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Development of liquid and solid-phase extraction sample treatment methods for quantitation and purity assessment of recombinant Kunitz domain proteins in *Pichia* fermentation culture by RP-HPLC

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Background: *Pichia pastoris* is a well-established cell system for the expression of recombinant proteins. However, the complex pigments and native proteins concurrently produced by this organism have presented significant challenges when attempting to determine protein product titer and purity in fermentation samples. **Results:** A methodology of treating *P. pastoris* fermentation samples using either liquid extraction or solid-phase extraction is presented, which consistently yields over 90% recovery for recombinant Kunitz domain proteins produced via high cell density *P. pastoris* fermentation. The developed sample treatment method allows subsequent HPLC assay to not only provide similar specificity and accuracy to that offered by a validated ELISA assay for titer analysis, but also to deliver product purity information and the benefit of lower assay variability. **Conclusion:** The use of the novel sample pretreatment methods described in this work can significantly improve the viability and outcome of subsequent analyses. This includes enabling the use of reliable and easily automated HPLC assays to perform quantitative and qualitative analysis of recombinant protein products produced by *P. pastoris* cultures during fermentation process development and manufacturing control.

Kunitz domains are a family of small structured proteins characterized by a common 3D structure that is stabilized by three internal disulfide bonds [1]. Many Kunitz domains function as inhibitors of serine proteases and as such serve as potential therapeutic candidates directly or as a result of protein engineering efforts. Dyax have built several highly diverse Kunitz domain phage display libraries from which potent and specific proteinase inhibitors could be selected [2–4]. The therapeutic candidates, Kunitz domains X and Y, are both proteinase inhibitors, and expressed from *Pichia pastoris* fermentation.

P. pastoris is an attractive expression system for producing recombinant proteins because of its ability as a eukaryote to provide appropriate post-translational modifications such as human-like glycosylation, proper 3D folding and disulphide bonding, and to readily secrete the protein of interest. *P. pastoris* is a robust organism that grows rapidly on minimal media and produces high yields of the desired recombinant protein on induction with ethanol [5–7]. However, it is a significant challenge to develop robust analytical methods to accurately quantify the amount of protein product in a *P. pastoris* high cell density fermentation culture because of the high cell mass in the culture and the complex matrix of the fermentation supernatant. SDS-PAGE and western blot, bioactivity assay and

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Key Terms

Pichia pastoris high cell density fermentation: Methylotrophic yeast *Pichia pastoris* is a eukaryote tool for recombinant protein production. *P. pastoris* can be cultured in simple inexpensive media and easily scaled up in large fermenter to permit growth of high cell densities exceeding 100 g l^{-1} cell dry mass. High cell density culture, together with strong and tightly regulated methanol-inducible AOX1 promoter, drives high-level expression of recombinant proteins for biochemical research and biopharmaceutical manufacturing.

Liquid extraction: Separation process by which analytes of interest are separated from a complex liquid mixture by contacting with a second liquid based on analytes relative solubility in the carrier liquid, in which the analytes are preferentially soluble.

Solid-phase extraction: Separation process by which analyte(s) of interest are separated from a complex liquid mixture using a stationary phase (membrane or resin) based on their physicochemical properties.

ELISA, if an anti-protein product antibody is available, are common methodologies used for product quantitation in such fermentation samples [8–10]. However, ELISAs and bioactivity assays, in general, can only provide information on total protein product or active protein level, respectively. Both assays do not distinguish among product and product-related substances to provide a purity assessment, which is important for upstream fermentation process development and production process control. Although SDS-PAGE and western blot can assist with product purity evaluation to a certain degree, these assays exhibit poor quantitation accuracy, and are often unable to distinguish product variants with close molecular weights [11]. HPLC methods are the most suitable analytical assays to assess product purity, and they have been used widely in process development and manufacturing production control for recombinant proteins expressed and purified from various host systems [12]. Unfortunately, HPLC assays are typically reserved for samples collected after the initial clarification and capture steps in the downstream process, to prevent column fouling and to allow accurate identification and quantification of the target molecule and any impurities present. **Figure 1** illustrates both the column fouling issue and impurity interference found when Kunitz domain proteins in crude supernatant from a *P. pastoris* fermentation culture were directly analyzed by RP-HPLC. The RP-HPLC chromatogram of Kunitz domain protein X **(Figure 1C)** shows that when injected *P. pastoris* fermentation supernatant sample without sample pretreatment, protein X main peak and two variants peaks are co-eluted with impurities derived from *P. pastoris* culture, which prohibits the accurate measurement of product quantity and quality at fermentation production stages. Variant 1 is an N-terminal nine amino acid extension form of product X and variant 2 is originated from N-terminal cyclization of pyroglutamic acid of product X with -18 Da mass difference. Monitoring the level of these product-related species is one of the important measurements to ensure consistent operation of fermentation manufacturing process. In addition, the chromatogram comparison of protein X standard that injected before **(Figure 1A)** and after **(Figure 1B)** the injection of *P. pastoris* fermentation supernatant sample clearly displays the reduction in column performance due to fouling.

In order to utilize HPLC assays for *Pichia* fermentation sample analysis, a sample pretreatment method needs to be developed to process crude samples prior to HPLC analysis without impact on the titer and purity of protein product of interest. Different HPLC sample pretreatment methods have been used to improve sensitivity and reproducibility of analyte measurements for samples of low concentration or for samples containing massive amounts of contaminants from a complex matrix, as well as for HPLC column protection. Among commonly used HPLC sample pretreatment methods are filtration techniques used to separate liquid-containing analytes from solid contaminants; ultrafiltration/diafiltration techniques able to concentrate analytes and exchange to a compatible buffer for subsequent HPLC column injection. Protein precipitation techniques are applied for massive protein interference removal. Liquid extraction (LE) and liquid–liquid extraction are methods to separate drug substances based on their solubility in different liquid phases, while solid-phase extraction (SPE) is a separation process by which analytes of interest are absorbed from a complex liquid matrix onto a stationary phase (membrane or resin) based on their physicochemical properties and then eluted for further HPLC analysis. With easy access to a variety of chemistries for the stationary phase and high-throughput device design, SPE has become the most powerful technique available for rapid sample preparation for HPLC and mass spectrometry analysis [13–17].

In this study, we describe the development of a methodology for pretreatment of *P. pastoris* fermentation samples to facilitate target Kunitz domain proteins X and Y analysis via RP-HPLC. A LE sample treatment method was first developed for protein X. When combined with RP-HPLC assay, the concentration of protein X in *P. pastoris* fermentation sample measured by LE-RP-HPLC method achieved good correlation with the concentration determined by a bioactivity assay but exhibited much better assay precision. The method was successfully transferred to a manufacturing site and used for process control monitoring. However, when the same method was applied for protein Y, large vari-

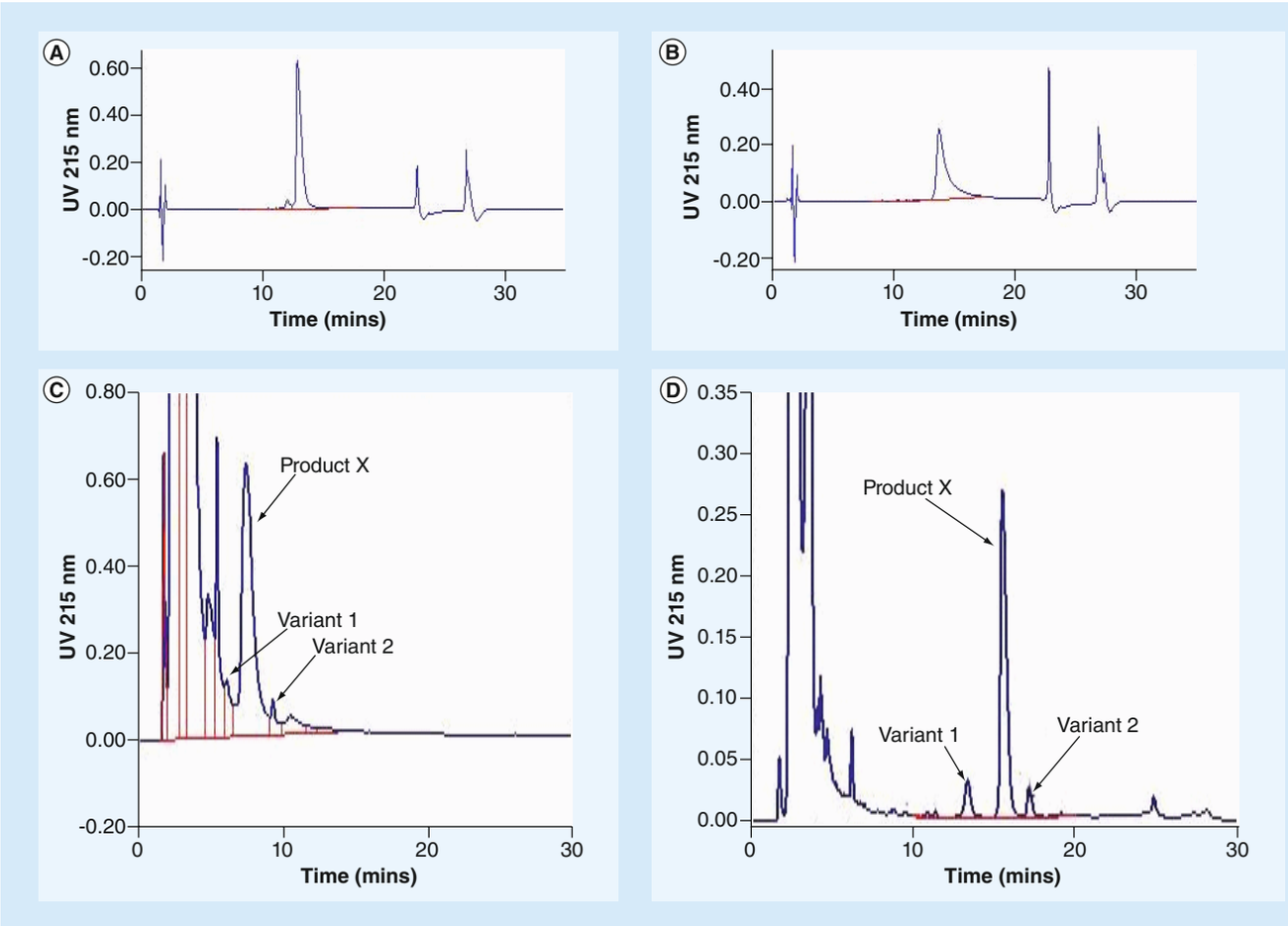

Figure 1. Chromatograms illustrating the significant column fouling and impurity interference typically observed when analyzing clarified *Pichia pastoris* fermentation supernatant samples via RP-HPLC. (A) The chromatogram obtained via RP-HPLC for a sample of highly purified Kunitz Domain protein X standard solution. **(B)** The chromatogram obtained for the same standard solution when the RP-HPLC column has previously been used to analyse a sample of clarified *P. pastoris* fermentation supernatant, demonstrating the significant and persistent column fouling produced by the fermentation supernatant sample. **(C)** The chromatogram resulting from RP-HPLC analysis of Kunitz domain protein X in clarified *P. pastoris* fermentation supernatant with no sample pretreatment. **(D)** The chromatogram of RP-HPLC analysis for Kunitz domain protein X in clarified *P. pastoris* fermentation supernatant post-liquid extraction sample treatment procedure, illustrating clear separation of protein X main peak and two variants peaks from impurities derived from *P. pastoris* culture.

ability in yield was observed, which made it difficult to detect problems of fermentation culture during manufacture and to make subsequent loading adjustments for the first capture chromatography step. A later study demonstrated that the LE method developed for protein X had a recovery issue for protein Y despite their structural similarity and similar *Pichia* fermentation operation conditions. A SPE sample preparation technique was then studied for product Y sample preparation to assist RP-HPLC analysis of *Pichia* fermentation culture samples. Cartridge type, loading and elution conditions, together with *Pichia* crude culture sample-handling protocols were systematically investigated, leading to an optimal SPE sample treatment procedure that allowed accurate measurement of protein Y levels in *Pichia* fermentation culture by RP-HPLC. A comparison study between the newly developed SPE-RP-HPLC method and a validated ELISA method for protein Y quantitation in *Pichia* fermentation samples was then conducted to confirm the reliability of SPE-RP-HPLC method results. Although our study is focused on a Kunitz domain protein product, the approach to developing a HPLC sample pretreatment method for protein products derived from a complex matrix, such as *Pichia* fermentation broth, can be extended and referenced for other applications.

Materials & methods

» Materials

Kunitz domain proteinase inhibitors (X and Y) were selected from Dyax Kunitz domain phage libraries. The gene of Kunitz domain protein was constructed

in pPIC vector and used to transform *P. pastoris* strain GS115. The recombinant Kunitz domain protein was produced by *P. pastoris* fermentation using basal salts medium with optimized glycerol and methanol feed strategy. To determine the titer of expressed Kunitz domain protein, the fermentation culture samples were subjected to the sample treatment method development described in each section.

SPE method development and analysis were performed using Oasis® MCX and Oasis HLB cartridge from (Waters, MA, USA), and Strata™-X-C, Strata-WCX and Strata-X cartridges from Phenomenex (CA, USA). RP-HPLC analysis employed Zorbax® SB-CN 3.5 mm, 4.6 × 150 mm analytical column with in-line Zorbax SB-CN analytical guard (4.6 × 12.5mm, 5 µ) column (Agilent, Santa Clara, CA, USA). 3-ml Luer-Lock syringes were from Beckman Dickenson and 0.2-µm syringe filters were from VWR. All chemical reagents were obtained from Sigma (MO, USA) or JT Baker (Mallinkrodt Baker, NJ, USA).

Analytical RP-HPLC was carried out on a Waters Alliance 2695 system (MA, USA). ELISA assay plates were analyzed using a SpectraMax M2 Plate Reader (Molecular Devices, CA, USA). A multi-syringe programmable syringe pump (Braintree Scientific, Inc., MA, USA) was used for SPE studies. Centrifugation was performed using Eppendorf centrifuges 5417R with F-45–30–11 rotor and 5804R with A-4–44 rotor.

» LE

Fermentation culture samples were centrifuged at 2880 × g for 10 min (Eppendorf centrifuge 5804R) and the supernatant decanted into a fresh 15-ml conical tube. An equal volume of 0.1% trifluroacetic acid (TFA) in acetonitrile was then added to the fermentation sample supernatant, mixed well and centrifuged at 10621 × g for 10 min (Eppendorf centrifuge 5417R). The extracted supernatant was collected and diluted by adding an equal amount of 0.1% TFA in water. The treated fermentation sample was now ready to inject on to RP-HPLC column for analysis.

» SPE

The initial SPE cartridge screening study employed several SPE cartridges with different specific separation chemistries. SPE was conducted using vendor-recommended loading, washing and elution conditions, which were modified based on knowledge of the specific Kunitz domain's chemical properties.

The final developed SPE method using Oasis HLB and Strata-X cartridges is described as follows: the cartridge was preconditioned with a volume of methanol followed by a volume of HPLC grade water. Fermentation sample supernatant pools, prepared as described in the 'Crude fermentation culture sample handling procedure' section, were loaded onto the preconditioned cartridge at defined flow rates. Bound protein was then eluted from the cartridge with 2 × 200-µl elution volumes of a 0.1% TFA, 40% v/v acetonitrile/60% water solution. Finally, the cartridge was flushed with 600 µl HPLC grade water and the flush solution was added into the elution vial to make a suitable dilution for RP-HPLC injection.

» Crude fermentation culture sample-handling procedure

A 2-ml aliquot of crude fermentation culture was serially diluted in 1:1 steps using phosphate saline buffer (PBS), pH 7.0, until the cell mass as a fraction of the total sample volume was significantly decreased to less than 1%. Supernatant samples were harvested from diluted culture samples by centrifugation and subjected to optimized SPE treatment and RP-HPLC analysis.

For a cell pellet wash study, a 2-ml aliquot of crude fermentation culture was centrifuged at 2880 × g for 5 min (Eppendorf centrifuge 5804R). The supernatant was collected. The cell pellet was sequentially washed five-times by re-suspending with 1-ml PBS buffer and centrifuging at 2880 × g for 5 min each time to collect wash solutions. The initial culture supernatant and the subsequent five wash solutions were pooled together, and filtered through a 0.2 mm filter before being subjected to optimized SPE treatment and RP-HPLC analysis.

For the finalized crude culture sample-handling protocol, a volume of crude fermentation culture (volume can vary depending on the concentration of the protein in the culture sample) was put into an appropriately sized conical centrifuge tube. 2.5-times the volume of PBS buffer was added. The tube was gently rotated end-over-end 20-times manually or for 5 min on a laboratory tube rotator. The sample was then centrifuged at 2880 × g for 10 min (Eppendorf centrifuge 5804R), the supernatant was collected and filtered through a 0.2-µm filter. The filtered supernatant was then subjected to optimized SPE treatment and RP-HPLC analysis.

» RP-HPLC analysis

The analytical RP-HPLC assay was performed on a Waters 2695 Alliance system using a Zorbax SB-CN 3.5 mm, 4.6 × 150 mm analytical column and Zorbax SB-CN analytical guard column 4.6 × 12.5 mm, 5 mm. The separation was performed with a gradient of 24–32% mobile phase B in 22 min at a flow rate 1 ml/min, where mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. Standard and test samples were injected onto

a preconditioned column and UV adsorption from 200–300 nm was collected by a PDA detector. The protein concentration in a test sample was determined from the sum of elution peak areas of product and all product-related species, based on the extracted absorption peak at 215 nm with Empower software according to a reference standard curve. The product purity of each fermentation sample was calculated as a percentage of the peak area of structurally intact protein main peak versus the integrated total area of product and contaminant peaks from the test sample.

» Spike-recovery study

Known amounts of purified Kunitz domain protein X or Y were spiked into *Pichia* fermentation supernatant or crude culture samples as a specific experiment defined to a predetermined concentration range, typically from 0.1 to 2 mg/ml. Spiked *Pichia* fermentation samples were then treated with either LE or SPE procedure prior to RP-HPLC analysis as each experiment described in results section.

» ELISA analysis

ELISA plates were coated with a specific rabbit anti-Kunitz domain antibody. After blocking with 1% BSA in PBS-Tween® solution (0.1 M phosphate buffered saline with 0.05% Tween-20), serially diluted reference standard and fermentation culture samples were added to the coated wells for 1 h incubation at room temperature. After washing the plate, a biotinylated anti-Kunitz domain secondary antibody was then added and incubated for 1 h. Followed by washing the plate, HRP-conjugated Streptavidin (KPL) was added and incubated for another hour. The amount of Kunitz domain protein presented in each well was quantified by enzyme–substrate colorimetric reaction following addition of TMP substrate solution based on OD450 reading results. The Kunitz domain titer in each test sample was calculated from the standard curve.

Results & discussion

» LE method for Kunitz domain protein X

In responding to the HPLC analysis challenge for Kunitz domain protein products in *Pichia* fermentation culture as shown in Figure 1, a reliable, reproducible and user-friendly sample pretreatment method was in demand to remove interference arising from the *Pichia* fermentation supernatant matrix and to allow RP-HPLC characterization of recombinant Kunitz domain product level and purity in whole culture broth from high cell density *Pichia* fermentations. The LE method was first explored due to its fast and easy operation, with a protocol suitable for tech transfer and use in a quality control laboratory at a manufacturing site. The procedure developed for Kunitz domain protein X was very simple, requiring 1:1 (v/v) extraction of the *Pichia* fermentation supernatant with 100% RP-HPLC mobile phase B (0.1% TFA in acetonitrile). After mixing and centrifugation, the extracted supernatant was diluted 1:1 (v/v) with 100% RP-HPLC mobile phase A (0.1% TFA in water), which resulted in a fourfold diluted fermentation culture sample that could be directly injected onto a RP-HPLC column for quantitative and qualitative analysis. To investigate any potential product loss caused by organic solvent extraction, known amounts of purified Kunitz domain protein X at 0.1, 0.25, 0.5, 1 and 2 mg/ml were spiked into *Pichia* fermentation supernatant samples. Spiked samples were then treated with the LE method prior to RP-HPLC analysis. Figure 2 presents the correlation of Kunitz domain protein X amount detected by RP-HPLC with that obtained by calculation. Satisfactory recovery for

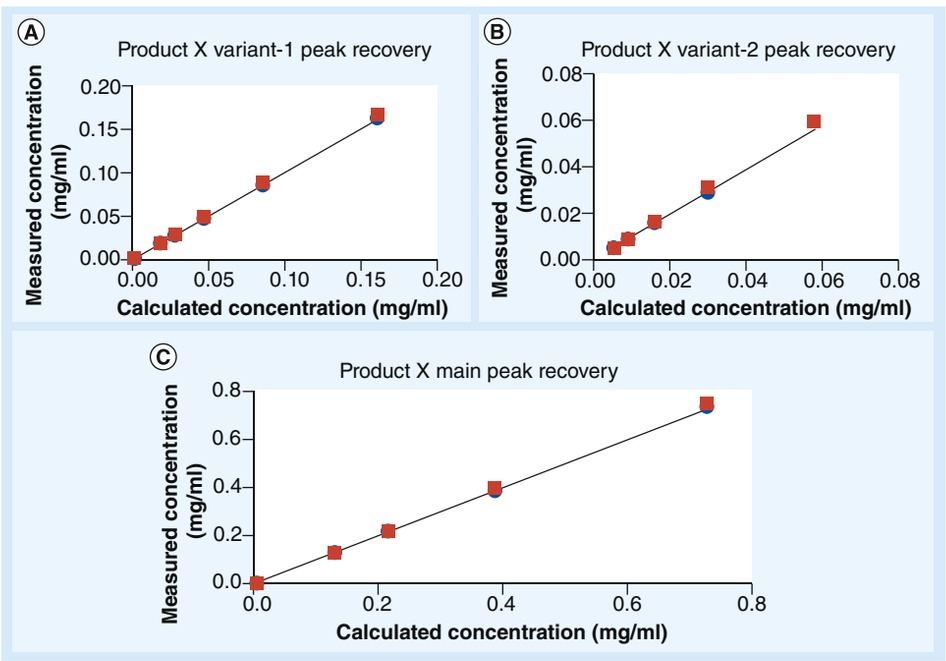

Figure 2. The recovery of the main peak and peaks of two variants of protein X by liquid extraction sample pretreatment coupled with RP-HPLC. The correlation of the amount of Kunitz domain protein X detected by RP-HPLC (red squares) with that obtained by calculation (blue circles) was plotted for the main product peak **(C)** and the peaks of two variants **(A and B)**, demonstrating the satisfactory recovery for all three peaks.

the three peaks (101.99–103.94% for variant-1 peak, 102.68–104.01% for main peak of Kunitz protein X, and 94.41–107.81% for variant-2 peak) demonstrated no significant loss of Kunitz protein X and its two variants in fermentation sample with LE sample preparation. As shown in Figure 1D (compared with Figure 1C), LE sample pretreatment and further optimization of the RP-HPLC separation gradient have dramatically improved the sensitivity and accuracy of RP-HPLC assay. Post-LE sample treatment procedure, protein X main peak and two variant peaks are well separated from impurities derived from *P. pastoris* culture, which allows the accurate measurement of product quantity and quality during upstream production process stages. The LE sample pretreatment method was transferred to the manufacturing site and coupled with RP-HPLC analysis to successfully monitor Kunitz domain protein X productivity in *Pichia* fermentation and facilitate the control of the levels of two product-related variants.

The LE method developed for Kunitz domain protein X was subsequently applied to Kunitz domain protein Y. However, the calculated capture step yield from manufacturing production batches varied from 60% to approximatley 200% when using protein Y titer in *Pichia* fermentation measured by LE-RP-HPLC method (Figure 3A). Further investigation found that when protein Y was spiked into either PBS or 50 mM Nacitrate buffer at pH 3.5 (similar to the *Pichia* fermentation harvest supernatant pH),

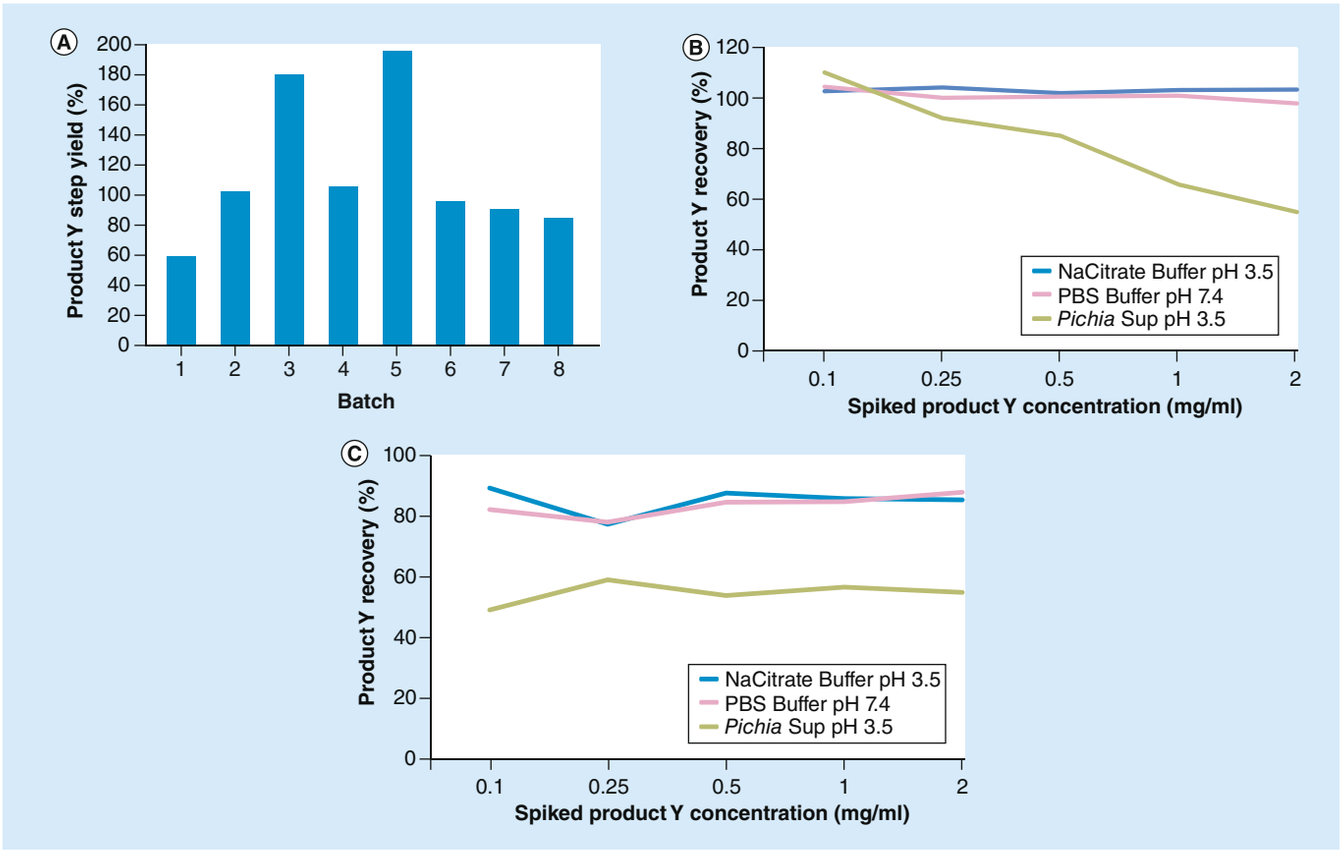

Figure 3. Matrix effects on RP-HPLC quantitation of Product Y following liquid extraction and solid phase extraction sample pretreatment. (A) Variation in capture step yield of the manufacturing production process when using RP-HPLC analysis coupled with liquid extraction sample pretreatment procedure. Protein Y recovery for the capture step was calculated using the amount of protein Y in *Pichia pastoris* fermentation culture divided by the amount in the capture step purified product bulk. **(B)** Matrix effect on the recovery of protein Y with liquid extraction sample preparation. Purified Kunitz domain protein Y was spiked into three different matrices: 1X phosphate saline buffer (red line), 50 mM NaCitrate buffer (blue line) at pH 3.5 and *Pichia pastoris* fermentation supernatant sample (green line) at the concentration of 0.1, 0.25, 0.5, 1 and 2 mg/ml. Spiked samples were then treated with the liquid extraction pre-sample treatment procedure prior to RP-HPLC analysis. The recovery of protein Y was plotted over the test concentration range among different matrices. **(C)** Matrix effect on the recovery of protein Y with solid phase extraction sample preparation. Similar to **(B)**, purified Kunitz domain protein Y was spiked into three different matrices: 1X phosphate saline buffer (red line), 50 mM NaCitrate buffer (blue line) at pH 3.5 and *Pichia pastoris* fermentation supernatant sample (green line) at the concentration of 0.1, 0.25, 0.5, 1 and 2 mg/ml. Spiked samples were then treated with an un-optimized solid phase extraction sample pre-treatment procedure using a Waters Oasis® HLB cartridge according to vendor manual instruction prior to RP-HPLC analysis. The recovery of protein Y was plotted over the test concentration range for three different matrices.

using the LE-RP-HPLC method the detected protein Y agrees very well with the expected recovery (CV% ± 5%). However, when protein Y was spiked into null strain *Pichia* fermentation supernatant, the detected protein level determined using the LE-RP-HPLC method decreased as the spiked protein amount increased from 0.1 to 2 mg/ml (Figure 3B). A clear indication that components of the *Pichia* fermentation supernatant matrix prevent the accurate measurement of protein Y level with the current LE sample treatment method despite the 58% protein sequence homology, 45% sequence identity and very similar *Pichia* fermentation process conditions between protein X and protein Y.

» SPE method evaluation for Kunitz domain protein Y

SPE is a commonly used sample preparation technique to remove interference from the sample matrix [18]. Most common SPE sorbents are categorized by the nature of their retention mechanism with the analyte of interest, including reverse phase, normal phase and ion exchange. Since Kunitz domain protein Y is an aqueous soluble small protein, both reverse-phase and ion-exchange SPE cartridges from two manufacturers (Waters and Phenomenex) were screened for their ability to bind protein Y and to remove interferences from the *Pichia* fermentation supernatant matrix. The SPE sample treatment together with in-process RP-HPLC methods were evaluated for protein Y detection in *Pichia* fermentation culture. Known amounts of purified protein Y were spiked into aliquots of null strain *Pichia* fermentation supernatant at concentrations of 0.05, 0.1, 0.25, 0.5, 1 and 2 mg/ml. Spiked supernatant samples were treated with different SPE cartridges according to the manufacturers' instructions [19,101], and cartridge eluates were subjected to RP-HPLC analysis. The recovery of protein Y was calculated using the concentration detected by the SPE-RP-HPLC method divided by the spiked amount in the sample. The reverse-phase SPE cartridge demonstrated the most promising recovery compared with various ion exchange SPE cartridges. Both strong and weak cation exchange SPE cartridges were later further studied for possible elution optimization with increasing organic solvent strength and pH optimization, and both strategies failed to yield desired separation of protein Y from interfering impurities [Chen J, Unpublished Data].

The reverse-phase SPE cartridge showed the best recovery among tested cartridges, however, the recovery of protein Y spiked in fermentation supernatant matrix was only approximately 50%, significantly lower than the recovery in a buffer matrix (both PBS and 50 mM NaCitrate buffer, pH 3.5), as shown in Figure 3C. It is clear that, similar to the LE method for protein Y, interfering components also exist in *Pichia* fermentation supernatant matrix for SPE method. The difference between LE and SPE for protein Y fermentation supernatant samples revealed that the SPE-RP-HPLC method was able to deliver consistent recovery across a range of spiked protein Y concentrations from 0.1–2 mg/ml, while detected protein Y concentration by LE-RP-HPLC decreases as spiked protein concentration increases across the same concentration test range (Figure 3B vs C). These observations suggested that, with additional method development to overcome the residual matrix interference, the SPE-RP-HPLC method has the potential to provide an accurate measurement of protein Y levels in the *Pichia* fermentation supernatants

» SPE method optimization for Kunitz domain protein Y

Elution buffer strength study

The low recovery of protein Y with LE sample treatment suggested that high organic acetonitrile solvent strength could precipitate protein Y when it was in *Pichia* fermentation supernatant. Therefore, elution conditions were investigated to determine the optimal organic solvent content in the elution buffer for SPE-HLB cartridge method development. Purified protein Y was spiked into null strain *Pichia* fermentation supernatant at a defined concentration close to the levels seen at production scale. The same amount of spiked fermentation supernatant samples was loaded onto a set of SPE-HLB cartridges in parallel and then eluted with solutions containing increasing amounts of acetonitrile. Figure 4A showed that when the acetonitrile percentage is 20% or below, bound protein Y cannot be eluted effectively from the SPE-HLB cartridge. On the other hand, with an acetonitrile percentage of 60% or higher, the amount of protein Y eluted from the SPE-HLB cartridge also decreased due to precipitation on the cartridge resin. The optimal acetonitrile solvent strength for protein Y elution was found in the range of 30 to 50%. The spike-recovery experiment was then repeated across wider concentration range from 0.1–2 mg/ml using a modified 0.1% TFA, 40% acetonitrile elution buffer. The protein Y recovery from *Pichia* fermentation supernatant matrix determined by SPE-RP-HPLC method increased to 80%, similar to the two buffer matrices shown in Figure 3C. Thus, optimized elution buffer allows 80% recovery of protein Y regardless of its matrix system. However, total recovery of protein Y was only approximately 80% thereby requiring further investigation and method optimization.

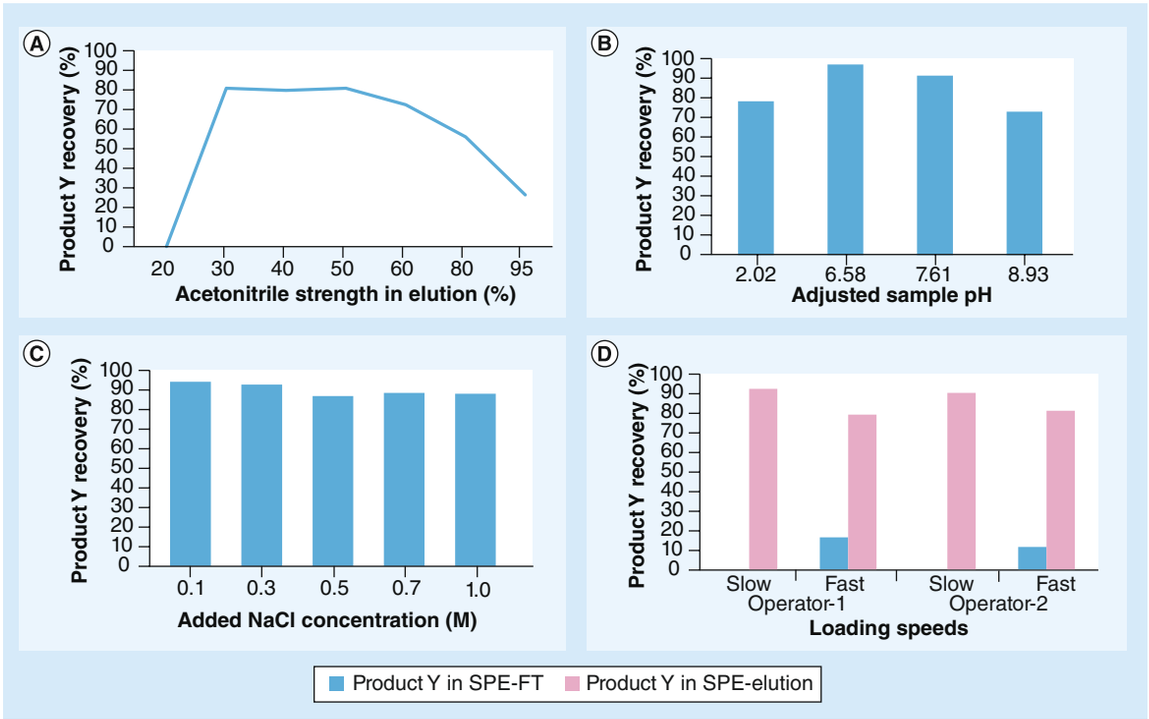

Figure 4. Optimization of the solid-phase extraction method for Product Y recovery. (A) Acetonitrile concentration optimization for eluting product Y from solid-phase extraction (SPE)-HLB cartridge. Purified protein Y stock was spiked into null strain *Pichia pastoris* fermentation supernatant. The same amount of spiked protein Y sample was loaded onto multiple SPE-HLB cartridges and then eluted with increasing amounts of acetonitrile, from 20 to 95% acetonitrile in water. The level of protein Y in SPE elution was measured by RP-HPLC and recovery calculated against the spiked amount in the sample. The recovery of protein Y was plotted against different elution conditions to illustrate the impact of organic solvent strength on protein recovery from HLB-SPE treatment. **(B)** Sample pH optimization for product Y binding on SPE-HLB cartridge. Same amount of purified protein Y was spiked into null strain *P. pastoris* fermentation supernatant samples. Sample pH was adjusted to the desired test pH in the range of 2-9 by addition of 1 M citric acid or 1 M Tris-base. Adjusted samples were then incubated at room temperature for 30 min before being centrifuged at 10,621 × g for 10 min. An equivalent amount of spiked protein Y sample was loaded onto an SPE-HLB cartridge and then eluted with optimized elution solution (40% acetonitrile/water). The protein Y level in the SPE elution was measured by RP-HPLC and recovery calculated against the spiked amount in the sample. The recovery of protein Y was plotted across different sample pH conditions to elucidate the effect of sample pH on protein recovery from HLB-SPE treatment. **(C)** Sample ionic strength optimization for product Y binding on SPE-HLB cartridge. Similar to the experiment described in **Figure 4B**, same amount of purified protein Y was spiked into null strain *P. pastoris* fermentation supernatant samples. Sample ionic strength was adjusted by sodium chloride addition in the range of 0.1–1 M. Adjusted samples were then incubated at room temperature for 30 min before being centrifuged at 10,621 × g for 10 min. An equivalent amount of spiked protein Y sample was loaded onto an SPE HLB cartridge and then eluted with optimized elution solution (40% acetonitrile/water). The protein Y level in the SPE elution was measured by RP-HPLC and recovery calculated against the spiked amount in the sample. The recovery of protein Y was plotted across different sample ionic strength conditions to show the effect of sample ionic strength on protein recovery from HLB-SPE treatment. **(D)** The impact of sample loading speed on protein Y recovery by SPE. Same amount of purified protein Y was spiked into null strain *P. pastoris* fermentation supernatant samples. The samples were treated using optimized loading and elution conditions with two defined loading speeds (slow and fast) by SPE procedure. Flow-through samples (blue bar) and SPE elution (red bar) were analyzed by RP-HPLC. The recovery of protein Y was determined as the percentage of the amount detected by RP-HPLC compared to the amount spiked into the sample.

Sample binding pH and ion strength study

pH and ion strength may play a role in affecting analyte retention on reverse-phase SPE cartridges. Depending on the pI value of the analyte, the binding pH for optimal retention may vary [20]. Therefore, the influence of both pH and ion strength on protein Y recovery using the identified optimal SPE elution condition were investigated. As was the case for the elution buffer study, purified protein Y was spiked into null strain *Pichia* fermentation supernatant at a final concentration close to the expected production level. Spiked fermentation supernatant samples normally have an ionic strength of approximately 22 mS/cm and pH approximately 3.5–3.6. Sample pH or ionic strength was then adjusted to either the desired test pH, in the range of pH 2 to pH 9, by addition of 1 M citric acid

or 1 M Tris-base, or to the desired test ionic strength by adding additional NaCl to the added concentration of 0.1 M to 1 M. Light precipitation was observed with higher pH-adjusted samples and these samples were centrifuged at 10,621 × g for 10 min (Eppendorf centrifuge 5417R) prior to analysis of the centrifuged supernatants. All sample volumes were normalized by dilution with water following pH and ionic strength adjustment before being subjected to SPE-HLB extraction and subsequent RP-HPLC analysis. **Figure 4B** shows that sample pH has significant impact on protein Y retention on the SPE-HLB cartridge and that the product recovery increases to almost 100% at an optimal pH of 6.5. **Figure 4C** shows that sample ionic strength also influences protein Y recovery and over 90% recovery can be obtained with additional 0.1 M NaCl supplement.

Since both pH adjustment and NaCl addition independently improve protein Y recovery from *Pichia* fermentation supernatant by SPE-RP-HPLC. A spike-recovery study was designed to compare the effect of both sample adjustments for SPE extraction over a full range of product concentrations. Known amounts of purified protein Y were spiked into null strain *Pichia* fermentation supernatant to produce final levels of 0.05, 0.1, 0.25, 0.5, 1 and 2 mg/ml. Spiked samples were:

- Directly loaded onto an HLB cartridge;
- pH adjusted to pH 6.5 and then loaded onto HLB cartridge;
- Additional 0.1 M NaCl added and then loaded onto HLB cartridge;
- pH adjusted to pH 6.5 and then added additional 0.1 M NaCl prior to HLB extraction.

All samples were subjected to SPE-HLB extraction using the optimized elution buffer and performed by RP-HPLC analysis. The results indicate that although both pH 6.5 and 0.1 M NaCl sample adjustments improved protein Y recovery to 90–100%, the influence of pH showed a dominant effect, and no additional benefit obtained with NaCl addition after pH adjustment. Therefore, pH adjustment alone was sufficient to achieve satisfactory recovery [DiLeo M, Unpublished Data].

SPE-HLB cartridge sample loading & elution speed study

Operation speed is one of the parameters that could impact on SPE recovery [21]. During the SPE method development program, there was a noticeable variation in measured product recovery when different operators used the same SPE extraction protocol. An SPE loading and elution operation speed study was conducted. Spike-recovery samples were subjected to SPE-HLB extraction with slow or fast speed operation for loading and elution. Slow speed was defined as sample loading at 5 µl/s or a 0.5 ml sample loaded in 100 s, while fast speed was defined as drawing the syringe at normal speed and completing a 0.5-ml load in no more than 20 s. RP-HPLC analysis showed that without sufficient contact time between product Y in the sample and resin in the SPE-HLB cartridge, protein Y would pass into the flow through causing a 10–15% lower recovery in the SPE elution sample **(Figure 4D)**. Later, a study was conducted to define the optimal operation speed, and a programmable multi-syringe pump system (Braintree Scientific Inc., MA, USA) was used to control the extraction process to achieve a highly reproducible assay results.

Crude high density *Pichia* fermentation culture sample handling

The Kunitz domain protein product is produced by high cell density *Pichia* fermentation, which at the end of the culture contains approximately 60% cell mass. It was, therefore, important to confirm that the developed analytical method was capable of accurately measuring Kunitz domain levels in real fermenter culture. Known amounts of purified protein Y stock were spiked into crude null strain *Pichia* fermentation raw culture at 0.1, 0.25, 0.5 and 1 mg/ml and allowed to equilibrate for 1 or 24 h. Supernatants were harvested by centrifugation and then subjected to SPE-RP-HPLC analysis. The recovery of protein Y from the *Pichia* whole culture sample was calculated as the percentage of measured amount of protein Y divided by the spiked amount of product. Although the method was demonstrated to accurately measure the protein Y level in *Pichia* fermentation supernatant, as described in previous sections, the result from the study showed the recovery of spiked protein Y in crude *Pichia* fermentation culture sample was significantly lower with a simple centrifugation harvest and supernatant analysis using the optimized SPE-RP-HPLC method **(Figure 5A)**. There was, however, no significant difference in product recovery between 1 and 24 h storage post-spiking.

The discrepancy between the recovery of protein Y measured by the SPE-RP-HPLC method in crude high cell density *Pichia* fermentation culture sample compared with *Pichia* fermentation supernatant, implied that protein Y was retained in the cell pellet following centrifugation, possibly through association with *Pichia* cells or lack of total recovery of the culture supernatant following centrifugation. Therefore, two

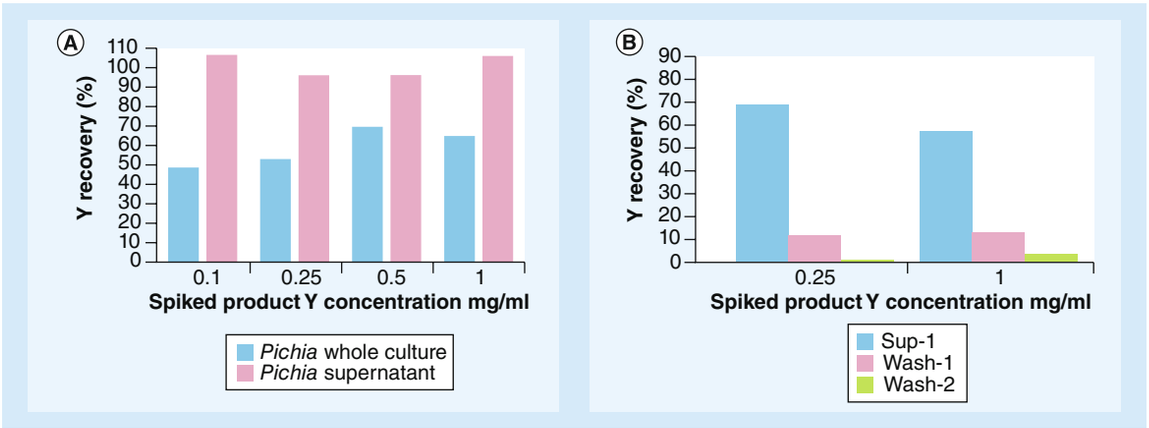

Figure 5. Effects of the presence of *Pichia* cells on Product Y recovery by the solid-phase extraction method. **(A)** The recovery comparison of protein Y in supernatant and crude culture matrix of *Pichia* fermentation. Purified protein Y was spiked into null strain *Pichia pastoris* fermentation supernatant or crude culture across the concentration range from 0.1 to 1 mg/ml. *P. pastoris* fermentation crude culture samples were then centrifuged after 1 h equilibration time post-spiking to collect the supernatant. All supernatant samples were subjected to the optimized solid-phase extraction protocol followed by RP-HPLC analysis. The recovery of protein Y was determined as the percentage of the amount detected by RP-HPLC in *P. pastoris* fermentation supernatant (red bar) or crude culture (blue bar) compared to the amount spiked into the sample. **(B)** The investigation of the cell-pellet wash procedure on the recovery of protein Y from *P. pastoris* fermentation crude culture. Purified protein Y stock was spiked into null strain *P. pastoris* fermentation crude culture at defined concentrations. Spiked culture sample was centrifuged to collect initial supernatant (blue bar). The cell pellet was then washed twice with 1 ml of phosphate saline buffer and the resulting wash solutions were also harvested by centrifugation (wash 1: red bar and wash 2: green bar). Supernatant and wash samples were subjected to optimized solid-phase extraction treatment and RP-HPLC analysis. The recovery of protein Y was determined as the percentage of the amount detected by RP-HPLC in different samples compared to the amount spiked into the sample.

experiments were designed to investigate the root cause, a cell pellet wash and serial dilution of the raw fermentation culture (method described in the section "Crude fermentation culture sample handling procedure"). A serial dilution experiment did not significantly increase the recovery, as cell mass decreased through dilution [Authors, Unpublished Data]. On the other hand, washing the cell pellet identified the source of protein loss. Higher product concentration in the culture sample required more washing steps to obtain satisfactory recovery (Figure 5B). Thus, the cell pellet wash study revealed that there is weak association between the secreted Kunitz domain and *Pichia* cell surface, which could be recovered through cell pellet wash steps. Therefore, a cycle of five washes of the cell pellet with 1-ml PBS following the initial supernatant harvest was incorporated into the sample preparation method. The entire pooled supernatant and wash samples were loaded onto an HLB cartridge and subjected to SPE-RP-HPLC analysis. The resultant measured product recovery from crude *Pichia* fermentation culture samples was now consistently between 95–100%.

There are several structural modified species of product Y generated during the fermentation process, including variant 1, methionine oxidized form of product Y, and variant 2, -18 Da form of product Y derived from N-terminal cyclization of pyroglutamic acid. Therefore, it is very important to have quick and accurate analytical method(s) to monitor the level of these product-related species during fermentation manufacturing process. RP-HPLC analysis showed that while the wash step increased product recovery, the additional handling resulted in increased oxidation of protein Y (Figure 6A). The level of oxidized variants increased as a result of the cell-pellet handling procedure. The cell-pellet handling protocol of the crude *Pichia* fermentation culture sample was therefore further modified. Instead of vigorously washing the cell pellet, the crude *Pichia* fermentation culture sample was diluted with wash buffer PBS (1:2.5 v/v), and gently rotated end-over-end 20 times manually. The supernatant was then harvested by centrifugation at 2880 × g for 10 min (Eppendorf centrifuge 5814R), and subjected to SPE-RP-HPLC analysis. Although the recovery using this protocol was slightly lower than an extensive cell pellet wash (90–95% vs 95–100%), the purity assessment is much more accurate (Figure 6B).

Lastly, all development studies described above used the reverse-phase HLB-SPE cartridge from Waters. A different SPE cartridge, Strata-X from Phenomenex, was evaluated as an alternate column. A spiked recovery study similar to that described in the 'Spike-recovery study' section, demonstrated Strata-X and HLB-SPE cartridges provided comparable outcome for product Y recovery [Chen J & Zhang Y, Unpublished Data].

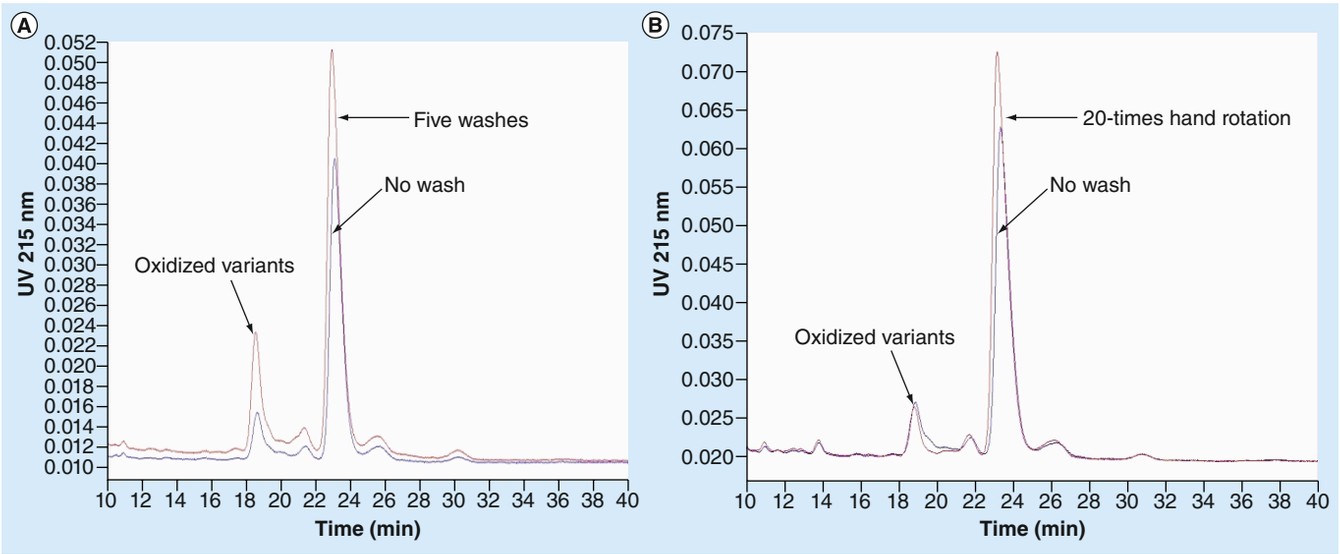

Figure 6. The impact of cell pellet washing on protein Y quality. Purified protein Y stock was spiked into null strain *Pichia pastoris* fermentation crude culture at defined concentration. The spiked culture sample was split into three equal samples. The first sample was simply centrifuged to collect the supernatant without manipulation (blue trace). The cell pellet of the second sample was washed five times with 1 ml phosphate saline buffer and the wash solutions were pooled together with initial supernatant (red trace in A). The third culture sample was diluted 2.5-fold with phosphate saline buffer and rotated 20-times before centrifugation to collect the supernatant (red trace in B). All three supernatant samples were then subjected to the optimized solid-phase extraction treatment and RP-HPLC analysis. The overlay of RP-HPLC chromatograms shows the increased recovery and the level of the oxidized variant of protein Y with cell pellet wash procedure in comparison of increased recovery but stable level of the oxidized variant of protein Y with culture rotation procedure.

» Cross-validation of SPE-RP-HPLC method & ELISA assay

Prior to SPE-HPLC, an ELISA assay has been developed and validated for measuring product Y yield directly for *Pichia* fermentation samples as described in the section "ELISA analysis". In contrast, the current SPE-RP-HPLC method evaluates the product Y yield using *Pichia* fermentation samples post-SPE. In order to confirm the accuracy and specificity of SPE-RP-HPLC method, the SPE-RP-HPLC method was compared with a validated ELISA assay according to the industry guidance for establishing accuracy [22–24]. The comparison study was conducted with spike recovery experiments at two different levels: *Pichia* fermentation whole culture and clarified supernatant samples derived from both null strain and protein Y product strain fermentation. Known amounts of purified protein Y stock were spiked into either fresh null strain or production strain *Pichia* fermentation whole culture or clarified *Pichia* fermentation supernatant at a concentration expected to be found in the manufacturing production culture. Spiked *Pichia* fermentation samples were split for either SPE-HPLC analysis or ELISA analysis. The concentration of detected protein Y determined by both analytical assays was plotted against the theoretical spiked protein Y values (Figure 7A–D). The study demonstrated that both SPE-RP-HPLC and ELISA methods exhibited consistent and good recovery in both matrices.

Although both SPE-RP-HPLC and ELISA methods provide consistent and satisfactory measurement of protein Y level in *Pichia* fermentation samples, the assay variation observed with the ELISA method is larger than that seen with the SPE-HPLC method. Table 1 shows that the assay variation of the newly developed SPE-RP-HPLC method is below 10% (1.89–8.57%) for detecting five different protein Y levels in the range of 0.1 mg/ml to 1 mg/ml over three different days by two operators. In contrast, the assay variation of the currently used validated ELISA method varies from 1.95% to 28.31%. In addition, the SPE-RP-HPLC method also provides product purity information and product variants distribution in the fermentation culture sample, which is important for manufacturing process control. Finally, SPE method is able to achieve similar or better assay sensitivity as ELISA method through sample loading volume adjustment based on product concentration in testing samples, which successfully assisted the capture resin binding capacity curve study during process development and optimization.

Future perspective

In this study, a systematic approach to the development of a pre-HPLC sample treatment method for recombinant protein quantification and purity analysis of samples from high cell density *Pichia*

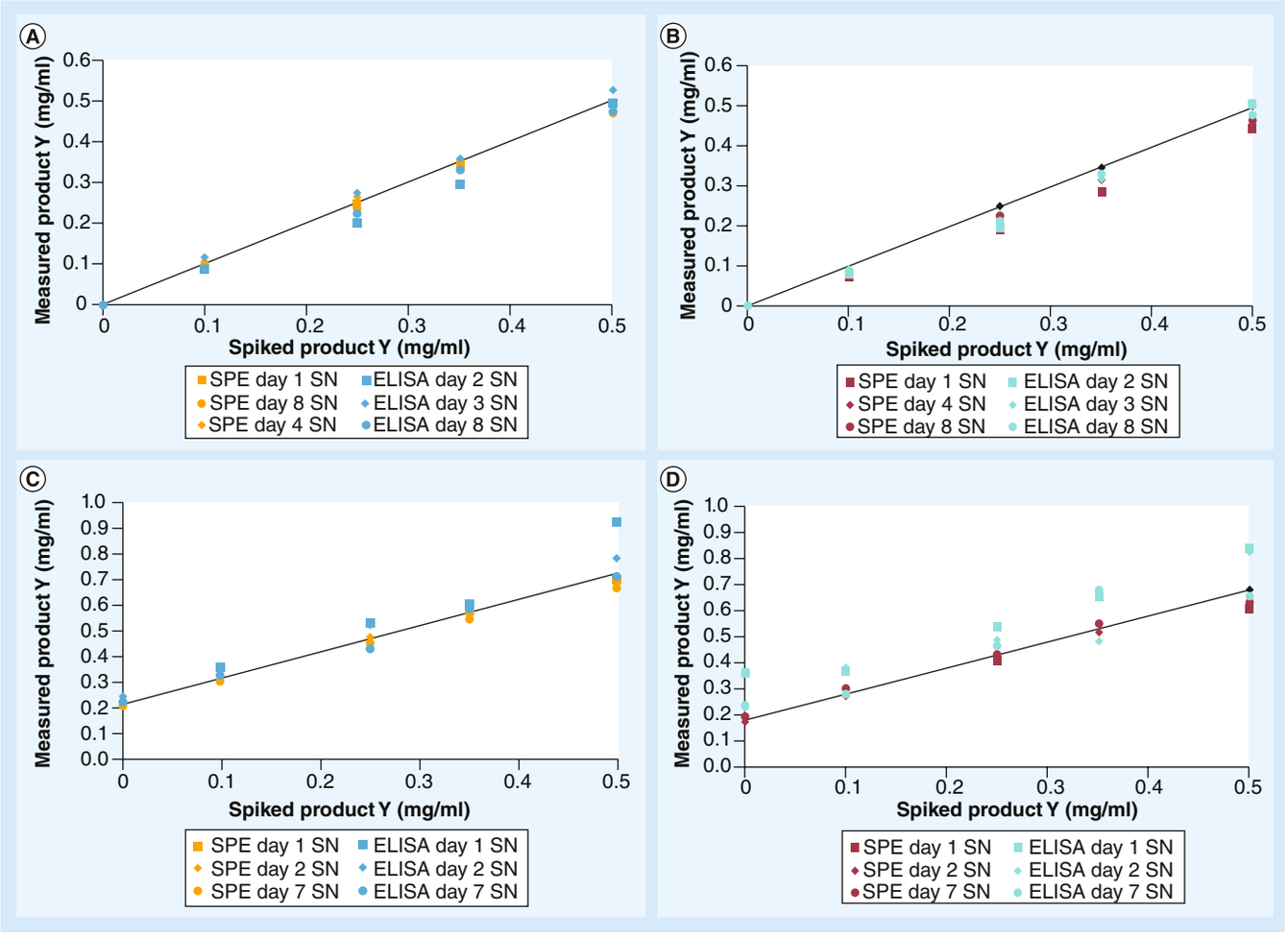

Figure 7. Comparisons of solid-phase extraction/RP-HPLC and ELISA methods for the quantitation of Product Y in *Pichia pastoris* fermentation supernatant and crude broth samples. (A) Comparison study of protein Y recovery in null strain *Pichia pastoris* fermentation supernatant and **(B)** crude culture determined by solid-phase extraction (SPE)/RP-HPLC analysis (red symbols) versus ELISA assay (green symbols). Purified protein Y was spiked into null strain *P. pastoris* fermentation supernatant or crude culture across the concentration range from 0.1 to 0.5 mg/ml. All samples were subjected to both ELISA and SPE using optimized protocol followed by RP-HPLC analysis. The amount of protein Y determined by either ELISA or SPE/RP-HPLC was plotted against the theoretic amount calculated by spiking. **(C)** Comparison study of protein Y recovery in manufacturing production *P. pastoris* fermentation supernatant and **(D)** crude culture determined by SPE/RP-HPLC analysis (red symbols) versus ELISA assay (green symbols). Purified protein Y was spiked into manufacturing production *P. pastoris* fermentation supernatant or crude culture across the concentration range from 0.1 to 0.5 mg/ml. All samples were subjected to both ELISA and SPE using optimized protocol followed by RP-HPLC analysis. The amount of protein Y determined by either ELISA or SPE/RP-HPLC was plotted against the theoretical amount calculated by spiking.

Table 1. Comparing assay variation (CV%) of solid-phase extraction-HPLC versus ELISA.

Product Y concentration (mg/ml)	Solid-phase extraction-RP-HPLC				ELISA			
	Null strain fermentation supernatant (%)	Product Y strain fermentation supernatant (%)	Null strain fermentation whole culture (%)	Product Y strain fermentation whole culture (%)	Null strain fermentation supernatant (%)	Product Y strain fermentation supernatant (%)	Null strain fermentation whole culture (%)	Product Y strain fermentation whole culture (%)
0	N/A	2.74	N/A	6.04	N/A	3.37	N/A	28.31
0.1	5.78	2.79	7.90	7.04	14.99	4.54	7.06	16.84
0.25	2.67	3.31	8.57	2.79	15.62	11.12	4.39	7.15
0.35	2.58	2.09	6.63	4.18	10.10	1.17	1.95	17.74
0.5	2.38	1.89	3.94	2.98	5.28	12.95	3.38	13.35

CV% was calculated based on protein Y concentration values determined by two analytical methods three-times across a 1 week period.

fermentation cultures is presented. Both LE and SPE sample preparation methods were investigated and demonstrated to be effective in the removal of interference from *Pichia* fermentation matrix. Despite the structural similarity among different Kunitz domain proteins, the choice of sample pretreatment methods could be quite different depending on the chemical properties of individual protein. In addition, a protein product–yeast cell association issue was identified and resolved by crude fermentation culture sample-handling procedures. The developed presample treatment methods that can consistently deliver over 90% recovery for recombinant Kunitz domain proteins in high cell density *Pichia* fermentation cultures. The results from the SPE-RP-HPLC method were compared with a validated ELISA method. It was shown that the assay accuracy of SPE-RP-HPLC method closely correlated with that of the ELISA assay, while it presents the additional benefit of lower assay variation along with product purity information. In comparison of the cost between SPE-HPLC and ELISA methods, SPE-HPLC assay does not require anti-protein product specific antibody and the cost of SPE cartridge is below $2/cartridge. With the common HPLC equipment in general bio-testing laboratories, SPE-HPLC is an easy and inexpensive assay method for implementation in R&D and quality control facilities.

In summary, LE and SPE sample treatment methods can provide vital tool in analytical method development, which allow highly sensitive and more accurate HPLC assays to be implemented in upstream cell culture process to provide critical monitoring for recombinant protein expression level and purity assessment in complex sample matrix including high cell density *P. pastoris* fermentation culture process. Many factors should be considered to develop an optimal sample treatment method. In particular, our study revealed that not only the general SPE parameters, such as cartridge chemistry, loading, washing and elution conditions need to be optimized, but also sample handling protocols are crucial to obtain maximum recovery and accurate purity measurements. Possible confounding effects, such as protein product – cell membrane association and chemical modification of protein structure during sample-handling are important to consider for investigation. The use of appropriately developed sample pretreatment methods with analytical HPLC assay(s), allows reliable and easily automated analytical assays to conduct fermentation process development, optimization, and manufacturing control.

Financial & competing interests disclosure

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Executive summary

Background

» *Pichia pastoris* is an attractive expression system for producing recombinant proteins because of its eukaryotic cellular machinery for protein processing, folding and post-translational modification, as well as its fast growth, easy manipulation and low cost of fermentation at manufacturing scale. However, it is a significant challenge to develop robust analytical methods to accurately monitor the expression level and purity of protein product in *Pichia* high-density fermentation culture because of the high cell mass in the culture and the complex matrix of the fermentation supernatant.

Materials & methods

» Different HPLC sample pretreatment methods were studied, liquid extraction and solid-phase extraction methods in particular for two Kunitz domain protein products. Cartridge type, loading and elution conditions, together with *Pichia* crude culture sample-handling protocols were systematically investigated, leading to an optimal solid-phase extraction sample treatment procedure.

Results & discussion

» Both liquid extraction and solid-phase extraction sample preparation methods were effective in the removal of interference from *Pichia* fermentation matrix. The choice of sample pretreatment methods depends on the chemical properties of individual protein.

» Many factors should be considered when developing an optimal sample treatment method, including cartridge chemistry, loading, washing and elution conditions and possible confounding effects such as protein product–cell membrane association and chemical modification of protein structure during sample handling.

» The use of appropriately developed sample pretreatment methods with analytical HPLC assay(s), allows accurate measurement of product titer and purity in *Pichia* fermentation culture, which greatly facilitates fermentation process development, optimization and manufacturing control.

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