The Secrets of Orthogonal Process Design

by Pete Gagnon, Validated Biosystems Inc., July 30, 2006

Orthogonal process design is the most fundamental concept in the development of successful purification procedures, but what does it mean in practical terms and how do you apply it? WordNet® defines orthogonal as having a set of mutually perpendicular axes (http://wordnet.princeton.edu/). As it relates to process development, this is generally understood to mean that multistep purification procedures should employ separation mechanisms that are distinct from one another; each step representing an axis in Cartesian space. A two-step process employing anion exchange and hydrophobic interaction chromatography (HIC) would be understood to be orthogonal.

Part of orthogonal design is simple math. If one separation mechanism removes 90% of the contaminants from a raw sample and another does the same, combined contaminant reduction should be 99%. If greater purification is required, a third method would increase the combined purification factor to 99.9%. These examples are based on an assumption that even through there may be substantial overlap in overall composition of contaminant subsets between the methods, individual contaminants will elute in different regions of the respective chromatograms.

This assumption is based on an even more fundamental assumption, that if a given contaminant has retention properties similar to the product in the first fractionation step, its retention properties will necessarily be different from the product in the second step. There is truth in this assumption but not universal truth. Reality comes closest to this ideal when separating different classes of molecules. For example, nucleotides have a unique cluster of properties characterized by their strong negative charge, absence of positive charge, and low hydrophobicity. They bind strongly to anion exchangers, flow through HIC media unretained, and are repelled so strongly by cation exchangers that they are excluded from the pores of the media and elute in the void volume. Few if any proteins manifest this cluster of properties, so the quality of nucleotide reduction actually does increase dramatically when orthogonal methods are combined.

Reality comes furthest from ideal with separations among closely related groups of molecules, such as product variants. Misfolds, deamidated forms, or enzymatic cleavage products may bear such a high degree of homology to the correct product form that even orthogonal methods may be challenged. It occasionally occurs that a single method will remove one type of product variant with good success but in general, the greater the similarity among the product and contaminants, the more essential orthogonal design becomes for achieving the necessary level of purification.

These examples emphasize an important feature of orthogonal process design: the purification capability of any one step is measurable only within the context of its partner(s). This is illustrated by comparison of Figures 1 and 2. In Figure 1, each method is capable of achieving 90% purification from raw product, but a particular contaminant co-elutes with the product in both. In Figure 2, the first method is the same as in Figure 1. The second method achieves only 60% purification from raw sample, but when combined with the first, the result is 100% purity because no shared contaminants co-elute with the product. This reveals the inherent fallacy in judging individual purification methods based solely on their average purification factor. Context is paramount.

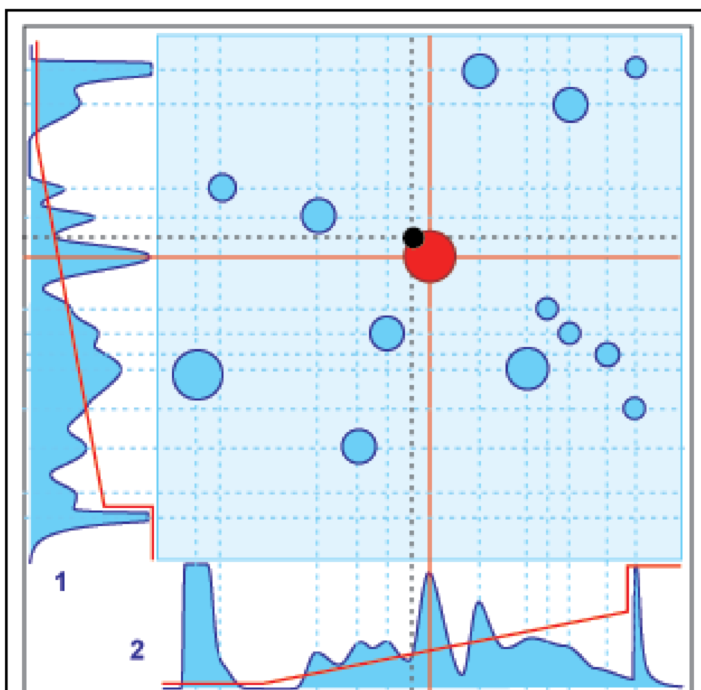

Figure 1. A Cartesian plot of results from two different chromatographic methods. In spite of good fractionation by both methods, a common contaminant (black) coelutes with the product (red). The plot shows that this combination of methods will not yield pure product.

Practical benefits of orthogonal process design

Orthogonal design and redundancy. Regulatory agencies and purification articles with a regulatory focus often highlight *redundancy* as a desirable feature of effective purification procedures, especially as it relates to DNA, endotoxin, and virus. This seems contrary to the concept of orthogonal process design but the distinction is semantic. Orthogonality refers to separation mechanisms. Redundancy is based on a contaminant-centric perspective: it refers not to repetition of the same separation mechanism, but to the compound ability of orthogonal mechanisms to reduce levels of a specific contaminant or class of contaminants. In practice, orthogonality is the foundation of redundancy.

Orthogonal design and process control. Figures 2 and 3 illustrate how maximizing complementarity through orthogonal design can reduce the impact of uncontrolled process variation, and thereby enhance process control. Equivalent purity is obtained in both but complementarity is greater in Figure 3, as indicated by the larger open area around the product. This implies that the result will be more insulated from material or process variations. The implication needs to be proven case by case but the probabilities are more favorable than for a process that is already operating close to its tolerance limits under the best of circumstances.

Orthogonal design and capacity. Maximizing complementarity through orthogonal design helps to achieve the highest capacity per unit of column volume. As column loads increase, elution peaks become broader, increasing the probability that the product will overlap with contaminants. The greater the complementarity among methods, the more tolerant the process will be of peak broadening in the individual methods, and the greater the productivity per manufacturing cycle.

Orthogonal design and product recovery. A frequently cited example in the field of process design describes the compound loss of product in multistep purification procedures. Even if recovery is 90% at each step, the compound loss in a three step process will be 27%. If better complementarity between steps allows the fractions to be cut more broadly, recovery can be improved. If greater complementarity also permits the purification to be achieved in fewer steps, so much the better. Beyond the savings achieved by better product recovery, elimination of a process step reduces hardware costs, media costs, buffer costs, labor costs, process time, and validation expense.

Orthogonal design and product integrity. Monoclonal antibodies provide an example of how orthogonal design can conserve product integrity. These proteins undergo post translational glycosylation, resulting in charge heterogeneity that shows up in isoelectric

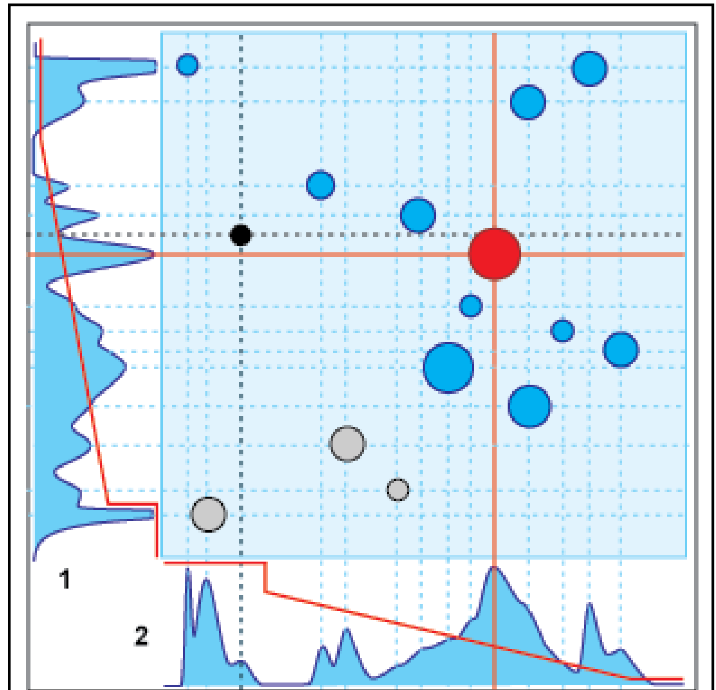

Figure 2. A Cartesian plot of results from two different chromatographic methods. Method 1 is the same as in Figures 1. Method 2 is different. Method 2 by itself produces a relatively poor fractionation but yields pure product in combination with method 1. This is indicated by the lack of contaminants overlapping the product.

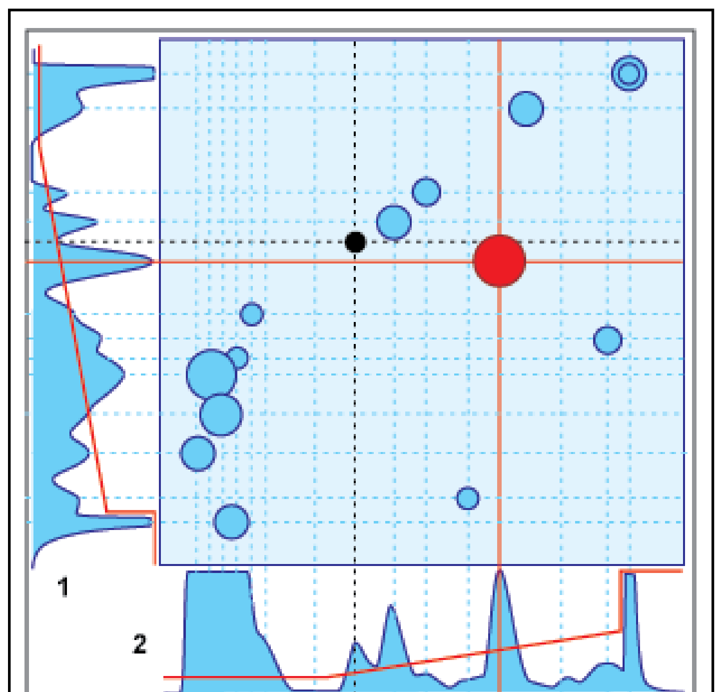

Figure 3. A Cartesian plot of results from two different chromatographic methods. In comparison to Figure 2, the larger open space around the product represents a higher degree of insulation from uncontrolled process variation.

focusing as a cluster of bands. Ion exchangers seldom resolve glycosylation isoforms into separate peaks, but fractions taken across an elution peak typically exhibit a cline from the most acidic to the most alkaline isoform. Individual isoforms may have different pharmacokinetic properties, so inadvertent reduction or removal of one or more isoforms in conjunction with removing a near-eluting contaminant has potential clinical and regulatory ramifications. This risk can be dramatically reduced if there is no need to shave peak boundaries.

Orthogonality versus complementarity

Figures 1-3 all fit the definition for an orthogonal process; each exploits a different separation mechanism in each step, but as illustrated, orthogonality alone is not enough. The choice of orthogonal steps needs to be driven by their relative complementarity.

Can there be different degrees of orthogonality between steps? Possibly, but complementarity remains a distinct issue. For example, it could be argued that a cation/anion exchange process is less orthogonal than a HIC/cation exchange process because it exploits only charge differences. The ion exchange process might nevertheless give better purification performance, but from a higher degree of complementarity, not a higher degree of orthogonality.

How do you optimize complementarity? With thoroughness. At the most basic level you should consider all of the separation mechanisms that are supported by media suitable for manufacturing the type of product you want to purify. For most protein applications this will minimally include anion exchange, cation exchange, HIC, possibly one or more mixed mode methods such as hydroxyapatite, and affinity. Size exclusion is generally avoided in protein purification but it is an important industrial tool in virus purification, where the exclusion of viral particles to the void volume allows shorter columns, larger sample volumes, and higher flow rates.

At the next level, differences in media of the same type can have substantial impact on the degree of complementarity among steps. Strong ion exchangers tend to dominate industrial applications, but weak exchangers sometimes provide just the selectivity required to solve a particular fractionation problem. In addition, there are substantial differences among exchangers with the same ligand, but on different physical supports, such as agarose, polymethacrylate, acrylamide, styrene, and various hybrids. These differences are compounded by variations in ligand presentation. Some ion exchangers present the ligand at the end of a simple spacer arm, forming a thin film on the surface of the media. Others employ tentacles. Tentacles tend to increase ligand density and produce a "deep layer" environment that may further distinguish selectivity. Hydrophobic media also differ substantially. Phenyl resins on the same kind of particle, made by the same supplier, can have very different properties depending on ligand density. Different physical supports may strengthen or weaken the net hydrophobicity of the product. Phenyl has a higher affinity for molecules with a high content of aromatic residues; butyl has a higher affinity for products with a high content of aliphatics, and so on.

Even the pore architecture of the media can be exploited to enhance overall complementarity. The pores on convective media like membranes and monoliths are so large (0.5-5 μm) that they are commonly referred to as channels. They are large enough to permit effective binding and high capacities even for large products such as plasmids and viruses. The pores on most particle based media are much smaller (600-1200 Å) and mass transport is limited by diffusion into and out of the pores. These materials provide their best performance with proteins. In the case of protein products, diffusion-based media can be focused on protein fractionation, while convection-based media can be used to enhance removal of DNA, endotoxin, and virus. With virus purification, diffusion-based media can be used to selectively extract protein contaminants and other "small" contaminants. If a particular protein contaminant co-elutes with a virus on a convective anion exchanger, it can be selectively removed by passing the sample over a diffusive anion exchanger with pores too small for effective binding of the virus.

Complementarity as it relates to buffer composition

Different buffers are well known to have a major effect on selectivity. In the case of HIC, the choice of binding salt may alter not only the degree of separation among sample components, but even the order of their elution. Buffer mediated differences can also be substantial on ion exchangers. Selectivity differences are frequently observed among zwitterionic buffers, anionic, and cationic buffers. The choice of eluting salt can make a significant difference as well.

One of the misconceptions that prevents aspiring protein chemists from becoming accountants is the notion that products and contaminants exist independently from one another in the full range of process environments employed for purification. However, most buffers are chemically extreme from a physiological perspective and they can promote transient nonspecific associations between the product and various contaminant classes. This can cause product-complexed contaminants to be carried through a fractionation step that should otherwise support outstanding reduction.

Cation exchange environments are a prime example. This technique is typically performed at low pH and

low ionic strength. Protein amino groups are fully charged under these conditions but the pH is usually not low enough to attenuate the negative charge on contaminants, especially the phosphoryl residues associated with DNA and endotoxins. At the low salt concentrations employed to maximize capacity, stable charge complexes can form, especially in the time interval during which the equilibrated sample awaits loading. As noted earlier, the negative charge on DNA should cause it to be repelled and elute in the void but not if it is bound to a carrier such as your product. In many cases the salt concentration for product elution may not reach a level sufficient to dissociate these complexes. The result is that cation exchange is typically inferior to most other methods for removing heavily phosphorylated contaminants, despite the fact that they should be eliminated easily.

Charge complexation is most severe at low pH but remains a potential concern with any method conducted at low ionic strength. This especially includes anion exchange but may include affinity, hydroxyapatite and other methods as well. However, low ionic strength is not the only problematic environment. The high salt concentrations employed for HIC have the potential to stabilize non-specific hydrophobic associations.

The practical solution is to design purification procedures including methods that support both high and low salt conditions. The combination of HIC and ion exchange is an obvious example but this does not mean that a HIC step is obligatory. Hydroxyapatite can be loaded at low ionic strength then washed at high ionic strength. Elution can be carried out at either high or relatively low ionic strength. Most affinity methods tolerate an even broader range of ionic strengths.

Complementarity with respect to buffer composition also provides a wider range of opportunities to develop procedures that support good process continuity. Process continuity refers to the ability to go directly from one purification step to the next without requirement for buffer exchange or other intermediate steps. Linking two ion exchangers in sequence works poorly if the product elutes at high conductivity in the first step because the high salt concentration in the product usually interferes with binding in the second step. The problem is relieved if the second step is salt tolerant, e.g. HIC, hydroxyapatite, or affinity. If a first step is chosen from which the product elutes at low ionic strength, such as HIC or affinity, the eluted product can be applied to an ion exchanger with little more than pH adjustment.

Conclusions

Consciously applying the concept of orthogonal process design to process development provides the best guarantee that your eventual purification process will reproducibly fulfill the needs of your application. Experience is a significant asset, but the most basic requirement is simply to investigate the full range of possibilities. Even the most experienced developers need data to develop a process, and they know that the more options they have, the better their chances to minimize the number of process steps, maximize continuity, recovery, and robustness.

The sheer amount of work to do this is daunting. Many developers soften the impact on their limited resources by managing it in stages. When assembling a process for initial product characterization and toxicology studies, it is common to evaluate the basic separation mechanisms on already established chromatography media within a limited range of conditions. If intial product performance merits a greater investment, complementarity enhancements can be investigated during development for subsequent clinical phases.

New tools are being developed that help make orthgonal process development more efficient. Sophisticated robotic systems are among these tools, but a generation of manual and semi-automated enhancements is emerging in parallel. However you choose to approach it, a fundamental commitment to orthogonal process design pays off. If you develop a process right the first time, you pay for it only once, and reap the benefits with every manufacturing cycle.

This article was reprinted from Validated Biosystems
Resource Guide for Downstream Processing
http://www.validated.com/
Copyright © 2006
Validated Biosystems Inc.
All rights reserved.