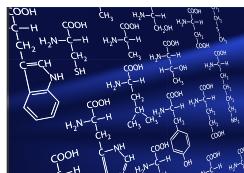


Insights into Optimizing Bioseparation Workflows for Critical Quality Attributes (CQA) Assessment

JULY 2018



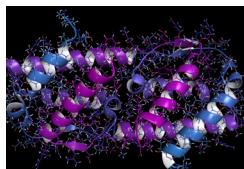
UV or MS? Derivatized or Not? Choosing and Optimizing a Workflow for Amino Acid Analysis of Spent Media

Anne Blackwell and Jordy Hsiao



Choosing the Right Column for Intact Protein Characterization: A Deeper Dive Into Column Characteristics

Andy Coffey

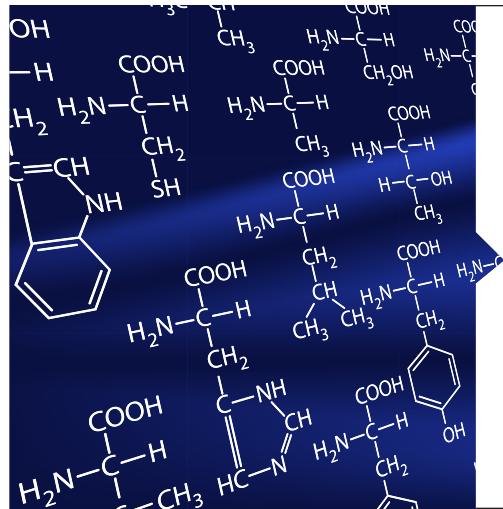


An Inside Look at Reversed-Phased Separations for Protein Analysis

An interview with Linda Lloyd

Sponsored by





SPONSORED CONTENT

UV or MS? Derivatized or Not? Choosing and Optimizing a Workflow for Amino Acid Analysis of Spent Media

There's more than one way to analyze amino acids in growth media. Which you choose depends on your equipment and what you want to measure.

Anne Blackwell and Jordy Hsiao

Overview

Analysis of spent media can provide invaluable information about the nutrient needs of a cell culture. The optimal growth of a culture requires that operators understand the culture's needs and when additional nutrients should be added. While liquid chromatography (LC) is a widely used technique for measuring amino acids, vitamins, metals, and other nutrients as well as the byproducts of production, there are still several decisions to make about the monitoring and analysis of the culture and spent media. Detection by ultraviolet (UV) absorbance or fluorescence needs relatively inexpensive equipment, but is somewhat limited in terms of throughput and what it can measure. Using a mass spectrometer (MS) for detection entails simpler sample preparation, but it comes at a higher cost and has less well-established separation modes. In the end, the method selected will most likely depend on what equipment is available, but it may also be influ-

enced by which compounds one needs to measure.

One might conduct spent media analysis for several reasons. For instance, a particular culture may be underperforming because of a shortage of a single nutrient. Tyrosine may be the limiting reagent for the production of monoclonal antibodies, but only an analysis of the spent media can determine which nutrient is running out first. Monitoring the concentration of tyrosine within the media helps operators know when the levels are getting low and allows them to maintain consistent optimal growth conditions. Testing of cell culture media is an important part of quality assurance and quality control processes, but it is also vital during the development and scale-up process.

UV Detection with Pre- or Post-Column Derivatization

The standard entry-level chromatographic system comes with UV detection. Fluorescence detection is also readily available. Unfortunately, most amino acids do not absorb ultraviolet (UV) light or

fluoresce. Detection with these systems, therefore, requires derivatization. The amino functionality provides the reactive group for most derivatization reactions. The reaction can take place either with the original sample (pre-column) or after the separation (post-column).

Post-column derivatization is less common because it has some challenging elements. The amino acids are typically separated in their native form on a cation exchange column. The column effluent is then mixed with ninhydrin reagent, which reacts with both primary and secondary amines to produce an optical absorbance signal. A specialized post-column reactor is required and some separation efficiency is always lost during the mixing.

Pre-column derivatization is an alternative method that can often be performed with existing laboratory equipment and offers several advantages. The reaction will take place at room temperature through the simple addition and mixing of reagents. An appropriately equipped autosampler, such as those made by Agilent, can perform the process automatically. A common technique involves reacting the sample with o-phthalaldehyde (OPA). The product is both UV-active and fluorescent, and has a half-life of about 90 minutes, which is more than enough time to perform the separation and detection. OPA will only react with primary amines. A second reaction step, adding fluorescamine (FMOC), will derivatize the secondary amines. Prepared kits of these reagents and the necessary calibration standards are available.

One advantage of pre-column derivatization is that it adds a large, relatively hydrophobic moiety to the molecules, meaning they can be separated by standard reversed-phase LC techniques. One consideration is that the optimal reaction pH is about 10, so highly acidic samples may need to be buffered first, and it is best to select columns that are resistant to high pH such as the Agilent AdvanceBio Amino Acid Analysis column. In addition to being ruggedized against high pH, its core/shell design provides high efficiency with relatively low back pressure, which is important for providing adequate resolution and high throughput.

Figure 1 presents the conditions for a separation of amino acids and **Figure 2** shows the resulting chromatogram. The OPA-derivatized primary amino acids elute before 9.5 minutes and are detected by UV absorbance at 338 nm. The detection wavelength is then switched to 262 nm for the detection of the FMOC-derivatized secondary amino acids, including proline, hydroxyproline, and sarcosine. If greater sensitivity is required, fluorescence detection could also be used.

This is a gradient elution with the organic component running from 2% to 57% for the elution of the compounds of interest, then jumping to 100% to clean the column of long-retained compounds, and finally followed by a re-equilibration period back at 2% before the next injection. It may become clear during method development that a minimum re-equilibration time is necessary, but that does not have to be the time programmed into the

FIGURE 1: CHROMATOGRAPHIC METHOD

AdvanceBio Amino Acid Analysis																	
Column	Agilent AdvanceBio Amino Acid Analysis																
Column Temp	40 °C																
Mobile Phase	A = 10 mM Na2HPO4 and 10 mM Na2B4O7, pH 8.2 B = Acetonitrile:methanol:water (45:45:10, v:v:v)																
Flow Rate	1.5 mL/min for 4.6 mm i.d. 0.62 mL/min for 3.0 mm i.d.																
Gradient Program	<table border="1"> <thead> <tr> <th>Time</th><th>% B</th></tr> </thead> <tbody> <tr><td>0</td><td>2</td></tr> <tr><td>0.35</td><td>2</td></tr> <tr><td>13.4</td><td>57</td></tr> <tr><td>13.5</td><td>100</td></tr> <tr><td>15.7</td><td>100</td></tr> <tr><td>15.8</td><td>2</td></tr> <tr><td>18</td><td>stop</td></tr> </tbody> </table>	Time	% B	0	2	0.35	2	13.4	57	13.5	100	15.7	100	15.8	2	18	stop
Time	% B																
0	2																
0.35	2																
13.4	57																
13.5	100																
15.7	100																
15.8	2																
18	stop																
Injection volume	1 µL, with 7s needle wash at wash port																
Detection	UV – 338 and 262 nm FLD – Ex λ 340 nm, Em λ 450 nm; Ex λ 260 nm, Em λ 325 nm																

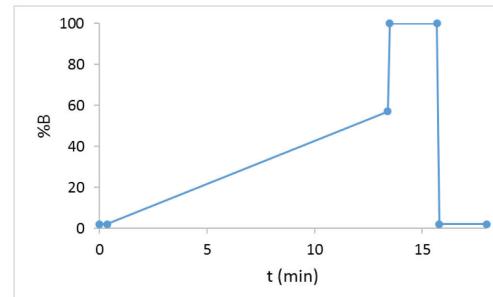
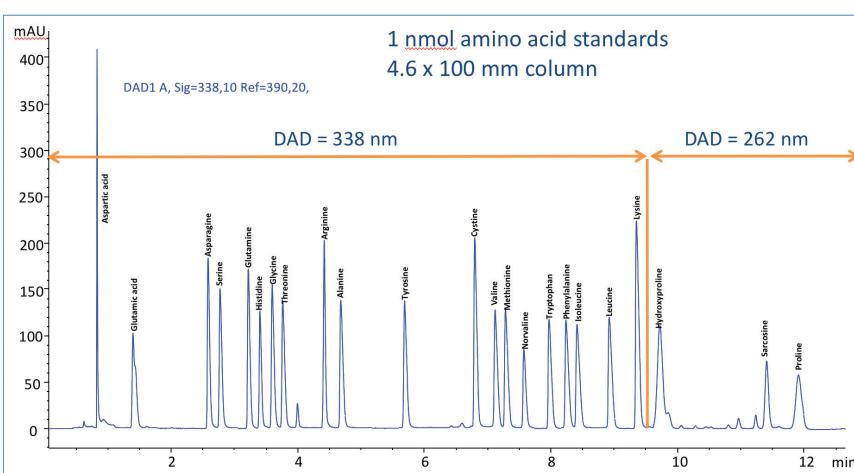


FIGURE 2: REPRODUCIBLE SEPARATIONS



- Retention time %RSD mostly under 1%
- Peak area %RSD mostly under 3%

Amino Acids	RT RSD (%)	Area RSD (%)
1. Aspartic acid	1.270	1.066
2. Glutamic acid	0.973	1.85
3. Asparagine	0.605	1.79
4. Serine	0.629	1.82
5. Glutamine	0.470	1.56
6. Histidine	0.430	1.22
7. Glycine	0.477	1.92
8. Threonine	0.440	1.95
9. Arginine	0.251	2.15
10. Alanine	0.280	3.06
11. Tyrosine	0.128	1.65
12. Cystine	0.067	1.9
13. Valine	0.084	2.47
14. Methionine	0.073	1.82
15. Norvaline	0.073	1.72
16. Tryptophan	0.054	1.57
17. Phenylalanine	0.051	1.66
18. Isoleucine	0.047	1.72
19. Leucine	0.03	1.7
20. Lysine	0.028	1.66
21. Hydroxyproline	0.021	4.13
22. Sarcosine	0.026	1.15
23. Proline	0.021	4.36



system. The derivatization process takes a finite amount of time during which the column is re-equilibrating. The system is essentially multitasking during this time. Taking advantage of this can significantly increase sample throughput.

Because media is a fairly complex sample, maintaining good resolution requires a well-assembled flow path. Poor peak shapes can result from improper selection of fittings. With Agilent InfinityLab Quick Connect fittings, no re-swaging is needed. It is a perfect fit every time. Other column manufacturers can have different-fitting geometries and re-swaging the connection may be necessary when switching between different manufacturers' columns. Extra-column volume should be kept to a minimum. Fronting or truncated peaks may be a sign that the sample is too concentrated and must be diluted prior to analysis.

If the signal intensity seems unusually low or the signal-to-noise ratio seems high, common culprits may include an old lamp or degraded derivatization reagents. Replacing the reagent on a standard schedule is a good idea. Similarly, borate buffers are prone to spoiling and should be replaced regularly. Sodium azide can be used to suppress bacterial growth, but is generally unnecessary if the buffer is being filtered and replaced with sufficient frequency. Occasionally, poor response may be caused by something as simple as a bubble in the vial insert. In this case, simply tapping the vial until the bubble is dislodged will immediately fix the problem.

One last point to remember is that even if columns are tolerant of high pH, they

are not totally impervious. They will not perform well if stored under even moderately high pH conditions. For short-term (e.g., overnight) storage, the columns should always be flushed with Mobile Phase B. For longer-term storage, columns are best stored in 50:50 acetonitrile:water.

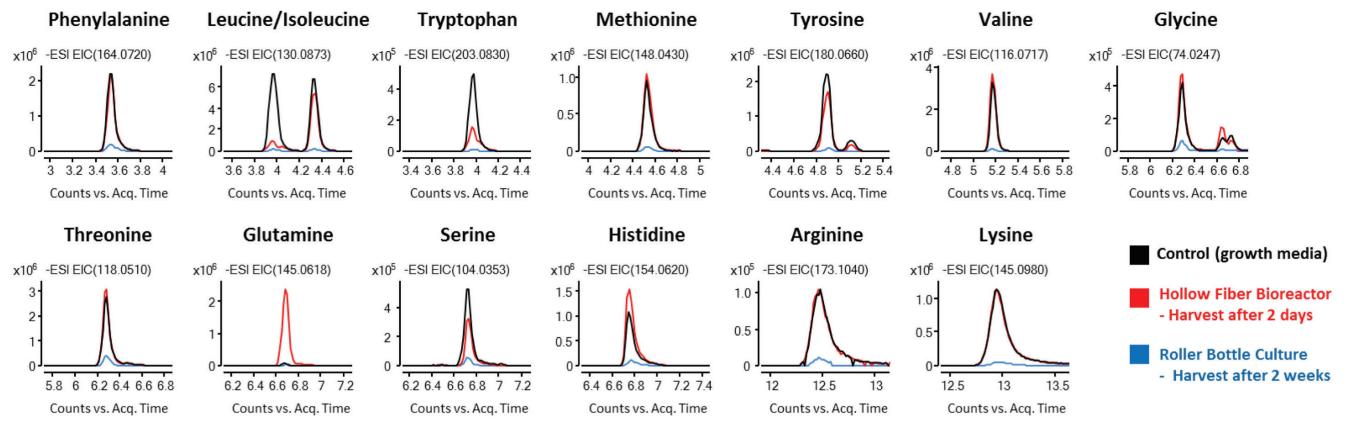
Even with the best treatment, columns will change over time and eventually become unsuitable. Reestablish retention times weekly. Large changes may indicate that the column is reaching the end of its lifespan. Similarly, monitoring the resolution of a critical pair of peaks will indicate column health. In a high-throughput environment, it is generally cheaper to err on the side of replacing a column somewhat sooner than necessary rather than repeat a long sample run. When the back pressure climbs too high, it is a good indication that it is time to replace the guard column.

HILIC Separation with Mass Spectrometric Detection

If available, detection using a mass spectrometer (MS) avoids the need for a derivatization step. In addition to being able to detect amino acids, MS instruments can be used to quantify other biologically important waste products and nutrients such as adenosine phosphates, sugars, and lactate.

Sample preparation is relatively simple in comparison. An aliquot of media is harvested and diluted 4:1 with 50% acetonitrile. This will precipitate larger proteins. A brief centrifugation step will then remove them from the suspension and allow a small sample (typically 1 μ L) to be injected.

FIGURE 3: ANALYSIS OF CELL CULTURE MEDIA – AMINO ACIDS



One downside of not derivatizing the sample is that non-traditional separation chemistries are needed to accomplish the retention and separation of hydrophilic analytes, such as hydrophilic interaction chromatography (HILIC). HILIC is a relatively recent addition to the chromatographer's toolbox. It is a subset of normal phase chromatography in which a polar solvent is used with a mobile phase consisting of a high concentration of organic solvent with a small amount of water. A water-rich layer forms on the surface of the stationary phase and separation is based on the liquid–liquid partition of solutes between the water-rich layer and the bulk of the mobile phase.

Several response peaks are displayed in **Figure 3** for a control, a sample of media from a hollow fiber reactor after two days, and a two-week time point from a roller bottle culture. For the separation, the aqueous portion of the mobile phase was 10 mM ammonium acetate at

pH 9. The organic component was 90% acetonitrile mixed with 10% of 100 mM ammonium acetate buffer. The resulting gradient, therefore, could have a varying concentration of the organic component, while maintaining a constant buffer concentration. The gradient ran from 90% B to 40% B at 10 minutes, then returning to 90% B for 8 minutes with a 0.25 mL/min flow rate. Under this mobile phase condition, the MS instrument was run in negative ion mode. The column was an Agilent AdvanceBio MS Spent Media column. This is a zwitterionic stationary phase in a PEEK-lined stainless-steel column. Observing the results, it is not surprising that the concentration of amino acids is reduced after two days and almost completely depleted in the two-week sample. The one exception is glutamine. Because it is unstable in solution, it is actively supplemented in the hollow fiber reactor, so that is the only sample where it is detected.

As an alternative, the aqueous portion of the mobile phase could be substituted with 20 mM of ammonium formate at pH 3, and the MS instrument can be operated in positive ion mode. Because mass spectrometry provides further separation and identification based on mass, the only critical pair that must be separated chromatographically is leucine and isoleucine. All the amino acids can be resolved using either method (see **Figure 4**), but there are differences in the ionization efficiency for various compounds that may inform the decision of which to use. Using the low pH method will generally give better ionization results for amino acid analysis.

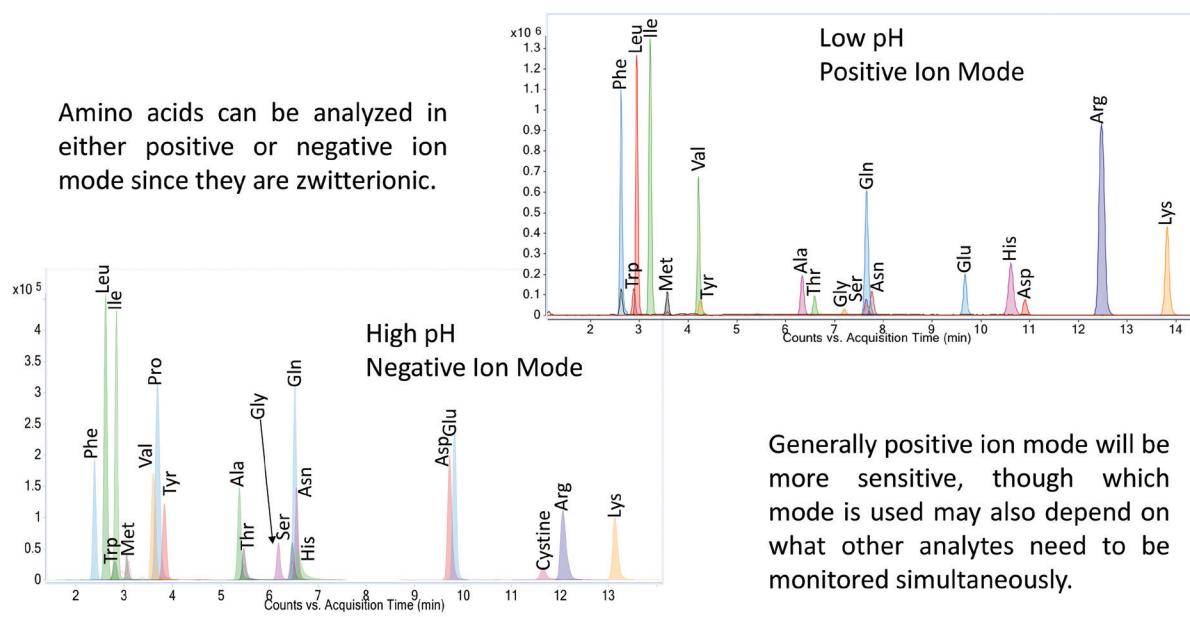
Polyamines will only be seen in positive ion mode, but also provide some additional challenges. Metal-sensitive compounds such as polyamines and phosphates will

interact strongly with stainless steel or other metal in the flow path, disrupting the efficiency of the separation. To improve both peak shape and sensitivity, metal should be removed from the flow path as much as possible. Any tubing should be PEEK or PEEK-lined tubing, and a PEEK-lined column is recommended. It may also be helpful to use plastic solvent bottles instead of glass, which can leach sodium ions.

Where some metal is unavoidable, a passivation protocol can reduce the interaction. This typically involves flushing the system with 0.5% phosphoric acid in 90% acetonitrile overnight. The MS nebulizer should be disconnected from the instrument for passivation so that phosphoric acid is not introduced into the mass spectrometer. After passivation, the

FIGURE 4: STANDARD AMINO ACIDS

Amino acids can be analyzed in either positive or negative ion mode since they are zwitterionic.



Generally positive ion mode will be more sensitive, though which mode is used may also depend on what other analytes need to be monitored simultaneously.



system should be fully flushed with mobile phase before the nebulizer is reconnected to the LC instrument.

Conclusion

Two ways have been presented to quantify amino acids and other compounds in spent media by LC analysis. The method selected may depend on a variety of circumstances, including the sensitivity that is necessary; how accurate the quantitation needs to be; if other compounds will also need to be quantified; and what equipment is available within the laboratory. Using MS instrumentation

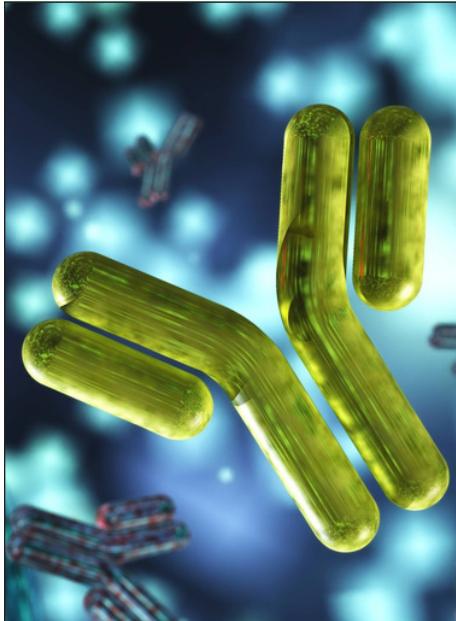
requires more expensive equipment with higher maintenance costs, but provides a higher throughput and allows for the quantitation of compounds beyond amino acids. Using derivatization with UV or fluorescence detection, on the other hand, can be performed with less expensive equipment; uses a more rugged separation mode; and (absent labeled internal standards) provides more precise quantitation. No matter which one is chosen, careful monitoring of growth media can ensure more reliable production and fewer headaches down the road.



Anne Blackwell
Applications Scientist
Agilent Technologies



Jordy Hsiao
R&D Scientist
Agilent Technologies



SPONSORED CONTENT

Choosing the Right Column for Intact Protein Characterization: A Deeper Dive Into Column Characteristics

How to properly evaluate key sorbent, instrument, and column characteristics as a whole, to select the best column for the analysis.

Andy Coffey

Introduction

Protein characterization comprises numerous techniques suitable for a wide variety of measurements, many of which require keeping the protein in its intact nature or even in its native state. For example, the determination of charge variants, accurate mass, purity level, and the identification of aggregates and impurities are all best performed on intact proteins. The chromatographic techniques commonly used include reversed phase, ion exchange (IEC), hydrophobic interaction (HIC), and size exclusion (SEC), coupled with mass spectrometry (MS) or ultraviolet light (UV) detection as needed, for the desired measurement. Since some of these techniques call for the use of organic solvents or ion-pair reagents, great care must be taken to keep the protein intact.

The factors that affect protein stability in these techniques can be grouped in three areas: instrument, sorbent, and column

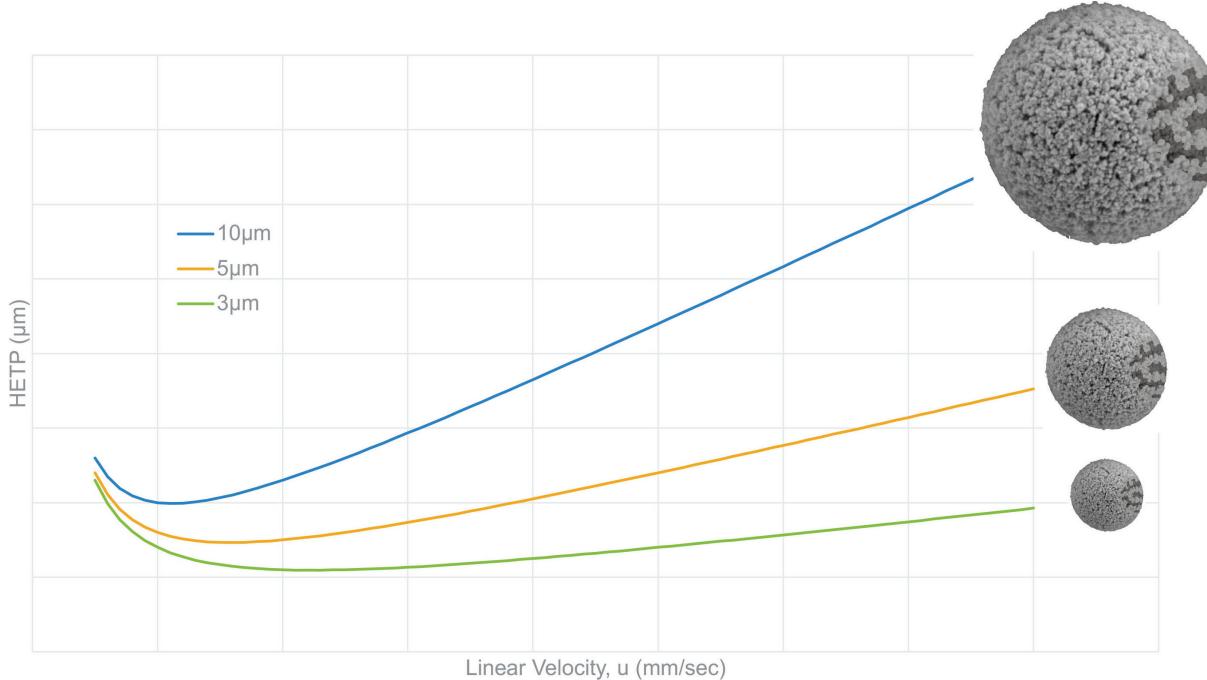
characteristics. They are all interconnected and ultimately, a combination of factors may give the best results for the specific molecule under consideration. This article focuses on the column and sorbent characteristics that have the greatest effect on proteins, with the objective of helping analysts choose the best column for the job.

Sorbent Particle Size

The stationary phase has a direct effect on several key parameters that determine the column performance. Specifically, the particle size of the material affects the plate count (efficiency) and the operating (back) pressure. The effect, unfortunately, works in opposite directions: while smaller particles result in higher plate count, which translates into higher efficiency and higher resolution, they produce higher back pressure, which creates instrumental limitations. Ultrahigh performance liquid



FIGURE 1: VAN DEEMTER PLOTS



chromatography (UHPLC) instruments can handle high back pressure. Meanwhile, for typical HPLC, the particle size cannot be much smaller than 2 μm , which corresponds to about 200,000 plates. Increasing the particle size to 3- μm cuts the plate count roughly in half; but means that frits used in column construction may be less likely to clog. Back pressure is also affected by column dimensions and eluent viscosity (and therefore operating temperature).

Plate count optimization can be done by using tools, such as a Van Deemter plot (**Figure 1**), in which the plate height (HETP) is plotted against the linear velocity of the eluent, u . The best operating

conditions are found at the curves' minimum. Comparing the three curves for 3, 5, and 10 μm easily shows the particle size effect. As the particle size decreases, the minimum moves toward lower HETP (which increases the plate count) and higher linear velocity.

In general, the curves can be described by an equation with three parameters that are related to the principal phenomena taking place inside the column: eddy diffusion (caused by the multiple path effect), molecular diffusion, and mass transfer within the particle (which results from the molecules moving in and out of the pores in the particle). These curves, however, are typically generated



using small molecules; a very different result is obtained with large molecules. For example, the Van Deemter plot for myoglobin under reversed-phase conditions is rather flat, dominated by mass transfer effects and resulting in a very different plate count from that of a small molecule. These mass transfer effects, fortunately, can be reduced by choosing the right pore size and particle morphology.

Pore Size and Particle Morphology

A good rule of thumb is that the pore size should be three times the size of the largest species being analyzed. For example, to look at aggregation states of intact monoclonal antibodies (mAbs) such as monomers, dimers, trimers, and higher order aggregates, size exclusion chromatography is the preferred choice. To optimize the analysis, the correct pore size must be chosen. As the molecular weight of the aggregates can be from about 150 kDa to 600 kDa, their hydrodynamic radius (R_h) can vary from around 5 nm to up to 10 nm. In this case, the appropriate pore size would be around 300 Å. Under these conditions, the analysis can be successfully run by isocratic separation under physiological conditions with UV detection.

With reversed-phase chromatography, the situation is a bit more complicated. The use of organic solvents and ion-pair

"A good rule of thumb is that the pore size should be three times the size of the largest species being analyzed."

reagents can result in slight denaturation and even unfolding of the proteins, which will increase their R_h . This would necessitate choosing higher pore sizes, up to 1000 Å, which would, in turn, result in very high mass transfer and prevent slow diffusion. Nonporous particles, useful in IEC, are not suitable for reversed phase because they would have very low capacity. Smaller particles would reduce the diffusion distance, but would result in very high back pressures. A way out of this dilemma is the use of particles that have a porous shell and an impermeable core (**Figure 2**). A 3.5-µm particle with a thin shell of pores of 450 Å will actually have a very short diffusion distance (0.25 µm) and perform as if it were only 0.5 µm in diameter, as far as diffusion is concerned. The resulting back pressure, however, will correspond with that of a 3.5-µm particle. The different morphologies available cover a wide range of effective diffusion distances, with particle sizes from less than 2 µm up to 5 µm. Fully porous particles are especially useful when large quantities of sample need to be injected, as when identifying minor impurities, without overloading the column.

Particle Construction and Bonding Chemistry

Silica, the most commonly used material for the stationary phase, has a surface rich in acidic silanol groups, which can lead to undesirable ionic interactions, since they are deprotonated under normal use conditions. The negative charges on the surface of the particle will interact with the proteins, which tend to have a positive charge. These interactions may cause peak tailing and the loss of resolution. Manufacturers usually bond a suitable alkyl ligand to the particle's surface for reversed-phase chromatography. For intact protein analysis, the best ligands are small, composed of 3, 4, or up to

eight carbon atoms (C3, C4, up to C8, respectively). An end-capping process often blocks the remaining silanols.

To further avoid undesired ionic interactions, ion-pair reagents are typically used. Trifluoroacetic acid (TFA) and formic acid (FA) are examples of commonly used reagents. Less common reagents, such as heptafluorobutyric acid and difluoroacetic acid, could be used if the method does not involve MS. FA is preferred when the analytical method involves MS separation and detection. FA, however, does not neutralize all the silanol groups but, in some cases, the addition of a very small amount of TFA (0.025%) to FA helps improve resolution. Ion pairs formed with

FIGURE 2: COLUMNS FOR INTACT PROTEIN ANALYSIS

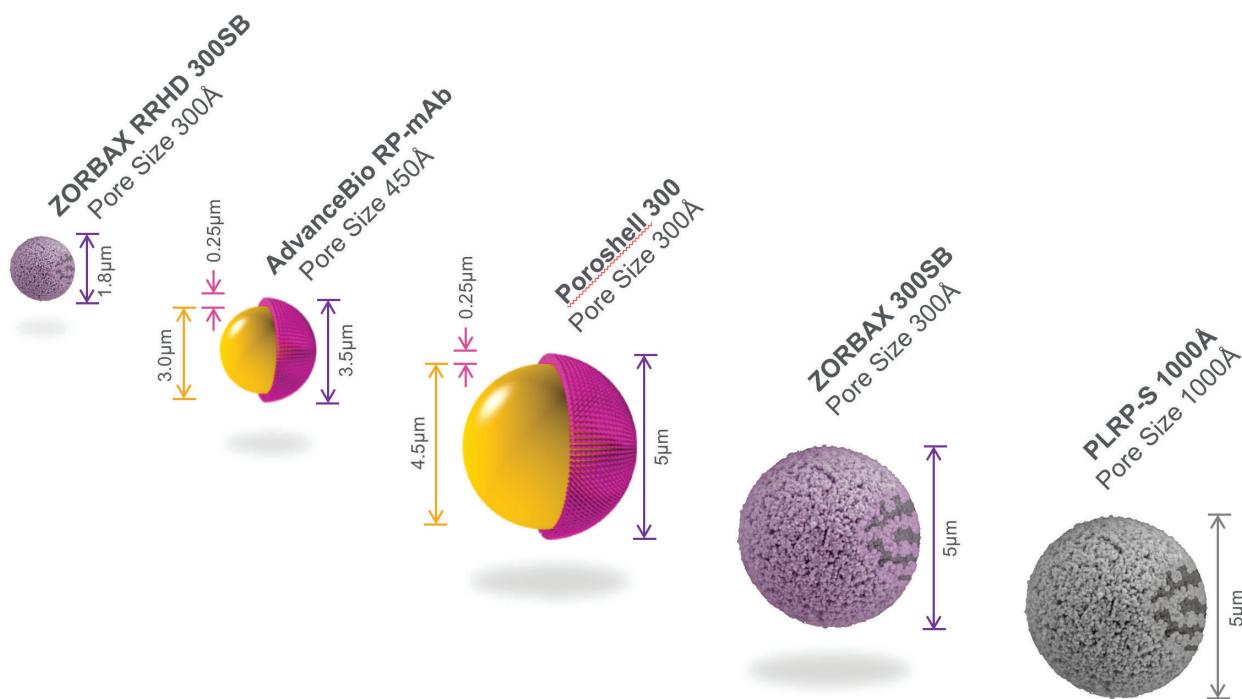
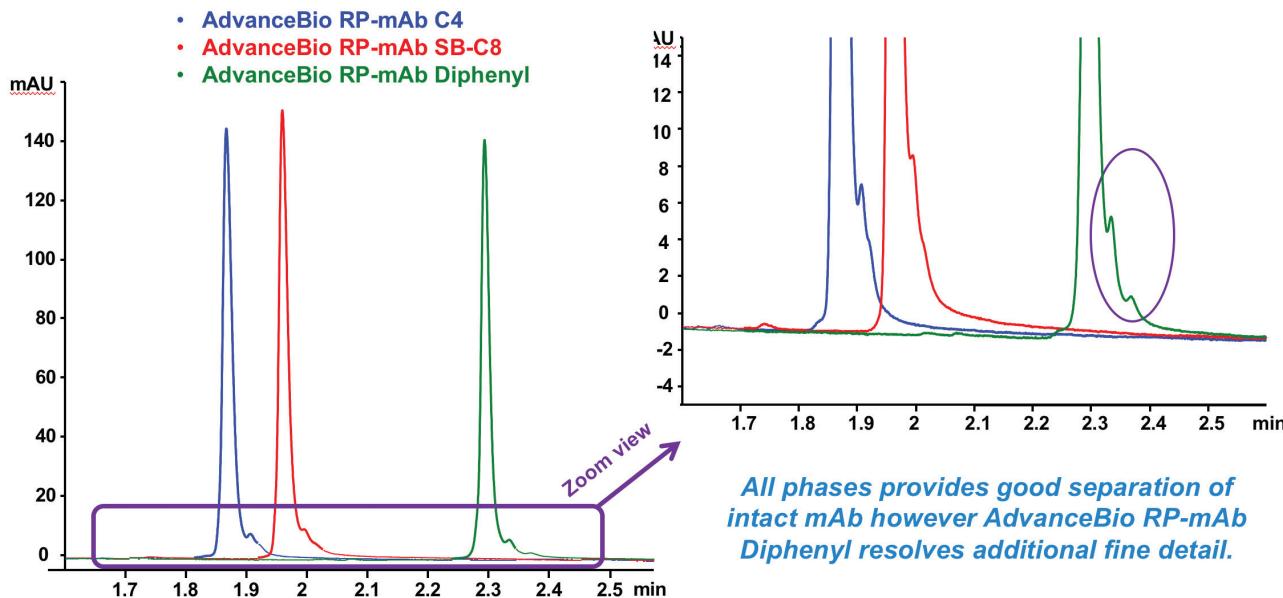


FIGURE 3: BONDING CHEMISTRY

FA are less hydrophobic than TFA and so protein peaks will elute earlier. The combined effect of using hydrophobic ligands and ion pairs can be fine-tuned to optimize peak shape, resolution, and retention time. In addition to C4 and C8 ligands, diphenyl materials are helpful for resolving fine details when analyzing mAbs, as shown in **Figure 3**.

Other types of stationary phases can also be used for reversed-phase chromatography of proteins. For example, macroporous polystyrene/divinyl benzene (PS/DVB) materials are useful because they do not contain silanol groups that cause peak tailing—no alkyl ligands are required; PS/DVB is sufficiently hydrophobic to retain most proteins, even when used with FA

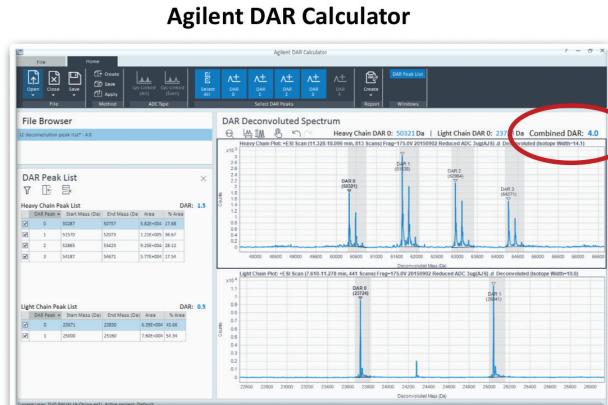
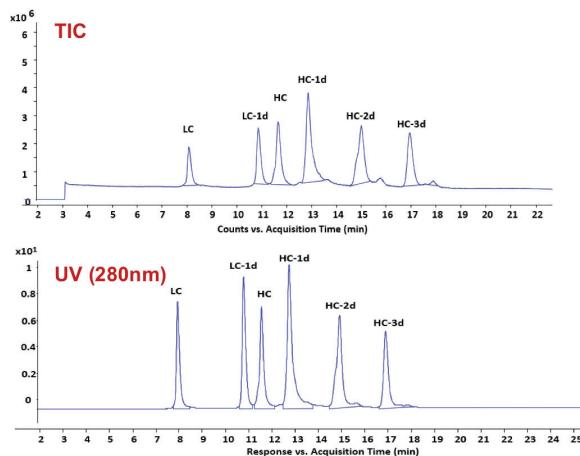
as an ion pair reagent. And, PS/DVB is very rigid and can be used at high flow rates without damaging the particles.

Polymer-based particles are often preferred over silica ones when FA is present in the mobile phase. An interesting example is the analysis of antibody-drug conjugates (ADCs) created by using cysteine to link drug molecules to the antibodies. These conjugates could perhaps be used to deliver drugs to a particular type of molecule or cancer cell. Each antibody can have 0, 2, 4, 6, or 8 drug molecules attached to it; measuring the amount of drug actually attached to the antibody is very important. Depending on the location and number of drug molecules actually linked to the antibody, a number

FIGURE 4: HIGH RESOLUTION ADC FRAGMENTS: LC/UV & LC/MS

Separation of different drug conjugated species

Reduced ADC (Cys conjugated)



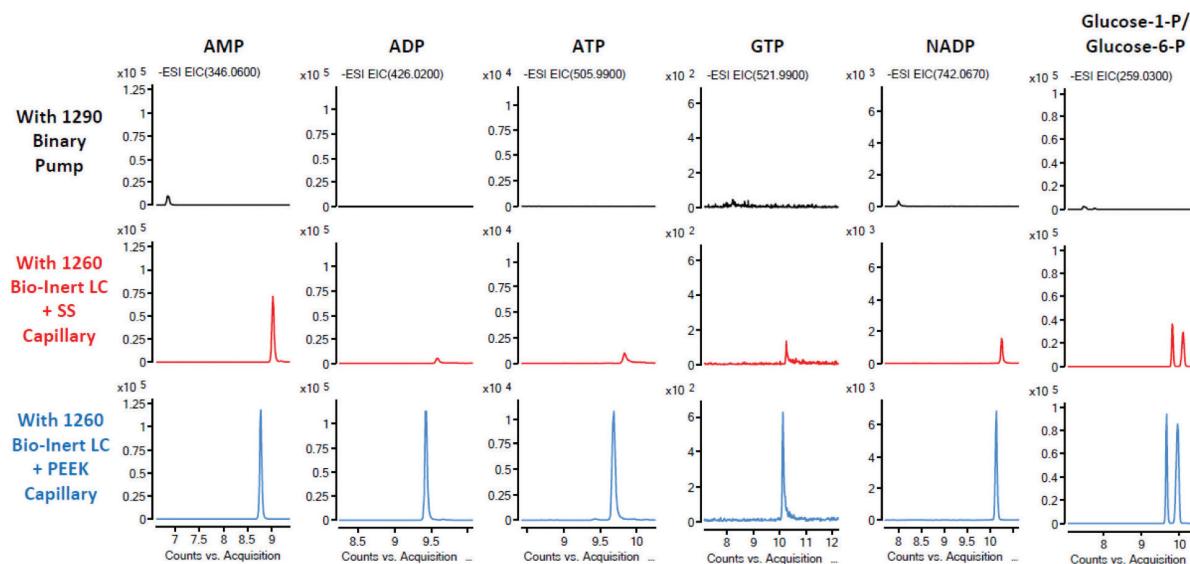
Method Parameters (Cys conjugated samples)
 Column dimensions: 2.1 x 50 mm
 MPB: 0.1% FA, 0.025% TFA in ACN
 MPA: 0.1% FA, 0.025% TFA in water
 Flow rate: 0.25 mL/min

Injection: 1 μ l
 Column temperature: 70 °C
 Gradient: 0min - 27% B, 3min-27% B, 25min-49% B, 26min-95% B, 31min-95% B, 31.5min-27% B

of positional isomers are obtained. Since the antibody molecules are susceptible to denaturing under the reversed-phase conditions, it is often simpler to reduce the ADCs all the way to light and heavy chains for quantization. The reduction generates a large number of fragments that need to be clearly resolved. This type of analysis is best carried out with a 50-mm column packed with fully porous, polymeric 5- μ m particles with a pore size of 1000 Å, such as the PLRP-S. The mobile phases A and B are composed of FA/TFA in ACN and FA/TFA in water, respectively. The drug-to-antibody ratio (DAR) can then

be determined by LC/MS and LC/UV methods. In this particular example, DAR was 4.0 (**Figure 4**).

PS/DVB columns are also useful as simple online desalting tools prior to MS systems, especially when the LC mobile phases involve components that are not volatile or lead to the suppression of the MS signal. Using a reversed-phase desalting cartridge can increase the sample concentration and eliminate the species that cause suppression, significantly increasing the ion count.

FIGURE 5: COLUMN AND INSTRUMENT MATERIALS**Nucleotide Phosphates on AdvanceBio MS Spent Media (HILIC stationary phase in PEEK lined SS hardware)**

A: 10 mM Ammonium Formate pH 6.8 in water, B: acetonitrile + 10 mM Ammonium Formate pH 6.8, 95-30% B in 10 minutes, 0.25 mL/min, 0.2 μ L injection (5 ng each on column).
MS Source: ESI-, m/z 191.02, 346.06, 426.02, 505.99, 521.99, 742.067, 743.067, 259.03

Column Construction, Mobile Phase, and Temperature

In some cases, the molecules of interest may interact with the stainless steel components of the equipment, whether they are part of the instrument itself or of the column. For example, nucleotide phosphates from cell culture spent media are not detected by HILIC using a regular stainless steel instrument. In contrast, a Bio-Inert LC instrument equipped with a polyether ether ketone (PEEK)-lined column readily shows peaks for adenosine mono-, di-, tri-, and higher phosphates, as well as nicotinamide adenine dinucleotide

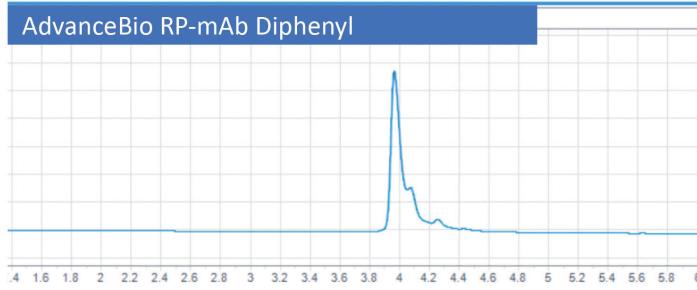
phosphate (NADP), and glucose variants (Figure 5).

When the protein analysis calls for fragmentation and partial reduction to obtain more detail or to look for impurities, it is important to prevent adherence to the column to avoid carryover and to recover 100% of the molecules. In this case, higher flow rates (0.8 to 1 mL/min) and longer columns (10 cm) are used. It is also important to properly design the mobile phase to improve recovery. For example, isopropyl alcohol (IPA) is often used, even though it is more viscous than acetonitrile (ACN) and it requires operating tempera-

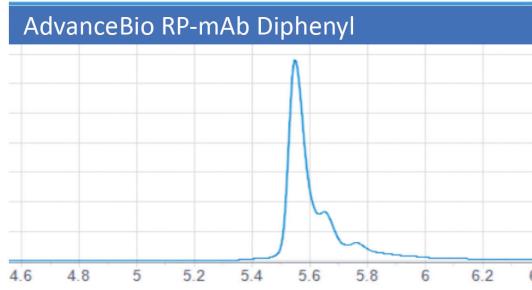


FIGURE 6: EFFECT OF ORGANIC MODIFIER

Column	AdvanceBio RP-mAb Diphenyl 2.1x50mm
Mobile phase	A: water:IPA 98:2 + 0.1% TFA B: IPA:ACN:MPA 70:20:10
Temperature	80 °C
Flow rate	0.5 mL/min
Gradient	25 – 45% B in 10 min



Column	AdvanceBio RP-mAb Diphenyl 2.1x50mm
Mobile phase	A: 0.1% TFA in water B: 0.08% TFA in ACN
Temperature	80 °C
Flow rate	0.5 mL/min
Gradient	25 – 45% B in 10 min



tures as high as 80 °C for a flow rate of 0.5 mL/min. In this case, diphenyl columns are often better suited for the analysis. Higher temperatures, when appropriate, result in sharper peaks (because of the improved mass transfer), lower operating pressures, and retention times of about four minutes. In contrast, using TFA/ACN at the same temperature and flow rate, the retention times are closer to five and a half minutes (see **Figure 6**). The downside of using high temperatures is that the proteins can undergo unwanted transformations, such as deamidation, so special care needs to be taken to find the optimal conditions.

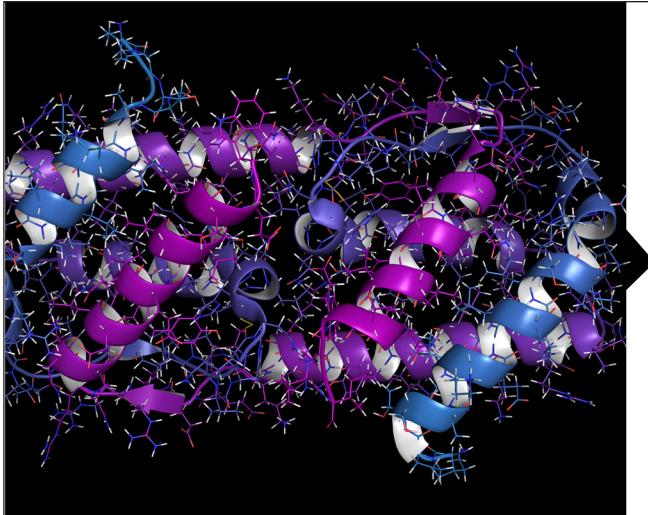
Conclusion

Choosing the right column for intact protein analysis involves a careful consid-

eration of the chromatographic technique itself, in combination with sorbent and column characteristics, as well as instrument characteristics and operating conditions. Each of these factors should not be considered just individually, as they are all interconnected. Ultimately, there may be only a small combination of factors that will give the best results for the molecules of interest.



Andrew Coffey, PhD
Senior Application Chemist
Agilent Technologies



SPONSORED CONTENT

An Inside Look at Reversed-Phased Separations for Protein Analysis

An interview with Linda Lloyd

Reversed-phase separations is a main technique used by analytical chemists for protein characterization. The method provides a lot of information about the critical quality attributes including glycosylation sites and amino acid sequence. Analysts can look at disulfide bond shifting, oxidation, deamidation, and more. Reversed phase is extremely powerful for confirming the identity of a protein, its absolute mass, its amino acid composition, and the impurity profile through a mass spectrometry workflow. In short, as Linda Lloyd, PhD, technical trainer, specializing in LC and biochromatography at Agilent Technologies, said in a recent interview with *LCGC's CHROMacademy*, "We would very much be struggling if we weren't able to use reversed phase for protein characterization." Linda's experience is based on over 35 years as a practical chromatographer with experience in R&D, production, technical support, application development, and product management.

CHROMacademy: What are some typical workflows for reversed-phase analysis of proteins?

Lloyd: Reversed-phase separations is an extremely powerful technique for characterizing proteins. It lends itself to several workflows rather than a single type of analysis. For instance, the technique could be used to look at the protein primary sequence, to look at amino acid deletions, to determine an absolute molecular weight via mass spectrometry (MS), or to see whether there are any modifications in the structure of those amino acids. You could be looking for site-specific glycosylation, for instance, if it's a glycoprotein like a monoclonal antibody (mAb).

Reversed-phase workflows could use either mass spectrometry or ultraviolet (UV) detection. MS instruments are routinely used with reversed phase because it enables the identification of the structure and any modifications to it and the mobile phases used enables direct coupling to MS detectors. In many workflows, UV is used when quantitation is needed such as in a

QA/QC environment.

There are sort of three levels of protein analysis starting with the intact protein, larger fragment analysis, and then looking at enzymatic digest down to the peptide level. Multiple levels of characterization can be done with reversed-phase systems.

CHROMacademy: What conditions affect reversed-phase separations?

Lloyd: Reversed phase is used for the analysis of denatured proteins. This type of separation uses an organic and aqueous combination for the mobile phase. It's a gradient separation—it's not done isocratically—with an increasing gradient of an organic component such as acetonitrile.

Occasionally, one might put some isopropanol or normal propanol into the organic part of the mobile phase if, for instance, the sample contains particularly sticky proteins and better peak shape and recovery are needed. An additive like trifluoroacetic acid (TFA), an ion-pair reagent, would be needed if you're working with UV.

Sometimes with mass spectrometry, TFA will be used, but the preferred additive for MS detection is formic acid because it doesn't suppress the ionization. Thus, it increases sensitivity when doing reversed-phase separations. Quite often, analysts are looking for a critical quality attribute at a very low level, so that sensitivity is needed.

Temperature is routinely used to improve the efficiency of separations involving denatured proteins. It also reduces the

viscosity of the mobile phase. Typically, temperatures are 60–80 degrees Celsius, depending upon the exact workflow.

CHROMacademy: Could you also talk about some of the challenges that analysts may face with reverse-phase separations and potential areas for improvement in the technology they work with?

Lloyd: There are quite a lot of challenges in reversed-phase separations. One of them is trying to maximize the amount of information obtained from a workflow. In terms of characterizing by reversed phase, you might do intact, fragmented, and peptide mapping. That's three workflows and three analyses. Even with the higher efficiency separations that are performed these days, these analyses can take an hour or more to perform.

Some analysts are looking at multiple-attribute methods based on reversed-phase separations and peptide analysis to home in on the critical quality attributes and maximize the amount of information that can be obtained from a single run.

Another challenge with reversed-phase separations is sample preparation. For intact proteins, sample prep is not too onerous, but once you start fragmenting a protein and doing a chemical or an enzymatic digest to get fragments, then the sample preparation method can be quite time consuming. There can also be issues with reproducibility of the sample prep, particularly with some of the enzyme methods.

As we move forward, we really need to look at how we can automate sample prep, simplify the number of steps involved, and



improve efficiency so we can reduce the total analysis time and get a more robust sample prep—and, in turn, sample analysis.

I should also talk about the mass spectrometry. There are some challenges in terms of column technology delivering clean mobile phase or samples into the mass spectrometer. There's a lot to be done in terms of looking at how we can better interface columns to MS instruments and making the material more compatible with mass spectrometry. We are looking at using mobile phases or mobile phase additives that don't affect the chromatography at all. This helps preserve the sample integrity and composition, resolution of critical pairs, and the sensitivity.

CHROMacademy: Can you elaborate more on what Agilent is doing to accomplish this?

Lloyd: Agilent has been looking at improving mass spec compatibility by using polymeric materials that don't have silanols. One issue with the formic acid ion-pair reagent is interaction with residual silanols on the silica, which results in peak tailing and reduced resolution and sensitivity.

So, Agilent has been looking at rigid polymers, the PLRP-S polymer, which can be made in very large pore sizes for the analysis of intact proteins and some larger fragments. Because it's a polymer, there are no residual silanols, so the peak shape is much better when you're using formic acid. Although they tend to be larger particle sizes, you can get much better separations due to improved peak shape even at high sample mass loadings.

CHROMacademy: What about reproducibility? What are vendors doing to ensure minimal variability in columns?

Lloyd: One challenge with bioseparations is the complexity of the molecules that you're separating. The molecular weight of an intact mAb, for instance, can be 150,000 Daltons—and that's the monomeric form. Moreover, mixed-mode interactions can occur with the column materials, so reproducibility is a concern across the separations for biomolecules.

Vendors are now recognizing the importance of the entire stationary phase. Going back to manufacturing particles and materials that are specifically designed now for bioseparations, we've seen some early materials that are essentially repurposed from other applications spaces from gel permeation chromatography (GPC)-size exclusion chromatography (SEC) for synthetic molecules or from the small-molecule liquid chromatography world.

Now, vendors are designing the particle with the correct porosity for large molecules. With stationary phases, it's important to control the size and shape of the pore. So, if we're looking at these large molecules, we need very large pore sizes. The control of the particle morphology—be it superficially porous or fully porous—in pore size is a key starting point.

Manufacturers are putting a lot of emphasis now on designing and manufacturing that base particle for the bioseparation and doing QC on the base particle. Instead of completely relying on the final product QC, they try to understand and consider the im-

pact of each components (i.e., particle QC). The chemistry is optimized for the bioseparation and optimized to minimize non-specific interactions to ensure that you aren't unintentionally introducing mixed mode phases through your chemistry. That chemistry must be clean, non-interactive, and, of course, not bleed or leach under the conditions that are used for the chromatography.

In terms of the final product, several application-specific QC tests must be conducted to look at particle characteristics, bonding density, and molecule performance (in terms of characterizing the type of stationary phase and the type of interaction). Over the last two or three years, we've seen this become far more important in biologics, especially to achieve reproducibility. Having test mixes that are representative of the application workflow used in the QC is important as well as, in some instances, having those test mixes available for customers to monitor performance and track it as a function of time.

Today, there is very much a focus on designing product for this space and addressing extremely complex separations so that QC testing will really probe the performance for the target applications. It's not something that a column user would necessarily be aware of, but quality and reproducibility must be built in from the very beginning of an R&D process.

CHROMacademy: As the biopharmaceutical industry progresses toward one of these next-generation therapeutics, how do you see reversed phase fitting in?

Lloyd: I think it will still be one of the primary

techniques that is used. One of the beauties of reversed phase is that there are so many options.

The challenges will be more complex in terms of the information needed from the separations, but the same principles will apply.

With reversed phase, we can look at synthetic peptides or materials manufactured as therapeutics with smaller pore sizes. Reversed phase will have the selectivity to drive the separations. And then if we look at RNAs, virus-like particles, then the ability to make very wide pore with reserve-phase materials is important. The PLRP-S at 1000 Ångström or even a 4000 Ångström pore size enables you to do some very high-performance separations on large molecules.

Where we would potentially see a limitation with reversed phase is when the molecule is unstable in an organic aqueous mix. If denaturing some of these more complex molecules actually makes it fall apart in a way that you don't want to happen, that could be a limitation.

But certainly something like antibody-drug conjugates, where you've got a small molecule that is covalently coupled to a monoclonal antibody, then reversed phase is a very powerful technique in looking at the drug-to-antibody ratio. It's one of the two techniques that can be used for such a calculation. The other is hydrophobic interaction chromatography.

As the field progresses, there will be some challenges in terms of optimizing those materials, but the capability and the technology is there to tune the reversed-phase columns as we go forward.



15 Biochromatography e-Learning modules now available at www.CHROMacademy.com/Bio

- Amino Acids, Peptides and Proteins
- mAbs - Introduction to Monoclonal Antibodies
- Biopharmaceuticals
- Introduction to Biopharmaceutical Analysis
- Reversed Phase - Introduction & Peptide Level Analysis
- Reversed Phase - Peptide Mapping LC Conditions & Effects
- Reversed Phase - Protein Level Analysis & Optimization
- HILIC
- Size Exclusion Techniques
- Ion Exchange Techniques
- Affinity Chromatography Techniques
- Mass Spectrometry for Biochromatography
- Introduction to Mass Spectrometry for Biomolecules
- Mass Spectrometry Basics for Biomolecule Analysis
- Mass Analyzers Used in Biomolecule Analysis
- Mass Spectrometry at the Protein Level

A lab staffed by more knowledgeable chromatographers will have less instrument downtime and team members who can foresee and prevent problems occurring.

CHROMacademy provides a reliable, effective and competitive training solution for your analysts.

Contact us to find out how we can help you develop the talent within your organization and make your team the most effective it can be.



All our Biochromatography modules have been developed with the help of the Research Institute for Chromatography

CHROMacademy – the world's largest e-Learning website for analytical scientists

Packed with practical information that will help improve the skills
and productivity in your lab

**Build a culture of learning with our help at
www.chromacademy.com**