Rapid Evaluation of Protein Product Quality Attributes from Lysate Samples *via* 2D-HPLC System

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Best Poster Award Participant

Introduction

A two-dimensional (2D) HPLC method was established to enable early product peak identification and to expedite product quality assessment of therapeutic proteins produced using The Pelican Expression Technology™ (PET), a bacterial recombinant expression system based on *Pseudomonas fluorescens*.

Purpose

This method enables the saving of both time and resources by accelerating analytical workflow during process development focused on quantitative and qualitative assessment of therapeutic proteins in lysate samples without the need to develop enrichment strategies prior to analysis.

Objective

To capture two example target proteins direct from clarified lysate by ion-exchange chromatography (e.g., AEX, CEX) in the first-dimension, and subsequently evaluate purity of the target proteins by reversed-phase chromatography in the second-dimension.

Experimental

2D-LC System Configuration: Agilent 1290 UHPLC Infinity II 2D-LC Solution **(Fig. 1)**

Columns				
	Manufacturer	**Model**	**Particle Size**	**Dimension**
First Dimension	Thermo Fisher	ProPac™ WCX-10	10µm	4.6x50mm
	Thermo Fisher	ProPac™ SAX-10	10µm	4.6x50mm
Second Dimension	Waters™	BioResolve RP mAb Polyphenyl	2.7µm, 450Å	2.1x150mm
	Agilent	PLRP-S	8µm, 1000Å	4.6x150mm

	Gradient	**Detection**	**Mobile Phase A**	**Mobile Phase B**
First Dimension	0-100% B in 4 min (CEX & AEX)	DAD at 280 nm	20mM Sodium citrate, pH 5.1 (CEX) 20mM Sodium phosphate, pH 8.0 (AEX)	20mM Sodium citrate, 1M Sodium chloride pH 5.1 (CEX) 20mM Sodium phosphate, 1M Sodium chloride, pH 8.0 (AEX)
Second Dimension	30-45% B in 9 min at 80°C (CEX-RP) 20-60% B in 10min at 70°C (AEX-RP)	FLD; Excitation at 280 nm Emissions at 317nm (AEX-RP) and 343nm (CEX-RP)	0.1% Formic acid, 0.02% Trifluoroacetic acid in Water (CEX-RP) 0.1% Trifluoroacetic acid in Water (AEX-RP)	0.1% Formic acid, 0.02% Trifluoroacetic acid in Acetonitrile (CEX-RP) 0.1% Trifluoroacetic acid in Acetonitrile (AEX-RP)

Sample Preparation and Loading: Protein 1 lysate sample was prepared via microfluidization and pH clarification of fermentation harvest, while Protein 2 lysate was processed by sonication and centrifugation using automation. Samples with titer <1mg/mL were injected neat at 10µg target protein load; Otherwise, samples were diluted to 1mg/mL with 1X PBS prior to injection.

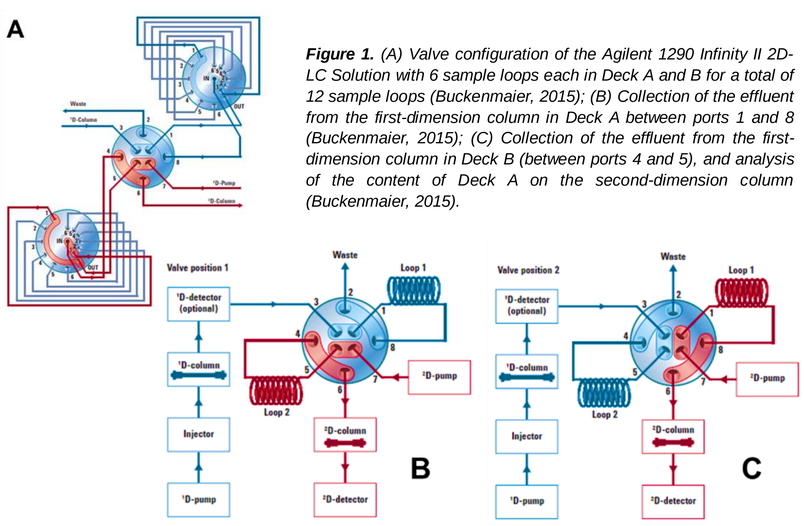

Figure 1. *(A) Valve configuration of the Agilent 1290 Infinity II 2D-LC Solution with 6 sample loops each in Deck A and B for a total of 12 sample loops (Buckenmaier, 2015); (B) Collection of the effluent from the first-dimension column in Deck A between ports 1 and 8 (Buckenmaier, 2015); (C) Collection of the effluent from the first-dimension column in Deck B (between ports 4 and 5), and analysis of the content of Deck A on the second-dimension column (Buckenmaier, 2015).*

Results

Protein 1: 48 kDa Protein

Strategies to assess product quality (PQ) of Protein 1 relied on bench-scale purification from fermentation pastes, which takes considerable time and limits the number of samples for early process development decision making.

Thus, a 2D-LC method was established to provide purity analysis direct from clarified lysate samples. In the first-dimension (Figure 2), the protein is successfully captured using cation exchange (CEX) chromatography column (ProPac™ WCX-10) (Figure 2A) and separated into two fractions using High-Resolution (HiRes) sampling (Figure 2B) for purity analysis by reverse phase (RP) column (Waters™ BioResolve Polyphenyl) in the second-dimension (Figure 3A and 3B). In HiRes sampling, target proteins can be determined by collecting several small fractions consecutively over a selected time range from the first-dimension chromatogram.

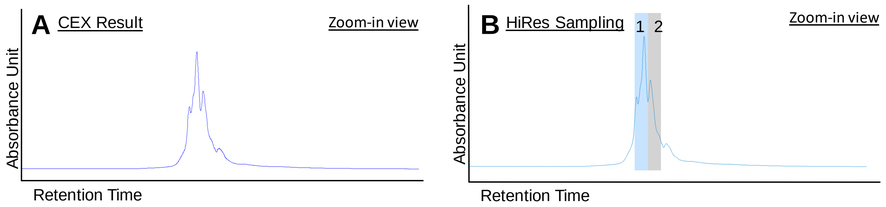

Figure 2*. Capture by CEX in the first-dimension. (A) Profile of the effluent from the first-dimension by CEX column; (B) HiRes sampling fractions of the effluent from the first-dimension by CEX column.*

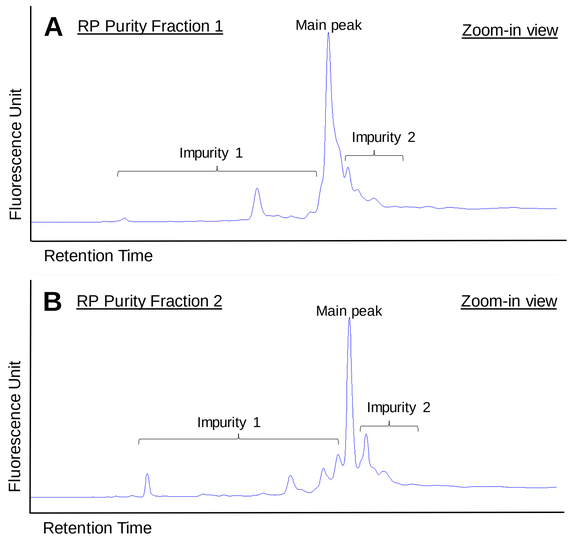

Figure 3. *Purity analysis by RP in the second-dimension. (A) Purity profile of the first fraction by RP column in the second-dimension; (B) Purity profile of the second fraction by RP column in the second-dimension.*

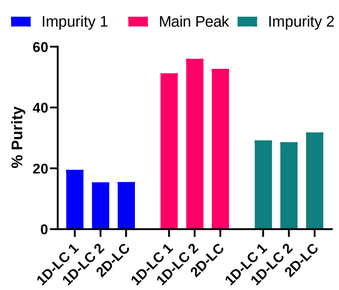

Figure 4. *Graph highlighting the comparison of protein percent purity between 1D-LC and 2D-LC methods.*

The purity result of the 2D-LC method was found to be comparable to the bench-scale purification samples analyzed by 1D-LC RP. Figure 4 features the comparison highlighting the comparable percent purity of the main peak and the impurities observed in these samples.

Therefore, the 2D-LC is a reliable and sufficient method for early identification and product quality assessment of this protein that provides excellent qualitative and quantitative results.

Protein 2: Enzyme (136 kDa)

To demonstrate that the 2D-LC method can be applied to other protein formats, an alternative capture method by anion exchange (AEX) chromatography in the first-dimension was used to address a wider range of protein isoelectric point (pI) values. Protein 2 has a more acidic pI value than Protein 1 and can be captured with anion-exchange (AEX) chromatography.

A 2D-LC method was established using AEX column (ProPac™ SAX-10) to capture Protein 2 in the first-dimension (Figure 5), then separated into several fractions using Multiple Heart-Cutting (MHC) sampling for purity analysis by RP column (Agilent PLRP-S) in the second-dimension (Figure 6). In MHC sampling, only fractions of selected peaks in the first-dimension are sampled for further analysis.

Results Continued

The chromatogram profile from the first-dimension (Figure 5A) showed multiple unretained peaks during the binding period, multiple product peaks of interest during the elution period, along with a single DNA-related impurity peak. Due to multiple peaks during the elution period, Multiple Heart-Cutting (MHC) sampling was performed (Figure 5B) to selectively collect several peaks, determine the target protein, and analyze its purity. The results of MHC sampling (Figure 6A) showed the purity profiles of each fraction, in which only the first and second fractions contained the target protein. The profiles of fraction 1 and 2 (Figures 6B & 6C) showed a strong main signal with smaller impurity peaks pre- and post-main peak.

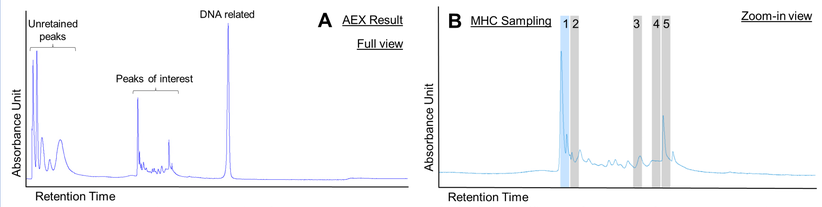

Figure 5*. Capture by AEX in the first-dimension. (A) Profile of the effluent from the first-dimension by AEX column; (B) MHC sampling fractions of the effluent from the first-dimension by AEX column. "F" indicates "Flush" to rinse the tubing prior to analysis of subsequent fractions.*

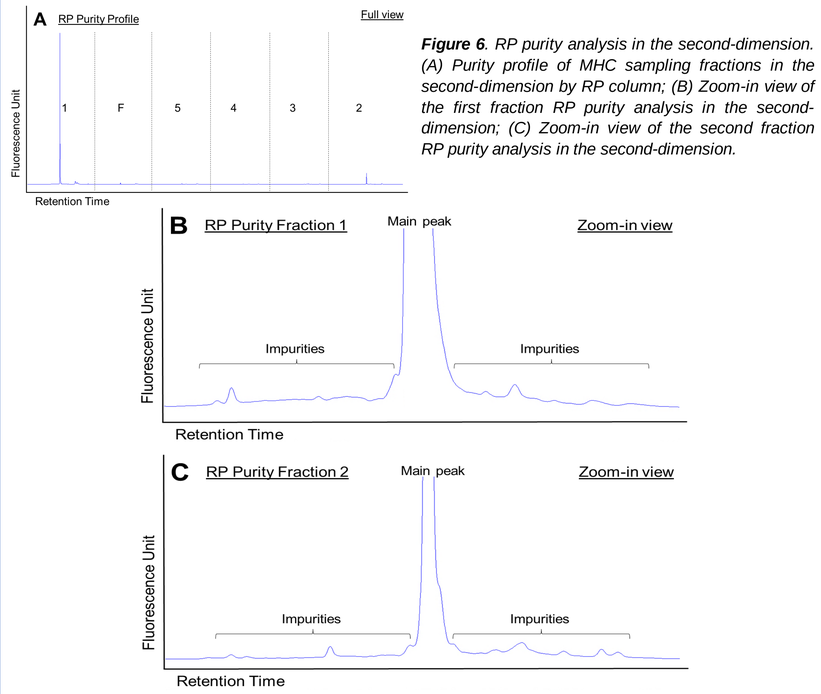

Figure 6*. RP purity analysis in the second-dimension. (A) Purity profile of MHC sampling fractions in the second-dimension by RP column; (B) Zoom-in view of the first fraction RP purity analysis in the second-dimension; (C) Zoom-in view of the second fraction RP purity analysis in the second-dimension.*

These profiles look comparable to a previous result from 1D-LC RP purity analysis of a bench-scale purification sample. The percent purity of fraction 1 in the 2D-LC method was found to be 99.1%, which is comparable to the 1D-LC RP purity of 99.0%. Therefore, an alternative capture method by AEX chromatography was shown to be a reliable and sufficient addition to the 2D-LC method platform with excellent qualitative and quantitative results for early peak identification and PQ assessment.

Conclusion

- A versatile 2D-LC method was established to assess purity of target proteins directly from lysate samples by ion-exchange chromatography capture (e.g., CEX & AEX) in the first-dimension and reversed-phase chromatography in the second-dimension.
- The 2D-LC method provided excellent qualitative and quantitative data comparable to the previously used two-step process entailing bench-scale capture purification followed by 1D-LC RP purity analysis.
- Overall, 2D-LC methodology has significant potential to accelerate analytical workflows by removing the need for resource-intensive enrichment strategies post-fermentation harvest.
- Future applications of the technology include coupling LC separation techniques in the first-dimension with mass spectrometry in the second-dimension for early-stage identity analysis.

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References

- Buckenmaier, S. (2015) *Agilent 1290 Infinity 2D-LC Solution for Multiple Heart-Cutting*. (Technical Overview No. 5991-5615EN). Retrieved from Agilent Technologies website: https://www.agilent.com/cs/library/technicaloverviews/public/5991-5615EN.pdf
- Krieger, S. (2014) *Automated Switching between 1D-LC and Comprehensive 2D-LC Analysis*. (Technical Overview No. 5991-4843EN). Retrieved from Agilent Technologies website: https://www.agilent.com/cs/library/technicaloverviews/public/5991-4843EN.pdf
- Stephan, S. (2016) *High-Resolution Sampling 2D-LC with the Agilent 1290 Infinity II 2D-LC Solution*. (Technical Overview No. 5991-7637EN). Retrieved from Agilent Technologies website: https://www.agilent.com/cs/library/technicaloverviews/public/5991-7637EN.pdf
- Stephan, S., Krieger, S. (2020) *High Resolution Sampling 2D-LC for Pharmaceutical Impurity Analysis – Detection of Impurities Hidden Under the API Peak at Relevant Levels*. (Application Note No. 5991-8082EN). Retrieved from Agilent Technologies website: https://www.agilent.com/cs/library/applications/5991-8082EN.pdf