Chromatofocusing: Does it Really Stack Up as A Process Tool?

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Chromatofocusing (CF) is a variant of ion exchange chromatography. It is fast. It supports high capacity. It gives high resolution. It provides unique selectivity. Given these characteristics, you would expect to encounter it as a frequent component of industrial purification processes, but it turns out to be surprisingly rare. Most of the reasons for its rarity are artificial, and the few hard-technical reasons are more a reflection on characteristics of particular proteins than on the technique itself. In practical terms this means that CF could be applied to advantage a great deal more often than it is. The goal of this article is to place the technique in a perspective that helps identify these opportunities.

Mechanism. CF involves the elution of ion exchangers solely by the mechanism of pH. In anion CF, proteins are bound to an anion exchanger at high pH. As the pH on the column descends, protein positive charge becomes stronger (more column-repellent) and protein negative charge becomes weaker (less column-attractive). The opposite situation prevails in cation CF. Either way, pH conditions in the column eventually reach a point where a given protein's net interaction with the column becomes zero, and it elutes. pH gradient elution partly defines CF, but the full definition is more restrictive. First, CF involves generation of a linear pH gradient *within* the column -- not an externally applied gradient. With anion CF, this is done by titrating the column initially to a pH high enough to bind the protein of interest, then applying a low pH titrating/elution buffer that contains buffer species collectively embodying a range of pKas. The range of pKas is selected to provide level buffer capacity across the entire pH amplitude of the intended gradient. When the buffer capacity of the eluting buffer is properly matched to the charge density of the column, a physical linear pH gradient is created inside the column. Proteins partition at the pH ostensibly representing their isoelectric point (pI). As the physical gradient migrates down the column, driven by the continuing flow of titrating/elution buffer, the proteins move with it, eluting when the pH of the buffer exiting the column is equal to their pI.

A second defining characteristic is that CF be conducted at low conductivity. This prevents salt-elution effects from skewing the selectivity. A third defining characteristic is that the column should maintain level ion exchange capacity over the pH range of the separation. This is an essential determinant of gradient linearity.

When all these conditions are met, some proteins do indeed elute near their pI; sometimes *very* near their pI; but many miss it by a wide mark. Discrepancies occur for several reasons. One is that proteins are 3-dimensional structures, while most ion exchangers present a generally 2-dimensional binding surface. This means that unless all of a protein's charges are localized on one small part of its surface, only a fraction of those charges will be in contact with the exchanger. If the distribution of charges is truly random, then the protein's desorption behavior may mimic its true pI. However, protein charges tend to be distributed unevenly, and only the most complementary surface interacts with the column. This phenomenon is called preferential orientation and its practical significance is that a protein's "contact pI" may be significantly different from its true pI. Elution behavior will deviate accordingly.

Another source of discrepancy arises from conductivity. The theoretical ideal would be

for the techique to be conducted at zero conductivity, but this is a practical impossibility. CF often makes use of zwitterionic buffers (including ampholytes) specifically because they have no conductivity. However, it remains necessary to adjust their pH with charged counterions (acids or bases). You can keep conductivity low by using low buffer concentrations, but you can't abolish it. Even these low conductivities cause proteins to elute prematurely, above their contact pI in anion CF, and below it in cation CF. The practical significance of these deviations is nil, and unavoidable in any case. It is important nevertheless to be aware that conductivity is an important variable, that it must be minimized to fully exploit the contact-pI differences among proteins, and that it must be carefully controlled to ensure reproducibility.

Uneven buffer capacity can change the shape of the pH gradient. For example, say that you are doing a gradient over 1.5 pH units. You have employed 3 zwitterionic buffers, representing an even progression of pKa by increments of 0.5 pH units. However, the buffer capacity of the middle component happens to be half that of the other 2. It will therefore be a weaker column titrant and the pH gradient will become flatter in the pH region of its influence. Like many of the aberrations in CF, this is not necessarily disadvantageous. You can deliberately exploit it to fine tune selectivity. If you do want to improve linearity, you can increase the concentration of the middle buffer, or reduce the concentration of the others.

A related source of deviations comes from not having adequate coverage of buffer pKas. Buffers generally lose about half their capacity within 0.5 pH units of their pKa. If you have excessively wide gaps between the pKas of the buffers in your mix, there will be less buffer capacity in these areas. Pharmacia has claimed that their CF polybuffers contain more than 1000 species (in the form of ampholytes) to guard against this particular problem. This is going overboard. You can blend combinations of "Goods" buffers (Hepes, MES, bicine and such) to achieve perfectly adequate coverage. Try to keep the pKa intervals within a half pH unit of one another.

The column can be another source of deviations. If you choose a weak exchanger, such as DEAE or CM, there may be changes in ion exchange capacity over the course of your pH gradient. Most DEAE exhangers start to lose charge at about pH 7.5, and have lost about half their charge by pH 8.5. Since the gradient is formed by pH titration of the column, a change in the column charge will affect the shape of the titration curve, making it progressively shallower as the charge on the exchanger increases. Again, this may not be disadvantageous. It may even fulfill your particular selectivity requirements better than a more uniformly charged matrix, but you need to be aware of it. In theory, its better to start with strong exchangers like QAE and SP, because of their resistance to titration.

Even among strong anion exchangers, you may encounter variations in selectivity from one product to another, due to variations in average charge density. If you change to a column with charge density higher than what you developed your method on, you'll have to compensate by increasing the buffer concentration or by increasing the gradient volume. Otherwise, the pH gradient will be shallower and probably fail to reach the specified endpoint within your specified gradient volume.

The last major source of deviations is the proteins you fractionate. Proteins have buffer capacity and especially in preparative applications, where at least one protein is likely to be present in high concentrations, that buffer capacity can be sufficient to skew the gradient, altering selectivity in that pH region. There are two ways to compensate (if you need to): either reduce column loading or increase the buffer concentration, but neither are as simple as they appear on their faces. Reducing the column load either increases the number of runs or the column size needed to achieve a certain level of productivity. Increasing the buffer concentration will simultaneously increase conductivity, which will

alter selectivity. The change may be beneficial, or it may not.

Separation characteristics . CF can be conducted over ranges of several pH units, in which case it is usually possible to separate proteins with resolution of about 0.1 pI units, or it can be conducted over ranges of less than a single pH unit, in which case it is sometimes possible to separate proteins differentiated by only a few hundredths of a pI unit. In contrast to salt-gradient ion exchange, where peak width has an unfortunate tendency to broaden as gradients are made more shallow, CF peaks tend to remain very sharp. It's not unusual for a protein to elute in a peak about the same width even when the gradient slope decreases by a factor of 10. Peak resolution also varies less in relation to column load than it does with salt-gradient elution.

Another positive attribute is CF's high capacity. Column loads of 100mg of product per mL of gel can be achieved. Unfortunately it doesn't necessarily follow that they can be eluted at that concentration. If the protein is highly soluble, then it probably will elute at a very high concentration, but if solubility is limited, eluting peak width will be determined by solubility rather than the pI of the protein. For example, you may be able to achieve a column concentration of 100mg/mL for your product. You will probably be able to see a stark white band of protein narrowly focused on the column. But if the protein's maximum free solution solubility is 20 mgs per mL, it's going to elute in at least 5 mL of buffer, and that 5 mL will tail out across a wider pH range in the gradient than if the load had been kept to something within the protein's solubility limits. This means it will coelute with contaminants that it would ordinarily be resolved from.

This turns out to be one of CFs worst and most ironic weaknesses. Proteins tend to exhibit minimum solubility at their pI to begin with, but beyond that, CF needs to be conducted at very low ionic strength, and this depresses solubility even more. This is especially true with very large proteins. It is also the reason why commercial literature on CF often illustrates results obtained with very small proteins. In general, solubility problems are more frequent with cation CF, due to the low pH to which the sample must initially be equilibrated. Increasing conductivity by addition of salt typically isn't an option because of its effects on selectivity. Fortunately, there are other additives that may enhance solubility without significantly affecting conductivity.

Among the most useful additives for preparative work are taurine, glycine, and betaine. Taurine is a glycine analogue that differs by having a virtually nontitratable sulfonic acid residue in place of the carboxyl. Betaine differs by having a virtually nontitratable quarternary amine in place of glycine's primary amino group. All three are powerful dielectric constant modifiers, elevating the polarity of aqueous solutions in proportion to their concentration. This increases the solubilizing capacity of the buffer, sometimes substantially. You can use these additives at concentrations of 1M to 2 M. Glycine covers most of the usable pH range. You can use it from pH 5 to 8. If your gradient extends below pH 5, omit glycine and use taurine. If your gradient begins above pH 8, omit glycine and use betaine. Within these ranges, the additives are zwitterionic and contribute nothing to conductivity. Outside the ranges, they become anionic or cationic, elevating conductivity, confounding pH control, and altering selectivity.

Urea at 1M to 2M is another useful solubilizing additive. Besides weakening hydrophobic interactions that tend to limit solubility, urea is a highly effective disruptor of hydrogen bonds. Hydrogen bonds have been shown to be a significant factor in self association of IgM at low ionic strength. The action of urea is complementary to the additives discussed above. Combining them will elevate solubility more than either alone.

If your product application permits, nonionic and zwitterionic detergents may be helpful as well. If the application is analytical, you can simplify your life by beginning with

detergents. They are usually the most effective solubility enhancers so long as you don't have to worry about removing them later.

No matter what solubilizing strategy you pursue, if your application is preparative you'll eventually have to do capacity studies. Dynamic capacity studies offer essentially nothing. Increase loading in a series of runs, measuring peak width as a function of column load. When you come to the point where you are losing necessary resolution, you've gone too far.

Another serious issue with CF involves separation of a single protein population into multiple subpopulations. The most frequent cause of this is the charge heterogeneity accompanying differential glycosylation. From a preparative perspective, this is highly undesirable. It causes the protein to elute in multiple peaks over a range of pH values. This both dilutes the protein and causes it to coelute with a wider diversity of contaminants. From an analytical/validation perspective fractionation of glycosylation morphs can be useful. To begin with, it's a very efficient way to capture sufficient material for detailed chartacterization. It can also be used to demonstrate stability of product composition across the duration of a cell production cycle, or to show that a purification scheme hasn't inadvertently altered the composition of a product.

Charge heterogeneity occurs for other reasons, such as folding variations and degradation. A point of qualification is necessary here. Strictly speaking, folding variants should not be heterogeneous with respect to charge, at least no more so than the parent population. However, the surface accessibility of charges may differ from one folding variant to another.

This brings us back to the difference between a protein's true pI and its contact pI. Because of this, CF can be extremely powerful for fractionating these forms, whether initially for their characterization or preparatively for their selective removal. For this particular application, CF is at its best with nonglycosylated proteins. The elution behavior of the target product form is better defined and the profiles are simpler.

Where to start. The easiest place to begin with CF is with a commercial kit (or components) coordinated specifically for the technique. These are available only from Pharmacia. Also try to obtain a copy of their manual entitled FPLC Ion Exchange and Chromatofocusing. Begin with the Mono-P column. It's more costly than the bulk agarose-based media, but much faster and gives higher resolution. Otherwise, follow the generic instructions, starting with a broad pH range. Use the results to identify a narrower range, then try that. In the few hours it takes to set up, conduct, and anlyze the results, you'll have enough information to determine whether CF has something special to offer you.

If the results look promising, you may want to try cation CF. The Mono-P column is appropriate only for anion CF, and Pharmacia doesn't market a cation CF column, so you'll have to improvise. Try any high performance strong cation exchanger. Equilibrate the column to 0.02M MES, pH 5.5. You can still use the Pharmacia polybuffers, but in this case you will titrate them to the high pH endpoint of the gradient. You may have to try a couple of polybuffer dilutions. Try to find one that completes the gradient in about 10 column volumes. Compare your results with anion CF. There will be differences. Pick the one with the selectivity that serves you best.

The next step will be to develop your own buffers, as suggested above. You may also want to change to a different chromatography medium. If you're going to use the technique analytically, then go with your favorite high resolution column. If you're plans are strictly preparative, pick your favorite there too. After you've selected your columns, expect to put some time into reformulating the buffers to reestablish the gradient characteristics you want.

The next round of experiments will address capacity. Capacity in this case refers to the amount of product you can load without the peak width exceeding your resolution

requirements. See how much capacity you have without any buffer additives. Be warned to allow the protein to sit on the column for an excessive time period in at least one experiment. Protein desolubilization at low ionic strength is progressive over time. The fact that you don't encounter a problem in a 15 minute development experiment doesn't mean there won't be a problem in the 2 hour full scale manufacturing procedure. If you do encounter solubility problems, explore solubilizing additives as necessary.

Where you place CF in a process depends on what you are using it for. It's a good concentrating step, but requires that the sample be equilibrated to column conditions in advance of application. If the sample is insufficiently soluble at the low conductivity conditions required for loading, equilibrate it offline to the lowest conductivity where it remains soluble, then do the final dilution online through the column pumps. If you are using CF for fractionation of bulk contaminants, put it near the front of a process. However, CF is probably at its best discriminating fine differences among closely related contaminants. In this case, you'll probably benefit from having it as one of your later process steps. Buffer restrictions make it generally unsuitable as a final process step. One last practical point: after completion of the pH gradient, strip the column with 1M NaCl. Depending on where the CF step is placed in your process, there may be a lot of residual contaminants remaining on the column.

Summary. Although CF is foreign to many process developers, its fractionation characteristics merit serious evaluation on a routine basis. It has limitations and it has a distinct set of development variables, but there are no more limitations nor variables than any other technique. They are simply different. For bulk separation, CF is just as powerful as salt-gradient ion exchange, hydrophobic interaction, or size exclusion chromatography. It is especially powerful for fine separations between degradation or folding morphs. On a preparative basis, the technique is at it's best with nonglycosylated proteins. Analytically, it offers valuable applications for both glycoslyated and nonglycosylated products. It won't be ideal for every purification, but you'll definitely benefit from making it a familiar member of your toolbox.

Recommended reading: If you plan to explore CF, it is definitely worthwhile to review the original description of the technique. It was published in a series of 4 articles by Sluyterman in J. Chromatography: 1978, 150 17; 1978 150 31; 1981 206 429; 1981 206 441. Note that where the current article suggests taurine, glycine, and betaine, Sluyterman synthesized taurobetaine. This is actually a better solution because of the broad pH range over which it can be used. However it is not readily available for doing preparative applications, and certainly not at a competitive price. Other than the Sluyterman references, consult Pharmacia product literature.

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