Application Note

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An Automated Sampling System for Online Monitoring as a Process Analytical Technology (PAT) Tool

The Agilent 1290 Infinity II 2D-LC interfaced with a Flownamics Seg-Flow system for control of upstream titer, product quality, and amino acid concentration

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Abstract

This application note demonstrates the use of the Agilent 1290 Infinity II 2D-LC system in combination with the Flownamics Seg-Flow system for automated bioreactor sampling as a UHPLC-based process analytical technology (PAT) tool for upstream titer and product quality assessments of mAbs and fusion proteins.

This combination enables the determination of multiple upstream critical quality attributes (CQAs) using a single setup and saves time and costs during the development of biotherapeutics. 2D-LC heart-cutting combined with a flow split after the first dimension enables shorter run time, improved peak shape, and therefore faster results to control the process.

As examples, the following CQAs have been determined for a mAb: titer, size variants, and charge variants as well as amino acid concentration of the bioreactor cell culture. Other potential applications include but not limited to online RP-HPLC, HIC, HILIC, affinity chromatography, reduced and nonreduced denaturing SEC, etc. to measure quality attributes of bioreactor samples.

Introduction

PAT has been defined by the FDA as a mechanism to design, analyze, and control pharmaceutical manufacturing processes through timely measurements of CQAs and CPPs (abbreviations are explained in the glossary). The goal is to ensure consistent final product quality. The principle behind the PAT is to enhance process and product understanding, and process control. This will help to ensure the desired final product quality with the use of statistical techniques to establish a design space. The implementation of PAT systems in the pharmaceutical industry is derived from the regulatory authorities' initiatives to improve and modernize the industry. This shall enhance product quality with the adaptation of quality by design (QbD)/PAT concepts. The common theme of the QbD philosophy is to build quality into the products instead of testing the product to ensure quality.[1]

QbD/PAT-enabled control strategies ensure the development of robust and efficient bioprocesses to deliver high-quality drugs with desired product quality and batch-to-batch reproducibility.

Online monitoring of titer and CQAs provides an opportunity to deploy appropriate engineering controls to keep the process within the design space.[9] Linking of online PAT data to DCS such as Delta-V or SCADA provides efficient feedback control to achieve consistent product quality to align with the expectation of QbD-enabled continuous bioprocessing. With the implementation of these technologies, it is now possible to align with the QbD philosophy of having product and performance quality built into the process design instead of having to test them in the final product.[9]

The PAT toolkit contains a series of resources to build quality into products while enhancing process understanding, increasing efficiency, and reducing costs. The PAT toolkit contains:

- **Multivariate analysis:** Principal component analysis (PCA) and partial least squares (PLS) for cluster analysis and predictive model generation
- **Process analysis:** Chromatographic and spectrometric techniques such as UHPLC, 2D-LC, Raman, NIR, and FTIR.
- **Process control:** Distributed control system (DCS) and architecture with a proportional-integral-derivative (PID) controller to establish feedback control

As size and charge variants are often considered CQAs for therapeutic proteins, monitoring them is imperative. Biotherapeutics are susceptible to aggregation, degradation, and other post-translational modifications that form size and charge variants. They can induce adverse immune responses, impacting drug safety and efficacy.[2] While high molecular weights (HMWs) of mAbs impact safety and efficacy[3], low molecular weights (LMWs) may cause immunogenicity, thus impacting pharmacokinetics.[4]

Charge variants of mAbs are typically caused by deamidation, isomerization, succinimidation, oxidation, sialylation, glycation, N-terminal pyroglutamilation, C-terminal pro-amidation, and C-terminal lysine clipping. These charge variants must be characterized to ensure safety and efficacy of the drug.[5]

This application note demonstrates the use of the 1290 Infinity II 2D-LC with its capability for high-resolution sampling to deal with large-volume peaks from the first-dimension separation of samples drawn from a bioreactor by means of the Flownamics Seg-Flow automated sampling system. The acceleration of analysis by a flow-splitting approach after the first dimension to make the approach PAT-applicable has been demonstrated. The determination of titer, size variants, and charge variants as well as amino acid concentration of the bioreactor cell culture are presented. The complete publication can be found in the scientific literature.[6]

Experimental

Instrument

The Agilent 1260/1290 Infinity II 2D-LC Solution comprised the following modules:

- Agilent 1260/1290 Infinity II High-Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with Infinity II Sample Cooler (Option #100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Two Agilent 1290 Infinity II Diode Array Detectors (G7117B) with 10 and 60 mm Agilent InfinityLab Max-Light Cartridge Cells (G4212-60008)
- Agilent 1260 Infinity II Fluorescence Detector (FLD) (G7121B)
- Agilent 1290 Infinity Valve Drive (G1170A) with a 2D-LC Valve (G4236A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Multiple Heart-Cutting Valves (G4242A) equipped with 40 µL loops

Software

- Agilent ChemStation edition rev. C.01.07 SR2 [255] or later version, with Agilent 2D-LC Software, product version A.01.03 [025] or later
- Flownamics FlowWeb control software

Instrumental configuration

Agilent 1290 Infinity II 2D-LC System with Seg-Flow Interface for Automatic Sampling and On-Line Analysis

- The 1260 Infinity II 2D-LC was interfaced with Seg-Flow to withdraw samples from the bioreactor.
- The drawn samples were sent to the built-in sample collection cup on the Seg-Flow.
- The scheduling feature of Flownamics FlowWeb Software allows the system to be programed according to the users' requirements.
- The open-source feature of Flownamics software allows the feedback control via the distributed control system (DCS).

Application of high-resolution sampling features of 1290 Infinity II 2D-LC System (Figure 1):

- The Agilent 2D-LC Software provides the option to collect multiple analytical cuts eluting from the first dimension into multiple sample loops, and inject one fraction at a time. This enables functions such as multiple heart-cutting 2D-LC (MHC 2D-LC), comprehensive 2D-LC, and high-resolution sampling 2D-LC (HiRes 2D-LC).
- The 2D-LC Software combines integrated peak areas of multiple cuts for quantitative results.
- Such aggregated results from multiple cuts correspond to results generated through conventional offline fraction collection.
- This approach overcomes the challenge of handling the higher peak volume associated with first-dimension protein A chromatography.

The use of a 2D-LC for product quality measurements of bioreactor samples is influenced by the large peak volumes of the protein A peak obtained from the first dimension. Using the high-resolution sampling mode of the 2D-LC Software of the 1290 Infinity II 2D-LC helps to overcome this, but an additionally included split after the first dimension further shortens the analysis time significantly.

Bioreactor sampling

The samples delivered to the 1260 Infinity II 2D-LC were drawn by the Flownamics Seg-Flow system through a Flownamics sterilized F-series 310 mm FISP probe from a 5 L bioreactor. This probe is capable of withdrawing sterile and cell-free samples from the bioreactor. The samples were sent to the built-in sample collection cup on the Flownamics Seg-Flow system and subsequently to a designated Agilent autosampler vial.

Columns

1. Protein A column
2. SEC UHPLC column, 4.6 × 150 mm, 200 Å, 1.7 µm
3. WCX column, 2.1 × 100 mm
4. SCX column, 2.1 × 100 mm
5. Agilent Agilent Poroshell HPH C18, 4.6 × 100 mm, 2.7 µm (part number 695975-702)

Methods

1. Online titer measurement of upstream bioreactor samples using protein A UHPLC method:
   - Flow rate: 1 mL/min
   - Solvent A: DPBS, pH 7.4
   - Solvent B: DPBS, pH 2.1
   - Gradient: 100% A from 0.0 to 0.5 min, 100% B at 5.1 min, 100% B to 0.2 min.
   - The effluent was split 1:10 and collected in a single loop for analysis in the second dimension.
2. Online size variants analysis of upstream bioreactor samples using protein A separation as first dimension (see above) and SEC as second dimension method:
   - Flow rate: 0.2 mL/min
   - Isocratic: 100 mM sodium phosphate, 100 mM sodium sulfate, pH 6.8

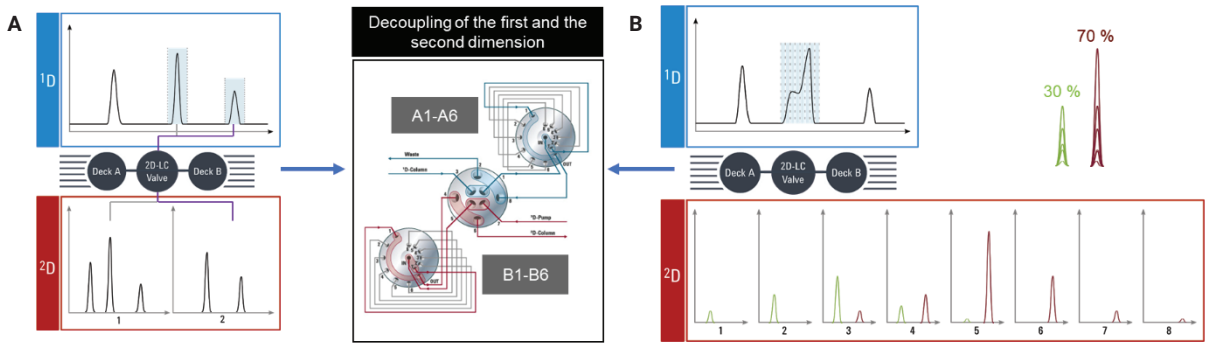

Figure 1. Peak-cutting scenarios for the Agilent 1290 Infinity II 2D-LC System: multiple heart-cutting 2D-LC (MHC 2D-LC) and high-resolution peak cutting (HiRes 2D-LC) with peak parking. (A) Store one or multiple peaks of interest and further analyze in the second dimension. (B) Make multiple cuts across one peak of interest, analyze later, and compare results at different positions.

3. Online charge variant analysis of upstream bioreactor samples using protein A as first dimension (see above) and IEX as second dimension method:
   - WAX: gradient: pH 5.0 to pH 8.5
   - SCX: solvents: A at pH 5.0 and B at pH 10.2
   - Gradient 5% B to 32% B in 54 minutes at a flow rate of 0.5 mL/min.
4. Online amino acid analysis using in-loop OPA and FMOC derivatization[7]
   - Flow rate: 0.2 mL/min
   - Solvent A: 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 5 mM NaN_3, pH 8.3
   - Solvent B: acetonitrile/methanol/water (45/45/10, v/v/v)
   - Gradient: 0 min: 2% B, 0.2 to 6.8 min: 2% to 57% B, 7.0 to 7.4 min: 100% B, 7.5 min: 2% B. Stop time: 9 min.
   - Column temperature: 40 °C
   - Wavelength for OPA derivatives: excitation 340 nm, emission 450 nm
   - Wavelength for FMOC derivatives: excitation 266 nm, emission 305 nm

Amino acid derivatization

Components of the Agilent OPA and FMOC derivatization kit (FMOC reagents: part number 5061-3337, OPA reagent: part number 5061-3335) were reconstituted and diluted according to the package instructions and loaded into the designated autosampler positions. Each calibration standard (amino acid analytical standards, part number 5061-2478) and sample were automatically derivatized with OPA and FMOC using the injector program features of the Agilent autosampler.

Injector program:

1. Draw 2.5 µL of borate buffer and draw 1.0 µL of sample from the designated vials.
2. Mix five times in the wash port.
3. Wait 0.2 minutes.
4. Draw 0.5 µL of OPA, add and mix in wash port.
5. Draw 0.4 µL of FMOC, add and mix in wash port.
6. Add 32 µL of a diluent.
7. Inject 20 µL from the resulting solution.

Solvents and chemicals

All solvents were LC grade. Acetonitrile was purchased from Merck, Darmstadt, Germany. Chemicals were from Sigma-Aldrich, Steinheim, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA).

Results and discussion

Online titer and product quality measurements of bioreactor samples

The high-resolution peak-cutting and peak-parking capability of the Agilent InfinityLab 2D-LC Solutions is an optimized solution to deal with the large peak volume coming out of the first-dimension protein A chromatography. The emerging peak is automatically fractionated, and each fraction is collected in individual sample loops prior to the second-dimension analysis. The high-volume peak from the first dimension and a representative IEX profile of high-resolution peak-cutting is shown in Figure 2.

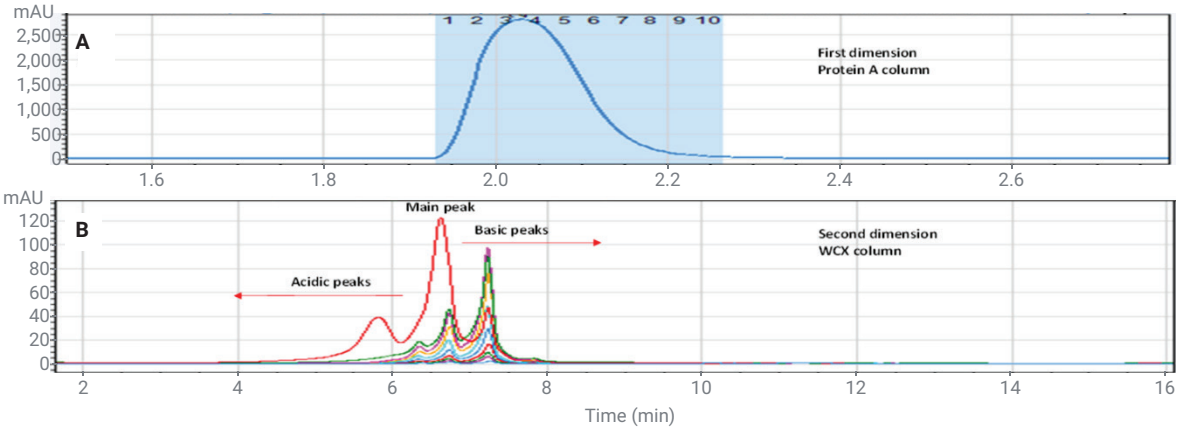

Figure 2. Two-dimensional analysis of bioreactor for charge variants. (A) First-dimension protein A chromatography. (B) Weak cation exchange (WCX) profiles of high-resolution peak cutting of first-dimension peak, demonstrating acidic and basic variants in early- and late-eluting fractions.

Despite its practical utility, this approach has the disadvantage of the long analysis time necessary for analyzing multiple peak cuts, which is not in alignment with the rapid analysis required by PAT. To overcome this disadvantage, the alternative is an approach of a 1:10 flow splitting of the protein A column eluate, filling the effluent only in one loop. This reduces the analysis time 10-fold, with a reduction of the number of chromatographic analyses to a single run.

Online size and charge variant analysis results generated using protein A chromatography in the first dimension followed by 1:10 flow splitting prior to the second-dimension SEC and CEX analyses worked well and have been demonstrated to be comparable to the results generated using offline test results. A representative chromatogram of protein A/SEC is shown in Figure 3.

A representative first-dimension protein A and second-dimension CEX chromatogram generated using weak and strong cation exchange columns are shown in Figures 4A and 4B, respectively. In Figure 4B, an overlay of the first-dimension protein A and the second-dimension WCX chromatogram is displayed.

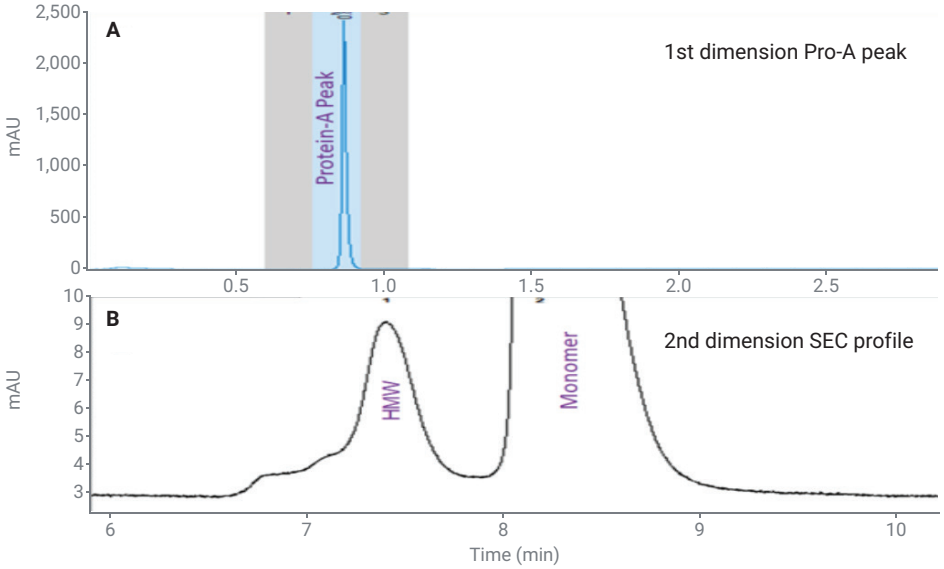

Figure 3. (A) First-dimension protein A peak. (B) Second-dimension SEC separation of mAb monomer and higher molecular weight compounds.

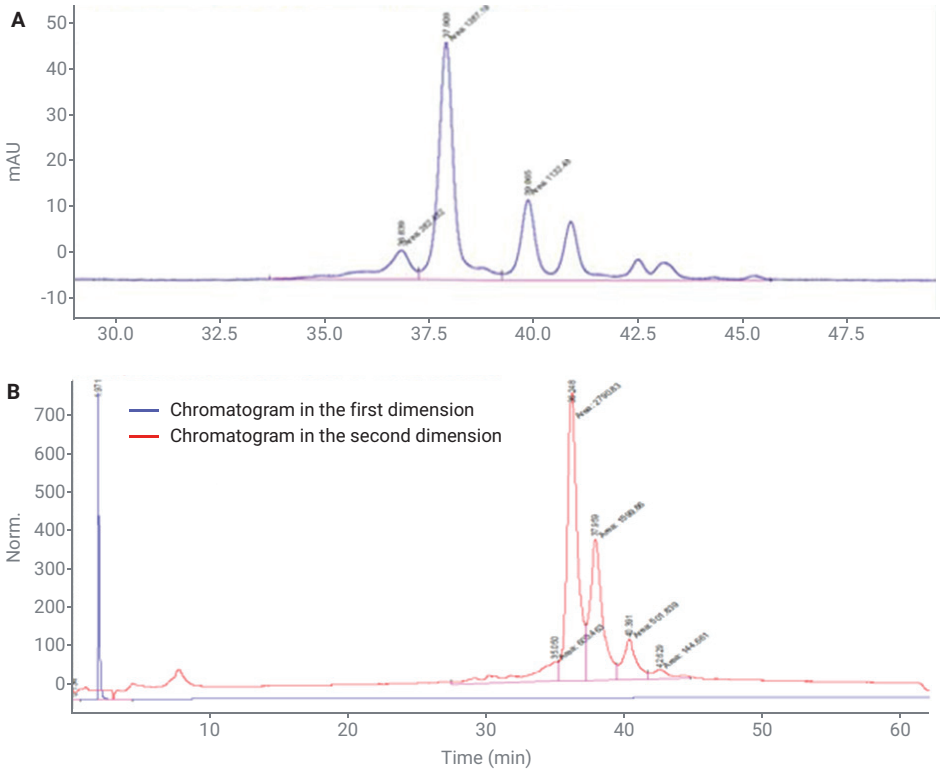

Figure 4. Representative chromatograms of bioreactor sample using the Agilent 1290 Infinity II 2D-LC with [1]D protein A and [2]D cation exchange chromatography (CEX) columns. (A) Strong cation exchange (SCX). (B) Weak cation exchange (WCX).

In addition to the product quality results, additionally generated titer results from the first-dimension protein A chromatography are an added advantage. A typical profile of online bioreactor titer measured from day 7 through day 15 is depicted in Figure 5.

Other potential chromatographic techniques, such as RP-HPLC, HIC, HILIC, affinity, and denaturing SEC (reduced and nonreduced) can also be performed for bioreactor samples using this Agilent 2D-LC/Seg-Flow platform.

Online amino acid analysis

The unique feature of Agilent autosamplers with programmability to carry out in-loop OPA derivatization in combination with Flownamics Seg-Flow for online sampling and dilution enables the system to be fully automated for online amino acid analysis (AAA). A typical online amino acid analysis profile generated using the 1290 Infinity II 2D-LC's first dimension with DAD/FLD and interfaced with a Flownamics Seg-Flow system is shown in Figures 6A and 6B. Figure 6A displays an overlay of OPA derivatized basal medium with and without a subset of spiked amino acids. Figure 6B displays a chromatogram of OPA-derived amino acids from basal medium only. Real-time AAA data of a bioreactor provide the opportunity to add missing AA into the bioreactor to maintain optimum cell growth conditions.

A typical online AAA profile of each amino acid during a bioreactor run from day 7 to day 14 is presented in Table 1.

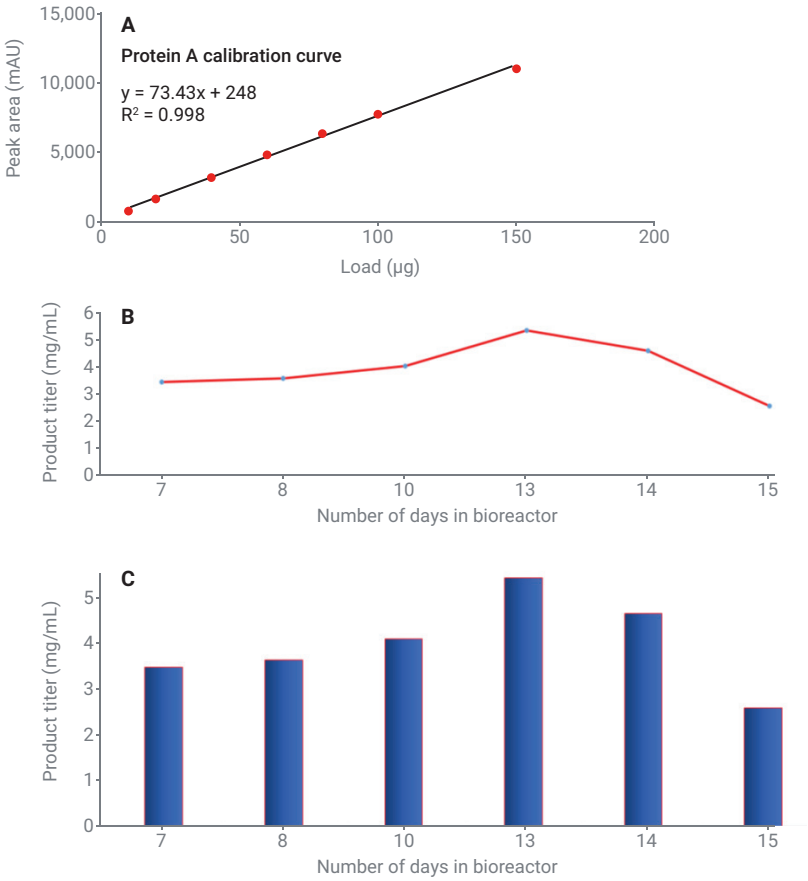

Figure 5. A typical online titer profile generated for mAb-X using the first dimension of integrated Agilent 2D-LC/Seg-Flow. (A) Representative titer standard curve. (B) Profile of bioreactor titer measured from day 7 to day 15. (C) Daily bioreactor titer for days 7 to 15.

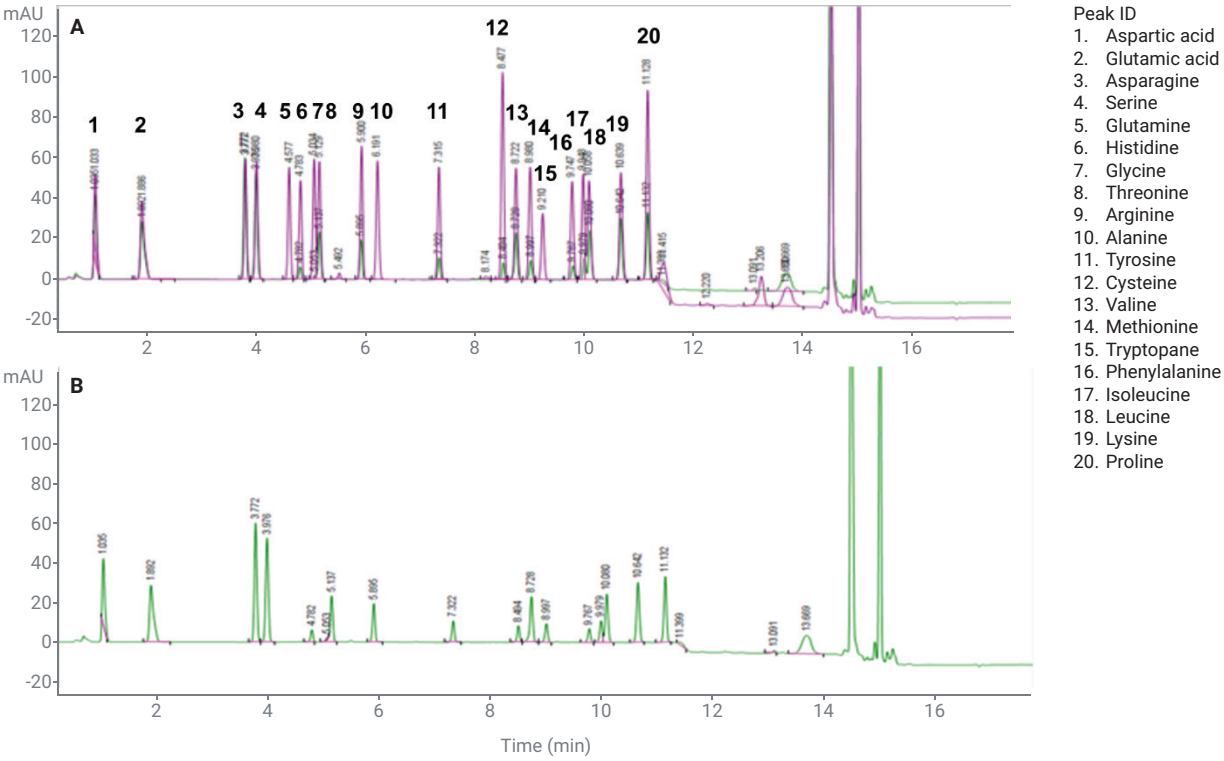

Figure 6. Online monitoring of amino acids in bioreactor. (A) Overlay of ainoacids in basal medium. (B) Basal medium. Both precolumn OPA derivatized.

Table 1. Online Flownamics Seg-Flow system AAA results of a typical bioreactor run using precolumn OPA derivatization.

Days	Asp	Glu	Asn	Ser	Arg	Ala	Tyr	Cys	NorVal	Trpt	Phy	Ile	Leu	Lys
7	4.1	11.5	5.9	14.7	4.1	5.3	5.3	6.0	3.2	3.7	2.7	11.2	12.2	4.6
8	4.2	11.7	2.8	18.4	2.9	2.1	2.1	6.7	3.0	2.7	2.1	12.1	9.0	4.5
10	4.6	11.1	6.0	15.9	3.0	1.5	1.5	7.5	2.1	5.4	ND	10.9	6.2	4.6
14	4.7	10.2	6.3	14.9	3.6	2.4	2.4	11.5	ND	ND	ND	11.9	7.2	5.4
15	4.4	10.4	5.4	14.6	4.2	2.3	11.1	13.1	4.0	ND	1.5	5.7	5.6	5.8
16	4.8	8.7	3.5	16.1	4.5	2.4	9.4	13.9	4.0	ND	ND	6.9	6.4	5.8

Conclusion

This application note demonstrated the utility of Agilent 1290 Infinity II 2D-LC System with the integration of postflow splitter and Seg-Flow automated sampling device for the following applications:

- Online titer measurement of upstream bioreactor samples using protein-A UHPLC methods
- Online size variants analysis of upstream bioreactor samples using a protein-A/SEC 2D-LC method
- Online charge variant analysis of upstream bioreactor samples using a protein-A/IEX 2D-LC method
- Online amino acid analysis using in-loop OPA derivatization

The integrated platform of Agilent 2D-LC with Seg-Flow is equipped to perform purification of mAbs and fusion proteins in the first dimension by a protein A column and product quality assessment in the second dimension. With the flow-splitting strategy, multiple injections needed for the current 2D-LC configuration can be reduced to a single injection to make the application PAT-amenable.

Agilent 1290 Infinity II 2D-LC System interfaced with Seg-Flow enables online CQA measurements such as charge variants and size variants as well as online amino acids analysis of bioreactor fermentation broth, providing the opportunity to control the process to achieve a predefined product quality.

FlowWeb control software acquires Seg-Flow integrated instrument data and exports the data to any OPC-enabled SCADA for enhanced process monitoring

and control.[8] This fully integrated architecture can be extended to other applications such as RP-HPLC, HIC, HILIC, affinity chromatography, reduced and nonreduced denaturing SEC, etc. for assessing product quality attributes of biopharmaceuticals directly from bioreactors.

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Glossary

- A distributed control system (DCS), or process control system (PCS), helps to control a procedural plant.
- A critical process parameter (CPP) is a process parameter that has an impact on CQAs and therefore should be monitored and controlled.
- Critical quality attributes (CQAs) should be understood as the measurable properties of therapeutic drugs critical for establishing the intended purity, efficacy, and safety of drugs.
- Multivariate data analysis (MVA) is a set of statistical models that examine patterns in multidimensional data by considering several data variables simultaneously.
- Object linking and embedding (OLE) for process control defines a common interface with a single designing stage and multiple reuses.
- Open platform communication (OPC)
- Process analytical technology (PAT)
- Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the data by retaining most of the variation.
- Partial least squares (PLS) regression is a widely used multivariate statistical technique in chemometrics to construct predictive models.
- Proportional-integral-derivative (PID) controller is a concept of feedback loop control for processes that require continuously modulated control.
- Quality by design (QbD)
- Supervisory control and data acquisition (SCADA) is a modern tool used for the control and monitoring of technological processes.

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