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Practical Strategies for Protein Contaminant Detection by High-Performance Hydrophobic Interaction Chromatography

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Hydrophobic interaction chromatography (HIC) occupies a unique niche in the field of analytical chromatography. A particular advantage of HIC is its unique selectivity. Whereas ion-exchange chromatography (IEC) principally reveals differences based on the surface charge of native proteins, HIC reveals differences based principally on their surface hydrophobicity. HIC is complementary to reversed-phase chromatography (RPC) in a different sense. Whereas HIC discriminates primarily on the basis of surface hydrophobicity, RPC principally reveals differences based on total hydrophobicity of all the hydrophobic residues of denatured proteins.

The retention mechanism of HIC is complex, involving hydrophobic interactions, which are in themselves strongly dependent on the character of the ligand, and the contribution of solvent–protein interactions. In spite of its mechanistic complexity the technique is simple to conduct. The column is generally loaded in a solution of high salt concentration, and elution is accomplished by reducing the salt concentration. Like both IEC and RPC, HIC is a high-resolution method, able under some circumstances to discriminate between proteins that differ by only a single hydrophobic residue.[1,2] These features make HIC an essential member in this triumvirate of powerful analytical technologies.

On a practical basis, analytical HIC is useful for aiding purification-process development, performing in-process monitoring, and assessing final product quality. It is commonly able to differentiate a protein product from its own variants, such as deamidated forms, terminal and intrachain enzymatic clips, and especially misfolded variants, as well as from a wide range of host cell protein contaminants. Likewise, in the case of protein conjugates, analytical HIC can discriminate and provide relative quantitation of unreacted materials, various conjugate morphs, and other reaction by-products. Obtaining the best performance from analytical HIC can be approached in two steps: minimizing variation in the analytical system and thoroughly evaluating the scope of potential selectivities. This chapter offers practical strategies addressing both sets of issues.

MINIMIZING VARIATION IN THE ANALYTICAL SYSTEM

Column Reproducibility

Ligand density is a primary determinant of selectivity in HIC.[3–5] This has two important ramifications: (1) minor variations in ligand density can significantly alter selectivity and (2) reproducibility of column selectivity is more difficult for manufacturers to achieve than it is for nonhydrophobic retention mechanisms. This makes it necessary for assay developers to document adequate lot-to-lot reproducibility of a given column medium before investing major resources in assay development.

For any column you are considering, obtain samples from at least three different production lots. Some column manufacturers set aside samples for this specific purpose, but you may obtain a better estimate of variability by simply purchasing columns over a period of time. An effective way to evaluate reproducibility among the columns is to run a standardized separation of a multicomponent sample cocktail. The cocktail should contain at least two different components that elute completely resolved from one another through the course of a shallow linear gradient. Column documentation from the manufacturer usually identifies an analytical cocktail with relative concentrations for each component.

Begin by specifying one of the column lots as the reference. Prepare enough buffer so that all runs can be conducted with the same buffer lot. Make sure that the chromatograph has been on long enough so that its internal temperature has stabilized. Run the reference and test columns in the shortest reasonable period of time. If your laboratory has tight temperature controls, you have more latitude with the length of time over which you conduct the runs. Otherwise, most laboratories warm up during the course of the day and at different rates and ranges according to the season. Also be aware that buffers will not equilibrate to temperature as rapidly as their surroundings. Selectivity in HIC is very sensitive to temperature, and uncontrolled variations may add a stratum of variation that can be misassigned to column variability.[5,6]

You can obtain an initial estimate of media reproducibility simply by overlaying the chromatograms. Align them first along the gradient trace. On columns with excellent reproducibility, the eluting peaks will overlap very closely. Vertical lines drawn through the respective peak centers will intersect the gradient trace at the same points. On columns with lesser reproducibility, the profiles may be shifted to the left or right. In this case, realign the chromatograms on the first eluting peak. There is no cause for alarm so long as the gradient offset is modest and the relative separation is maintained. If the offset between the gradient-aligned profiles is substantial and the quality of separation is significantly affected, then that particular column may not be qualified for analytical use.

For validation of columns that will be used for an official assay and to provide an unambiguous standard for qualifying future media lots, it is useful to employ more measurable comparative criteria than a simple overlay. Resolution, plate heights, and peak symmetry, as calculated by the classical formulae, should match very closely among test and reference columns (Figure 6.1, Figure 6.2).

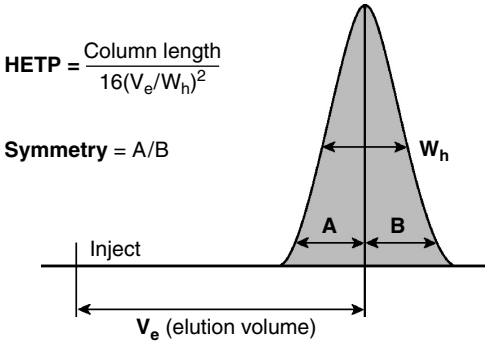

Figure 6.1 Calculation of HETP and symmetry. W_h equals width at half of the peak height. A and B are measured at 10% of peak height.

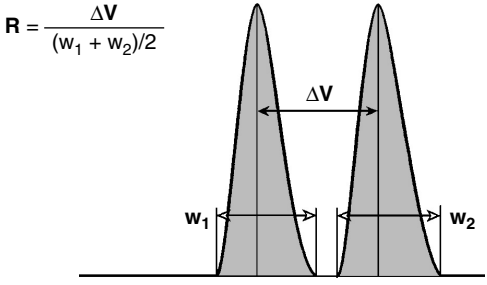

Figure 6.2 Calculation of resolution.

Plate height and symmetry calculations will provide an index of consistency in particle size, porosity, and packing quality. Resolution is affected by these variables, but will also provide an estimate of reproducibility for ligand density. Plate height and peak symmetry values calculated from gradient-eluted protein peaks will not be directly comparable with values calculated from the passage of nonadsorbed low-molecular weight solutes and should not be interpreted in the same way. They are nevertheless influenced by the same physical variables and will provide you with an unambiguous means of comparing the performance of different lots of chromatography media.

The degree of lot-to-lot reproducibility you require from a column is ultimately a function of the needs of a particular assay, which makes it impossible to state definite limits that will be appropriate in every case. Whatever the level of variation, it is important that it be documented. As new lots of media are brought into use over the course of years, their performance vs. the established reference should be included in a master database begun with the original qualification testing. Among other factors, this will allow you to track the column manufacturer's performance over time and possibly detect trends that could affect your assay performance — before a problem occurs.

It is also important to appreciate that adequate reproducibility is not the same as absolute reproducibility unless your assays employ isocratic elution. Low levels of variation among different lots of media can be offset by the use of linear gradient separations. Linear gradients are good insurance with HIC assays in any case because they also buffer routine variations in mobile-phase composition or ambient temperature.

The second step of qualifying a column is to characterize its stability. Different columns are affected to varying degrees by exposure to routine cleaning and maintenance procedures. This is a particular concern for hybrid chromatography media where an extremely hydrophobic base matrix is coated with a neutral material and then derivatized to incorporate hydrophobic groups.[7] Such compound construction creates more complex degradation pathways than encountered with homogeneous media, the most troublesome of which is loss of coating and subsequent exposure of the base matrix.

Figure 6.3 compares nonspecific protein adsorption on a new HIC column vs. the same column after 50 wash cycles. In each case the column was equilibrated, injected with 10 µg of purified IgG, washed, eluted in a linear gradient, and then cycled four more times for a total of five consecutive runs. Eluted peak height was fairly consistent on the new column. Results on the wash-cycled column indicated that significant amounts of protein were being adsorbed. The effect diminished with successive injections so that by the fourth injection, performance was roughly on par with the new column. However, by this time the column had adsorbed about 15 µg of protein.

In addition to assessing nonspecific adsorption with a simple system such as just described, it is recommended that postwash-cycle testing be performed with the same qualification methods and criteria used to evaluate new column

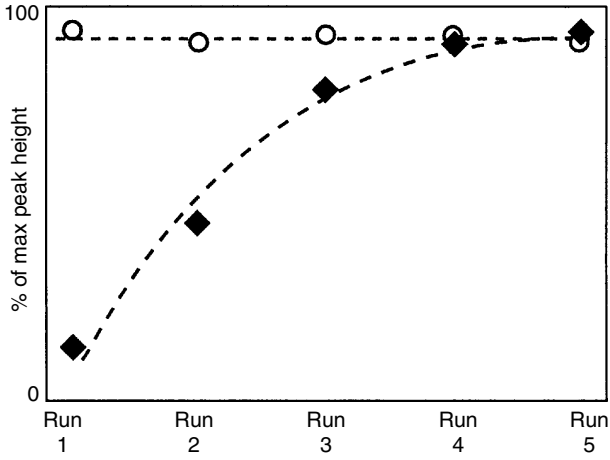

Figure 6.3 Nonspecific protein adsorption as a result of column degradation. White circles indicate eluted column peak heights of a new column. Black diamonds indicate eluted peak heights after the column was treated with 50 wash cycles. See text for discussion. (Data from P. Gagnon, 1997, *Validated Biosystems Quarterly Resource Guide for Downstream Processing*, 2(1), 1, http://www.validated.com/revalbio/library.html.)

reproducibility: resolution, plate height, and peak symmetry. Consult manufacturers for limitations on column chemical exposure, then apply the most severe conditions the columns are certified to withstand. The sample need not be applied during the wash cycling; only the cleaning agents need be added. Automated chromatographs allow this to be performed on an overnight basis. If possible, monitor system pressure throughout the treatment, as increased pressure may also be a sign of media degradation.

A short stability curve is not necessarily a reason to reject a particular column. If the unique selectivity of a given column is essential for achieving a particular analytical separation, it can be used so long as its performance is validated to persist for a specified number of runs and a column log is maintained to document that its usage is limited accordingly. Periodic analyses to document that it is still within functional specification may also be prudent. The point is to ensure that assay performance does not fall victim to an undetected source of progressive variation.

Controlling Sample Variation

Variations in sample composition other than those toward which an assay is directed may detract from the performance and reproducibility of HIC assays. Controlling these influences can significantly improve resolution and sensitivity as well as reproducibility. As with IEC, product complexation with other solutes can be an important source of aberrant retention behavior.

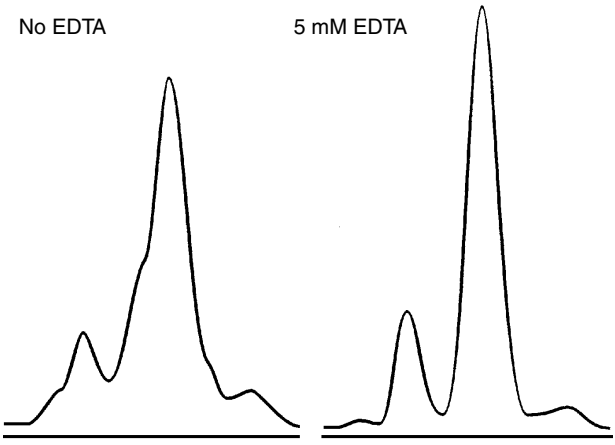

Figure 6.4 Loss of resolution due to sample complexation with contaminating metal ions. Profiles of a 30 KD protein on Tosoh Phenyl 5pw. Elution in ammonium sulfate. EDTA was added to the sample and both buffers.

HIC has a major advantage over IEC in that product binding tolerates high salt concentrations. Charge complexes are dissociated as a by-product of loading conditions. Protein–metal complexes, however, easily survive high salt concentrations and if not addressed can substantially degrade analytical performance (Figure 6.4). Fortunately, HIC's tolerance of high salt provides a way to eliminate most metal complexation problems as well. Metal-scavenging agents such as EDTA and imidazole can be added to the sample prior to loading.

The inclusion of these agents in buffers is also strongly recommended. Many of the binding salts commonly used in HIC are highly contaminated with metals. This creates a risk of protein–metal complex reformation after elution. Inclusion of these agents in buffers is also valuable for scavenging metals that may leach from stainless steel components of the chromatography system. The high salt concentrations used in HIC are notorious for their corrosive effects. Whether or not metal-scavenging agents are included in buffers, stainless steel chromatography systems used for critical analytical procedures should be passivated periodically as a part of their routine maintenance. Passivation is believed to work by creating a thin corrosion-resistant oxide film on exposed stainless steel surfaces. Run a solution of 0.2 to 0.5 *M* citric acid through the system at a low flow rate for 10 to 20 min. Rinse with water, then 0.01 *M* NaOH, and then the storage solution normally used. The obvious alternative is to avoid stainless steel chromatography systems for HIC.

EVALUATING SELECTIVITY

Overall selectivity in HIC is a composite effect created by several different mechanisms, each of which affect protein retention in a different way. Different

HIC supports manifest different balances among these mechanisms. This makes column selection a fundamental aspect of your assay development strategy.

One of the component mechanisms is, of course, hydrophobic interactions. Retention is proportional to column hydrophobicity, and elution order is expected to generally follow solute hydrophobicity. However, it is important to keep in mind that proteins bind preferentially to columns by their dominantly hydrophobic surface. Two proteins with very similar average surface hydrophobicity may exhibit very different retention characteristics due to differences in their respective distribution of hydrophobic residues.[1,2]

Interactions between proteins and salts in the binding buffer are also a major determinant of selectivity. Salts that are strong retention promoters in HIC are excluded from protein surfaces by repulsion from their hydrophobic amide backbones and hydrophobic amino acid residues.[8,9] This causes the mobile phase to exert an exclusionary pressure that favors the association of proteins with the column, regardless of stationary-phase hydrophobicity.[10–12] Because this mechanism involves the entire protein surface, the degree of exclusion is proportional to average protein hydrophobicity, regardless of the distribution of hydrophobic sites.

The balance between stationary-phase hydrophobicity and solvent exclusionary forces is the major determinant of selectivity on nonphenyl columns. On strongly hydrophobic columns, hydrophobic interactions between the proteins and the column are most likely to be the dominant determinant of selectivity. The low levels of binding salts required to achieve retention exert relatively less influence. On weakly hydrophobic columns, the high levels of binding salts cause solvent exclusionary effects to exert a greater influence.

Phenyl columns represent a third mode of selectivity. Pi–Pi bonding, properly speaking, is independent from hydrophobic interactions, but it has been shown to exert substantial influence on retention. Solutes with accessible ring structures are retained much more strongly than they are on similarly hydrophobic nonphenyl columns.[5,13]

These selectivity differences provide a rational basis for a three-column initial screening strategy: a strongly hydrophobic column, a weakly hydrophobic column, and a phenyl column. Commercially available columns are not indexed in a manner that easily allows column selection based on hydrophobicity; however, butyl columns are good candidates for strongly hydrophobic columns. Columns with names like ether-, iso-, and isopropyl are good candidates for weakly hydrophobic columns.

Initial screening conditions are suggested in Table 6.1. Multiple pH values are included because mobile-phase pH can significantly affect retention. Major selectivity shifts such as transpositions in elution order are fairly common; changes in resolution are much more so.[2,14–16] Changes in retention due to pH variation relate to protein hydration. Proteins are minimally charged at their isoelectric points (pIs). This means that they carry the minimum of electrostricted hydration water. Both protein surface hydrophobicity and HIC retention should therefore reach their maximum at a protein's pI.[6] As pH is either increased or

Table 6.1 Buffers for Initial Selectivity Screening

pH 8.5, binding buffer
0.05 *M* Tris, 2 *M* potassium phosphate, 5 m*M* EDTA, 5 m*M* imidazole
pH 8.5, eluting buffer
0.05 *M* Tris, 10% ethylene glycol[a,b]
pH 7.0, binding buffer
2 *M* potassium phosphate, 5 m*M* EDTA, 5 m*M* imidazole
pH 7.0, eluting buffer
0.05 *M* potassium phosphate, 10% ethylene glycol
pH 5.5, binding buffer
0.05 *M* MES, 2 *M* potassium phosphate, 5 m*M* EDTA, 5 m*M* imidazole
pH 5.5, eluting buffer
0.05 *M* MES, 10% ethylene glycol

[a] The level of ethylene glycol may be increased if necessary on strongly hydrophobic columns.

[b] 1–2 *M* urea may be substituted for ethylene glycol.

decreased, protein charge increases and electrostrictive hydration with it. Retention of that protein should decrease regardless of the direction of pH change.

Note that all of the eluting buffers in Table 6.1 contain 10% ethylene glycol. This has little effect on protein elution from weakly hydrophobic columns but can substantially improve peak sharpness on stronger HIC columns. This is especially true of later eluting peaks. You may also find that strongly retained proteins do not elute quantitatively from strongly hydrophobic media in the absence of competing additives. Thus the inclusion of ethylene glycol may improve not only resolution, but also sensitivity and accuracy.

Note also that ammonium sulfate is absent from the recommended binding buffer formulations despite its general popularity in the field. Ammonium ions become fully titrated at alkaline pH and convert to ammonia gas. Buffer pH may become unstable as a result and causticity of the free ammonia may partially hydrolyze the proteins in a sample, creating a source of assay variability.[6,17,18] At small buffer volumes used for analytical applications, liberated ammonia gas may not be a significant health hazard, but precautions may still be necessary to meet regulations. For all these reasons, ammonium salts are best avoided at alkaline pH.

Table 6.2 describes a linear gradient configuration that can be used for initial screening. Although the gradient segment is conspicuously long, this configuration has proven to provide a good initial balance between separation potential and dilution. If promising results are encountered, gradient slope and interval can be optimized to support the best results. Note that sample volumes up to about 2% of the column volume can be injected without preequilibration when the sample is at roughly physiological conditions. You can often increase the relative sample volume by adding dry NaCl directly to the sample. Most proteins

Table 6.2 Separation Conditions for Initial Screening

Equilibrate column: 5 column volumes (CV) 100% binding buffer[a]
Inject sample: 2% CV unequilibrated sample[b]
Wash: 2 CV binding buffer
Elute: 20 CV linear gradient to 100% elution buffer
Strip: 5 CV elution buffer[c]

[a] Starting salt concentration can be reduced to 1 *M* for strongly hydrophobic columns.

[b] Larger volumes can be applied in some circumstances. See text.

[c] If elution is incomplete, increase concentration of ethylene glycol.

will remain soluble up to at least 4 *M* NaCl. For strongly hydrophobic columns, 1 *M* NaCl added to the sample may allow injection volumes of 5% of the column, 2 *M* twice that. For weakly hydrophobic columns more salt will be required to achieve the same effects. Salts that are stronger retention promoters can achieve the same effects at lower concentrations, but involve a much higher risk that the protein in the sample will precipitate.

SELECTIVITY WILD CARDS

There are a number of methods by which you can modify the surface hydrophobic characteristics of proteins or otherwise modulate hydrophobic interactions. How they will affect a given separation is unpredictable, but they may be useful.

BINDING SALTS

The ability of various salts to mediate HIC retention for any given protein is well known to correlate strongly with the additive ranking of a salt's component ions in the Hofmeister series.[19–22] However, this does not mean that all proteins exhibit the same magnitude of response to different binding salts. Out of a given group of proteins, some may be more or less responsive to a particular salt. As a result, it frequently occurs that different binding salts produce significant changes in resolution, and sometimes produce transpositions in elution order.[6] Because any differential effects are dependent on the individual proteins, there is no way to predict which salts, if any, may produce useful results in a given situation.

Surveying different salts is simple but can be time consuming. For phenyl and butyl columns, because of the modest salt concentrations required to achieve retention for most proteins, the potential range of candidates includes dozens of options. The range is somewhat more restricted for weakly hydrophobic columns because fewer salts will be able to achieve good retention; however, the range is still substantial. Even NaCl may serve at concentrations of 4 to 5 *M*.

As a baseline, choose the column and operating pH that have so far provided the most promising selectivity. Prepare a nearly saturated solution of the salt you

wish to evaluate and run a 20-CV linear gradient, with your sample, from 100 to 0%. Determine the minimum concentration of salt necessary to ensure retention on that particular column. To determine if the particular salt will give you the selectivity you seek, run another gradient from that concentration to 0%. Use the same gradient length as your baseline run.

Boronate Complexation

Cis-boronate derivatives have the ability to form covalent bonds with *cis*-diol sugars at alkaline pH. Unlike lectins, which typically bind only the terminal sugar in a complex carbohydrate, boronates can bind any physically accessible *cis*-diol on a glycoprotein.[23–25] When hydrophobic boronate derivatives are used, the hydrophobicity of each complexation site is enhanced. To the extent that the protein components of a sample are differentially glycosylated, this can provide a tool for exploiting those differences.

A wide variety of substituted boronates are available commercially. For phenyl supports, use a phenyl or biphenyl (napthalene) derivative. For nonphenyl supports, evaluate both weakly and strongly hydrophobic alkyl derivatives (straight chain or branched). Raise sample pH to at least 8 and add the boronate derivative of choice to a concentration of at least 1 m*M*. The sample need not be buffer-exchanged prior to injection. Column buffers should be at least pH 8.

Organic and Inorganic Modifiers

As explained above, ethylene glycol was added to all of the initial screening buffers to improve peak sharpness. However, it also exerts an effect on selectivity. Other additives can achieve the peak-sharpening effect, but alter selectivity in different ways. Urea (1 to 2 *M*) in the eluting buffer occasionally gives selectivities different from ethylene glycol.[6] This may relate to the fact that urea is a chaotrope that binds to proteins and reduces their surface hydrophobicity, whereas ethylene glycol is excluded from protein surfaces but reduces solvent polarity and thereby weakens hydrophobic interactions in the system as a whole.[6,26–28]

Ethylene glycol is unusual among organic solvents because it is protein-stabilizing up to concentrations of about 50%.[26,29] Other organic solvents such as alcohols may alter selectivity in different ways, but with a coincident risk of protein precipitation. The addition of chaotropic salts to the elution buffer may alter selectivity in yet different ways, but with a different risk. Protein precipitation is unlikely to be an issue, but salt precipitation may occur. Mix aliquots of your binding and elution buffers at a 1:1 ratio in a test tube before you commit them to the chromatograph. If salt crystals precipitate due to a low solubility product constant for some combination of the ions in your buffers, then look for a different chaotrope. If there is some compelling reason why you still want to evaluate that particular salt, perform a more complex set of mixing experiments in which you reproduce a series of points in the range of mixing proportions that

the buffers will experience through the course of the intended gradient. If salts still precipitate, you can also try reducing the concentration of the chaotrope.

Detergents are a unique class of organic modifiers. They have occasionally been used with HIC for special applications such as purification of membrane proteins, but they have not been evaluated systematically as selectivity modifiers.[30] Nevertheless their ability to alter protein surface hydrophobicity makes them potential candidates for this application. It is likely that different classes of detergents will have different effects on selectivity. For example, anionic detergents should have a higher affinity for hydrophobic sites that include or are adjacent to positively charged residues. Cationic detergents should prefer hydrophobic sites of electronegative character. Detergents with ring structures may show a preference for aromatic residues, whereas alkyl detergents may show a preference for alkyl protein residues.

If you decide to pursue these possibilities there are several cautionary points you should take into consideration. First, detergents also bind to HIC supports, especially strong HIC supports. Unless the interaction between your proteins and detergent is extremely strong, you will need to include detergent in your binding buffer. Otherwise, the column may competitively displace it from your proteins, creating a source of uncontrolled variation in your assay. It will also be necessary to extend column equilibration until the concentration of detergent exiting the column equals the concentration entering the column. Second, high salt concentrations severely depress critical micelle concentrations (CMC).[31] In order to prevent micelle formation you will need to keep detergent concentrations very low. How low will depend on the individual detergent and the high salt buffer composition. Consult with detergent manufacturers about the micellar properties of their products. Third, detergents may be difficult to clean from your column. An organic solvent wash will certainly be necessary. If you are evaluating several different detergents you will need to clean the column thoroughly between each treatment because each may confer a different selectivity. Fourth, some nonionic detergents such as Tweens and Tritons are heterogenous in composition and are often contaminated with peroxides that can damage proteins and affect assay results. If the detergent solution is anything less than water-clear, do not use it. Ultrapure detergents are available from a number of suppliers.

There are several formats in which you can conduct your experiments, each of which may produce a different selectivity. The simplest is to add detergent to the eluting buffer, as with other agents as discussed previously. Another is to conduct the entire run at a fixed low concentration of detergent. Contrary to expectations, some proteins actually bind more strongly under these conditions than they do in the absence of detergent.[32,33] A more complex approach is to elute with an increasing detergent gradient while the salt concentration is held constant. In order to minimize the risk of micelle formation during elution, you should first reduce the salt concentration to a level just sufficient to retain the proteins of interest in your assay.

TEMPERATURE

Hydrophobic interactions are very sensitive to temperature. Retention of most proteins increases with temperature, but for some the opposite is true, and the magnitude of response is highly individual in any case.[34–36] If you elevate temperature sufficiently (56°C and above) you may begin to denature proteins in the sample. This may expose more hydrophobic sites and alter selectivity to a greater degree. Whether or not you exploit temperature as a selectivity factor, good temperature control is essential for assay reproducibility.

COLUMN DIFFERENCES

Different HIC columns are well known to support different selectivities, even when they use the same ligand. Among same-ligand supports (for example, among phenyl columns), selectivity differences are chiefly attributed to differences in ligand density. Differences in the surface hydrophobicity of the support matrix and spacer also contribute to selectivity. The differences you observe among same-ligand supports can be characterized generally as frame shifts. Elution order typically remains fairly consistent. The primary exception to this pattern lies with supports that present the ligand in a different steric configuration. Tentacle-type supports, for example, may allow a given protein to bind by a larger subset of its hydrophobic residues. This may alter resolution and elution order among a series of sample components.

CONCLUSIONS

HIC is more challenging than some analytical chemistries. It requires more attention to environmental controls, to equipment maintenance, and to column qualification. The retention mechanism is both more complicated and less intuitive than other methods. And, there are fewer practical guidelines concerning how to exploit the technique to its full potential. Despite these limitations, the bottom line is that HIC provides selectivities that are not obtainable with any other analytical method.

Begin by constructing a foundation based on evaluation of the three-column/multiple-pH strategy outlined above. Optimize the gradient interval and slope of the most promising candidates. Before proceeding with more exotic variables, compare your initial results with those obtained from alternative analytical technologies, such as IEC and RPC. If a different method offers more promising opportunities, start there. If you do come to the point of exploring selectivity wild cards with HIC, there is no way to predict which, if any, will produce the selectivity that you want. Most will probably fail to do so. On the other hand, it is fairly certain that you will encounter substantial variations in selectivity and that you will learn a great deal. Whether or not the results serve your immediate needs, the insight you gain from the exercise is likely to prove valuable in future assay development projects.

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