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We are living in exciting times, when researchers like you are exploring new frontiers in drug development. Biotherapeutics have already dramatically changed many peoples' lives, and the potential for new treatments—and even cures for some of our most intractable diseases—is boundless. It's rewarding to be able to be a part of the new discoveries taking place.

At Agilent, we are committed to developing the instruments, chemistries, and workflow support that make new discoveries and innovations possible. Working together with scientists in all parts of biopharma, we are finding ways to make the processes of discovery, development, and manufacturing biologics and biosimilars faster and easier.

In this e-book, we explore the steps it takes to find and evaluate an effective biosimilar, looking at protocols for Herceptin. The techniques discussed here are not so different from the tools used to develop and evaluate innovator drugs. Our large family of biocolumns and leading-edge UHPLC and LC/MS technologies support these analyses.

We hope to partner with you to support your important work!

Thank you,
The Agilent Team

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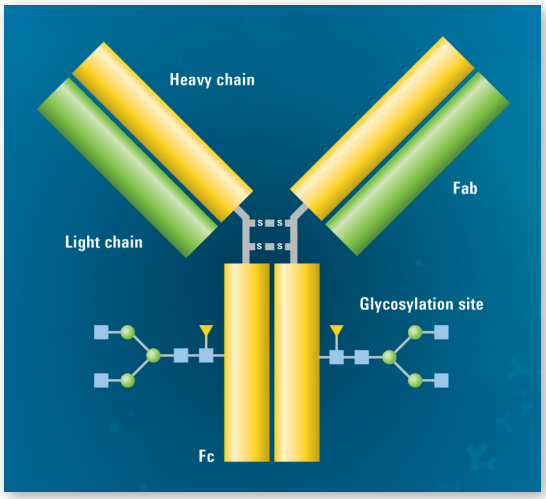

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Key Biosimilar Issues

and Emerging Trends

Angelo DePalma, Ph.D.

In a 2012 presentation Annelie Sköld, Ph.D., program manager for biosimilars at GE Healthcare, identified five drivers for biosimilars development. The three "positive" factors were regulatory framework, treatment costs, and the entry of many large pharmaceutical and biopharmaceutical players. Contra-trends were delays resulting from legal and intellectual property activity, and lack of prescribing interchangeability between biosimilars and originator molecules.

According to early European Medicines Agency guidances and current worldwide practice, manufacturing processes for biosimilars stand on their own and need not duplicate the originator's production methods. "Instead, the focus is on extended characterization efforts to show that the biosimilar is 'highly similar' to the reference medicine. That can really cut clinical efforts and save developers a lot of money," says Fredrik Sundberg, Ph.D., global director of strategic market development, GE Healthcare Life Sciences.

It also presents opportunities for greater process economy through improved production technology as, for example, through the implementation of single-use equipment, more rigorous process monitoring, process intensification, and other improvements.

Characterization and Quality

Regulators have been encouraging the use of sophisticated analytical tools, particularly as biosimilars have picked up steam. FDA's guidance, Quality Considerations for Biosimilar Products (Feb. 2012), mentions surface plasmon resonance, microcalorimetry, and classical Scatchard analysis to provide information on binding kinetics and thermodynamics. "Such information can be related to the functional activity and characterization of the proposed biosimilar product's higher order structure," according to the guidance.

"These data, as well as immunogenicity as measured by anti-drug antibodies, are key regulatory and technical challenges for biosimilars and other novel drugs," adds Dr. Sundberg.

Characterization and quality control may be significantly improved with state-of-the-art analytics that provide more data, more rapidly or in real time, provided this occurs during early development. Companies should apply the same methodology for

selecting the best clones, assessing higher-order structure, and evaluating function and physicochemical attributes as they would for any well-characterized biological product, says Dr. Sundberg.

"One hurdle for smaller players may be access to several batches of the reference medicine, which are required to establish a threshold for the originator's process variation—which can sometimes be greater than its dissimilarity with the biosimilars," he points out.

U.S. Lagging Behind?

Biosimilars have been available in Europe for more than six years, but have not yet been approved for the U.S. market. Lisa Skeens, Ph.D., corporate vp, global regulatory affairs, at Hospira, attributes the late U.S. entry to the fact that many U.S. patents for key biologics don't expire until mid-decade.

This past July Sandoz reported that the FDA accepted its Biologics License Application for filgrastim, which was filed under the new biosimilar pathway created in the Biologics Price Competition and Innovation Act (BPCIA) of 2009. The company was the first to file for approval via the BPCIA process.

Hospira, which expects to file initial applications for biosimilars with the FDA within the coming year, is the only U.S. company already marketing biosimilars in Europe and Australia. "Our experience suggests that once physicians become aware of the safety and efficacy, including from post-marketing studies, they will gain confidence in prescribing biosimilars," Dr. Skeens tells GEN.

Hospira believes that biosimilars will lower the cost of expensive biologics and expand access. "The U.S. market will be more knowledgeable when biosimilars debut here," Dr. Skeens says. "U.S. physicians and payors in the United States have had the advantage of observing European experience, and there is a multitude of studies supporting biosimilars."

It Comes Down to Economics

As noted in a 2011 IMS report, Shaping the Biosimilars Opportunity, biosimilars are expected to increase their market share within biopharmaceuticals from 1% in 2014 to 10% by 2020, representing a market value increase from $1.9 billion to as much as $25 billion.

Because the European Union enjoyed a five to seven-year head start on issuing guidance related to biosimilar development, virtually all sales of biosimilars today occur in Europe. The comparison between European and U.S. approvals resembles a lopsided football score: EMEA 21, FDA 0.

IMS Health attributed the slow uptake of biosimilars in the U.S. to legislation allowing originator companies to delay approval of new biosimilars. Once products begin breaking through these legal and regulatory barriers, however, the U.S. is expected to be a major market for biosimilars.

Another IMS study, commissioned by the Generic Pharmaceutical Association in 2011, calculated that generic drugs saved the U.S. health system more than $150 billion in 2010 alone, and $931 billion during the preceding decade. IMS estimated that biosimilars could save as much as $108 billion over their first decade of U.S. availability.

Analysis by the German asset management firm DVFA indicates that biosimilar development will require approximately half the number of patients and just 60% of the time to market compared with originator biologics. Even more attractive: development costs are just 15% to 20% of those for biologics, with a probability of success that is ten times higher (50% vs. 5%).

Similar numbers no doubt emboldened Hartmann Willner, an FDA regulatory affairs consulting firm, to predict, in 2007, that biosimilar sales would reach $16 billion by 2011. Actual sales that year were about $600 million. Gabriel Morelli, Spain Country Manager at IMS Health, recently wondered if industry analysts were not "expecting too much" at that early point but if the time for optimism has indeed returned. Morelli concludes that biosimilars "are approaching a turning point…but still much has to change."

On the plus side, biologics sales have doubled since 2006, and growth has been two to three times as rapid as that for all pharmaceuticals. Global sales increases of 14% in 2012 for biologics suggest significant pent-up demand, even as health systems look to cut costs. Yet growth in biosimilars is expected to be strongest in advanced economies, particularly the U.S., Europe, and Japan, while the most rapid growth (based on extremely modest baseline sales) will occur in emerging nations like Brazil, Mexico, and China.

Conversely, the economics of biosimilars are by no means cut-and-dry. Small molecule generics lose approximately 90% of their value compared with the innovator medicine, and their introduction does not increase utilization. Cost reduction estimates for biosimilars may vary from 10% to as much as 60%. Prices will differ by large factors between established economies and developing ones, complicating cross-border sales efforts. Add to this the uncertainties of a huge number of entrants and regulatory disharmony outside of advanced countries, and market predictions become reckless, even parlous.

All in all, Morelli believes that factors positively and negatively affecting biosimilars, including IP and legal challenges, will turn increasingly positive, re-affirming his company's prediction of $25 billion in sales by 2020.

Utilization

Nathan Wei, M.D., director of the Frederick, MD-based Arthritis Treatment Center, predicts that biosimilars will be priced about 30% lower than originator drugs. Dr. Wei, who has participated in biosimilar studies for rheumatoid arthritis, is cautious about utilization predictions. "Insurance companies will probably mandate the use of biosimilars ahead of brand name products," he says, "but as with other generics, some people will be leery about taking them. I know endocrinologists who will only use branded Synthroid, for example, instead of the generic."

Dr. Wei believes that skepticism will be higher for biosimilars than for generics because "biologics are more difficult to manufacture." Moreover the lower prices will not likely lead to greater utilization. "Insurance companies will still stonewall, and docs who aren't prescribing biologics now still won't," he predicts.

The price drop for biosimilars in Europe relative to innovator molecules has been 20%–30%, and most experts expect a similar discount in the U.S. Because the U.S. is the world's largest market for biologics, pharmacy benefits manager Express Scripts has estimated that once biosimilars hit the U.S. market the healthcare system could realize savings of up to $250 billion in the first decade of availability. "The overriding need to bring down healthcare costs will drive lower prices," emphasized Hospira's Dr. Skeens, "particularly once physicians become comfortable with these drugs." ■

Setting Out on the Journey to Find a Biosimilar

First Steps in Biosimilar Characterization Using Affinity Chromatography

Koen Sandra, Ph.D., Research Institute of Chromatography
Maureen Joseph, Ph.D., Agilent Technologies

Monoclonal antibodies (mAbs) are a rapidly growing class of therapeutics with increasing numbers of these drugs being approved. The knowledge that the top-selling mAbs are, or will become, available to the market in the near future as "generics" has driven an explosion of interest in biosimilars. The complexity of these molecules and their sensitivity to changes in the manufacturing process creates an increased need for advanced analytical tech-niques to thoroughly characterize and compare biosimilar versions to originator drugs.

Chromatographic techniques are powerful analytical tools for examining intact and fragmented mAbs. These techniques include reversed-phase liquid chromatography (RPLC) and liquid chromatography mass spectrometry (LC/MS) for studying primary structures and post-translational modifications. Size exclusion chromatography (SEC) is used to assess aggregation, and ion-exchange chromatography (IEX) to examine charge variants. For glycan analysis, hydrophilic interaction chromatography (HILIC) chromatography is becoming the technique of choice.

Before these additional chromatographic techniques can be used one needs to isolate the mAb from cell-culture supernatants and this is typically done with affinity chromatography, often with a Protein A column. In this article, the focus is on the use of affinity chromatography as a first step to evaluate a Herceptin biosimilar. Other steps are covered in subsequent articles. Table 1 shows the different chromatographic techniques used.

Affinity chromatography, making use of Protein A, is the gold standard in therapeutic monoclonal antibody purification. It typically represents the first chromatographic step in downstream processing. At the analytical scale, affinity chromatography is used early in the development of monoclonal antibodies for the high-throughput determination of mAb titer and yield directly from cell-culture supernatants. Affinity chromatography also enables early assessment of biocomparability, and purifies µg amounts of material for further measurements, for example by mass spectrometry (MS) or by other chromatographic techniques such as RPLC, SEC, and IEX.

Using affinity chromatography to begin mAb characterization

Protein A has a very strong affinity for the Fc domain of immunoglobulins (IgG), allowing its capture from complex matrixes such as cell-culture supernatants. The Agilent Bio-Monolith Protein A column contains a highly cross-linked poly (glycidyl methacrylate-co-ethylene dimethacrylate) monolithic disk coated with native Protein A from *Staphylococcus aureus*. The column combines the advantages of monoliths, i.e., fast and efficient separations with limited carry-over, with the selectivity of the Protein A receptor for the Fc region of IgG. As such, it represents an ideal tool for the high-throughput determination of mAb titer and yield directly from cell-culture supernatants, and for purifying mAbs at analytical scale for further measurements, with excellent robustness. In addition, the column can be used as a tool to facilitate cell-culture optimization. As changes are made to the cell line or media to optimize production and structure of the mAb, for example, to bring the structure and glycosylation profile of the biosimilar to within originator specifications, the Protein A column can be used to again isolate the mAbs of interest. Therefore the Protein A column is an important tool for mAb titer analysis, and to successfully guide clone selection and cell-culture optimization.

Developing affinity methods to isolate proteins of interest

In this method, the use of the Protein A column in the determination of absolute mAb concentrations in Chinese hamster ovary (CHO) cell-culture supernatants, will be illustrated with data from a trastuzumab biosimilar project. Trastuzumab, marketed as Herceptin since 1998, is used in the treatment of HER2 positive breast cancer and comes off patent in 2014 and 2018 in Europe and the United States, respectively.

A Protein A separation is relatively quick and simple. The unbound material is easily separated from the bound mAb. Figure 1

Biosimilar Analysis	Chromatographic Technique
Titer determination and purification	Affinity chromatography
Protein identification and impurity profiling	Reversed-phase liquid chromatography (RPLC
Glycan analysis	Hydrophilic interaction chromatography (HILIC)
Charge variant analysis	Ion exchange chromatography (IEX)
Aggregation analysis	Size exclusion chromatography (SEC)

Table 1. Types of biosimilar analyses and their corresponding chromatographic techniques

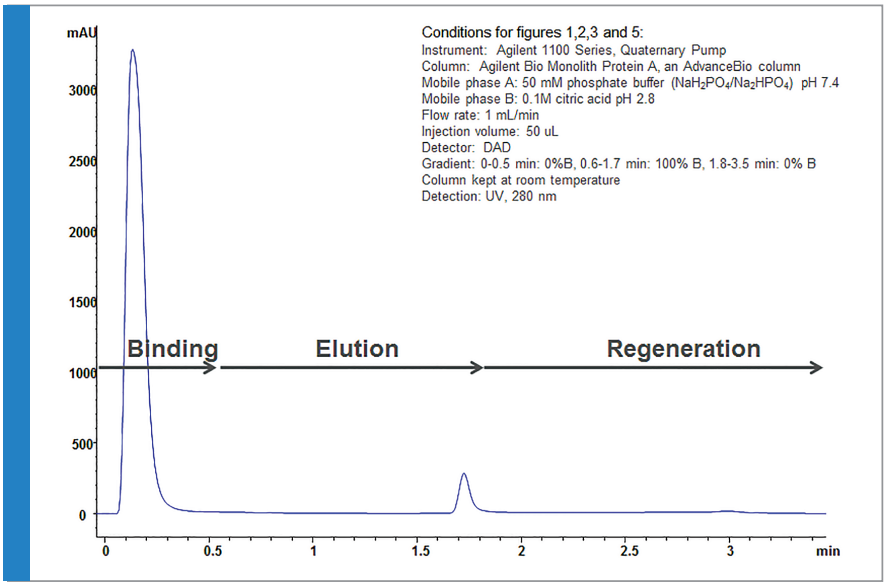

Figure 1. Protein A chromatogram from the supernatant of a trastuzumab-producing CHO clone, showing the typical 3 minute gradient separation.

	Citric acid	
	RT (min)	Peak area
1	383	1.666
2	372	1.666
3	365	1.665
4	389	1.667
5	383	1.666
6	378	1.666
7	379	1.668
8	377	1.666
9	376	1.667
10	377	1.667
Mean	378	1.667
S	6.52	0.001
%RSD	1.73	0.060

Table 2. Retention time and peak area RSD values obtained for the ten-fold analysis of a Herceptin originator at 0.5 mg/mL (5µL injection volume).

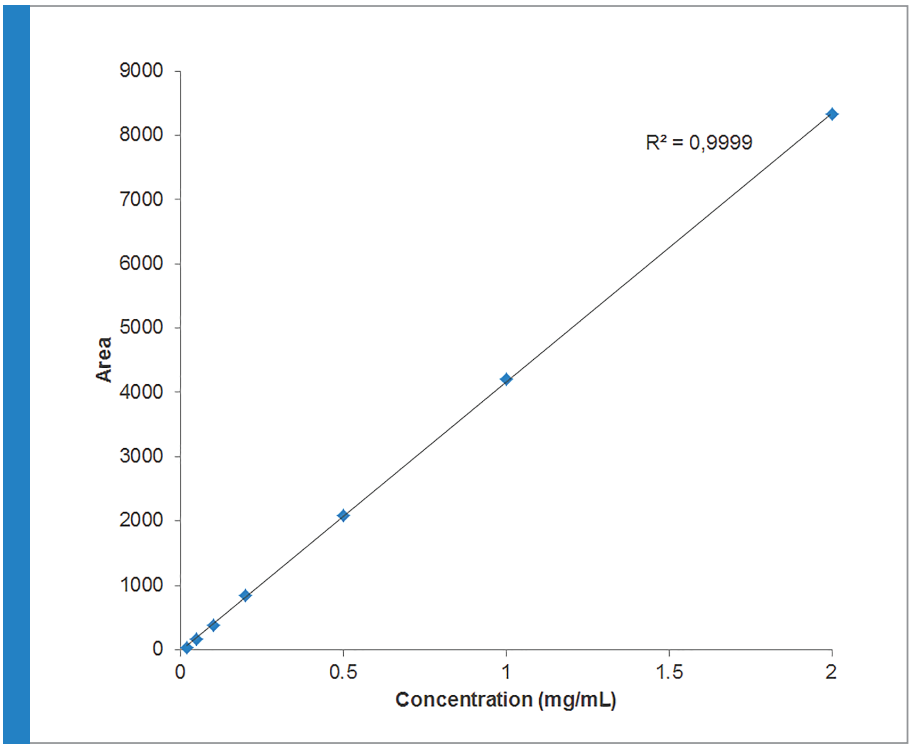

Figure 2. Herceptin Protein A calibration curve (0.02 to 2 mg/mL) using citric acid as elution buffer.

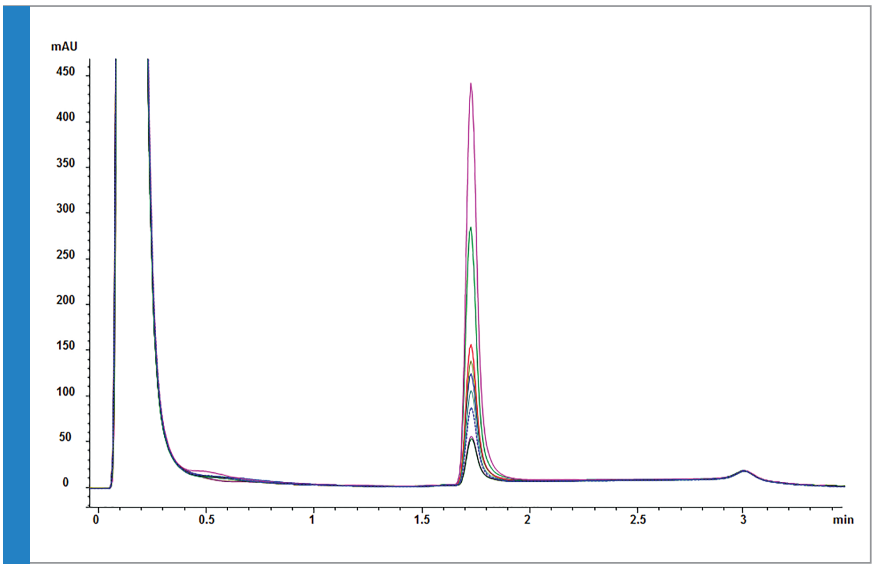

Figure 3. Overlaid Protein A chromatograms of nine trastuzumab-producing CHO cell clones using citric acid as the elution buffer.

CHO Clone	Concentration (mg/mL)
3	0.210
8	0.256
9	0.494
10	0.757
24	0.262
25	0.098
26	0.090
28	0.173
32	0.144

Table 3. Absolute mAb concentrations determined in the different trastuzumab-producing CHO clones.

shows a typical chromatogram from the Protein A column, from the cell-culture supernatant of a specific trastuzumab-producing CHO clone. The unbound material is eluted in the flow-through while the mAb is retained at neutral pH (binding) and is only released (elution) after lowering the pH upon applying a step gradient. When developing a new method for a Protein A column, both binding and elution buffers should be optimized. For binding buffers, 50 mM Na phosphate, pH 7.4, is a good starting point, and can be optimized between pH 7 and 8. For elution buffers, the 100 mM citric acid used here is a good starting point and provides excellent results. Other possible elution buffers are 500 mM acetic acid, pH 2.6, 100 mM glycine HCl, pH 2.8, and 12 mM HCl, pH 1.9.

Precision, linearity, carry-over, and injection size

Precision is critically important in the determination of a mAb titer. Table 1 shows the peak area and retention time repeatability that can typically be expected when injecting a Herceptin originator ten times.

In mAb titer determination, it is critical to be able to determine absolute mAb concentrations. These can be found by linking the peak areas measured in cell-culture supernatants to an external calibration curve constructed by diluting a mAb standard. For this Herceptin biosimilar project, the standard was found in the originator product, which was accurately formulated at 21 mg/mL.

Carry-over and buffer background can be simultaneously assessed by injecting a buffer blank after the mAb injection sequence. Using a ten-fold 2.5 µg column load, carry-over appears to be nonexistent, which we attribute to the use of a monolithic support. If the buffer system shows any background then a simple baseline subtraction will result in efficient chromatograms.

Determining the mAb titer of different clones

Our method possesses all the characteristics for the determination of mAb titer in cell-culture supernatants. It is fast, precise, and linear in the expected mAb concentration range, as can be seen in the calibration curve of a dilution series of Herceptin originator using citric acid as the elution buffer (Figure 2).

To illustrate the power of the method, nine trastuzumab-producing clones were analyzed using the Bio-Monolith Protein A column to determine absolute mAb concentrations. Results are presented in Figure 3 and Table 3.

Comparison with the originator molecule

The next step is to assess the structural characteristics of the preferred clone, typically the high titer clones, and compare these with the originator molecule. Therefore, Protein A fractions are collected and measured by high-resolution MS, following disulfide bond reduction, giving rise to the light and heavy chains. This strategy allows verification of the amino-acid sequence and reveals the glycosylation pattern. To reduce the mAb directly in the collection vial containing acidic buffer, TCEP was chosen due to its reducing capacities over a broad pH range. Reduced fractions are delivered to the MS system following online desalting. The LC/MS used is an Agilent 1290 Infinity Binary LC coupled to an Agilent 6540 Ultra High Definition Accurate-Mass Q-TOF.

Comparing the MS profiles of the heavy chain of the Herceptin originator and 2 high producing clones (Figure 4), it can be concluded that products are similar from a qualitative perspective, yet quantitative differences are observed in the glycosylation profile, i.e. the clones are undergalactosylated

The Agilent Bio-Monolith Protein A column can then be used to guide cell-culture optimization, evaluating changes in titer with tuning of the cell culture media to bring the glycosylation profile of the biosimilar to within the specifications of the originator. Optimization was done by feeding with uridine, galactose, and manganese chloride at different concentrations. These are the substrates and activators of the galactosyltransferase responsible for donating galactose residues to G0F and G1F acceptors.

Figure 5 shows an overlay of the Protein A chromatograms of the supernatant of the trastuzumab-producing clone (clone 9) grown at different concentrations of galactose, uridine, and manganese chloride (denoted as 0x, 4x, 8x, 16x, 24x). The unbound material elutes in the flow-through while the mAb is retained and released after lowering the pH.

The mAb titer is determined making use of a calibration curve generated with the Herceptin originator, and structural characteristics are revealed by LC/MS and compared to the originator molecule. It is observed that the ratio G1F/G0F increases with increasing concentration of galactose, uridine and manganese chloride (Figure 6). From these results it can be concluded that conditions can be found that adjust the glycosylation of the biosimilar to within the originator specifications. As can be retrieved from the chromatograms displayed in Figure 5, a drop in antibody concentration is noticed with increasing galactose, uridine and manganese chloride concentrations. Hence, a balance has to be found between desired structural characteristics and mAb titer.

Step one completed: on to further characterization

In affinity chromatography with the Protein A column, several method parameters can be optimized for the best method, including binding and elution buffers and injection size (volume and concentration). Once you have an optimized method and the best clone, you can then use the column to isolate a mAb in order to further understand biosimilarity, including glycosylation. The mAbs can be tuned to fall within the originator specifications with appropriate adjustments of the culture media.

It is important to select columns that will support confident decisions at each phase of your biosimilar characterization, and prepare you to move into development, once comparability is assured.

Affinity chromatography is a powerful technique most commonly used as a first stage in the purification of recombinant proteins. We next turn our attention to reversed-phase LC, to examine intact structures and produce peptide maps and, when coupled to MS, to increase confidence in your results. ■

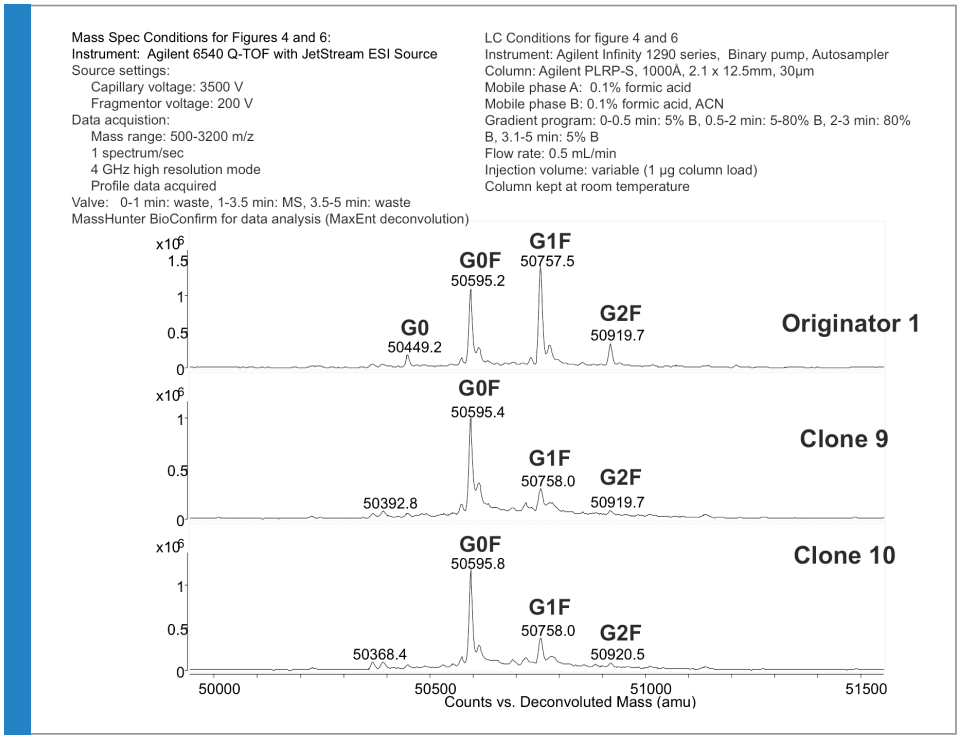

Figure 4. Deconvoluted heavy chain spectra of a Herceptin originator and two trastuzumab-producing clones. The abbreviations G0, G0F, G1F, and G2F refer to the N-glycans attached to the mAb backbone.

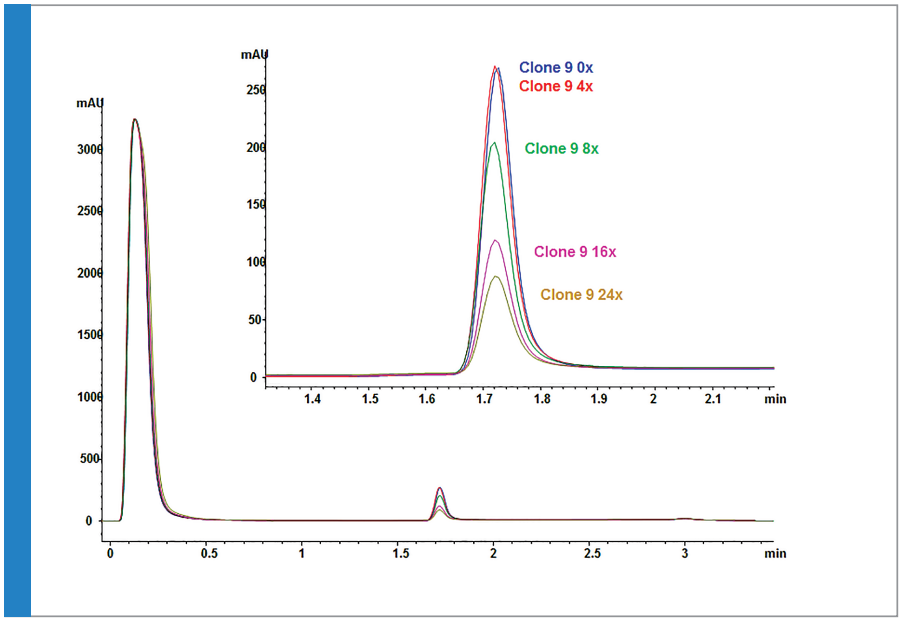

Figure 5. Overlaid Agilent Bio-Monolith Protein A chromatograms of a trastuzumab-producing CHO clone grown under different cell culture conditions, with increasing concentrations of galactose, uridine, and manganese chloride.

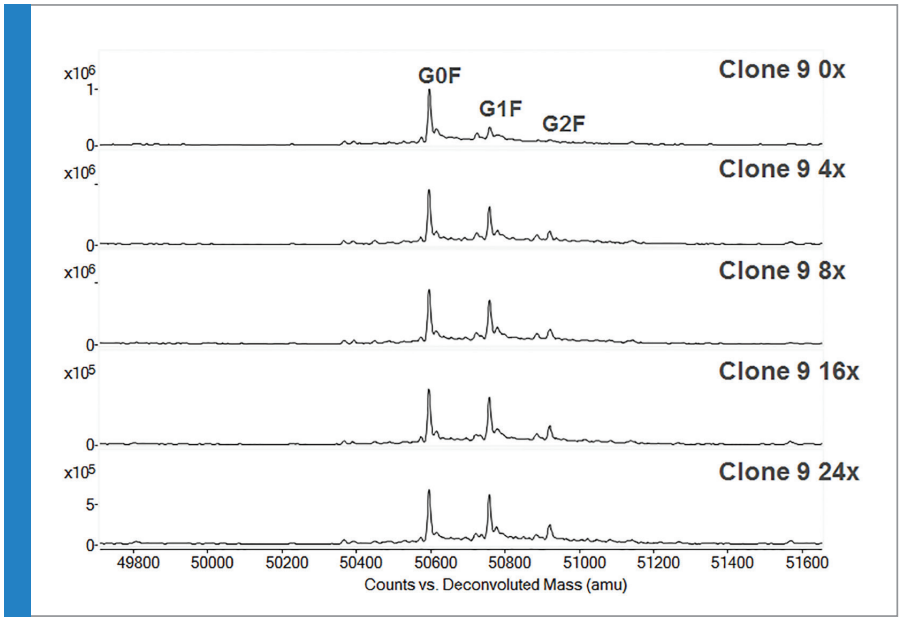

Figure 6. Deconvoluted heavy chain spectra obtained by growing a selected trastuzumab-producing CHO clone at different galactose, uridine, and manganese chloride concentrations.

Getting Acquainted with Your "Vehicle": Reversed-Phase Chromatography of Herceptin

Koen Sandra, Ph.D., Research Institute of Chromatography
Maureen Joseph, Ph.D., Agilent Technologies

Reversed-phase LC (RPLC) provides the more "fundamental" data necessary on the intact mAb, which will support further characterization of mAbs and biosimilars. It is used with common UV detectors in QC monitoring and product release testing, but it is also used with powerful MS detectors to characterize everything from the intact antibody down to the peptide fragments in a digested antibody. Reversed-phase columns with smaller particle sizes and superficially porous particles provide additional resolution and time savings when characterizing mAbs and biosimilars.

The present contribution focuses on the reversed-phase separations of intact, heavy chain/light chain, Fab/Fc fragments and tryptic peptides of Herceptin and a biosimilar in development by reversed-phase LC and LC/MS using UHPLC columns packed with sub-2 µm fully porous particles or HPLC columns packed with 2.7 µm superficially porous particles. The biosimilar has been purified by Protein A as shown in the first article. The goal in all of these separations is identity and purity determination for characterizing and comparing innovator and biosimilar therapeutics data in the least amount of time. Figure 1 shows an example of the types of RPLC chromatograms obtained with these samples.

Looking first at the separation of the intact mAb, a typical reversed phase gradient method is used for the separation. Minor changes in the organic solvent will substantially impact the separation; therefore the method uses a shallow gradient (low %B change/min) over a narrow total organic range. Figure 2 shows an example of the separations of the Herceptin originator and biosimilar. Several peaks are identifiable in the UV chromatogram due to the high resolution provided by the 1.8 µm Agilent ZORBAX 300SB-C8 column fitted to an Agilent 1290 Infinity LC. Given the compatibility between RPLC and mass spectrometry,

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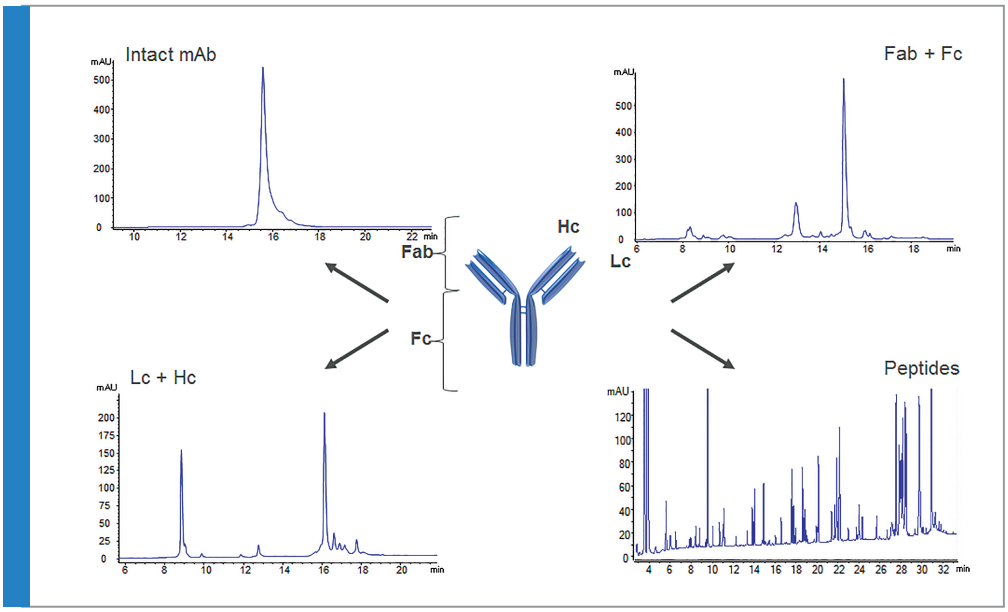

Figure 1. Examples of RPLC analyses used to characterize biosimilars

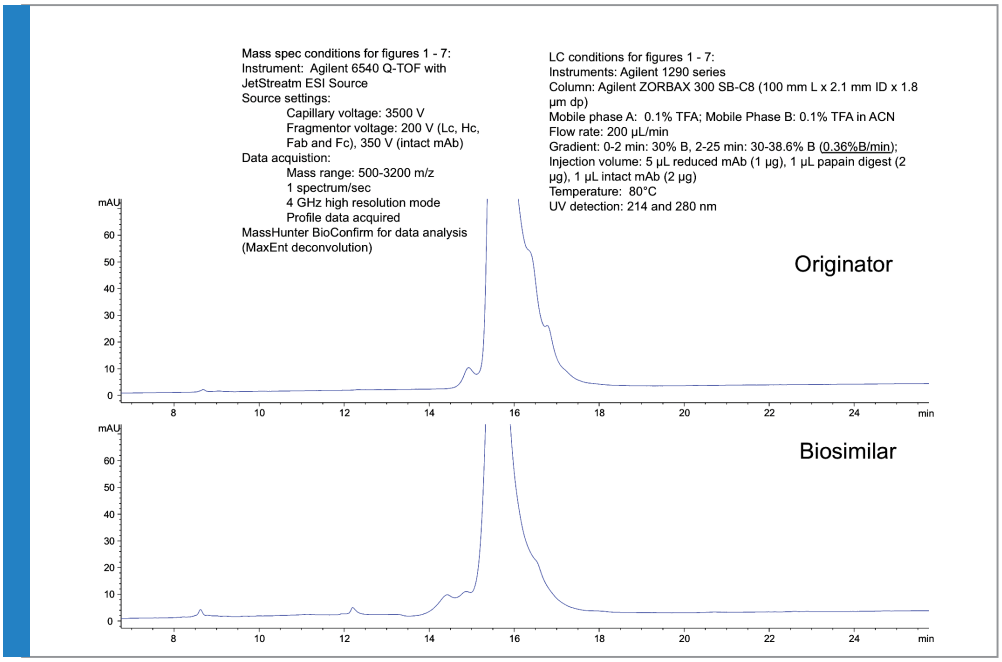

Figure 2. RPLC gradient separation of intact Herceptin and biosimilar on ZORBAX RRHD 300SB-C8, 2.1 x 100mm, 1.8 µm

peaks can be identified using the latter technology. Figure 3 shows the mass spectral data associated with the main peaks in both the originator and biosimilar. Quantitative differences in glycosylation are observed.

Figure 4 shows the reversed-phase LC separation with UV detection of the heavy and light chains of the mAb originator and biosimilar. The main light and heavy chain peaks are identical but several differentiating peaks are observed in the separation of the biosimilar. Considering the peaks in the light chain area of the chromatogram, the reversed-phase ZORBAX 300SB-C8 column provides sufficient resolution to identify the peaks A-D, using mass spectrometry. MS data was acquired simultaneously with the UV data using the Agilent MassHunter software. After extracting the raw mass spectra, deconvolution took place using the Maximum Entropy algorithm incorporated in the Agilent MassHunter BioConfirm add-on software. Deconvoluted spectra are provided in Figure 5.

It can be deduced from the data that peaks A and B originate from the light chain plus 2 hexose units, as well as the light chain plus 1 hexose. This potentially originates from a glycation event which appears to be nonexisting in the originator mAb upon comparing the two chromatograms displayed in Figure 4.

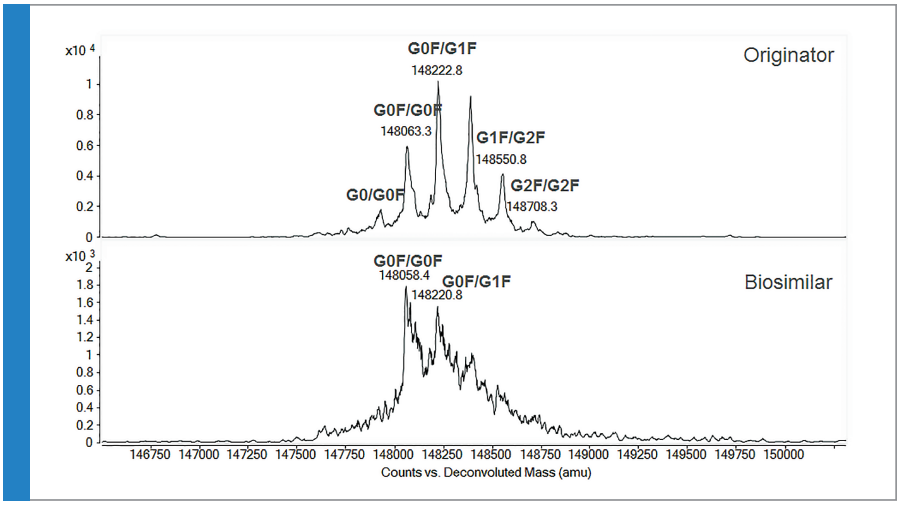

Figure 3. Mass spectra of main Herceptin peak and biosimilar from Figure 2

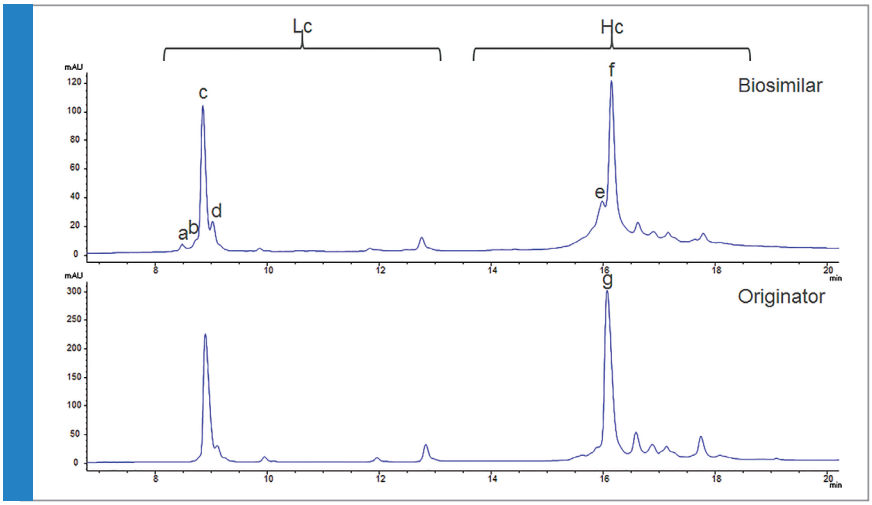

Figure 4. Reversed-phase light chain/heavy chain separation of reduced Herceptin and a biosimilar on a ZORBAX RRHD 300SB-C8, 2.1 x 100 mm column

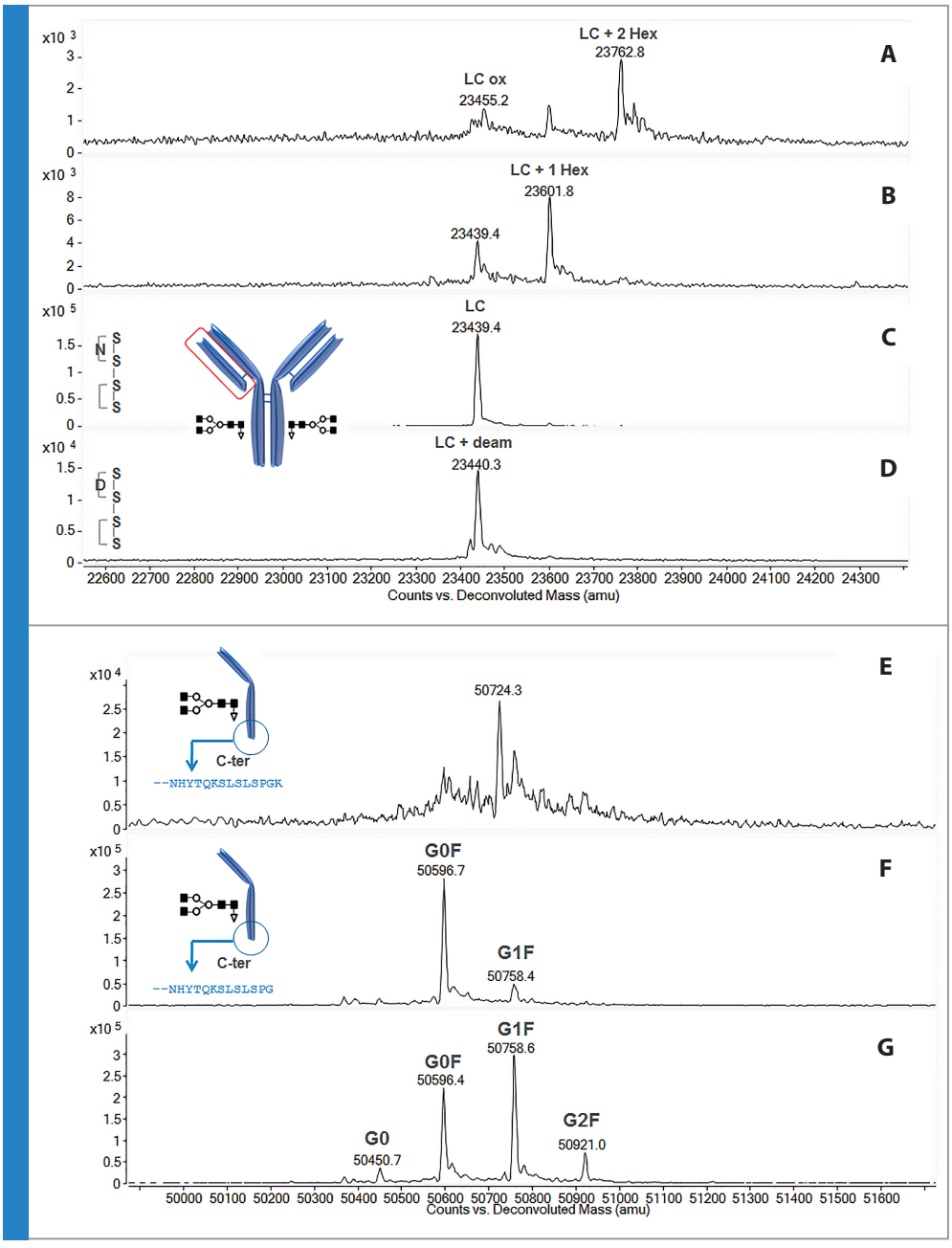

Figure 5. Mass spectra from heavy chain/light chain peaks (A–G) from Herception and a biosimilar on a ZORBAX RRHD 300SB-C8, 2.1 x 100 mm column

The MW associated with peak C exactly matches the cloned light chain sequence and is identical in both the biosimilar and originator. Peak D shows a 1 Da mass increase compared to the main light chain peak and can be traced back to a deamidation event taking place in the light chain. This event is apparent in both the originator and biosimilar with an increased occurrence in the latter (see UV chromatogram).

Upon considering the heavy chain area in the chromatograms shown in Figure 4, the biosimilar shows a more dense region eluting in front of the main peak. Upon consulting the corresponding deconvoluted spectrum (Figure 5), this peak corresponds to the glycosylated heavy chain with the C-terminal lysine still attached. To provide some more background on this particular event, the heavy chain is cloned with a lysine residue at the C-terminus. During protein maturation, this lysine is removed by host cell carboxypeptidases. Apparently, this process is more dominant in the host cell producing the originator product than in the host cell producing the biosimilar mAb. Figure 5 furthermore shows the deconvoluted spectra associated with the peaks F and G. These peaks correspond to the glycosylated heavy chains. The heavy chains in the originator and biosimilar match from a qualitative perspective, yet quantitative differences are observed in glycosylation. The biosimilar appears to be enriched in the N-glycan G0F while a more even distribution between G0F and G1F is observed in the originator mAb. A similar observation was made at the intact protein level (Figure 3). This finding immediately demonstrates the strength of incorporating MS in the characterization workflow.

The third reversed-phase separation is that of the Fab/Fc fragments of the mAb obtained from a papain digest. Figure 6 shows the RPLC-UV separation of Herceptin and the biosimilar. Differences between the two products are readily apparent and the mass spectra in Figure 7 from the Fc region peaks once more show the undergalactosylation of the biosimilar.

The reversed-phase separations finish with peptide mapping. Peptide mapping can provide an enormous amount of detail on the

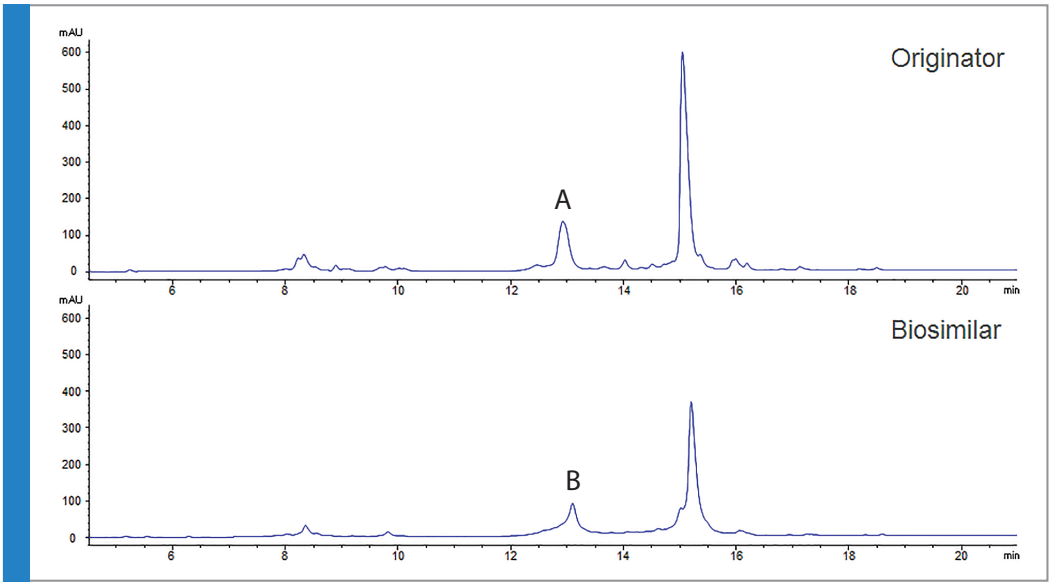

Figure 6. Reversed-phase separation of Fab/Fc fragments from a papain digest of Herceptin and a biosimilar

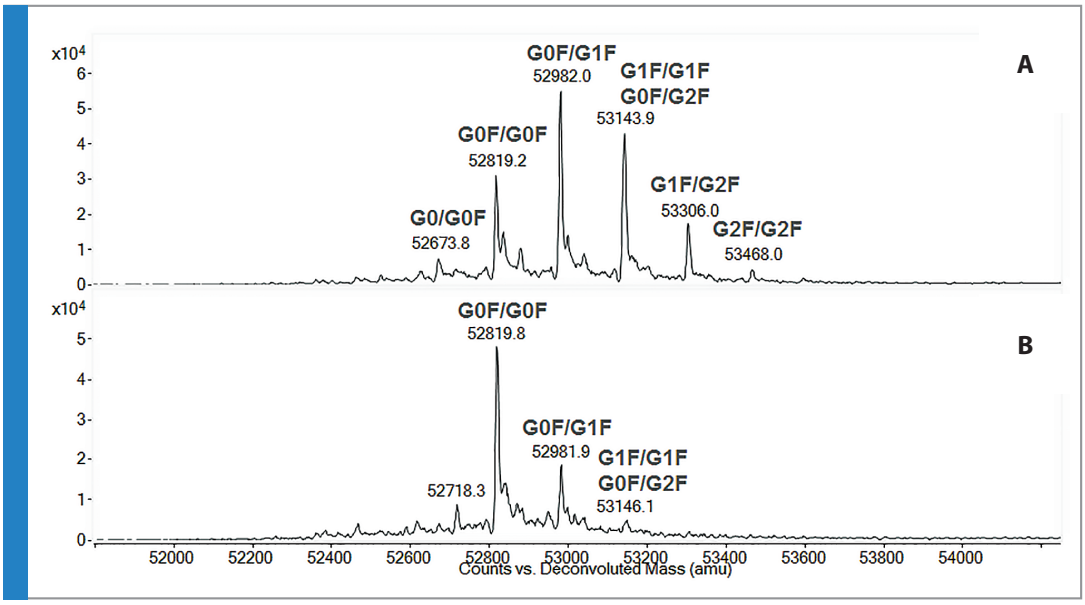

Figure 7. Mass spectra from the Fc region peaks from the papain digest of Herceptin and a biosimilar

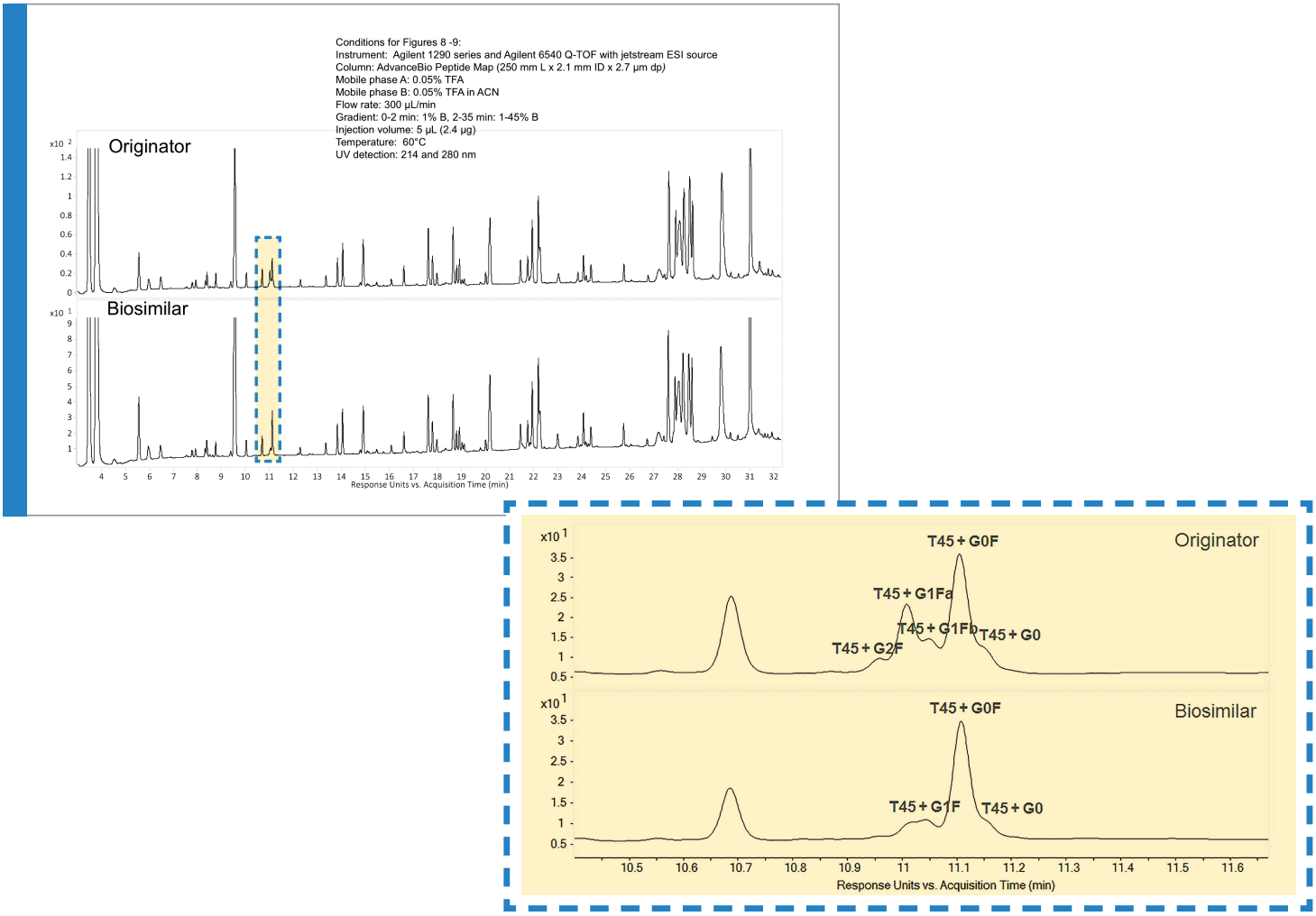

Figure 8. Reversed-phase peptide map of Herceptin and a biosimilar on AdvanceBio Peptide Mapping column, 2.1 x 250 mm, 2.7 µm with enlarged inset of 11-minute region showing undergalactosylation of the biosimilar

primary structure and post-translational modifications allowing extensive characterization and comparison between an originator and biosimilar. To extract the most information, high resolution is critical and columns packed with 2.7 µm superficially porous particles are preferred over traditional 3 and 5 µm totally porous particles for peptide separations. Figure 8 shows the UV peptide maps of both the originator and biosimilar obtained on a 2.1 x 250 mm long column. The peptides were generated using trypsin digestion and separated in a 35 minute gradient separation. Taking advantage of MS over 99% of the peptide sequence could be covered in both the originator and biosimilar thereby confirming identity. In addition, various post-translational modifications could be highlighted.

Differences in glycosylation between originator and biosimilar were already revealed using the previous reversed-phase methods in combination with MS. At peptide level, the different glycosylated variants are nicely resolved chromatographically and observed at UV level. Again the undergalactosylation of the biosimilar becomes apparent (Figure 8). Several other impurities identified from the peptide mapping data include lysine truncation, methionine oxidation, and asparagine deamidation. All of these modifications are minor and in low quantities but could be critical to potency and safety of a mAb. The extracted ion chromatograms of native and modified peptides are shown in Figure 9. The deamidation revealed, corresponds to the deamidation observed above upon separating the light chain (Figure 4 and 5).

These four examples of reversed-phase LC and LC/MS of Herceptin and a biosimilar in development — intact mAb, reduced heavy chain/light chain, Fab/Fc fragments and peptide mapping — are key separations to characterize and compare the mAbs. High resolution, optimized gradient LC methods with sub 2 µm porous and 2.7 µm superficially porous columns combined with UV and accurate mass Q-TOF provide the advances needed to correctly determine molecular weight, identity and purity. ■

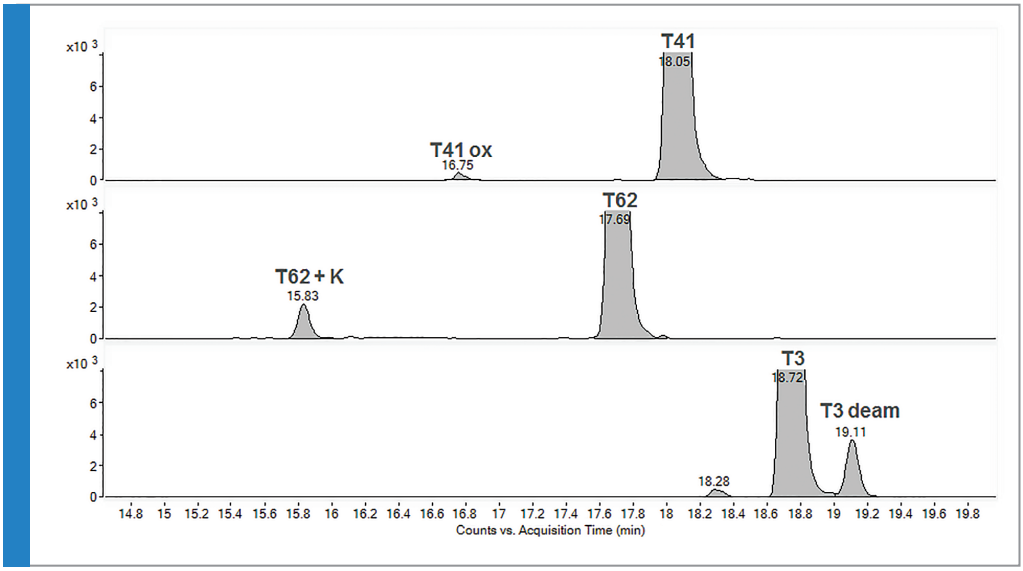

Figure 9. Reversed-phase separation and MS detection of protein impurities — lysine truncation, oxidation and deamidation on the AdvanceBio Peptide Mapping column, 2.1 x 250 mm, 2.7 µm

Stops Along the Way: Glycan Analysis, Aggregate Analysis, and Charge Variant Analysis

Koen Sandra, Ph.D., Research Institute of Chromatography
Andrew Coffey, Ph.D., Agilent Technologies

It is easy to think of proteins purely in terms of their amino acid sequence (primary structure), how the peptide chains fold into helices, sheets, and random coils (secondary structure) and how these larger assemblies are arranged (tertiary and quaternary structure). However there are other important sources of structural variation that are equally important to understand, measure, and control for reliable production of therapeutic mAbs.

Many proteins undergo post-translational modifications to incorporate oligosaccharides onto their surface. These carbohydrates known as glycans are enzymatically attached to the side chains of key amino acids such as asparagine (N-linked) and serine or threonine (O-linked), and may play a major part in ensuring correct protein folding or be involved in molecular recognition or signaling pathways. Although the level of glycosylation in therapeutic mAbs is relatively low (typically around 3-4% by weight), some therapeutic proteins have much higher concentrations (the glycan content of erythropoietin is as high as 40% w/w). Characterizing and controlling the glycan structure is an essential requirement for therapeutic mAb production given its potential role in antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC); it is not only affected by the cell line used for protein expression, but also by the fermentation conditions (levels of dissolved oxygen and carbon dioxide and even reactor design can all influence the structure of the glycan).

In order to characterize protein N-glycosylation, the glycan is first cleaved enzymatically from the denatured protein using PNGase F (Figure 1). The separation of the released glycans is then achieved by hydrophilic interaction chromatography (HILIC) using a purposely designed glycan analysis column. The gradient

elution conditions are compatible with MS detection, although it is more common for the carbohydrate to be labelled using 2-aminobenzamide (2-AB) prior to analysis, with a fluorescent detector incorporated into the HPLC system. The latter derivatisation procedure furthermore increases the electrospray ionization efficiency of the glycans. Newer choices in HILIC columns allow high-resolution, fast UHPLC and HPLC separations of glycans. Figure 2 shows the detailed HILIC separation of the 2-AB labeled N-glycans enzymatically liberated from Herceptin using either sub 2 µm fully porous particles or 2.7 µm superficially porous particles.

Differences in glycan structure may also play a role in another important source of mAb heterogeneity: charge variants. Glycans may contain different numbers of sialic acid residues resulting in differences in the number of net negative charges, however for a mAb with a molecular weight of 150,000 Da there are several

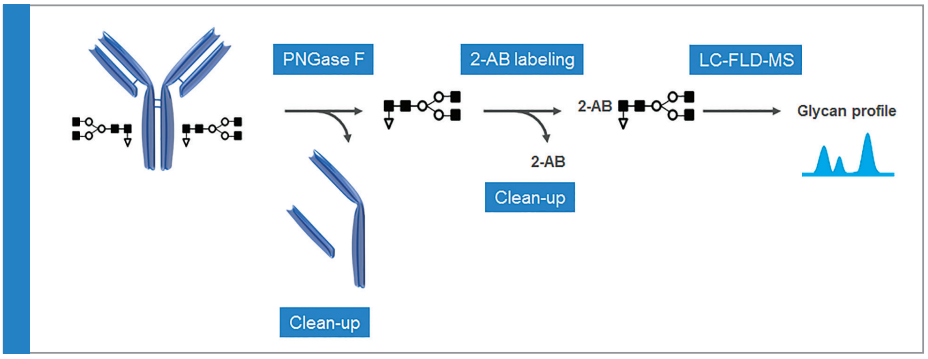

Figure 1. Schematic of N-glycan analysis process incorporating enzymatic release, 2-AB labelling, HILIC separation and fluorescence and MS detection

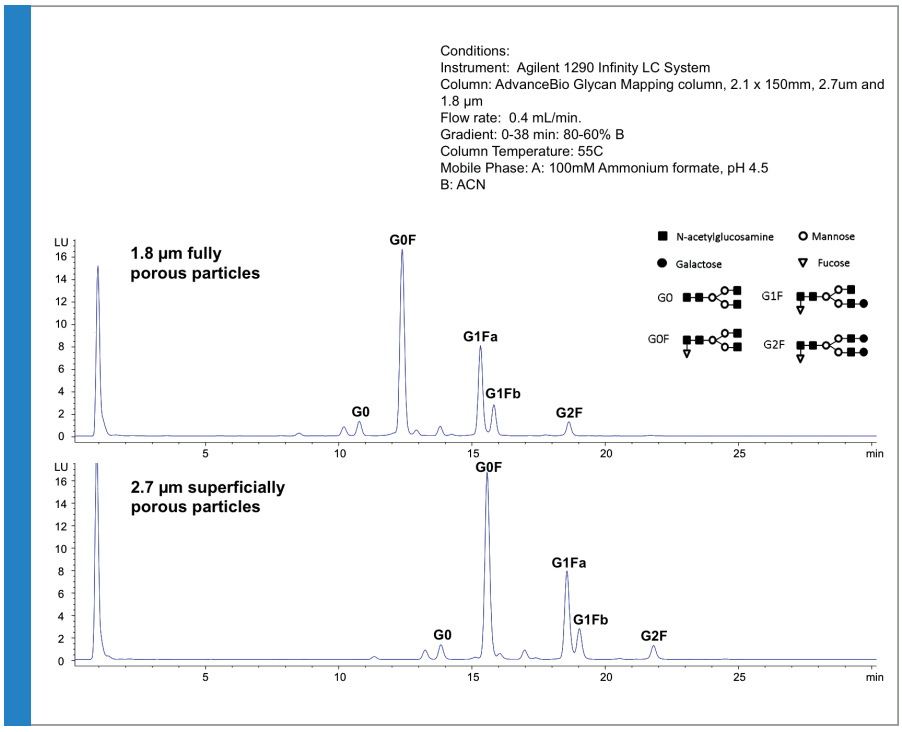

Figure 2. Herceptin N-glycan separation on both 1.8 µm and 2.7 µm HILIC columns

other sources of charge variants. A typical mAb molecule contains over 1,300 individual amino acids. The actual amino acid composition will vary from protein to protein but it is likely that there will be a higher proportion of amino acids containing basic side chains (arginine, lysine and histidine) than there will be acidic amino acids (aspartic acid and glutamic acid). The overall net charge of the molecule at neutral pH could be +50. As well as differences in sialic acid content, charge variants arise from other chemically or enzymatically driven modifications, including:

- Succinimide formation from aspartic acid residues
- Deamidation of asparagine residues
- Loss of C-terminal lysine residues
- Cyclization of the N-terminus with the formation of pyroglutamate

Measuring and quantifying the level of charge variants from such a large, complex molecule is therefore an immense challenge. The most appropriate technique is ion exchange chromatography (IEX), and since most therapeutic mAbs have a higher proportion of basic residues, cation exchange chromatography is most commonly used. The advantage of using cation exchange chromatography is the fact that the protein does not need to be denatured; the mild aqueous conditions allow the intact mAb to be analyzed. In order to maximize resolution it is often necessary to use long columns with shallow gradients, and weak cation exchange (WCX) columns will often give better selectivity than strong cation exchange columns. Some weak cation exchange columns are specifically optimized for mAbs. No matter which IEX column you select, method development and optimization is still necessary for each product and a rigorous "Quality by

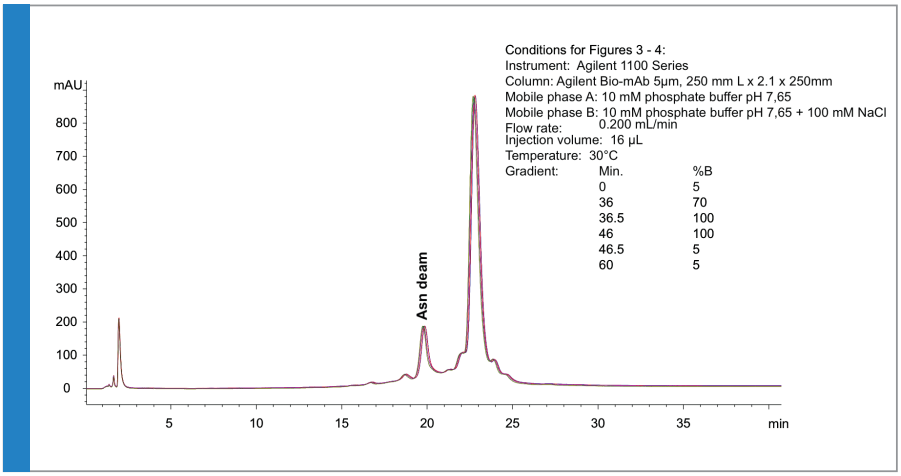

Figure 3. Replicate analysis (n=5) of intact Herceptin on WCX

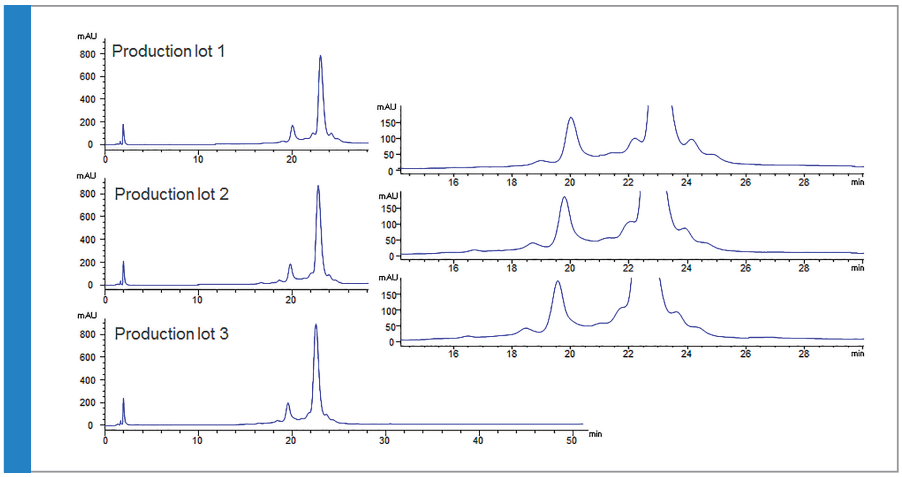

Figure 4. Comparison of 3 different production batches of Herceptin by WCX

Design" approach covering mobile phase ionic strength and pH is essential. Once the optimum method is determined it will be used to evaluate different batches of originator or biosimilar product. Figure 3 shows the replicate analysis of Herceptin on WCX with resolution of the asparagine deamidation noted before the main Herceptin peak. The precision offered makes the technology highly attractive for the comparison of different production batches (Figure 4) and to compare innovator biopharmaceuticals with biosimilars.

Ion exchange chromatography is also used to look at the Fab and Fc fragments of the mAb. While the general approach of using a weak cation exchange column may still be the same, separate method optimization takes place. The mobile-phase pH and buffer may change substantially to separate the Fab and Fc fragments as shown in Figure 5. The separation clearly resolved the deamidation seen in the reversed-phase separations.

Perhaps the most critical attribute for product efficacy and safety for therapeutic mAbs requires yet another HPLC technique: size exclusion chromatography (SEC). Proteins may form dimers or larger aggregates, or even degrade into fragments such as the heavy and light chain components characteristic of IgG molecules. It is essential to measure and control aggregation particularly since it is recognized that aggregates may stimulate immune responses and could potentially lead to an adverse event such as anaphylactic shock. In the production of biosimilars, it is critical for the high and low molecular weight (HMW and LMW) variants to be very similar to the originator in order to avoid potential adverse events. Figure 6 shows the comparison of the SEC separation of Herceptin and a potential biosimilar, displaying differences in the HMW and LMW profile.

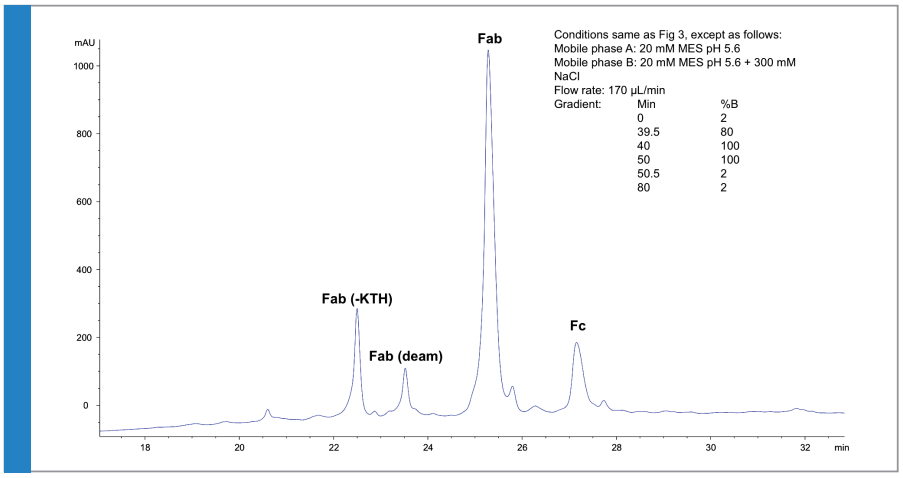

Figure 5. WCX separation of Fab and Fc fragments of Herceptin using a mobile phase pH of 5.6

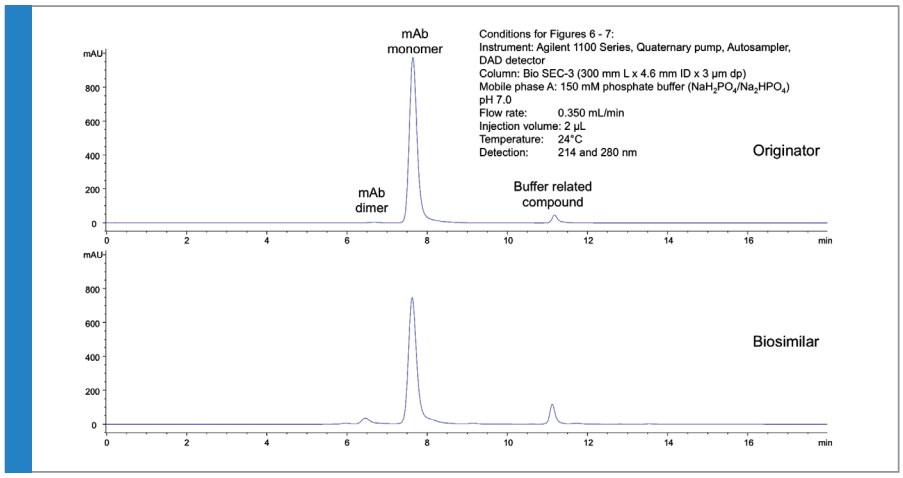

Figure 6. Comparison of SEC separations of Herceptin and a biosimilar

Aggregation can arise at many points during the recombinant protein manufacturing cycle: during fermentation, purification, formulation, storage, and shipping. It usually arises when a protein is subjected to stress conditions that include changes in temperature (exposure to heat, or during freeze-thaw), changes in concentration (during isolation, purification or formulation) and even mechanical agitation can cause aggregation. Figure 7 shows the increase in the aggregates of Herceptin that occurred under oxidative stress conditions.

Size exclusion chromatography is ideally suited to the separation of protein monomers from their larger dimers or aggregates and can be conducted under mild, nondenaturing conditions. Even so it is essential to ensure that the analysis technique (including sample preparation) does not increase or decrease the level of aggregation.

Analyzing protein biopharmaceuticals involves a wide range of methodologies and techniques and typically requires measurements at the protein, peptide, and glycan level. Combining RPLC, IEX, SEC, and HILIC allows one to obtain great insight in the characteristics of a mAb. Moreover, the robustness associated with these technologies allow for their use in routine QA/QC environments for clinical and commercial release of materials. ■

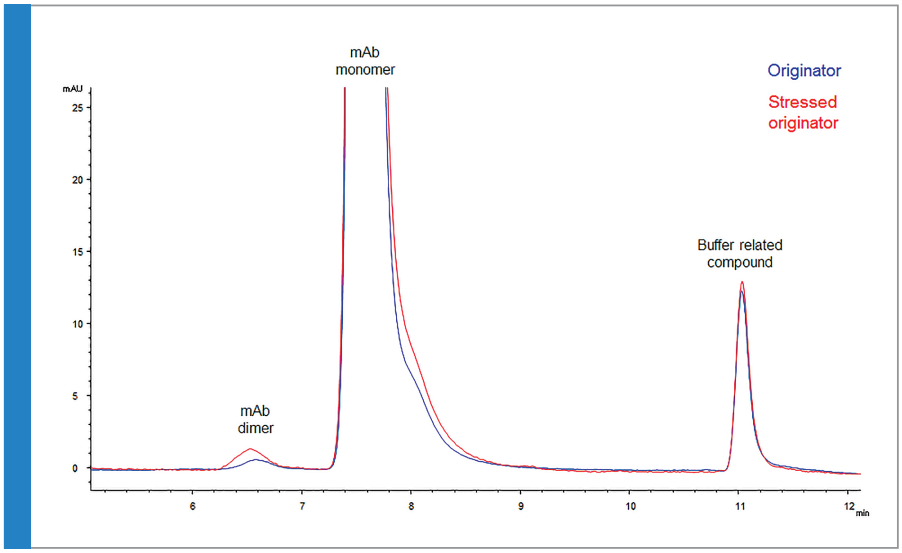

Figure 7. Comparison of aggregation by SEC in non-stressed and oxidatively stressed Herceptin

Maps to Guide Your Journey: The Importance of Powerful Software

Ning Tang, Ph.D., Agilent Technologies

As described at the outset of our "journey", biologics generally are more complex and sensitive to changes in manufacturing processes. Therefore, reference to the innovator product is an integral component of the biosimilar development. LC/MS is a very important part of the workflow, and is used to ensure comparability, a key attribute in the analytical characterizations of biosimilars. In the other article in this guide, we've discussed the various chromatographic techniques that are used to characterize the innovator and biosimilar molecules and shown some LC/MS data. This article will focus on the LC/MS data analysis tools that streamline the comparison between the biosimilar and originator and will ultimately improve productivity and speed up development of the biosimilar.

LC/MS software is critical to the success of your analysis, as it couples multiple powerful data processing algorithms with sophisticated sequence and database matching tools to enable fast, accurate identification of intact proteins, peptides, and their variants in simple and complex mixtures. Agilent MassHunter BioConfirm software has some unique capabilities that set it apart for biosimilars analysis. First, the software provides several different mass spectral deconvolution algorithms that are optimized for intact proteins and peptides, as well as for different sample complexities. Using the electrospray ionization conditions employed for the analysis of proteins, mass spectra exhibit a charge envelope that contain a number of multiply charged peaks (Figure 1A). An example of the zoom-in single charge state is shown in the Figure 1A. Agilent's state-of-the-art Maximum Entropy deconvolution algorithm converts these complex spectra to simple zero-charge spectra (Figure 1B) that directly provide the molecular weights of proteins and their variants. Maximum entropy charge deconvolution is a well-known and powerful data analysis algorithm for

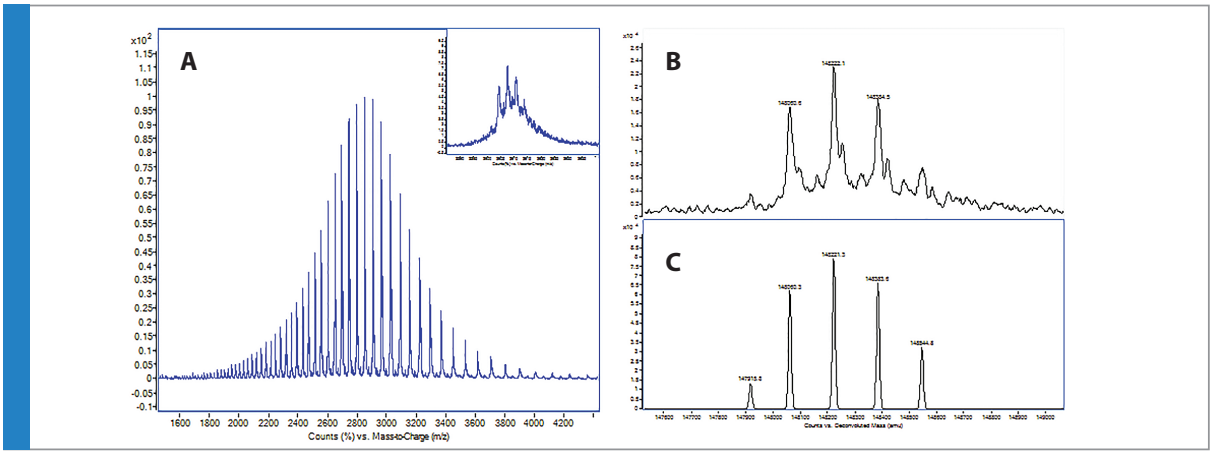

Figure 1. A) Charge envelope of intact Herceptin (trastuzumab) acquired by LC/MS. B) Maximum entropy deconvolution result of trastuzumab. C) Peak modeling deconvolution result of trastuzumab.

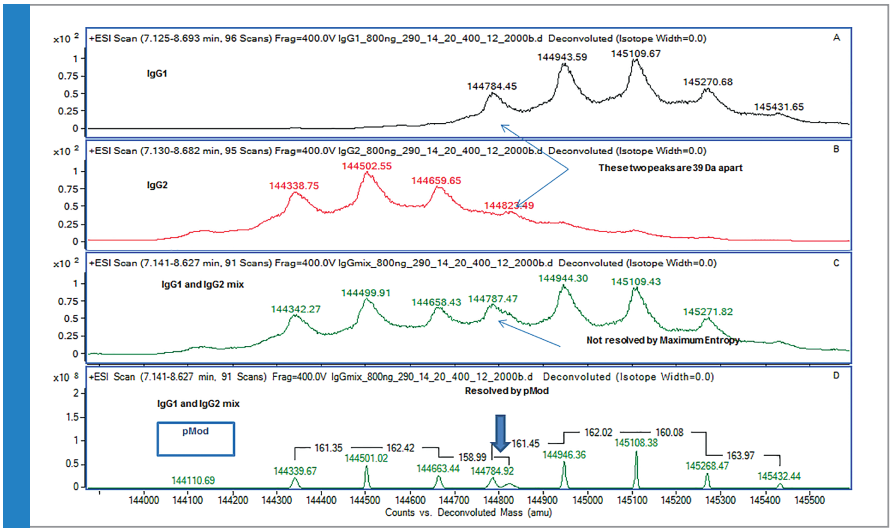

Figure 2. A) Maximum entropy deconvoluted spectrum of IgG1. B) Maximum entropy deconvoluted spectrum of IgG2. C) Maximum entropy deconvoluted spectrum of mixture of IgG1 and IgG2. D) Peak modeling deconvoluted spectrum of IgG1 and IgG2 mixture.

determining neutral mass for intact proteins using electrospray mass spectra. This method transforms an m/z raw spectrum of one or more intact proteins into the most probable zero-charge mass spectrum. Maximum entropy deconvolution works reliably for low complexity protein data or a relatively simple protein mixture.

BioConfirm also offers another advanced data deconvolution algorithm, peak modeling deconvolution (pMod) that improves deconvolution results by applying an automatic peak modeling technique, delivering the most probable deconvolution results that are virtually free of artifacts. The peak modeling deconvolution method results in improved signal-to-noise ratios and enhanced resolution of protein peaks for precise analysis (Figure 1C). The pMod deconvolution algorithm starts with maximum entropy deconvolution. Then, based on the maximum entropy result, pMod automatically generates mass spectra peak models without manual intervention and applies these models through fitting and validating procedures. Spectral data that does not fit the model is rejected as noise. Therefore, the pMod result is much cleaner than the maximum entropy deconvolution result and produces fewer artifacts. Using this process pMod generates a highly resolved

zero-charge spectrum and a set of mass precision assessments for each peak.

The peak modeling deconvolution result has an enhanced resolution which leads to overlapped peaks frequently being well-resolved. In the sample IgG1 and IgG2 mixture (Figure 2), peaks with masses at 144784 and 144823 are 39 Da apart and not well resolved by Maximum Entropy deconvolution. The mass peaks are much narrower and exhibit better resolution after pMod has been applied. In addition, the width of the peaks provides the precision of the mass measurement for that protein. Overlapped peaks from IgG1 and IgG2 are well resolved from each other and can be identified as separate proteins. This has enabled the differentiation of small modifications from the main heterogeneous glycoprotein profile with much greater clarity.

Mirror plots are another powerful tool for biosimilars comparison. During the biosimilar development, comparison of the molecular similarity between the biosimilar and the originator is critical. Unlike small molecule drugs, biologics are heterogeneous and present multiple modifications such as glycosylation. Measuring the protein masses accurately and identifies all the variants are only the first step in the biosimilar characterization. Comparing the relative abundance of different variants is also important. Often a visual comparison gives the most direct confirmation of the similarities between two biologics. BioConfirm generates a mirror plot of two samples as shown in Figure 3. The originator and biosimilar were analyzed by LC/Q-TOF. Protein molecular weights were accurately confirmed and the major glycoforms were identified. The deconvoluted mass spectra were mirror plotted for easy comparison. The black trace is Herceptin (trastuzumab) and the red trace is the biosimilar. The blue dotted trace is the difference between the two samples. The nearly flat blue dotted line showed the two samples have great similarity at the intact level.

Peptide mapping is an essential step in characterizing biologics. It confirms protein sequence, identifies post-translational modifications (PTM) and sequence variants, quantifies the relative

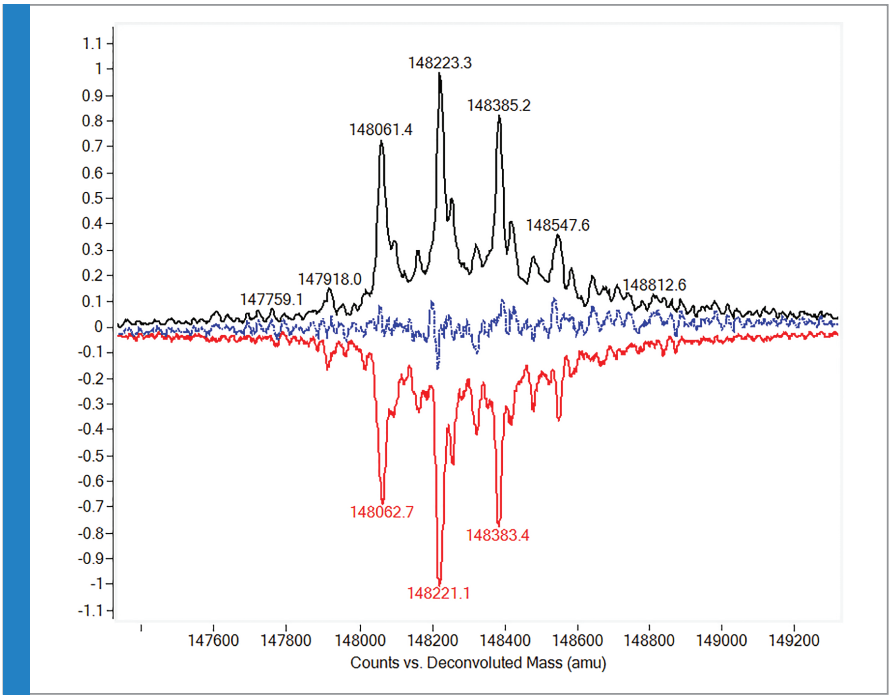

Figure 3. Mirror plot of intact originator and biosimilar.

abundance of different PTMs. For peptide mixtures that contain many components, manual analysis of LC/MS results can become time consuming. To automate the process, BioConfirm provides the Molecular Feature Extraction (MFE) algorithm, which finds the peptides in the LC/MS chromatogram, and determines their masses via resolved isotope deconvolution. MFE not only uses the resolved isotopes to directly determine the charge states for the peptides, but also looks for related ion clusters that represent the same peptide with neighboring charge states. Within the peptide mapping workflow, compounds are first extracted by MFE, and then matched to the target protein digest sequence. The Comparative Analysis module of MassHunter BioConfirm is important for the analysis of two different samples such as biosimilar and originator. This module enables direct visual comparison of different LC/MS runs from two peptide mapping samples. Mirror plots and tabular comparisons make it easy to visually compare the samples. The tryptic peptide map base peak chromatograms of Herceptin (trastuzumab) and the biosimilar samples are compared by mirror plot to allow easy visualization of any differences (Figure 4). The table in Figure 5 shows the compound-centric comparison between two samples. The MS and data-dependent MS/MS spectra of the tryptic peptides are stacked for comparison. The b, y and immonium ions in the MS/MS spectra are labeled with different colors (blue, red, and green).

In this study, Herceptin (trastuzumab) and its biosimilar were compared using LC/MS/MS both at the intact level and the peptide level. Software algorithms designed specifically for accurate and fast extraction and identification of protein/peptide masses are the foundation for biosimilar characterization workflow. Furthermore, comparability modules such as mirror plot and peptide mapping comparison tables allow direct and visual comparison of two samples. These software tools greatly increase productivity and speed up biosimilar development. ■

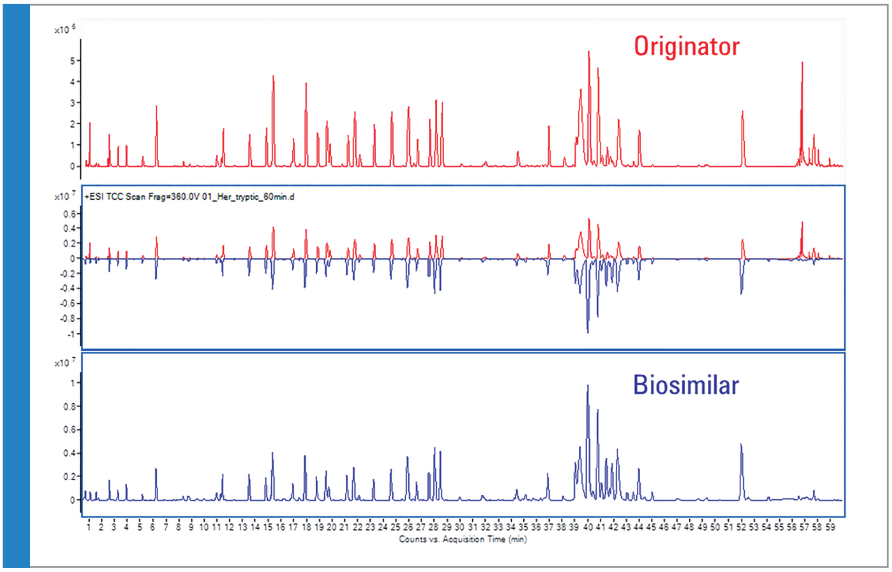

Figure 4. Mirror plot of tryptic peptide mapping chromatograms.

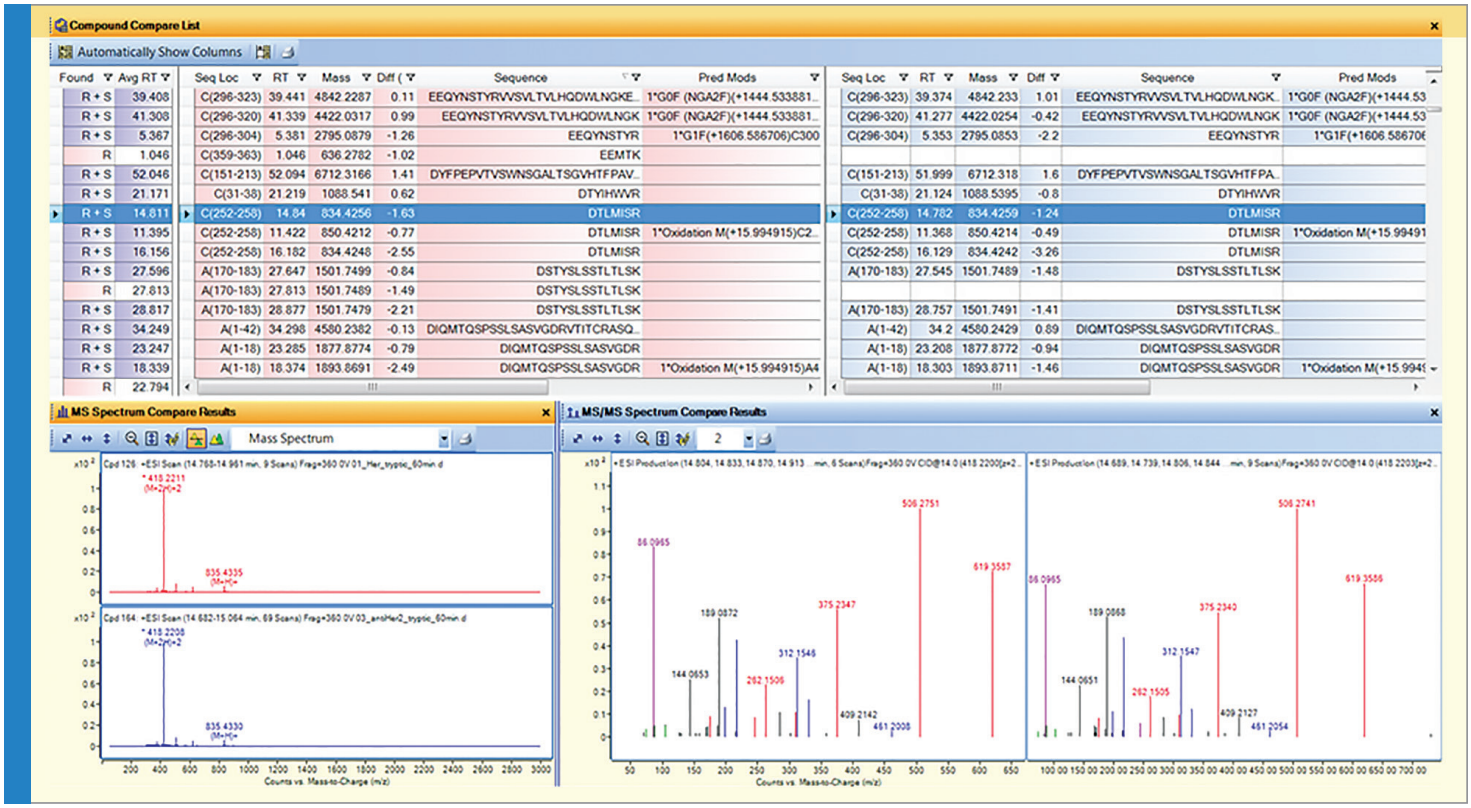

Figure 5. Trastuzumab and biosimilar tryptic peptide mapping comparison view.


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Printed in the USA, October 29, 2014
5991-5493EN

Agilent Technologies