of the 2x YTN preculture. Initially, bacterial proliferation occurs, and after the depletion of oxygen, L-alanine synthesis commences.

Subsequently, hourly samples were collected, along with an initial sample taken at the beginning of the experiment.

Bioreactor sampling

The samples delivered to the 1290 Infinity III Bio Online LC were drawn by the Flownamics Seg-Flow system through a Flownamics F-series 310 mm FISP probe from the 5 L bioreactor. This probe is designed for the safe and precise extraction of cell-free samples from the culture broth. The samples were sent to the built-in sample collection cup on the Flownamics Seg-Flow system and subsequently to a designated vial in the Agilent 1290 Infinity III Bio Online Sample Manager.

Sample derivatization with injector program

1. Draw 5.00 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 the default speed using the default offset.
4. Wash the needle as defined in the method.
5. Draw 1.00 µL from location 2 (OPA reagent) with the default speed using the default offset.
6. Wash the needle as defined in the method.
7. Mix 7.00 µL from air with the default speed 10 times.
8. Draw 0.40 µL from location 3 FMOC reagent) with the default speed using the default offset.
9. Wash the needle as defined in the method.
10. Mix 7.40 µL from air with the default speed 10 times.
11. Draw 32.00 µL from location 4 (injection diluent) with the maximum speed using the default offset.
12. Wash the needle as defined in the method.
13. Mix 20.00 µL from air with the maximum speed five times.
14. Inject.

Methods

Table 1. Chromatographic conditions for analysis of derivatized amino acids.

Parameter	Value
Column	Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm
Solvent	A) 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$ in water, pH: 8.2 B) Acetonitrile/methanol/water (45:45:10; v:v:v)
Gradient	Time (min) %A %B 0 98 2 0.40 98 2 13.60 43 57 14 0 100 Stop time: 17 min Post-time: 3 min
Flow Rate	0.600 mL/min
Temperature	40 °C
Detection	DAD: Signal A: 338 nm, bandwidth 10 nm Reference wavelength: 390 nm, bandwidth 20 nm Signal B: 262 nm, bandwidth 16 nm Reference wavelength: 324 nm, bandwidth 8 nm Peak width: > 0.1 min (2.5 Hz)
Injection	Use sample preparation method (injector program) for derivatization of amino acid. Outer wash: 3 s with acetonitrile: 0.1 M HCl (50:50; v:v) Draw speed: 100 µL/min Eject speed: 400 µL/min Wait time after draw: 1.2 s Sample temperature: 10 °C

Table 2. Online LC Monitoring Software conditions.

Parameter	Value
Sampling	Pure to vial
Retain Volume	240 µL
Sampling Source	Vial
Sampling Speed	Setting 1 Draw speed: 130 µL/min Dispense speed: 155 µL/min
Transport Solvent	S1: Mobile phase A
Schedule	With Flownamics Flow-Web Software Sampling frequency: 60 min Repeats: 24 (+1 at start)

Results and discussion

For the exemplary chosen bioprocess, an engineered derivative of *V. natrigens*, namely *V. natriegens Δdldh Δlldh Δpfl Δmdh*, was used. This strain was genetically modified for the enhanced anaerobic production of L-alanine, which can be directly analyzed in the culture broth.

Amino acid calibration

To quantify target analytes in chromatograms, calibration with respective standards is necessary. The calibration standards were prepared manually (as described above), and the derivatization was carried out with the injector program. Calibration was performed by injecting the standard solutions from vials with automated derivatization by using the Agilent 1290 Infinity III Bio Online LC without the Flownamics Seg-Flow S3 and Sample-Mod S3.

Figure 2 shows a chromatogram obtained from the analysis of the amino acid calibration standards containing 900 pmol/µL of amino acids and 500 pmol/µL of ISTD, which were derivatized using the 1290 Infinity III Bio Online Sample Manager and injector program. The chromatogram shows a successful separation of 20 amino acids and two ISTDs within 17 minutes. In addition, the calibration function (Figure 3) was determined to quantify the concentration of L-alanine during the bioprocess.

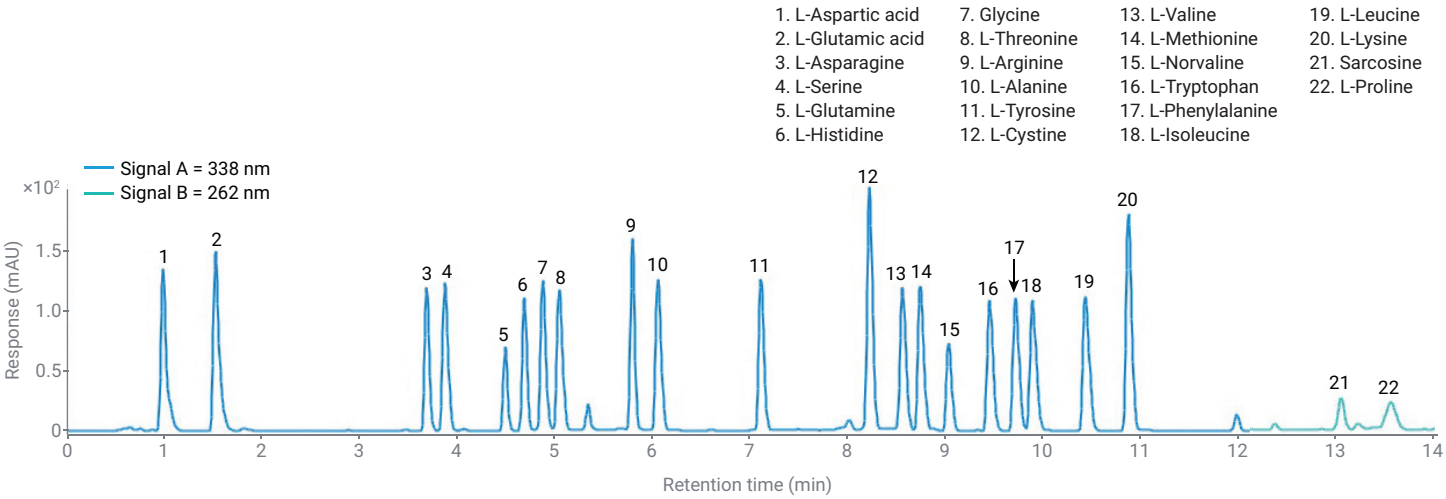

Figure 2. Chromatogram from an analysis of 900 pmol/µL amino acid mixture and 500 pmol/µL ISTD.

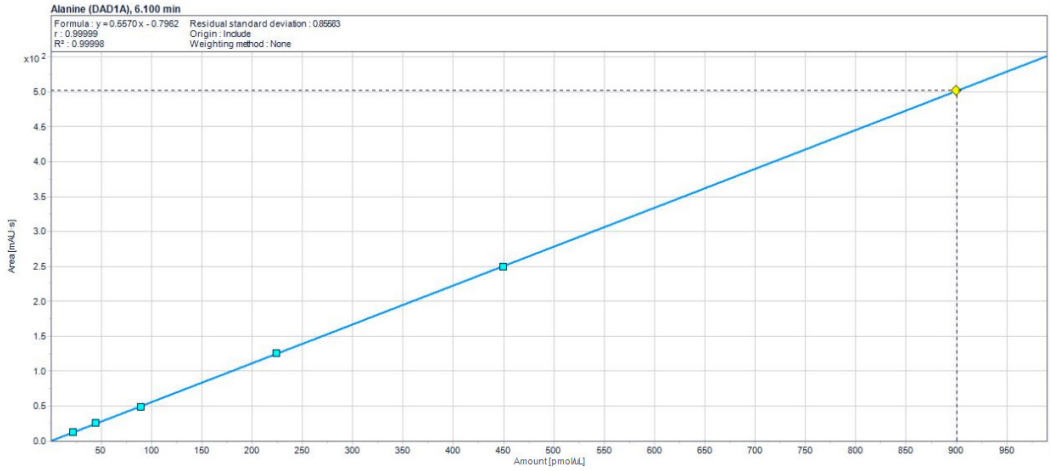

Figure 3. Calibration curve obtained for L-alanine with the Agilent 1290 Infinity III Bio Online LC.

Online monitoring of L-alanine production during the bioprocess

The analysis was performed automatically, including sampling, derivatization, and analysis, as well as data processing. This was made possible by using the Agilent 1290 Infinity III Bio Online LC with the Flownamics Seg-Flow S3 and Sample-Mod S3. The data was displayed using the Agilent Online LC Monitoring software (see Figure 5), which illustrates the evaluated data in a trending plot.

A closer look at the trending plot (Figure 5) and the chromatograms at different time points (Figure 4) reveals that the concentration of L-alanine increases continuously during the bioprocess. After 24 hours, a significant amount of L-alanine was produced. However, it is also apparent that a certain amount of L-alanine is already present at the beginning of the experiment. This is since the 2x YTN medium used for preculture and incubation also contains L-alanine. In addition, further peaks can be seen in the chromatogram. This is due to the fact that the 2x YTN medium is complex, and the challenging matrix consists of many ingredients that can also absorb at 330 nm wavelength. This makes the effectiveness of this configuration in facilitating monitoring particularly noteworthy.

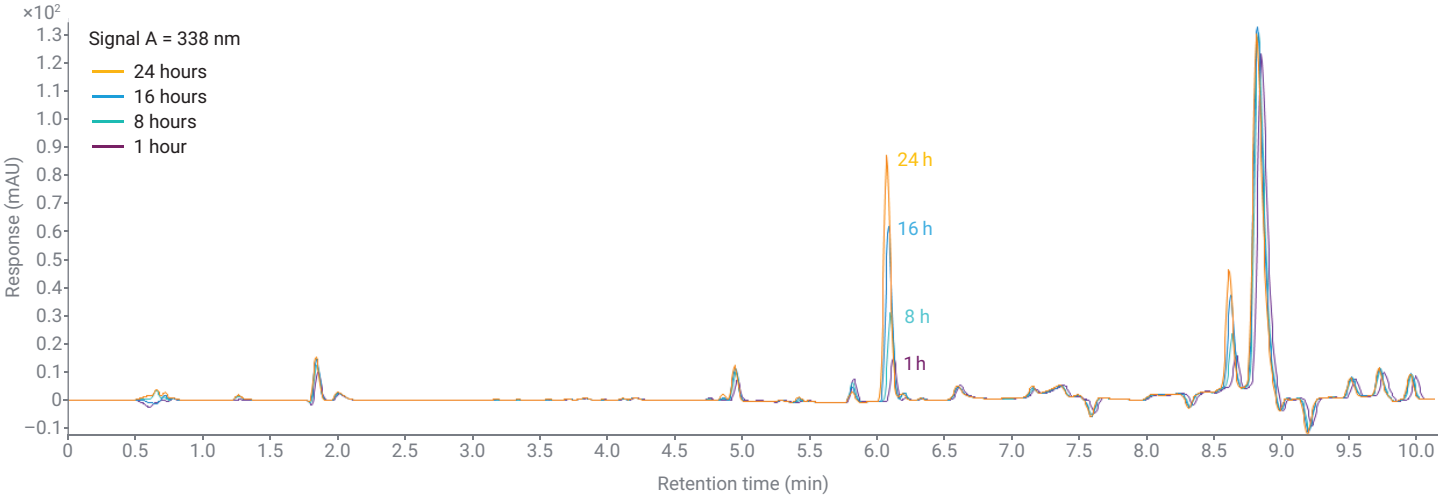

Figure 4. Comparison of L-alanine concentration in the bioreactor at different time points.

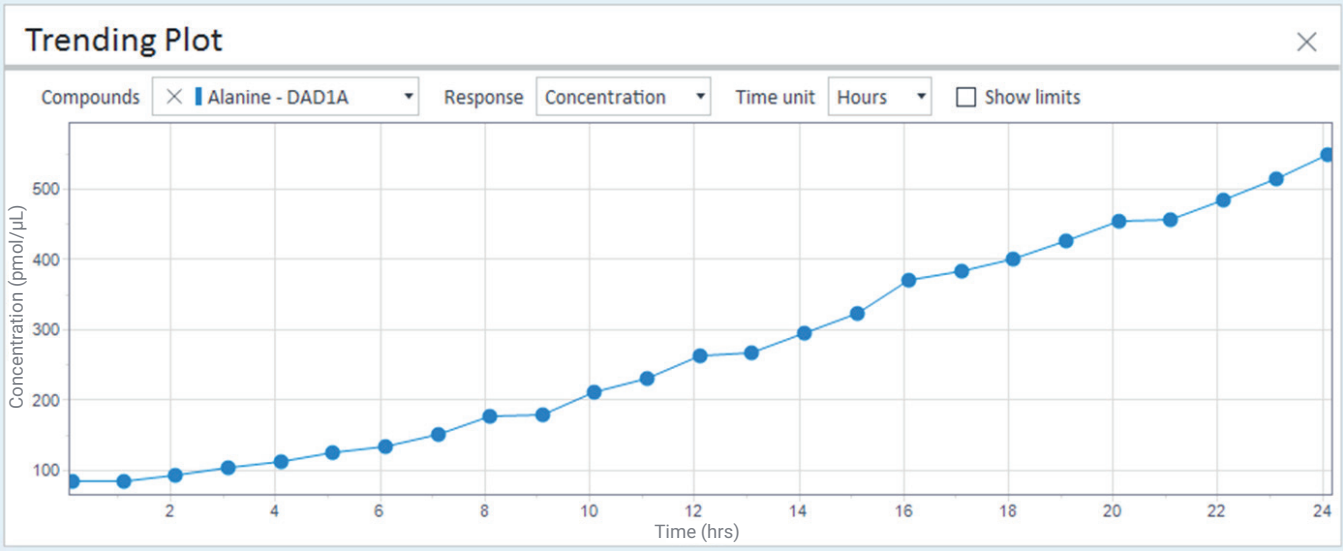

Figure 5. The trending plot of the Online LC Monitoring Software illustrates the increase in L-alanine concentration in the bioreactor over a 24-hour period, with hourly sampling and measurement.

Conclusion

For online bioprocess monitoring, a fully automated setup consisting of a bioreactor, a Flownamics sampling system, and an Agilent 1290 Infinity III Bio Online LC solution was successfully established. This setup is capable of taking aseptic samples from a bioreactor, preparing, analyzing, and processing samples without the need for manual interaction. In addition, the added value of the Agilent Online LC Monitoring software was evident in the display method and the ability to track it quickly.

References

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DE-011179

Printed in the USA, December 12, 2025
5994-8865EN

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