Online Monitoring and Control of Upstream Cell Culture Process Using 1D & 2D-LC with SegFlow Interface

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ABSTRACT

The biopharmaceutical industry is transitioning from currently deployed batch-mode bioprocessing to a highly efficient and agile next generation bioprocessing with the adaptation of continuous bioprocessing, which reduces the capital investment and operational costs. Continuous bioprocessing, aligned with FDA's quality-by-design (QbD) platform, is designed to develop robust processes to deliver safe and effective drugs. With the deployment of knowledge based operations, product quality can be built into the process to achieve desired critical quality attributes (CQAs) with reduced variability. To facilitate next generation continuous bio-processing, it is essential to embrace a fundamental shift-in-paradigm from "quality-by-testing" to "quality-by-design", which requires the deployment of process analytical technologies (PAT). With the adaptation of PAT, a systematic approach of process and product understanding and timely process control are feasible. Deployment of PAT tools for real-time monitoring of CQAs and feedback control is critical for continuous bioprocessing. Given the current deficiency in PAT tools to support continuous bioprocessing, we have integrated Agilent 2D-LC with a post-flow-splitter in conjunction with the SegFlow automated sampler to the bioreactors. With this integrated system, we have established a platform for online measurements of titer and CQAs of monoclonal antibodies (mAbs) as well as amino acid concentrations of bioreactor cell culture.

KEYWORDS

Process Analytical Technology (PAT), Quality by design (QbD), 2D-LC, Monoclonal antibody (mAb), Continuous bioprocessing, SegFlow

INTRODUCTION

The move towards next generation of QbD-driven continuous bio-processing is intended to deliver high quality drugs at a reduced cost with the adaptation of PAT-enabled technologies for real-time measurements of CQAs. Biopharmaceutical companies are shifting priority from productivity improvement to product quality enhancement to reinforce the safety and potency of drugs (Sokolov et al., 2017). As optimized batch processes are capable of yielding product titers as high as 10 g/L titer (Handlogten et al.), improving product quality is the current industry focus (Sokolov et al., 2017; Bruhlman et al., 2015). QbD-based architecture, designed to develop products with a defined product quality, requires PAT as an enabling technology to facilitate real-time monitoring. Deployment of PAT tools helps to understand the process so that we can ensure consistent product quality. Science-based approaches such as continuous bioprocessing serves as an important platform to achieve desired product quality (Allison et al., 2015). As an emerging field, PAT requires continued evolution in methodological and technological innovations (Croughan et al., 2015). Although physicochemical measurements such as UV and pH have been deployed for continuous bioprocessing, online measurement of CQAs is a gap. PAT enabled online CQA measurement is essential for bridging this gap (Croughan et al., 2015).

To align with FDA's PAT guidance to enhance process understanding, biopharmaceutical companies are leveraging multivariate analysis (MVA) tools to make a transition from the traditional approach of "quality-by-testing" paradigm to "quality-by-design" approach (Federal Drug Administration, 2004; Ferreira & Tobyn, 2015). Partial least squares regression and principal components analysis are effective PAT tools for establishing predictive models for real-time product quality measurements (Sokolov et al., 2017). As FDA has stated, real-time monitoring of CQAs using PAT tools and timely control of critical process parameters (CPPs) are

crucial to ensure desired product quality (Raj & Gupta, 2016). Biopharmaceutical companies are moving towards developing cost effective and time efficient processes without compromising product quality (Bhambure, Kumar, & Rathore, 2011). Continuous bioprocessing with the deployment of PAT tools is better equipped to adapt such anticipated paradigm changes. The QbD philosophy to augment process and product understanding provides efficient control strategy to accomplish cost-effective manufacturing (Mascia & Trout, 2015).

As the Director of FDA shared at the AAPS annual meeting in October 2011, the current manufacturing paradigm will undergo a transformation in the next 25 years to take precedence of more efficient, cleaner and flexible continuous manufacturing (Chatterjee, 2012). FDA promotes QbD based drug development and manufacturing to improve product quality by expanding the overall scope of analytical testing (Yu et al., 2014). Integrated continuous manufacturing (ICM) is intended to be cost effective with the miniaturization of facility foot-print and minimization of product variability along with improved productivity and efficient delivery of high quality drug at a reduced costs (Mascia & Trout, 2015; Croughan et al., 2014). Rapid product removal of continuous perfusion culture preserves high quality of drugs with enhanced safety. With the significant reduction in bioreactor time, proteins are protected from degradation pathways such as proteolysis, aggregation, oxidation, etc. (Pollock et al. 2017).

Considering the large number of monoclonal antibodies (mAbs) under development and their relatively higher dosing-regime, requirements of a low cost production of these drugs is inevitable (Subramanian, 2014). Continuous bio-processing with disposable single-use technology can reduce investment cost while increasing manufacturing flexibility. With the minimization of facility footprints and high level of automation along with higher product titer leads to significant reduction in capital expenses (Croughan et al., 2014). The larger equipment

size seen in batch processing leads to a higher suite cost of $255K per batch versus $120K per batch for the smaller highly utilized continuous equipment (Pollock et al., 2017). Continuous process is more flexible and cost effective than batch processes (Yang et al., 2019).

Health authorities are in favor of continuous bioprocessing to improve product quality (Alper & Rapporteur, 2019; Kornecki et al., 2019). Regulatory agencies have advised companies to take advantage of QbD and PAT applications for new process approvals (Zobel-Roos et al., 2019). Due to the lack of PAT tools available to support continuous bioprocessing, the FDA is encouraging companies to develop PAT tools. Implementation of QbD-enabled technologies such as PAT is critical for developing safe and efficacious drugs (Rathore & Winkler, 2009). With the modernization of drug manufacturing, the current practice of product quality testing at the finish-line of the process will be superseded with DOE-based statistical modeling, enabled by the integration of PAT tools (Bayer, 2014).

The motivation behind this study is attributed to the increased interest in accoomplising process and product understanding to administer process control, which is designated as a pre-requisite for QbD enabled continuous bioprocessing. Although chromatography tools are the most widely used techniques for product quality analysis in traditional in-process testing during biopharmaceutical development, utilization of these techniques as online PAT tools requires automated-sampling technologies (Wasalathanthri et al., 2020). Waters UPLC with PSM autosampler has been demonstrated to be a valuable tool for online titer measurements of bioreactor samples and product quality measurements of downstream unit operations (Chemmalil et al., 2020). However, Waters 2D-LC with PSM-autosampler exhibited poor peak resolution resulted from the large protein A peak volume entering into the 2nd dimension. The Dionex 2D-LC system overcame the peak volume issues attributed to its ability to collect the entire peak into

the autosampler and the option to inject a desired volume into the 2nd dimension. The Dionex 2D-LC system may be interfaced with a choice of automated inline samplers for online product quality analysis, but lacking the capability to perform online amino acid analysis at this time.

With the integration of Agilent 2D-LC with SegFlow automated sampler and the addition of a post-flow-splitter we established online methods for measurements of CQAs and protein titer of bioreactor samples as well as online measurements of amino acids in bioreactors, which is crucial for enhancing process robustness and product understanding. This is further augmented by the regulatory initiatives to incorporate PAT tools to enhance product quality via increased process & product understanding and control (Jenzsch et al., 2018). This work was intended to fill the current gap in PAT tools for real-time engineering control, discussed by Vargas et al. (2018). As Kornecki and Strube (2018) specified, real-time monitoring and control of product attributes are critical for an efficient and well controlled robust bioprocess.

Our initial exploration demonstrated that size exclusion chromatography (SEC) measurements using Agilent 2D-LC in its default configuration was not comparable to the offline results attributed to the large 1st dimension protein A peak-volume, in agreement with the publication by Dunn et al. (2020). Although the system's high resolution peak cutting/peak parking feature can overcome this issue, the lengthy analysis time makes it inadequate for PAT application. Our innovative approach of performing flow-splitting prior to the 2nd dimension analysis helps to overcome this challenge. This study has demonstrated that Agilent 2D-LC integrated with flow splitter and a SegFlow interface is suitable for online measurements of titer and CQAs of bioreactors samples for timely control. Agilent system's pre-column derivatization feature coupled with SegFlow's online sampling capability can facilitate online monitoring of amino acids, the main nutrients in bioreactors, with the option to establish a feedback control.

2. Materials and Methods

As product quality assessments of upstream samples require protein A purification prior to product quality measurements, 2D-LC was utilized for Protein A purification on the 1st dimension followed by product quality measurement on the 2nd dimension. While the 1st dimension protein A chromatography serves as a protein purification step, it also serves as a valuable source for titer information. Online sampling can be achieved via interfacing 2D-LC with automated samplers. We evaluated the following 2D-LC/auto-sampler combinations: (1) Agilent 2D-LC with SegFlow (2) Waters 2D-LC- with PSM autosampler (2D-PATROL™) (3) Dionex 2D-LC with the potential to integrate with integral or Numera autosamplers. Waters 2D-LC with PSM in its default configuration is not suited for online product quality measurement of bioreactor samples as the 1st dimension protein-A peak volume exceeds the recommended maximum limit of the 2nd dimension SEC or IEX analysis. Agilent and Dionex 2D-LC systems offer alternative option to address the peak volume issue. In addition to the online measurements of titer and product quality, 1st dimension of Agilent 2D-LC with SegFlow is suitable for online monitoring of amino-acids using in-column OPA derivatization, which prompted us to move forward with Agilent 2D-LC for further evaluation.

The various peak-cutting scenarios of Agilent 2D-LC include: (1) comprehensive analysis in witch eluent entering from the first dimension is continuously being introduced into the second dimension for enhanced peak resolution; (2) conventional heart cutting option in which the selected peaks are either cut and sent directly to the second dimension or heart cutting and peak parking option with the peak is being cut and stored in the sample loop before injection into the second dimension; and (3) high-resolution peak cutting that involves fractionation of large 1st dimension peak followed by collection and storage of each fraction in individual sample

loops prior to 2nd dimension injection of individual fractions. Agilent chromatography data system (CDS) can then integrate the data and deliver it as a consolidated data package. While the high-resolution peak cutting and peak parking offers a viable solution for dealing with the large protein A peak volume, it also means a lengthy analysis time. This was addressed by using a post flow-splitting strategy described below.

To reduce the analysis time associated with high-resolution peak cutting and peak parking, a flow-splitter strategy was adopted using commercially available mass spectrometry (MS) flow splitters. In the flow-splitter set-up, first dimension protein A effluent is split into a desired ratio, based on the peak volume. The use of a 1:10 flow splitter helped to reduce the analysis time by 10-fold. Once the analysis time issue was resolved, the next step in the process was to implement an automated online sampling system. The SegFlow system from Flownamics was selected as the interface, and custom scripts were developed. The integrated system is capable of withdrawing samples automatically from multiple bioreactors. Samples are sent to a SegFlow sample vial and subsequently to a designated Agilent autosampler vial. Scheduling can be customized according to the user requirements and the open-source features enable feedback control via a distributed control system (DCS). The Agilent 2D-LC system interfaced with the SegFlow and flow-splitter has served as the online PAT tool for measuring upstream titer and product quality. The 1st dimension of Agilent 2D-LC with SegFlow is also a suitable PAT tool for online measurements of amino acids in bioreactors.

2.1 Online Titer & Product Quality Measurements of Bioreactor Samples

Agilent 2D-LC consisting of 1260 U-HPLC and 1290 UPLC, equipped with SegFlow interface (from Flownamics) was used for online measurements of titer and product quality. For product quality assessments, 1st dimension eluate was subjected to size and charge variant

analyses on the 2nd dimension. Use of 2D-LC for product quality measurements of bioreactor samples is challenging due to the peak dispersion resulted from the large 1st dimension protein A peak volume entering the 2nd dimension. High resolution peak-cutting and peak-parking features of Agilent 2D-LC help to overcome this obstacle with the option of dissecting multiple segments of the Protein A peak and to collect each fraction in individual sample loops prior to the 2nd dimension injection. Although this approach resolves the large peak volume issue, the analysis time increases in proportion to the number of cuts across the protein A peak. We took an innovative approach to overcome the issue with the introduction of a post-flow-splitter to reduce the 1st dimension peak volume by 10-fold to transform the method to be PAT-amiable.

Agilent 2D-LC was interfaced with SegFlow to draw samples from a 5L bioreactor with a scheduled time-table. Sterilized F-series 310 mm FISP probe from Flownamics, capable of withdrawing sterile, cell-free samples from bioreactors, was immersed in bioreactor fermentation broth for drawing cell-free samples for online analysis. The drawn samples were sent to the built-in sample collection cup on the SegFlow and subsequently to a designated Agilent auto-sampler vial prior to the 1st dimension injection. To fully automate the sampling and analysis work flow for these emerging applications, a custom software tool was scripted to facilitate the connectivity between the FlowWeb (Flownamics) software and the Agilent OpenLab CDS. Samples were analyzed from day-7 through day-14 for titer and product quality.

For performing the titer assay, a POROS™ Protein-A column from Thermo Fisher Scientific was maintained at 1mL/min flowrate. Following a 0.5 minutes hold at 100% Mobile phase A (1× D-PBS, pH 7.4), a steep gradient of 100% Mobile phase B (1× D-PBS, pH 2.1) was applied at 0.51 minute, and maintained at100% B for 0.2 minutes. For the conventional approach, the high resolution peak cutting and peak parking option was chosen to send 40 µl of

each fraction of 1st dimension peak into multiple sampling loops and subsequent injection of each fraction into the 2nd dimension. For the non-conventional flow-splitting approach, the Protein A effluent was subjected to 1:10 flow-splitting using a flow splitter prior to the collection of the entire volume into a single sample loop and subsequent injection into the 2nd dimension.

For online size variant analysis, eluate from the 1st dimension Protein A chromatography was split 1:10 before performing the 2nd dimension SEC analysis. Analysis was carried out using Waters BEH 200 SEC UPLC column (4.6 x 150 mm, 200 Å, 1.7 µm) under isocratic conditions at a flow rate of 0.2mL/min using phosphate mobile phase (100 mM Sodium Phosphate, 100 mM Sodium Sulfate, pH 6.8). For charge variant analysis, eluate from the 1st dimension Protein A chromatography was split 1:10 before performing 2nd dimension CEX chromatography. For weak cation-exchange method, Sepax nonporous WCX column (2.1x100mm) was used on the 2nd dimension, applying a pH gradient from pH 5 to 8.5. For Sepax SCX method, SEPAX SCX-NP5 column (2.1x100mm) was used. Mobile phases A and B used for the analysis were commercially available 10X buffer concentrates from Waters with catalogue numbers 186009063 & 186009064, respectively. A shallow gradient from 5%B to 32%B was applied over the course of 54 minutes at a flow rate of 0.5 mL/min. **Figure1** illustrates the schematics of 2D-Agilent/SegFlow/FISP probe/Post-flow-splitter integration architecture.

Figure 1 about here

2.2 Online Amino Acid Analysis

In-column derivatization of primary and secondary (1° and 2 °) amino acids using o-phtalaldehyde (OPA) and fluorenylmethoxycarbonyl (FMOC) furnishes quick and fully automated analysis using the Agilent injector program. With the coupling of SegFlow sampling device to the Agilent LC, the integrated system offers the opportunity for online monitoring and

control of free amino acids in bioreactor culture broth. Agilent 1260 Infinity U-HPLC pump (1st dimension of Agilent 2D-LC) equipped with diode array detector (DAD) and fluorescence detector (FLD) was utilized. Agilent Poroshell HPH-C18 column used for the analysis was maintained at 40ºC at a flow rate of 0.2 mL/min. Mobile phases A and B used for the analyses were 10 mM Na_2HPO4 10 mM Na_2B4O7, 5 mM NaN_3 pH 8.2 and acetonitrile: methanol: water (45:45:10, v: v: v), respectively. Components of the OPA derivatization kit from Agilent were reconstituted and diluted according to the package insert instructions and loaded into the designated auto-sampler carousel position. Each calibration standard and sample was automatically derivatized with OPA and FMOC using programmable feature of the Agilent injector. While excitation and emission wavelengths of 340 nm and 450 nm, respectively were used for monitoring OPA derivatives, FMOC derivatives were monitored at excitation and emission wavelengths of 266 nm and 305 nm, respectively. The injector program was established to draw 2.5 µL borate buffer and 1.0 µL sample from designated autosampler vials prior to the 5-times mixing in the wash-port. Subsequent to a 0.2 minute waiting period, 0.5 µL of drawn OPA was added and mixed in wash-port, proceeded by the addition of 0.4 µL FMOC and sequential mixing at a default speed. Consecutively, a 32 µL diluent was added, and from that a 20 µl was injected. For chromatographic analysis, a shallow chromatographic gradient was applied.

3. RESULTS

Based on our initial evaluation, Waters 2D-LC system was determined to be non-optimal in its original configuration for upstream product quality measurements. This was due to the encountered peak broadening resulting from the entry of large peak-volume discharged from the 1st dimension into the 2nd dimension, leading to poor peak rsolution due to dispersion induced peak broadening. The Dionex 2D-LC system can overcome the peak volume issue attributed to

its unique feature to collect the entire 1st dimension peak into Dionex autosampler vial and subsequent injection of the desired volume into the 2nd dimension. The Dionex system can potentially be interfaced with a choice of automated samplers for online 2D-LC analysis of titer and product quality assessments. Agilent 2D-LC has a comparative advantage over other systems as it is capable of doing online amino acid analysis of bioreactor samples in addition to the online titer and product quality measurements. As shown in sections 3.1 and 3.2, integrated Agilent 2D-LC with the SegFlow has been demonstrated to be an ideal platform for upstream online titer and product quality measurements as well as for amino acid analysis.

3.1 Online Titer & Product Quality Measurement of Bioreactor Samples

Agilent 2D-LC's high resolution peak-cutting and peak parking feature is a viable solution to deal with the large peak volume coming out of the 1st dimension protein A chromatography. With this approach, the protein A peak with a large peak volume is automatically fractionated across the protein A peak and collect each fraction in individual sample loops prior to 2nd dimension injection. Agilent's open-lab CDS can conjoin the data from multiple fractions to deliver consolidated final results, representing the overall profile of the sample. A representative IEX profile of high resolution peak cutting is shown in **Figure 2**, demonstrating the dominance of acidic and basic variants in early and late eluting fractions, respectively. The alternative approach of 1:10 flow-splitting of protein A eluate reduces the analysis time by 10-fold with a reduction of number of chromatographic analysis to a single run.

Figure 2 about here

Online size and charge variant analysis results generated using protein A chromatography in the 1st dimension followed by 1:10 flow splitting prior to the 2nd dimension SEC and CEX analyses worked well and has 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 protein A/CEX chromatogram generated using weak and strong cation exchange columns are shown in **Figures 4 and 5,** respectively. In addition to the product quality results, concurrently generated titer results from the 1st dimension protein-A chromatography is an added advantage. A typical profile of online bioreactor titer measured from day-7 through day-15 is depicted in **Figure 6**. Other potential chromatographic techniques such as RP-HPLC, HIC, HILIC and denaturing SEC (reduced & non-reduced) can also be performed for bioreactor samples using this Agilent 2D-LC/ SegFlow platform.

Figures 3, 4, 5 & 6 about here

3.2 On-line Amino Acid Analysis

The unique feature of Agilent injector program to carryout in-column OPA derivatization in conjunction with the capability of SegFlow for inline sampling enables the system to be fully automated to do online amino acid analysis (AAA). A typical online amino acid analysis profile generated using the Agilent 1st dimension1260 interfaced with a SegFlow is shown in **Figure 7**. Comparison of the OPA method versus the legacy AccQ-Tag method suggests that the apparent differences in results between the two methods are within the inherent variability of the AAA method. Shown in **Table 1** are the results of spike and recovery study performed by spiking amino acids in NAOH, demonstrating satisfactory recovery achieved for both methods. **Figure 8** exhibits the spike and recovery results of amino acids spiked in cell culture media, demonstrating comparatively better recovery achieved for OPA method. A typical online AAA profile of each amino acid during a bioreactor run from day-7 to day-14 is presented in **Table 2**.

Table 1&2, Figure 7&8 about here

4. DISCUSSION

The work presented in this study is intended to fill the current gap in PAT tools to support continuous bioprocessing. Implementation of PAT in the pharmaceutical industry has been encouraged and supported by the regulatory authorities' initiative to improve and modernize the industry to enhance product quality. The common theme of QbD philosophy is to build quality into the products instead of testing the product to ensure quality. Quality cannot be tested into products; it should be built-in or should be by design. As the FDA has indicated, PAT is a mechanism to design, analyze, and control pharmaceutical manufacturing processes through timely measurements of CQAs and CPPs with the goal of ensuring consistent final product quality. The principles behind the PAT initiative is to enhance process and product understanding, and control the process to achieve desired product quality. The flexibility to operate within the established design space is intended to build desired product quality into the product. The QbD/PAT enabled control strategies ensure the development of robust and efficient bioprocesses to deliver high quality drugs with desired product quality at a reduced variability.

Four building blocks of PAT framework are multivariate analysis, process analysis tools, process control and continuous improvement (Guidance for Industry: PAT, 2004). Multivariate statistics and design of experiments (DOEs) are the foundation for establishing QbD based design space involving the use of multidimensional interactions such as CPPs that are impacting CQAs (Guidance for Industry: PAT A, 2004). A scientific understanding of relevant multi-factorial relationships between CPPs and CQAs requires multivariate analysis and simultaneous modeling of multiple variables. Biopharmaceutical companies transitioning from a "quality-by-testing" philosophy to a "quality-by-design" paradigm are establishing multivariate analysis tools to increase product and process understanding (Guidance for Industry: PAT, 2004). Process

analyzers, automatic in-line or online tools controlling the flow from the process equipment to the analyzer, are intended for timely measurements and control of CQAs to keep the process within the design space to attain desired final product quality. Based on FDA's guidance, process parameters, raw materials, and intermediates that are impacting CQAs must be monitored and controlled to keep the process in a state of control (Guidance for Industry: PAT, 2004)

Implementation of online process analytical tools for timely measurements and control of CQAs is central to QbD-enabled continuous bioprocessing. Developing online tools for measuring CQAs of bioreactor samples is challenging due to the requisite for protein purification prior to product quality assessments. With Agilent 2D-LC in conjunction with SegFlow and a flow-splitter, this study demonstrated the prospect of online monitoring of titer, CQAs and amino acids in bioreactor samples to control the process timely. While SegFlow with FISP probe is furnished to draw sterile cell-free samples from bioreactors, Agilent 2D-LC is equipped to perform purification of mAbs and fusion proteins in the 1st dimension and product quality assessment on the 2nd dimension. Flow-splitter effectively reduces the volume injected into the 2nd dimension and help to minimize the dispersion induced peak broadening.

As size and charge variants are often considered as CQAs for therapeutic proteins, controlling them is imperative. Bio-therapeutics are susceptible to undergo aggregation, degradation, and other post-translational modifications to form size and charge variants. These variants can induce adverse immune responses, impacting drug safety and efficacy (Moussa et al., 2016), hence considered as CQAs. While HMWs of mAbs are impacting safety and efficacy, (Polumuri, Haile, Ireland, & Verthelyi, 2018), LMWs may cause immunogenicity, thus impacting pharmacokinetics (Wang, Liu, Yan, Daly, & Li, 2018). Therefore, it is critical to monitor and control size variants. Charge variants of mAbs are typically caused by deamidation,

isomerization, succinimidation, oxidation, sialylation, 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 (Kaschak et al., 2011). Commonly used techniques such as imaged capillary isoelectric focusing (icIEF) and IEX require offline purification prior to testing. With the utilization of online 2D-LC, it become possible for purification and charge variant measurements to be carried out on the 1st and 2nd dimensions, respectively.

Waters 2D- PATROL UPLC system in its original configuration was not suitable as a PAT tool for online monitoring of upstream product quality. Recent modification of the system with the addition of multiple sample collection loop similar to Agilent 2D-LC can provides the possibility to perform high resolution peak cutting and peak parking option, and eliminate peak dispersion issue. However, lengthy analysis time is still a challenge. To transform the system to be PAT amiable with reduced analysis time, implementation of post-flow splitting strategy applied for Agilent 2D-LC is a viable solution. Dionex 2D-LC is suitable for product quality measurements of bioreactor samples as the large peak volume from the 1st dimension can be collected into an autosampler vial, and from which a desired volume can be injected into the 2nd dimension. Dionex 2D-LC can potentially be interfaced with Integral System form Dionex or Numera from ProAnalytics, but the option to do online amino acid analysis is a gap. As depicted in **Figures 3, 4, 5, and 6,** Agilent 2D LC system interfaced with SegFlow with a flow-splitting strategy is suitable for online measurements of titer and product quality. As shown in **Figure7**, with in-column derivatization feature of the Agilent system in conjunction with the SegFlow interface provides the opportunity to carryout online amino acid analysis.

In summary, this study has demonstrated the proof of concept of the capability of Agilent 2D-LC with post-flow splitting and SegFlow integration to serve as a viable PAT platform for

online titer measurement and product quality assessment of upstream cell culture process. In addition, the 1st dimension of Agilent 2D-LC in conjunction with SegFlow serves as an online amino acid analysis platform by harnessing the automatic in-column derivatization capability of Agilent injector program. Maintaining the right amount of individual amino acids in bioreactors is critical for optimal cell growth and protein production. In this fully automated architecture, the UPLC signal can be sent to Delta-V or other comparable distributed control systems (DCS). The integrated system can then control the Agilent 2D-LC and the bioreactor to facilitate a feedback control to ensure desired product quality.

5. CONCLUSION

Online CQA monitoring and real-time control of upstream bioreactor samples are critical for enhancing process and product understanding to keep the process in a state of control. We have demonstrated the proof of concept that the fully integrated Agilent 2D-LC interfaced with SegFlow and a flow splitter can serve as an ideal online PAT platform for measuring upstream titer and product quality. We have also shown that the in-column derivatization feature of the Agilent system in conjunction with the SegFlow is suitable for online analysis of amino acids directly from bioreactors. This new platform provides comparative advantages in terms of speed, sample integrity and convenience, while providing an opportunity to make timely decision.

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List of Figures

Tables

Table 1. % recovery of amino acids spiked in NaOH

Amino Acid	AccQ-Tag method (500 mM spike)	OPA method (125 mM spike)	AccQ-Tag method (500 mM spike)	OPA method (125 mM spike)
Tyrosine	95.7%	98.2%	102.6%	114.3%
Valine	101.9%	102.5%	108.2%	118.2%
Iso-Leucine	95.3%	101.2%	101.0%	116.6%
Leucine	101.3%	109.2%	107.7%	125.5%
Phenylalanine	90.3%	93.4%	97.4%	109.4%
Tryptophan	90.7%	92.8%	96.6%	107.6%

Table 2. Online AAA results of a typical bioreactor run using in-column 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

Figures:

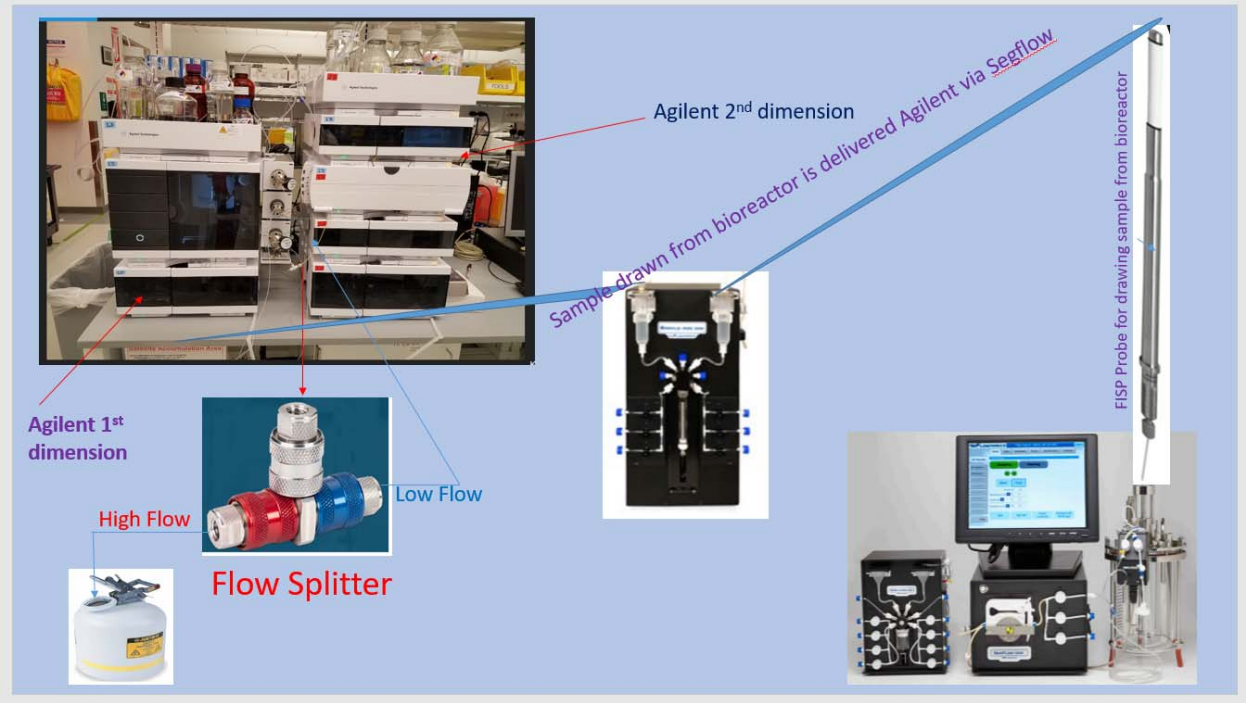

Figure 1: Schematic of 2D-Agilent/SegFlow/FISP probe/Post-flow-splitter integration architecture

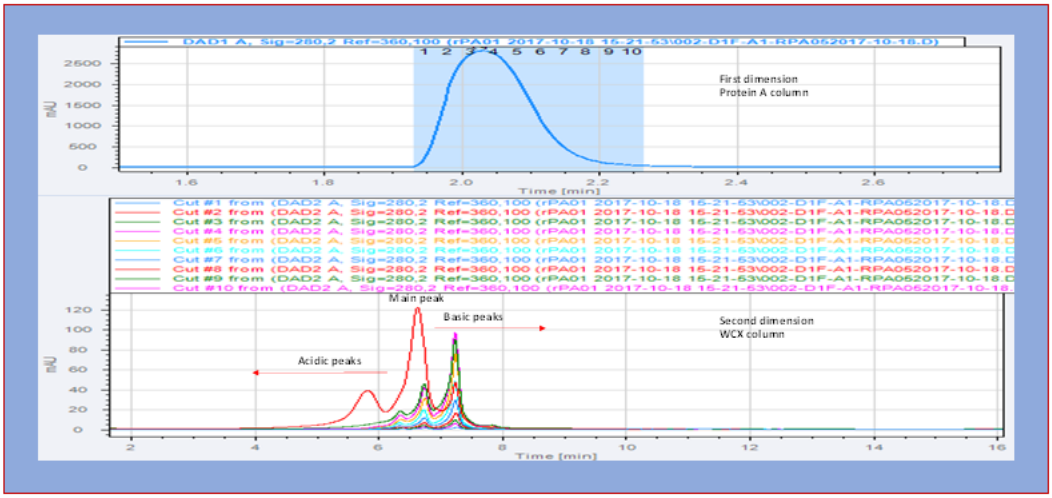

Figure 2: Representative 2nd dimension IEX chromatograms of individual fractions of 1st dimension protein-A peak, resulted from high resolution peak cutting

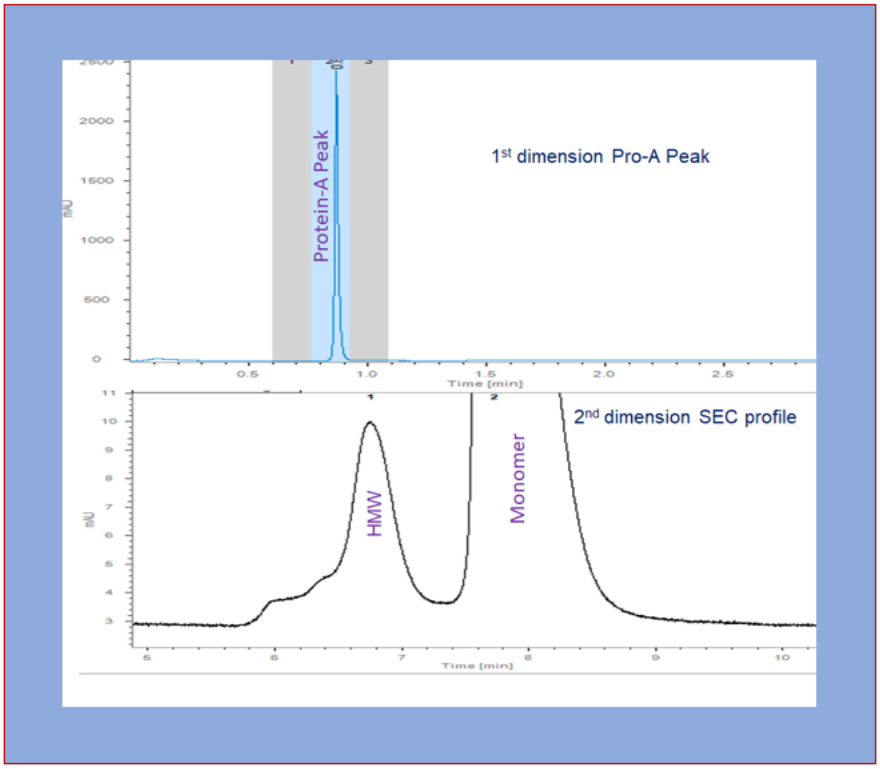

Figure 3: Representative online SEC chromatogram of Bioreactor sample using Agilent 2D-LC with ^{1}D Pro-A and ^{2}D SEC

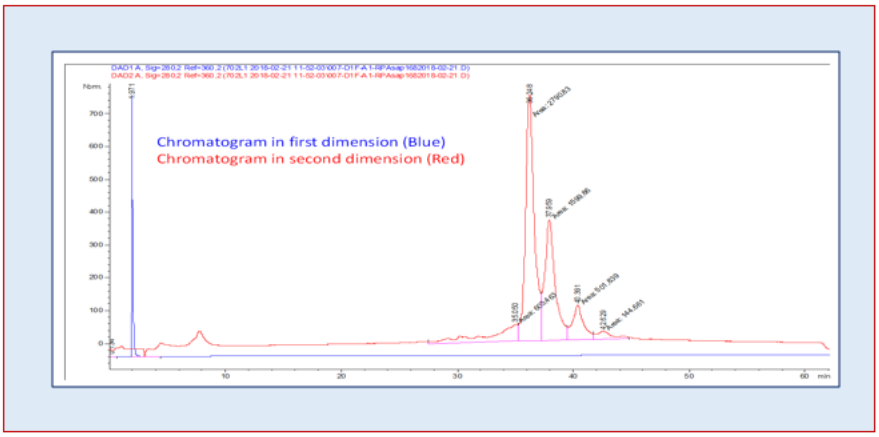

Figure 4: Representative online Weak Cation Exchange (WCX) chromatogram of Bioreactor sample using Agilent 2D-LC with 1D Pro-A and 2D CEX

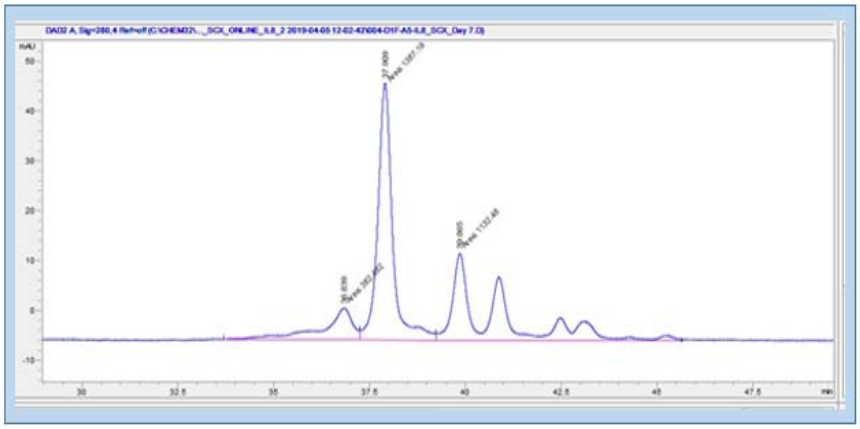

Figure 5: Representative online Strong Cation Exchange (SCX) chromatogram of Bioreactor sample using Agilent 2D-LC with ^{1}D Pro-A and ^{2}D CEX

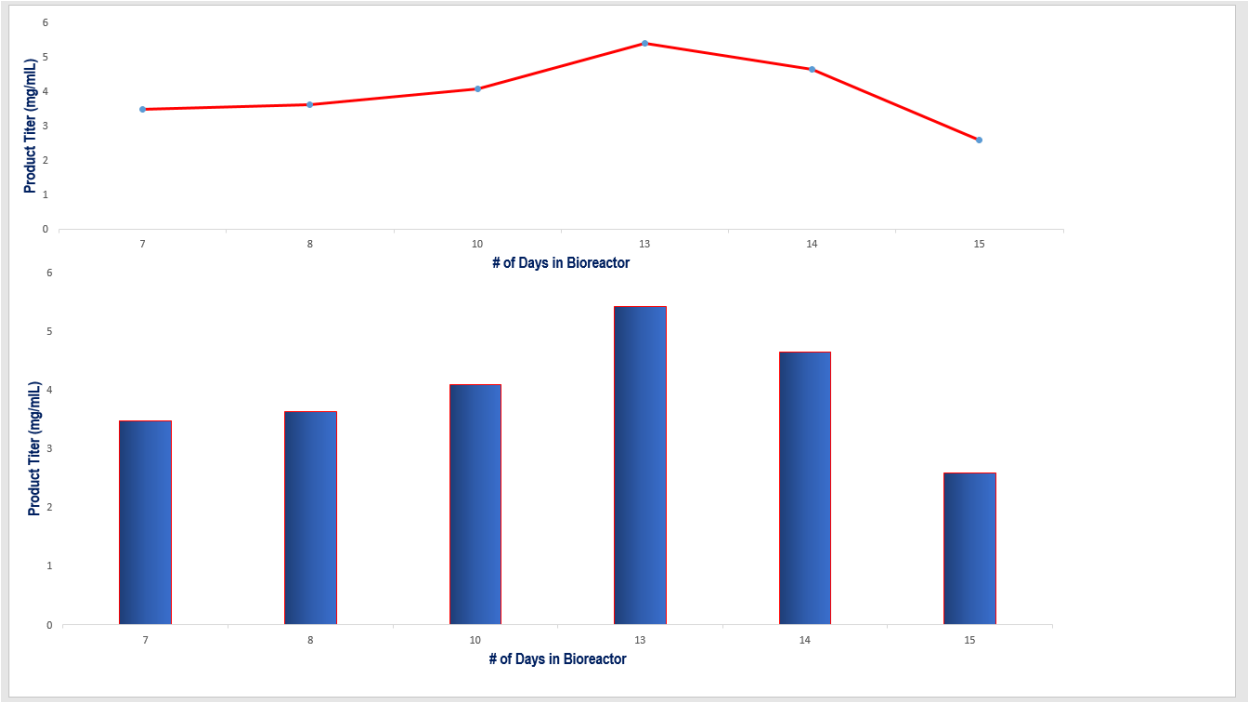

Figure 6: A typical online Titer profile generated for mAb-X using the 1st dimension of integrated Agilent 2D-LC/SegFlow

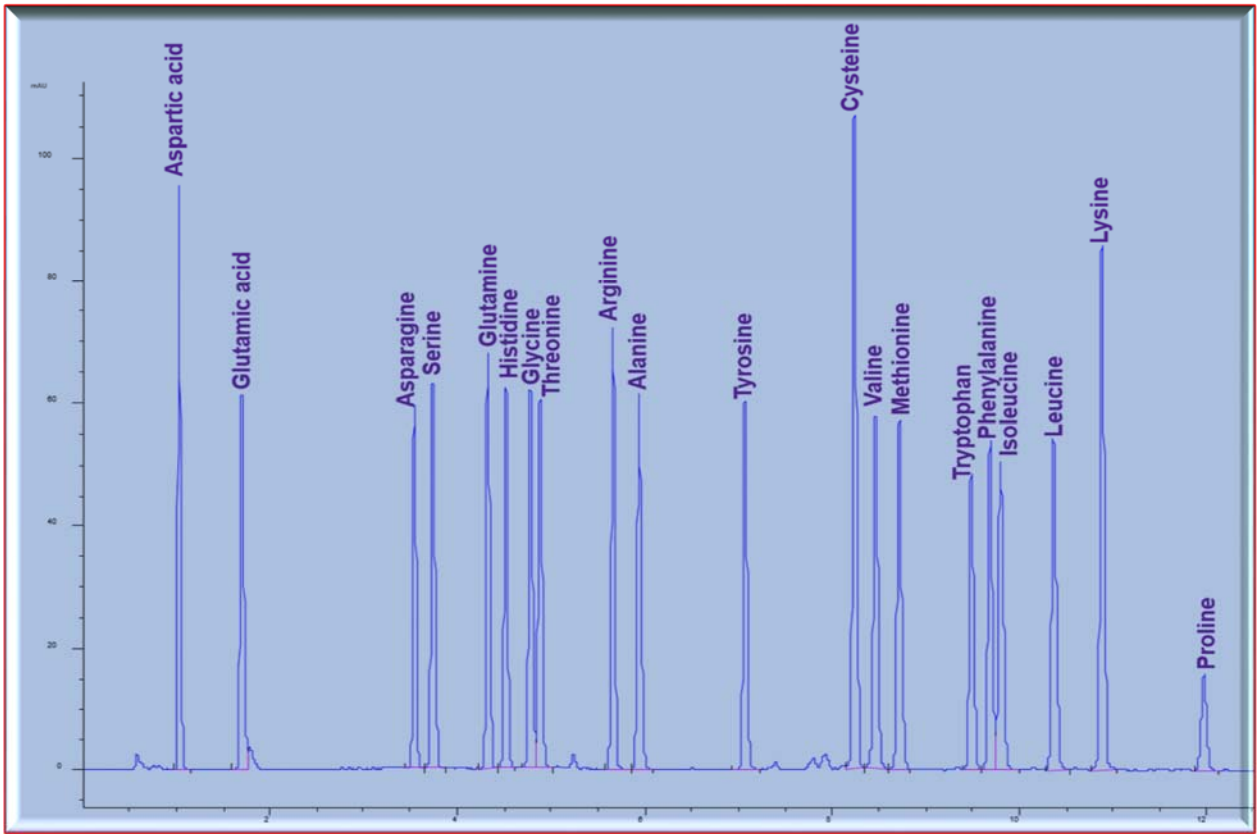

Figure 7: Representative profile of in-column OPA derivatized aminoacid standards

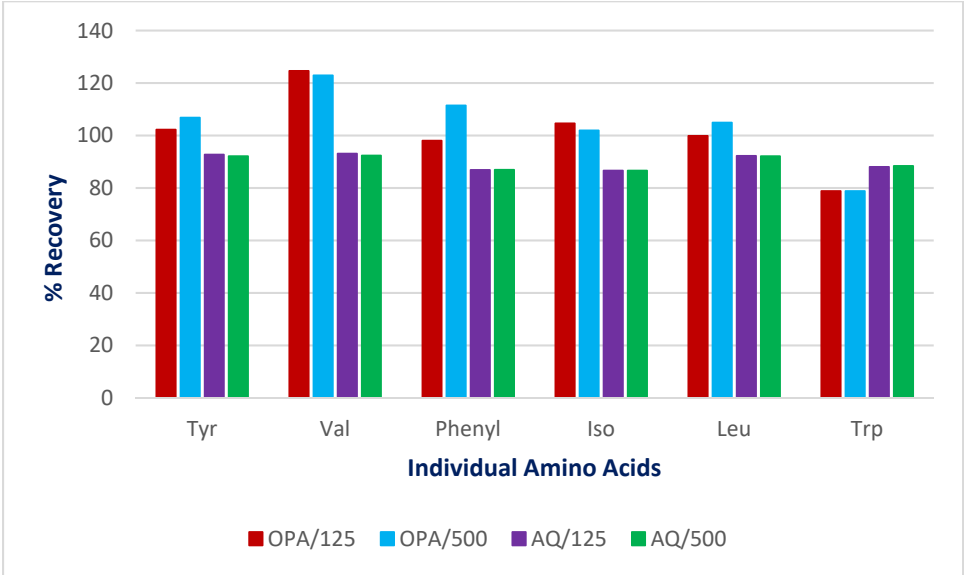

Figure 8 Spike and recovery results of amino acids spiked in cell culture media using AccQ-Tag and OPA derivatization methods