

# An Automated System for At-Line Process Analysis of Biopharmaceutical Fermentation Reactions

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## ABSTRACT

Monitoring amino acid profiles in fermentation reactors is a crucial factor for maintaining biopharmaceutical processes at optimal performance, with the analytical results used to control the feed to the reactor. Many laboratories perform small-scale parallel fermentation reactions in order to maximize experimental throughput. Typically, 40–60 minutes are required to run a full amino acid profile by HPLC. Using this method, each fermentation reactor can require the use of one complete HPLC system in order to produce a single data point per hour, per reactor.

Here, the authors demonstrate a solution for fast, at-line amino acid profile analysis using the Dionex UltiMate® 3000 Rapid Separation LC system and Acclaim® 2 µm particle size columns. This method resolves 21 amino acids in 5 to 10 min, depending on the resolution required. In addition, the autosampler automatically performs a precolumn derivatization, allowing more controlled experiments and significantly reducing run time.

## INTRODUCTION

With increasing interest in red biotechnology, research and development of efficient fermentation processes has become the focus of many pharmaceutical companies. These companies all strive to achieve the fastest experimental throughput in order to reduce the time to market for new products. One way to achieve this is to automatically control the feed of the fermenters based on process monitoring data. Like pH or oxygen content, the amino acid profile of a fermentation reactor is crucial for its efficiency and must be monitored continuously. This presentation focuses on an application of a major pharmaceutical company that runs six research fermenters in parallel, and requires one amino acid profile per hour, per reactor vessel.

Their goal is a total solution for:

- Automated sampling from six fermentation reactors for one sample per hour, per reactor (solution not shown in technical detail on this presentation)
- Fully automated analysis of one amino acid profile per fermentation reactor per hour, on one HPLC system

An HPLC system with precolumn derivatization enables simultaneous determination of all 21 biogenic amino acids; however, conventional LC methods require up to 1 h analysis time and can therefore support only one out of six fermenters. U-HPLC technology can reduce the analysis cycle time by up to a factor of 10. However, the need to derivatize amino acids using OPA and FMOC methods prior to injection onto an LC system introduces another bottleneck into the process analysis workflow. The derivatization process—which is performed manually—can also be error prone.

Therefore, we have developed a solution that comprises:

- Sequential, direct introduction of the six samples from six fermenters by another vendor's probe system into a storage loop of our autosampler injection valve
- Automated autosampler-based derivatization with OPA and FMOC for primary and secondary amino acids analysis in parallel to an analysis in progress
- LC analysis of all biogenic amino acids with a cycle time  $\leq 10$  min

## INSTRUMENTAL CONFIGURATION

UltiMate 3000 RSLC system supporting pressure up to 800 bar and flow rates up to 5 mL/min, consisting of the following modules:

- HPG-32000RS Binary Pump
- WPS-3000RS Split-Loop Well Plate Sampler with micro sample loop (25 µL), with on-line sample introduction (optional) (Figure 1)
- TCC-3000RS Column Compartment
- VWD-3400RS Variable Wavelength Detector with semimicro flow cell (2.5 µL)
- Eluent preconditioner (2 µL), in-line filter with (0.2 µm SST frit)

Chromeleon® Chromatography Data System, Version 6.80, SP 5-, (preliminary version)

## Sampling

The instrumental solution for probing the fermentation reactors will be provided from another vendor. As a final selection has not been made, it is neither referred to in this poster, nor described in technical detail.

Figure 1 shows the schematic instrumental setup, with the details of the autosampler fluidics. The samples from the six fermenters can be transferred sequentially into the storage loop by the delivery pump of the probe system, while the autosampler valve is in the inject position. After actuating the valve, the autosampler syringe supports the sample transfer to the sample loop of the split-loop device in reversed-flow direction. This allows the needle to move from the needle seat to the reagent vials. In this way, the derivatization reagents can be drawn in and mixed with the sample according to the protocol outlined in the derivatization procedure section below. Once the derivatization reaction that occurs in the autosampler needle (parallel to the LC analysis) is finished, it can be injected onto the column after the needle has been

returned to the needle seat. This is accomplished by switching the valve back to the inject mode.

The system is then ready to introduce the next fermenter sample into the storage loop of the autosampler valve.

## DERIVATIZATION PROCEDURE

### Reagents

- Sample: Amino acid standards (in 0.1 N HCl) or fermenter samples
- Reagent A: Borate buffer (0.2 M  $\text{Na}_2\text{HB}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ , pH 10.2)
- Reagent B: OPA/MPA reagent (75 mM o-phthalaldehyde, 225 mM 3-mercapto-propionic acid in 0.1 M borate buffer, pH 10.2)
- Reagent C: Fmoc solution (9-fluorenylmethoxycarbonyl chloride, 2.5 mg/mL in acetonitrile)
- Prep Vial: Injection diluent (50 mL Eluent A + 750  $\mu\text{L}$  phosphoric acid (85%))

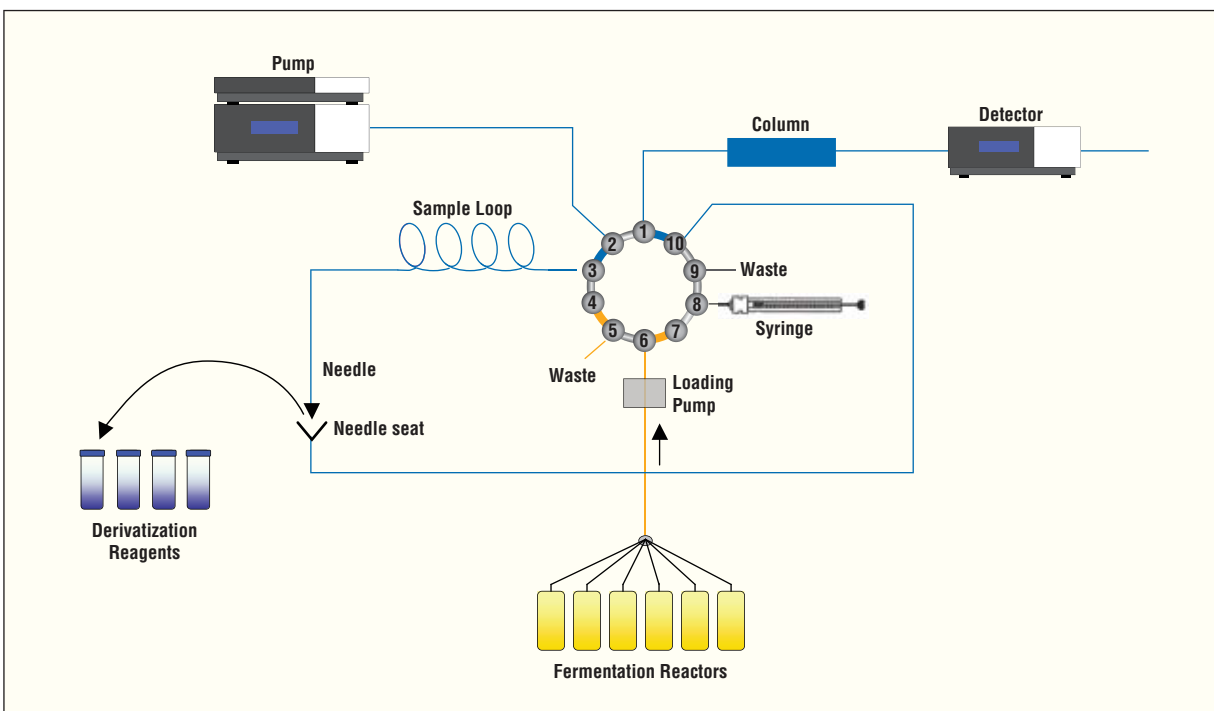


Figure 1. Automated sampling from six fermentation reactors with direct introduction of sample into the storage loop of autosampler injection valve for subsequent automated derivatization.

## Protocol

Small volumes of OPA/MPA reagent, borate buffer and sample are mixed. After a reaction time of 60 seconds, FMOC solution is added. The reaction is stopped by mixing with injection diluent. The entire derivatization procedure takes place in the needle. The detailed steps are as follows:

1. Draw borate buffer (50  $\mu$ L)
2. Draw OPA/MPA reagent (2.5  $\mu$ L)
3. Needle wash (100  $\mu$ L)
4. Draw borate buffer (2.5  $\mu$ L)
5. Draw sample (0.5  $\mu$ L)
6. Draw air (6  $\mu$ L)
7. Mix in needle 5 $\times$  (6 $\mu$ L)
8. Wait 60 s
9. Needle wash (100  $\mu$ L)
10. Draw FMOC solution (1  $\mu$ L)
11. Draw air (7  $\mu$ L)
12. Mix in needle 7 $\times$  (7 $\mu$ L)
13. Needle wash (100  $\mu$ L)
14. Draw injection diluent (14  $\mu$ L)
15. Draw air (15  $\mu$ L)
16. Mix in needle 9 $\times$  (15  $\mu$ L)
17. Inject

## CHROMATOGRAPHIC CONDITIONS

Column Temperature: 40 °C  
Eluent A: 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Na<sub>2</sub>HB<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O, 0.5 mM NaN<sub>3</sub>, pH 8.2  
Eluent B: CH<sub>3</sub>CN/MeOH/H<sub>2</sub>O (45/45/10)

### Conventional LC Method

Column: Dionex Acclaim 120 C18, 5  $\mu$ m, 250  $\times$  4.6 mm  
Injection Volume: 23  $\mu$ L  
Flow Rate: 1.5 mL/min (maximum pressure: 198 bar)  
Gradient: 0–40 min: 2–57% B, 40–44 min: 57–100% B, 44–54 min: 100% B, 54–55 min: 100–2% B, 55–60 min: 2% B  
Detection Parameters: 0–31.5 min: 338 nm, 31.5–60 min: 263 nm, data collection rate: 10.0 Hz, time constant: 0.18 s

### Fast LC Method for High-Resolution Amino Acid Separation for Fermentation Process Monitoring\*

Column: Dionex Acclaim 120 C18, 2  $\mu$ m, 100  $\times$  2.1 mm with 0.2  $\mu$ m in-line filter  
Injection Volume: 3  $\mu$ L  
Flow Rate: 0.710 mL/min, maximum pressure: 504 bar  
Gradient: 0–6.98 min: 2–57% B, 6.98–7.80 min: 57–100% B, 7.80–9.49 min: 100% B, 9.49–9.72 min: 100–2% B, 9.72–10.00 min: 2% B  
Detection Parameters: 0–6.04 min: 338 nm, 6.04–10.00 min: 263 nm, data collection rate: 10.0 Hz, time constant: 0.18 s

\*Developed using the Dionex Method Speed-Up Calculator

## RESULTS AND DISCUSSION

### Implementation of Fast Method for At-Line Process Monitoring

- By using a smaller particle size column with shorter length, and working at a higher linear velocity, the run time was reduced from 60 to 10 min with baseline resolution of all amino acids (Figure 2A).
- This method represents a total solution that supports:
  - Sequential introduction of samples from six different fermenters into the storage loop of an autosampler injection valve
  - Subsequent automated transfer from this loop to the autosampler, where derivatization takes place (with OPA and FMOC) for primary and secondary amino acids (derivatization time: 5 min)
  - High speed/high resolution LC analysis of 21 amino acids with a cycle time of 10 min
- Sampling and derivatization are performed parallel to analysis in-progress, and therefore do not add to the overall LC cycle time
- This solution requires only a single LC system, minimizing investment, running costs, and laboratory bench space.

## Acceleration Potential for Higher Analysis Throughput

2  $\mu\text{m}$  Acclaim columns combined with the high-pressure capabilities of the instrument provide potential to accelerate the separation to achieve analysis times under 10 min. Using the Dionex Speed-Up Calculator, the method can be optimized to achieve a total run time of 7 min, with acceptable resolution for process monitoring (Figure 2B). With a sampling time of 1 min and a derivatization time of 5 min, this method achieves maximum theoretical throughput, as sampling and derivatization runs parallel to the separation. With a cycle time of 7 min, the HPLC system can either support at-line cycle time monitoring of up to nine reactor vessels in parallel, or provide more than one data point per hour for six reactors.

## Application of the Method to Fermentation Broths

A major pharmaceutical company kindly provided samples from their fermentation reactors for this experiment. Samples were taken at different time points, and the amino acid content of the complex nutrient solution was determined with high-resolution using a cycle time of only 10 min as described in the Experimental section. This allows consumption of nutrients during the fermentation process to be easily monitored. Figure 3 shows the nutrient content of the growth medium at the beginning of the fermentation process (Figure 3A) and the decreased amino acid content at the end point. (Figure 3B). This fast amino acid analysis provides the opportunity to control the feed more precisely for maximum yield of the fermentation.

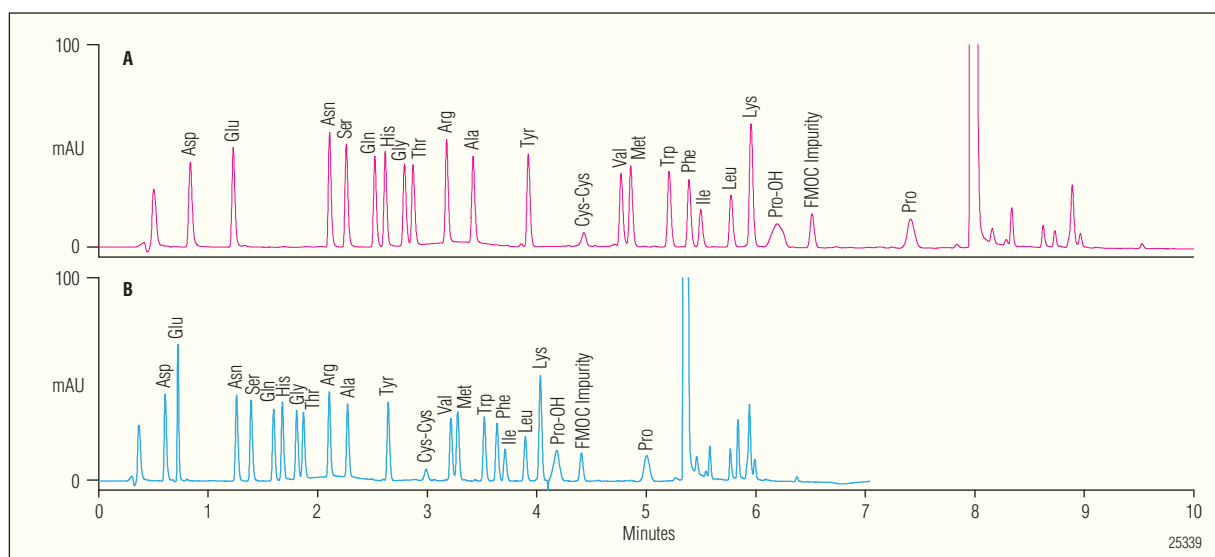


Figure 2. Fast, high-resolution separation of amino acid derivatives on Acclaim 120 C18,  $100 \times 2.1 \text{ mm}$ ,  $2 \mu\text{m}$  column (A) and the high-speed LC method (B).

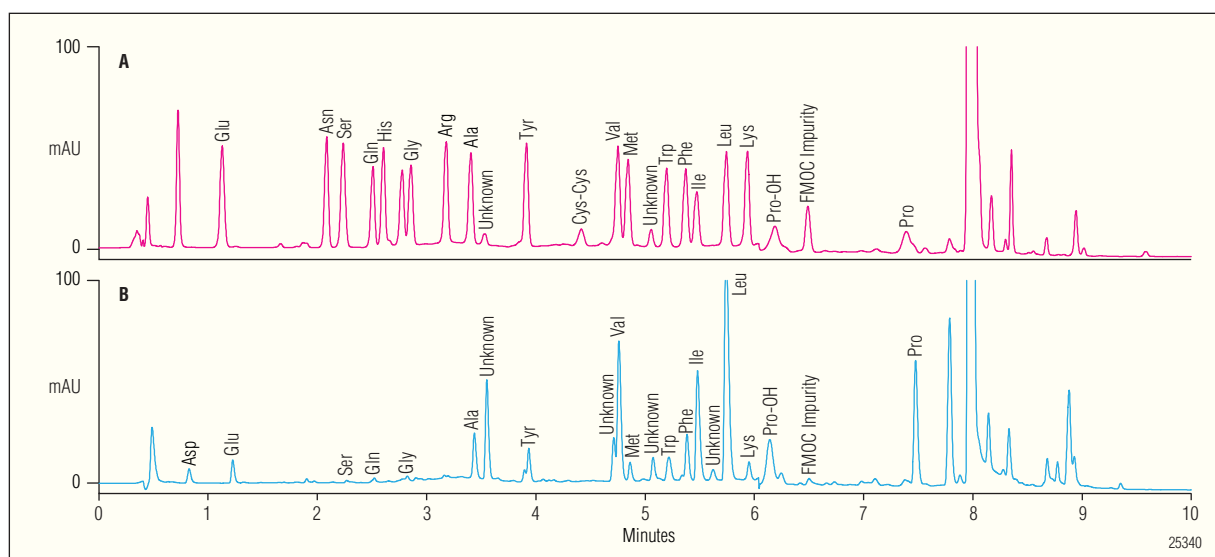


Figure 3. Comparison of samples from a fermentation reactor drawn at the starting point (A) and end (B) of the fermentation process.

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## CONCLUSION

The high pressures and high flow rates supported by the Dionex UltiMate 3000 RSLC system, along with the excellent efficiency demonstrated by the 2 µm particle size Acclaim columns, allow development of high-speed/high-resolution chromatographic methods. By combining these new methods with automated sampling and derivatization processes, an effective process monitoring solution has been achieved. The main benefits demonstrated by this solution are:

1. Automated sampling and precolumn derivatization allowing unattended process control of fermentation experiments
2. High-speed/high-resolution chromatography with run times under 10 min, providing improved control of the fermentation experiment
3. A total solution using only one LC system, providing lower investment and running costs

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