Large Scale Process Development for Hydrophobic Interaction Chromatography, Part 2: Controlling Process Variation

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Abstract
The scale-up of hydrophobic interaction chromatography (HIC) relies on users successfully anticipating the nature and magnitude of variations that affect the final process. Part 2 of this 4-part article examines both direct and indirect sources of variation in HIC processes and suggests ways to control them. These concepts can be used to improve scale-up and on-line manufacturing performance of HIC and other chromatographic methods.

Introduction
Scale-up failures, or failures of on-line manufacturing processes, are occasionally caused by external circumstances such as mechanical or electrical failure. However, most problems result from uncharacterized variations in process chemistry, raw materials, equipment, and the manufacturing environment. These problems are generally predictable and controllable. Controlling variation begins with identifying its underlying causes. Process developers can then assess the magnitude of variation. Some sources can be eliminated; others can be controlled. Remaining variations can usually be accommodated by altering process specifications. Adopting this proactive strategy significantly enhances scale-up success and helps to ensure dependability and reproducibility of on-line manufacturing processes. In addition to supporting good process economics, this strategy provides an invaluable foundation for process validation.

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Materials and Methods
As noted in Part 1, we obtained Source 15ETH, 15ISO, and 15PHE hydrophobic interaction prepacked columns and bulk media from Pharmacia Biotech AB (Uppsala, Sweden) (1). All three media are based on 15-mm d_p monodisperse spheres with a pore-size distribution suitable for large proteins. The base matrices are composed of poly(styrene–divinylbenzene) coated with a hydrophilic polymer. We obtained different monoclonal antibodies from Becton Dickinson Immunocytometry Systems (San Jose, CA) for this study and used them as process models. We purchased buffers and salts from Sigma Chemical Company (St. Louis, MO). All buffer components were American Chemical Society (ACS) grade or better. Process water was prepared using reverse osmosis and deionization. We filtered buffers through a 0.22-mm filter immediately after formulation and assigned five-day expirations. The experimental methods are described in the figure legends.

Results and Discussion
Process-related variations. The most important factors influencing the robustness of HIC scale-up are gel selection, binding conditions, and sample-application methods. These issues were discussed in Part 1 of this article (1). Temperature is another process-related source of variation. For most proteins, retention on HIC media increases with temperature (2–4). This appears to be a compound effect, resulting both from protein conformational changes and from modification of the degree to which salts alter water structure (2,5,6). As a result, HIC processes are benerally more sensitive to tem-

perature variations than other methods. Figure 1 shows that maintaining the selectivity of an antibody fractionation across a temperature reduction from 23 °C to 4 °C required increasing the ammonium sulfate concentration by approximately 25%. Other proteins may be affected to greater or lesser extents and should be evaluated on a case-by-case basis.

The most common cause of temperature-related variations in HIC processes is insufficient equilibration of the sample. When using samples as small as a few microliters during early process development, even cold samples will equilibrate rapidly enough that temperature effects are negligible. However, as process development proceeds into evaluations of capacity and other parameters that require larger sample volumes, temperature effects are amplified in parallel. At full process scale, a too-frequent cause of process failure occurs when sample is taken directly from cold storage for a room-temperature process (Figure 2). Failures of this sort pose the worst obstacle to subsequent investigations because the evidence is lost as soon as the sample temperature reaches equilibrium.

Avoiding this type of problem is largely a matter of education for both process developers and manufacturing staff. It is important that temperature specifications be included in manufacturing standard operating procedures (SOPs). SOPs should emphasize that *all* process materials—buffers and raw product—must be at a specified temperature before beginning a process.

Minor temperature variations between development and manufacturing laboratories are unlikely to cause serious process deviations, but they should not be dismissed. Consult facility records to determine the range and average temperatures of manufacturing areas—then develop processes to those specifications.

Variations from raw materials. Raw materials for manufacturing include raw product, buffer components, process buffers, and gel media. Routine variations in these materials seldom result in process failure, but they do have an impact, so it is important to characterize and accommodate them.

Raw product. Typically, the raw product is the least controlled component in a purification process. Even under the best circumstances, significant variations in product concentration and the proportion of contaminants are likely. Very often, users conduct early process development with materials that are inconsistent with materials to be used in the final production process. The worst possible situation is when process developers use a failed production lot—such as one with micro-

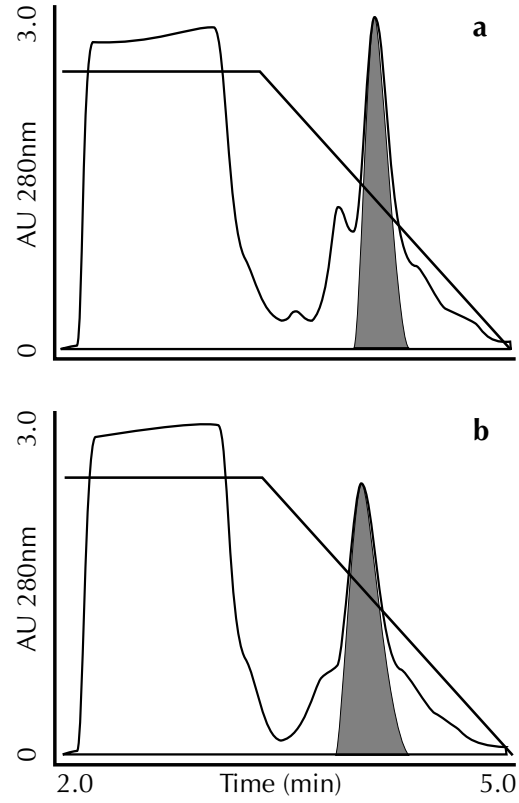

Figure 1. Maintaining selectivity by increasing the binding salt concentration after reducing temperature from (a) 23 °C to (b) 4°C. Column: 30 mm x 6.4 mm Source 15ISO; buffer A (a): 1.55 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer A (b): 1.95 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); flow rate: 940 cm/hour (5 mL/min); sample: 1 mL mouse IgG_1 ascites. Conditions: equilibrate column with 5 mL (5 column volumes) 80% A; load sample by on-line dilution, 20% sample, 80% A; wash with 2 mL 100% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B. Hatched areas indicate elution position of the MAb.

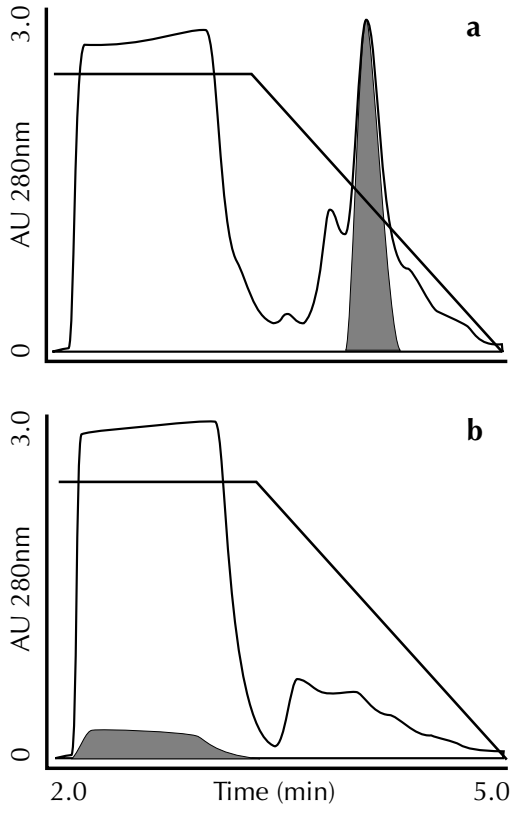

Figure 2. HIC process failure caused by non-equilibration of sample temperature. Shown are profiles generated (a) with both the sample and system at 23 °C and (b) with the sample at 4 °C and the system at 23 °C. Other conditions were the same as in Figure 1a. Hatched areas indicate elution position of the antibody.

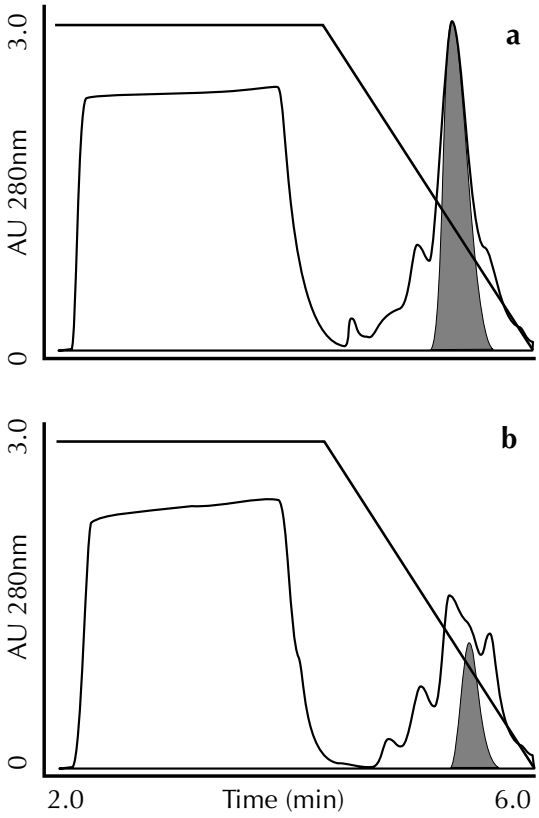

Figure 3. Process variation caused by varying the antibody concentration of the cell culture supernatant. Shown are profiles generated with (a) 90 and (b) 20µg/mL levels of antibody. Buffer A: 1.5 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); sample: 2 mL mouse IgG_{2a} cell culture supernatant. Other conditions were the same as in Figure 1a. Hatched areas indicate elution position of the antibody.

bial contamination—for method development. Such media often contain elevated levels of nucleotides, endotoxins, and proteases. These contaminants can foul chromatography media or cause other interference problems that would not be encountered normally, The product itself may even be altered.

Before a process proceeds to scale-up, process developers should finalize the production SOP and characterize multiple lots of product for routine variations. It must be demonstrated that the purification method functions adequately at the extremes of the range (see Figure 3). If production media representing these extremes are unavailable, process developers can simulate them by spiking samples with partially purified product or with product-free growth media. To obtain an indication of the amount of variation for a particular cell line in the absence of production-behavior data, users can consult data from production histories of similar established products.

Chromatography media. Users often take for granted that commercial chromatography media offer identical performance characteristics from lot to lot. In fact, chromatography media are manufactured to meet specified ranges—not fixed values—and lot-to-lot differ-

ences can have significant effects on process reproducibility. This is especially true of HIC media because of the dependence of selectivity upon both ligand density and hydrophobicity. (7–11). It is also true that the QC tests used by gel manufacturers can never reveal all of the performance characteristics relevant to the range of poteintial user applications.

For any given chromatography product, users should evaluate media from three or more lots to characterize variation. Capacity, selectivity, and resolution are the key variables. Process developers can test mixtures of commercially available model proteins but should also include the product of interest in crude or purified form. The main point of this testing is to obtain an estimate of matrix variability that has meaning in the context of your specific requirements and operating conditions (see Figure 4). Acceptable ranges of matrix variation are process dependent, and some processes are more tolerant than others. However, as a general guideline, lot-to-lot variation greater than 5% is cause for concern. Ranges greater than10% will almost certainly result in significant process variation. Such variations may require lot-specific method adjustments to ensure adequate reproducibility.

Conducting process development on one particle size and scaling up to another on the same medium increases the likelihood of gel-associated process variation. It is important to characterize the variation of the different media individually and then compare ranges and averages. If the differences between particle size ranges are of the same approximate magnitude as the differences within each medium, the problems should be no greater than those encountered when developing and scaling up using the same medium. If the differences are significant, users should exercise caution. Users should also be aware that media with different matrix and ligand chemistries can yield significantly different results for endotoxin, virus, and nucleotide clearance, even if their protein fractionation capabilities are similar.

Buffer components. The quality and consistency of buffer components can affect the selectivity of HIC separations significantly. Users should be aware of heavy metal contamination. Heavy metal binding by proteins generally increases their hydrophobicity (12,13).

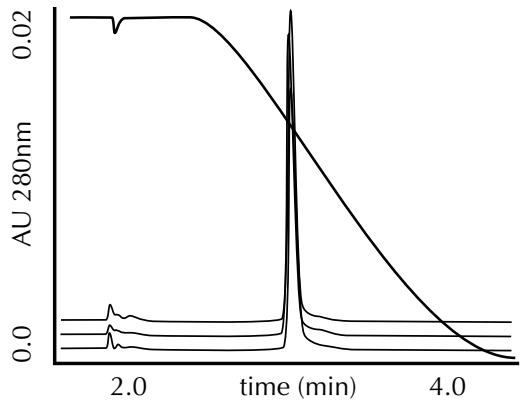

Figure 4. Reproducibility of selectivity using three lots of HIC media. Columns: 30 mm 3 6.4 mm Source 15ISO, lot numbers 225234/5, 27227/2, and P34Ep(A)12PG7; buffer A: 1.5 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); sample: purified mouse IgG_1 (0.5 mg/mL). Conditions: equilibrate column with 5 mL (5 column volumes) 100% A; inject 20 µL of sample; wash column with 2 mL 100% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B.

Buffer salts that contain high levels of heavy metals and vary from lot to lot can cause process variation. Not every separation will exhibit detectable sensitivity to this parameter, so process developers should test for it with known high and low metal controls. Laboratory and ACS grade salts can serve this purpose. Using ethylenediaminetetraacetic acid (EDTA) in process buffer formulations can suppress heavy metal–induced variability, but the best practice is to purchase salts that are controlled for metal content. Users should obtain certificates of analysis for critical buffer components as a matter of routine.

Buffer preparation. Differences in buffer formulation conventions ae udually not a problem within either Development or Manufact-uring, but problems occasionally occur when processes are transferred from one to the other. Development staff must remember that formulating hundreds or thousands of liters of buffer at a time may impose constraints on process buffer formulation. The methods used by the manufacturing staff should be downscaled and

used consistently during process development. Development staff should also use manufacturing SOPs for buffer formulation, adhering explicitly to the same chemical formulations (counterion, hydration number) and buffer component grades. Development buffer storage and expiration protocols should strictly adhere to Manufacturing conventions. Development instrumentation—balances, pH and conductivity meters—should be calibrated and maintained according to the programs in place for Manufacturing equipment. Both departments should keep buffer logs that note buffer pH and conductivity values on a lot-by-lot basis. Those logs serve as useful tools to distinguish routine variability from loss of process control.

Variations from process equipment. Variations due to differences in equipment between Development and Manufact-uring often necessitate process adjustments after scale-up. Characterizing these differences in advance may not eliminate last-minute refinements, but it can help avoid the large deviations that send processes back to development. Users should check the following primary equipment features: composition of wetted parts, mixer efficiency, accuracy of solvent proportioning, and system internal volumes.

The composition of wetted parts is a particular concern for two reasons, both of which cause more problems with HIC than with other chromatography methods. Because HIC routinely uses high salt concentrations, the corrosion of stainless steel surfaces and the subsequent leaching of metal ions are a perpetual concerns. The other problem stems from the hydrophobicity of ruby check valves and sapphire push rods in some laboratory-scale equipment. In HIC methods in which samples are applied through the pump, hydrophobic proteins and lipids often foul the outer surface of these components, which can lead to variations in flow precision, and in turn, to gradient aberrations or clogs. Periodic cleaning with sodium hydroxide and methanol helps to minimize this problem, but performing regular diagnostic procedures to detect flow abnormalities is important.

Mixer efficiency is more important for HIC than for most other methods because of the high differential viscosity between high- and low-salt solutions (14). If samples are loaded using on-line dilution (see Part 1), mixer efficiency becomes especially important because poor mixing of samples and binding buffer will prolong the duration of the high salt–protein interface and encourage precipitation of protein in the lines. Mixer efficiency must be at least as good on manufacturing chromatographs as on process development systems.

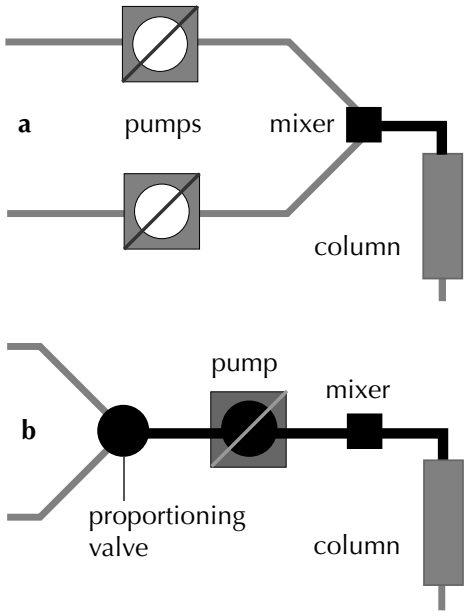

Figure 5. Diagrams illustrating (a) post- and (b) prepump solvent-proportioning formats. The black area in each diagram represents the internal system volume from the point of solvent proportioning to the column.

Variations in solvent-proportioning accuracy between chromatographs are frequent sources of scale-up problems. When methods are developed and scaled up on postpump solvent-proportioning systems usually no problems occur; however, methods that are developed and scaled up on prepump solvent-proportioning systems or transferred from one format to another frequently require process corrections. Figure 5 is a diagrammatic comparison of pre- and postpump solvent-proportioning systems. The internal fluid volume between the point of solvent proportioning and the column is the key variant. With postpump proportioning, this volume is typically small, but it can be very high with prepump proportioning.

Figure 6 shows variations in gradient accuracy using pre- and postpump solvent-propor-

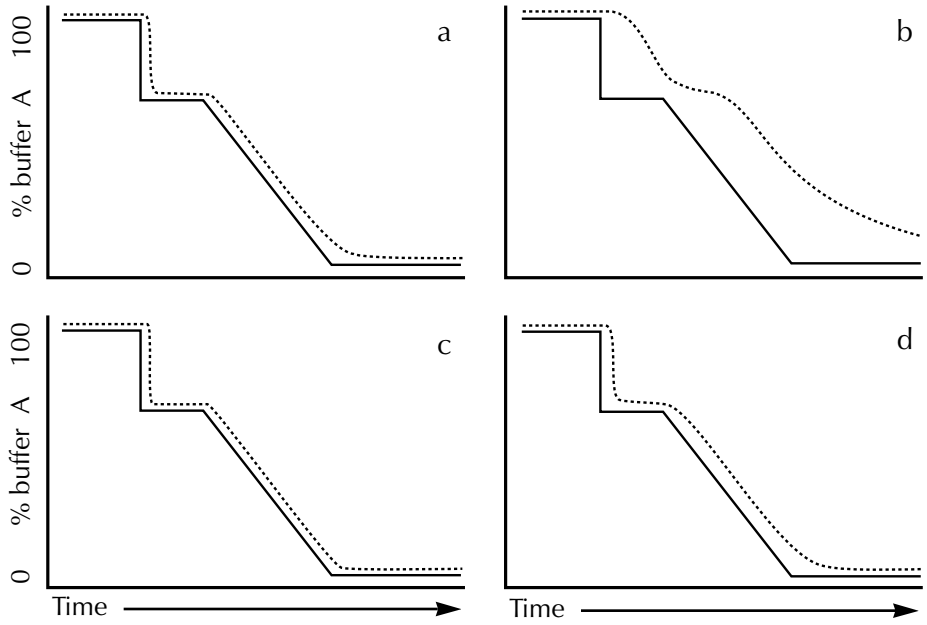

Figure 6. Comparison of gradient accuracy for (a,c) post- and (b,d) prepump solvent-proportioning systems. Column volumes: (a,b) 1 mL and (c,d) 25 mL; total run volumes: (a,b) 30 mL and (c,d) 750 mL. The dashed and solid lines represent the actual and programmed gradients, respectively.

tioning systems at different process scales. We generated these profiles by programming gradients between water (buffer A) and 1% acetone in water (buffer B) and compared the optical profiles at 280 nm with the programmed gradients. We corrected the position of the optical gradients relative to the programmed gradients for the volume between the column and the monitor on both systems. Actual profiles thereby reflect the gradient at the column. We used the same proportional gradient configuration for each profile: five column volumes 100% A; five column volumes 65% A; 10–column volume linear gradient to 100% B; and 10 column volumes 100% B.

Note that conformance of the gradient to programmed specifications improves with increasing relative process volume on both solvent-proportioning formats, especially on the prepump format. When the column volume is small in the prepump system, actual gradient accuracy (compared with the gradient program) is extremely poor. Figure 6b shows a time lag and a gross aberration of gradient shape. Both are functions of the ratio of system internal volume to process volume.

Reproducibility of the lag and the aberration are excellent when this ratio is kept constant. Problems arise when the ratio changes (for example, during scale-up). If a product is eluted near one of the set points in a linear gradient, a scaled-up process will likely vary significantly from specifications. The effects may range from a simple shift of the product's elution position to a change in relative purity. If a process employs a narrow-interval step gradient, scale-up is likely to fail outright.

The ratio of internal system volume to column volume can also affect the efficiency of sample equilibration using on-line dilution. Figure 7 contrasts the clearance profile of a protein solution introduced through the proportioning valve in a prepump solvent-proportioning system with the profile of a solution introduced through a dedicated line in a postpump proportioning system. Clearance time is important because it parallels the precolumn residence time during which proteins loaded through a pump will be exposed to binding concentrations of salt. The longer the precolumn residence time, the higher the risk of precolumn aggregation or precipitation. As Figure

7 shows, an approximately 7-fold differential between the two systems' precolumn residence times occurs at 5 mL/min.

To avoid these problems, minimize the ratio of system internal volume to column volume and keep that ratio constant across process scales, or as close to it as possible. If its not possible to maintain a constant ratio, the next best solution is to characterize the ratios. Knowing what to expect may make it possible to develop generic rules of thumb for applying process adjustments between nonmatching systems.

Developing safety margins. In spite of the fact that different sources of variation act on HIC methods by different mechanisms, most variations have the net effect of either increasing or reducing product retention. This fact provides a simplistic but practical basis for modeling the effects of variations and determining appropriate safety margins. Regardless of their source or mechanisms of action, most variations can be standardized by expressing their effects in terms of the change of salt molarity required to create a similar variation. Users can determine this relationship by measuring the left- or right-hand shift of the product peak in a linear-gradient elution profile. For example, a decrease of 5 °C may produce approximately the same effect as reducing the ammonium sulfate concentration by 0.075 M (Figure 1). If the sources of variation have been identified and characterized, this measurement provides a reasonable method for expressing the cumulative variation that can affect a process.

Table 1 shows how this information can be used to estimate process extremes, against which the process can be modeled before scale-up. The magnitude of the cumulative variation from center in each direction represents the minimum safety margins that must be added to insulate the process from this variation. Figure 8 compares a set of profiles that lack adequate safety margins with a set of profiles from a protected process. Linear gradients are especially effective for buffering process variation because safety margins can be added simply by extending the set points. The original gradient selectivity can be preserved by proportionately increasing gradient volume. The worst aberration that can occur under actual run conditions will be a variation in gradient slope. Assuming that the product is eluted near the middle of the gradient, the relationship between the product and the contaminants will remain fundamentally unchanged. Step-gradient processes can be partially insulated from process variation, but adding safety margins involves a compromise: broader step intervals increase asurance that the product will elute fully within the boundaries of the appropriate step, but the price may be lower purity.

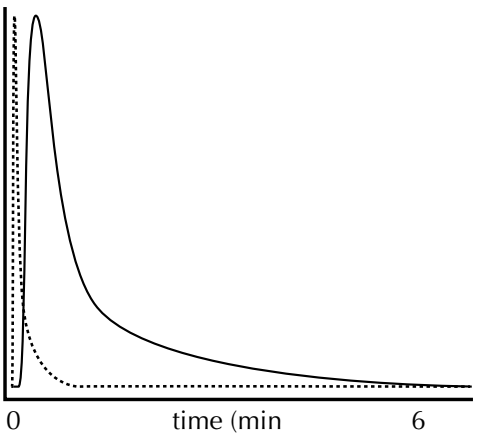

Figure 7. Precolumn residence–time profiles for post- (solid line) and prepump (dashed line) solvent-proportioning systems. The postpump system profile was generated by introducing a pulse of about 100 mL of 1% acetone through pump B at 1 mL/min. Flow was stopped briefly while the system was programmed to 100% pump A (water). Flow was started at 5 mL/min, and the signal was allowed to return to baseline. The prepump system profile was generated using the same solvents. The pump B line was first purged to bring the acetone solution up to the proportioning valve. The system was switched to 100% A (water) and rinsed until the signal was restored to baseline. A pulse of approximately 100 mL of 1% acetone was introduced through line B at 1 mL/min. Flow was stopped briefly, the system was switched to 100% A, flow was started at 5 mL/min, and the signal was allowed to return to baseline.

Conclusions

The scale-up processes that provide the best results and the manufacturing processes with the highest consistency of conformance to specifications are the ones in which process developers have fully anticipated the range of

Table 1. Estimating cumulative process variation

Variable	Range	Mean	Process equivalent[a]	
Process temperature	21–25 °C	23°C	-0.015 M	+0.015
Sample temperature	18–25 °C	21°C	-0.03 M	+0.03 M
Buffer pH	6.8–7.2	7.0	—	—
Binding salt molarity	1.45–1.55	1.50	-0.05 M	+0.05 M
Gradient precision (%)	62.0	—	-0.03 M	+0.03 M
Gel variation (%)	62.5	—	-0.04 M	+0.04 M
Total			-0.165 M	+0.165

[a]Expressed as the change in ammonium sulfate concentration required to produce a comparable effect.

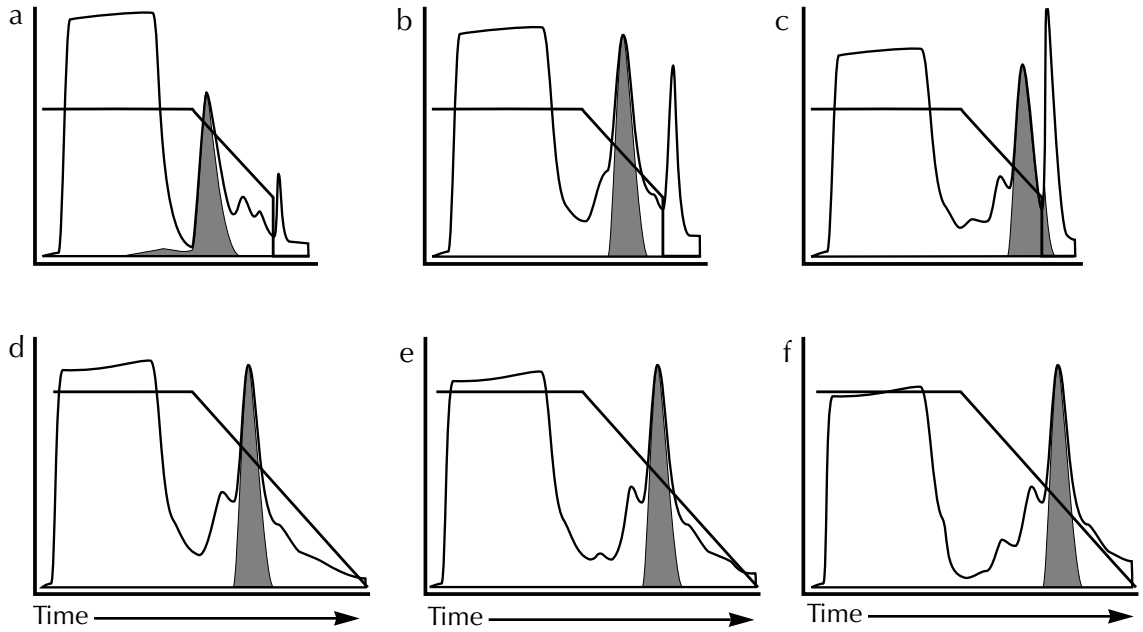

Figure 8. HIC profiles generated by unprotected (top) and protected (bottom) processes. Column: 30 mm 3 6.4 mm Source 15ISO. Buffer A: (a) 1.35 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); (b) 1.50 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); (c) 1.65 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0). Buffer B: 0.1 M sodium phosphate (pH 7.0). Flow rate: 940 cm/h (5 mL/min); sample: 1 mL mouse IgG_1 ascites; detection: UV absorbance at 280 nm (3.0 AUFS). Unprotected conditions: Equilibrate column with 5 mL 65% A; load sample by on-line dilution: 35% sample, 65% A; wash with 2 mL 65% A; elute antibody with a 5-mL linear gradient to 27% B; strip column with 10 mL 100% B; total run time: 5 min. Protected conditions: Equilibrate column with 5 mL 75% A; load sample by on-line dilution: 25% sample, 75% A; wash with 2 mL 75% A; elute antibody with a 10-mL linear gradient

variations the process will face. Some sources of variation are unique to a given method, such as the sensitivity of HIC to temperature variations. Others are more generic, but all sources of variation can be identified and evaluated. Some can be eliminated or reduced. The rest can be modeled to define safety margins that will protect the purification process.

This approach expands the role of process development to include tasks such as characterizing lot-to-lot variability of chromatography media. It also constrains process development to following manufacturing SOPs and conventions for buffer preparation. But ultimately, it provides the greatest assurance that scale-up and manufacturing will perform predictably and consistently.

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