Process Scale-up of Protein and Polypeptide Separations

A. Calculating the Desorbing Solvent or Salt Concentration

Adsorption of proteins and polypeptides on reversed-phase columns is an all-or-nothing phenomenon. Their separation virtually always requires a gradient. Each polypeptide has a specific "desorption concentration" – the solvent concentration at which the polypeptide releases from the stationary phase and moves rapidly off the column. In general, the shallower the gradient (Δ solvent concentration / Δ mobile-phase volume), the better the resolution of closely eluting polypeptides.

In analytical and bench-scale prep, where the objective is to resolve many peaks, it is customary to run shallow gradients over a wide range of solvent concentrations. In process chromatography, however, the objective is usually to achieve maximum purification and throughput for a single target molecule.

The recommended strategy

(1) Load the sample in as dilute a solution as practical at a solvent concentration 10% to 20% below the desorption concentration for the target molecule. This minimizes aggregation but assures adsorbtion to the stationary phase.

(2) Elute the column using a very shallow gradient in the region of desorption of the target molecule. This shallow gradient can be preceeded by a more rapid ramp to a solvent concentration just below the desorption concentration.

(3) Once the target molecule has eluted, ramp rapidly to a much higher solvent concentration to remove later-eluting contaminants, then back to the starting concentration for loading the next sample.

Determining the desorption concentration for a target molecule is an important step in designing a process purification. It is not as trivial as it may seem. Due to system delay volumes, the programmed gradient usually does not directly reflect the concentration at which a polypeptide is released. The following procedure will give the correct value.

Procedure

Run a small scale separation on a column with the same stationary phase chemistry as the process column. Use a linear gradient over a fairly wide solvent concentration range and empirically determine the elution times of the injection peak (RI blip or unretained material) and the target polypeptide.

Determine the gradient delay time of the system used for this separation as follows: Replace the column with a short piece of small ID tubing (negligible volume) between the injector and detector. Run a linear gradient of acetone in water, from 0 to 0.3%, at the same flow rate used for the separation. Monitor UV absorbtion at 254 nm. The resulting profile of acetone concentration with time will reveal the gradient delay time of the system (Figure 1).

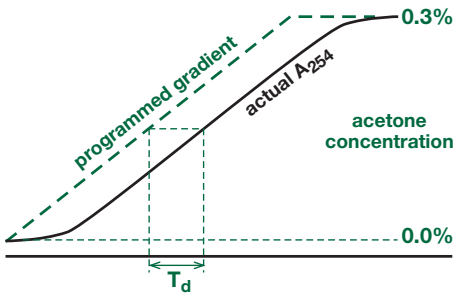
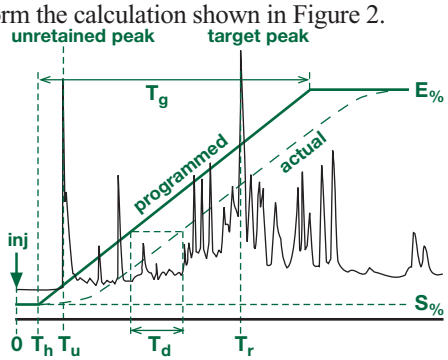


Figure 1 . Run a gradient from 0 to 0.3% acetone to determine the gradient delay time of the system. The column should be replaced with a short piece of small diameter tubing. The flow rate should be the same as used for the chromatogram. The gradient delay is the time displacement of the measured absorbance vs. the programmed concentration at the mid-rise concentration. NOTE: A simple programmed step will also yield the same value as the linear ramp shown.

Calculations

Perform the calculation shown in Figure 2.



Calculations:

$$T_c = T_r - T_u - T_d - T_h$$

where

T_c = corrected retention time of target peak
T_r = measured retention time of target peak
T_u = measured time of unretained peak
T_d = gradient delay time of system
T_h = hold time to start of programmed ramp

$$D_{\%} = S_{\%} + \frac{T_c}{T_g}\left(E_{\%} - S_{\%}\right)$$

where

$D_{\%}$ = desorbing solvent concentration
T_g = time duration of gradient ramp
$S_{\%}$ = solvent % at start of ramp
$E_{\%}$ = solvent % at end of ramp

Figure 2. Calculating the desorbing solvent concentration for a target peak.

The corrected elution time of the target peak (Tc) takes into account offsets due to

- the transit time (Tu) of an unretained substance, resulting from the volume between the column inlet and the detector

The Nest Group • 45 Valley Road • Southborough, MA 01772.1306 • USA
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- the gradient delay (Td), resulting from the volume between the mixer and the column inlet
- any programmed hold time (Th) between the injection and the start of the linear gradient ramp for which the calculation is made

Applying this procedure carefully will save time and material by allowing you to come close to optimum conditions for process chromatography on the first try. The same procedure will work for ion-exchange chromatography. Just substitute salt concentrations for solvent percentages.

B. Example: Developing a Process for Purification of Insulin

Partially purified porcine insulin provided by a Vydac customer was approximately 90% pure, with several peaks greater than 1% of the main peak at 280 nm. The objective was to design a process for reducing total impurities to less than 1%.

The TFA mobile phase system from a USP analytical method for human and porcine insulin was adopted as a starting point. From initial runs and the method in Section A, the main peak was determined to elute between 28% and 32% ACN.

A short 300Å, 5 micron, C4 analytical column (Vydac 214TP5415, 4.6mmID x 150mmL) was selected for analysis and quantitation of fractions during process development. The analytical column was operated using a shallower gradient than that specified by USP in order to reveal in greater detail impurities eluting near the insulin peak.

For preparative separations (Fig. 3), a 10mmID x 250mmL column (Vydac 214TP101510) with the same C4 chemistry but 10-15 micron packing was selected. For an initial run, 26 mg of sample was sonicated into solution and pumped onto the column in 30 mL of 20% ACN/0.1% TFA (v/v). (Loading sample by pumping a dilute solution reduces aggregation and produces better separation than loading a smaller volume at higher concentration.) Gradient elution from 20% to 35% ACN established that the insulin peak began to elute at 28.5% ACN. Subsequent runs with gradient conditions modified to bracket the insulin peak succeeded in increasing loading to 60 mg with good results (Fig. 4).

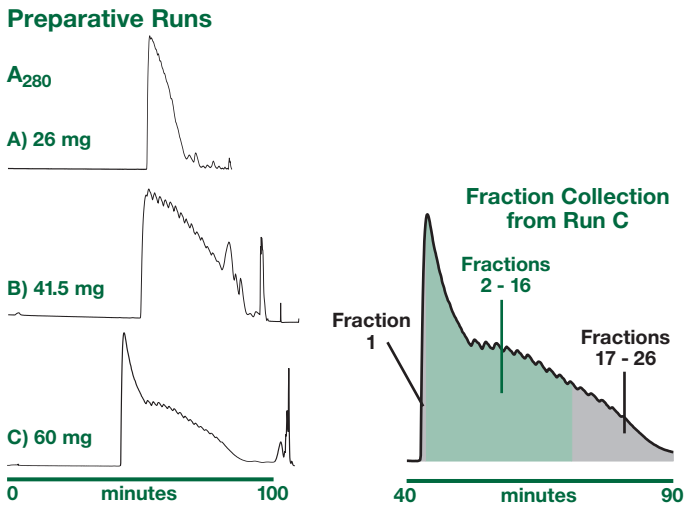

Figure 3. Preparative chromatography of porcine insulin.
Column: Vydac 214TP101510 (C4, 10-15µm, 10mmID x 250mmL).
A) Sample: 26 mg partially purified porcine insulin in 30 mL of 20% ACN/0.1% TFA (v/v), pumped on at 8 mL/min. Elution: 4 mL/min. Gradient from 20% - 35% ACN in 0.1% TFA (v/v) over 90 minutes.
B) Sample: 41.5 mg in 42 mL of 20% ACN/0.1% TFA (v/v), pumped on at 8 mL/min. Elution: 4 mL/min. Gradient from 20% - 27% ACN in 35 minutes, hold 27% for 10 minutes followed by 27% - 28% in 20 minutes, 28% - 35% in 10 minutes, and 35% - 95% in 5 minutes, all in 0.1% TFA (v/v).
C) Sample: 60 mg in 60 mL of 20% ACN/0.1% TFA (v/v), pumped on at 8 mL/min. Elution: 4 mL/min. Gradient from 20% - 27% ACN in 35 minutes, hold 27% for 10 minutes followed by 27% - 28.5% in 30 minutes, hold 28.5% for 20 minutes.
Note: Periodic irregularities in the peak profile are due to steps in the elution gradient generated by the low-pressure-mixing solvent delivery system. They do not adversely affect product purity.

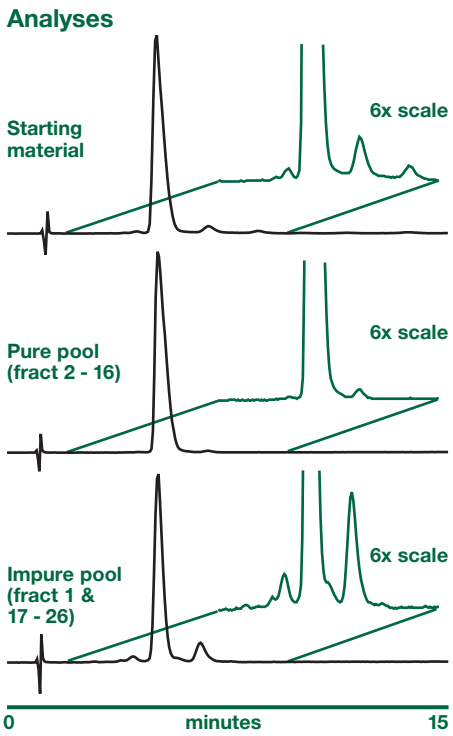

Figure 4. Analyses of starting material and pooled fractions from preparative column. Column: Vydac 214TP5415 (C4, 5µ, 4.6mmID x 150mmL). Samples: Starting material and pooled fractions of preparative run C (Fig. 5), as indicated. Detection: 215 nm. Elution: 1.5 mL/min. Gradient from 30% - 33% ACN in 0.1% TFA (v/v) over 15 minutes.

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