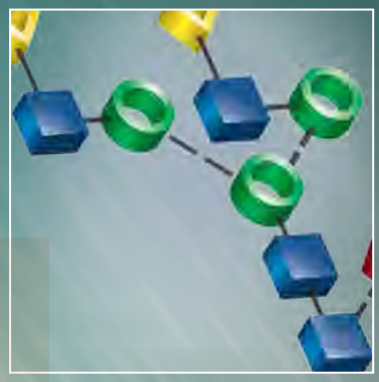
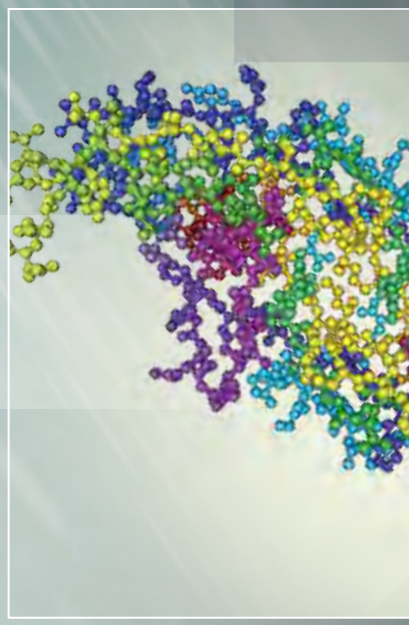
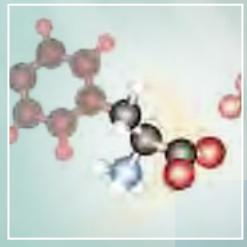
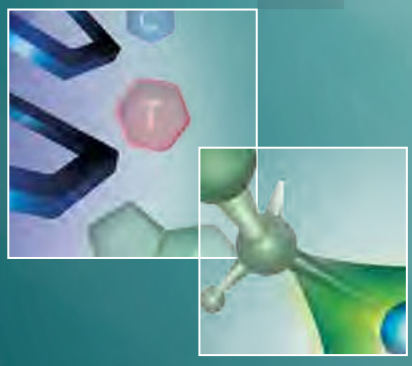




BIOSEPARATIONS

Tools, Techniques, and Insights into Biopharmaceutical Analysis





Waters

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INTRODUCTION

Biopharmaceuticals have emerged as a dominant class due to their specificity and efficacy. The production of biopharmaceuticals, however, is distinctly different than traditional small molecule therapeutics in that they are often produced by living cells. Small changes in manufacturing process conditions can cause biologics to undergo several post-translational modifications, yielding products that are highly heterogeneous both in primary and tertiary structures.

Due to this complexity, biotherapeutic characterization and quality control assays require many orthogonal techniques to accurately define and assess critical quality attributes (CQAs) related to safety, stability and efficacy of the product. Of the common techniques, liquid chromatography (LC) plays a pivotal role in accurately characterizing and monitoring product heterogeneity throughout the product life cycle.

LC-based methods are often used as foundational assays for the analysis of complex biotherapeutics. Multiple modes of separation can generate rich primary and secondary structural information early in development, which can save time and cost throughout development and production.

Waters' latest innovations in sample preparation, LC, LC-MS, and informatics are designed to address the multi-faceted analytical needs of large molecule characterization – from the discovery and development of innovator biopharmaceuticals such as antibody drug conjugates (ADCs) to the successful commercialization of biosimilars/biobetters that satisfy international biotherapeutic drug regulatory requirements.

Bioseparations Techniques

This application notebook is organized by technique as shown in the graphic below. **HOVER** and **CLICK** on any one of the sections to quickly link to the appropriate set of application notes.

1 SEC FOR CLIPS AND AGGREGATES	Size-exclusion chromatography (SEC) for biotherapeutic aggregate and clip determinations
2 IEX FOR CHARGE VARIANTS	Ion-exchange chromatography (IEX) for biotherapeutic charge variant measurements
3 REVERSED-PHASE FOR PROTEIN SEPARATIONS	Reversed-phase LC separation designed to detect protein modifications
4 REVERSED-PHASE FOR PEPTIDE MAPPING	Reversed-phase LC for peptide mapping of complex protein therapeutics
5 REVERSED-PHASE FOR AMINO ACID ANALYSIS	Reversed-phase LC for amino acid analysis of hydrolyzed protein or peptide drugs
6 HILIC FOR RELEASED GLYCANS AND GLYCOPROTEINS	Hydrophilic interaction liquid chromatography (HILIC) for separation of released glycans and intact glycoprotein species
7 HIC FOR HYDROPHOBIC MOIETIES AND DRUG ANTIBODY RATIO (DAR)	Hydrophobic interaction chromatography (HIC) to separate hydrophobic moieties and to determine drug antibody ratios (DAR) in Antibody Drug Conjugates
8 MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY (2D-LC)	Multidimensional Liquid Chromatography (2D-LC)

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SEC for Clips and Aggregates

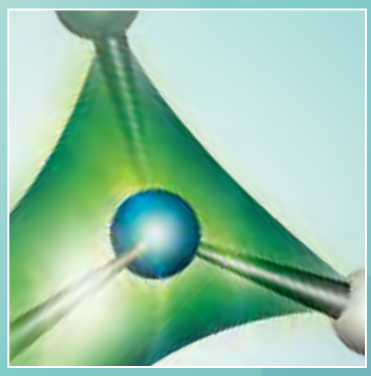
The presence of aggregates can compromise safety and efficacy in protein based bio-therapeutics. Given these factors, protein aggregates are typically monitored during drug discovery, throughout development, and as a final manufacturing quality control release test. However, each stage of development may have different assay requirements, including robustness, method sensitivity, ease of use, and throughput needs.

While a variety of analytical techniques have been used to analyze soluble aggregates, the dominant technique for routine and validated analyses continues to be size-exclusion chromatography (SEC). The primary application for SEC in the biopharmaceutical industry is the routine monitoring of peptide, protein or modified protein (protein-drug conjugates, pegylated proteins, etc.) aggregation and potential lower molecular weight clip forms.

Solution highlights

Challenges in SEC method development often revolve around poor peak shape, reproducibility, loss of resolution and throughput needs. With the **ACQUITY UPLC H-Class Bio System** and **sub-2-µm ACQUITY UPLC Protein BEH SEC Columns and specially formulated protein standards**, SEC separations can be obtained reproducibly, reliably, and in shorter analysis time with minimal method development requirements. Methods can be easily developed with the system's quaternary solvent manager and **Auto•Blend Plus™ Technology** so that less time is spent preparing buffers.

The ACQUITY UPLC Protein BEH SEC Columns are available in different pore sizes for separations across wide molecular weight ranges: 125Å (1000 Da-80 KDa), 200Å (10 KDa-450 KDa), 450Å (100 KDa-1500 KDa). The available BEH125Å, BEH200Å, BEH450Å Protein Standard Mixes are the same materials that Waters uses to batch test and approve each lot of BEH SEC material. Their use is recommended to benchmark the ACQUITY UPLC Protein BEH SEC Columns and confirm proper LC system performance prior to sample analysis.



The packing material found in these new columns is based on Bridged Ethyl Hybrid - capitals (or BEH) technology which provides a chemically and mechanically stable particle for use in SEC applications and also exhibits significantly less secondary interactions than 100% silica based columns.

This Waters total UPLC-based system solution, combining an inert, low-dispersion system with a chemically-stabilized BEH SEC column provides optimal resolution and sensitivity, while enhancing analytical robustness over traditional silica and polymer based SEC separations.

Future Proofing the Biopharmaceutical QC Lab: Benefits of Automating Mobile Phase Delivery to Improve pH Consistency in Size-Exclusion Chromatography Methods

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Using the ACQUITY UPLC® H-Class Bio System to perform size exclusion chromatography (SEC) in biopharmaceutical quality control (QC)
- Experimental approach for converting conventional mobile phase delivery to Auto•Blend Plus™ SEC assays

WATERS SOLUTIONS

ACQUITY UPLC H-Class Bio System

BioSuite™ SEC Column

ACQUITY UPLC Protein BEH SEC Column

BEH200 SEC protein standard mix

Empower® 3 Chromatography Data Software

KEY WORDS

Size exclusion chromatography, SEC, quality control, QC, AutoBlend Plus, automated mobile phase delivery, automated buffer management

INTRODUCTION

Large molecule separations that require buffered mobile phases represent a challenge in analytical labs due to the potential sensitivity of analytes to changes in pH and salt concentration. One such large molecule assay includes size exclusion chromatography (SEC), which is typically used to measure the extent of aggregation in protein-based therapies.

Mobile phases for SEC separations have historically been prepared by combining individual components of the mobile phase followed by adjustment to the desired pH using an appropriate acid or base. In this scenario, calibration of the pH meter and the associated accuracy of pH measurements can directly influence the final pH of the mobile phase, affecting the quality of the final separation. As a result, subtle changes in the preparation of mobile phase can lead to differences in chromatography in situations where pH differs between mobile phase preparations.

In this application note, we continue our discussion of using the ACQUITY UPLC H-Class Bio System for size exclusion chromatography[1] by demonstrating the benefits of Auto•Blend Plus Technology – which is included with all ACQUITY UPLC H-Class instruments – for consistent and reliable delivery of pH-dependent mobile phase for SEC-HPLC and SEC-UPLC.

Compared to manual approaches where mobile phase delivery is defined by percent composition of each solvent line, Auto•Blend Plus allows the user to define individual method steps based on the desired pH and salt concentration. This enables the analyst to explore an extensive list of method parameters in a single set of buffer preparations.

Auto•Blend Plus can be particularly advantageous in QC environments, where methods are expected to be accurate, precise, and robust. Variability in mobile phase preparation due to inconsistencies with pH can potentially lead to erroneous outcomes that can otherwise be controlled using automated chromatographic tools such as Auto•Blend Plus.

EXPERIMENTAL

LC conditions

ACQUITY UPLC H-Class Bio System, comprised of:

- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
- ACQUITY UPLC H-Class Bio Sample Manager (SM)
- ACQUITY UPLC Tunable UV Detector with Ti flow cell
- Extension loop: 100 µL (p/n 430002625)
- BioSuite SEC 10 µm, 250 Å Column, 7.5 mm x 300 mm (p/n 186002170)
- ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225)
- BEH200 SEC protein standard mix (p/n 186006518)

Column temp.:	Ambient
Seal wash:	10% acetonitrile in H_2O
Conventional mobile phase:	20 mM phosphate, 200 mM NaCl, pH 6.8

Auto•Blend Plus

Mobile phase A:	A: 100 mM NaH_2PO_4
Mobile phase B:	100 mM Na_2HPO_4
Mobile phase C:	1 M NaCl
Mobile phase D:	H_2O
Detection wavelength:	214 nm
Syringe purge:	H_2O
Syringe wash:	H_2O

HPLC conditions

Injection vol.:	20 µL
Flow rate:	0.400 mL min^{-1}
Method length:	35 min

UPLC conditions

Injection vol.:	4 µL
Flow rate:	0.885 mL min^{-1}
Method length:	3 min

In this application note, we demonstrate how a conventional SEC method is converted to an Auto•Blend Plus-enabled method using the ACQUITY UPLC H-Class Bio System. There is no disruption to mobile phase composition when performing this conversion: Auto•Blend Plus delivers identical chromatography to that obtained using mobile phase prepared and delivered in a conventional manner. The results presented in this application note show robust, precise, and reliable chromatography for both SEC-HPLC and SEC-UPLC, supporting the prospect of Auto•Blend Plus as a technology that can be successfully deployed in large molecule QC environments.

RESULTS AND DISCUSSION

Experimental design of conventional and Auto•Blend Plus assisted SEC

Preparation of aqueous, pH dependent mobile phases can be a cumbersome aspect for both method development experiments as well as high-throughput assay environments where mobile phase is used in high volume. In the latter case, each preparation of new mobile phase can be susceptible to variability due to differences in pH meter calibration and accuracy, pH adjustment of the mobile phase, and general differences in the way analysts prepare mobile phase.

To get around this inconsistency, control of mobile phase preparation can instead be accomplished using Auto•Blend Plus Technology. Solutions of appropriate acid, base, salt, and water can be prepared separately as concentrated stocks and mixed together using Auto•Blend Plus, which combines the necessary proportions of each solvent required for delivering a specified pH and salt concentration. This strategy is made possible by the ACQUITY UPLC H-Class System's Quaternary Solvent Manager, which can combine four separate solvents to form a desired mobile phase composition.

To evaluate the similarity between conventional SEC-HPLC and Auto•Blend Plus assisted SEC-HPLC, we created two sets of mobile phase for each SEC assay. For conventional SEC, the mobile phase consisting of 20 mM phosphate with 200 mM NaCl adjusted to pH 6.8 was prepared at the bench. For Auto•Blend Plus assisted SEC, four separate stock solvents of 100 mM NaH_2PO_4 buffer, 100 mM Na_2HPO_4 buffer, 1 M NaCl, and pure H_2O were prepared.

In each case, a BioSuite SEC 10-µm 250 Å Column (7.5 x 300 mm) was used for comparison. Two separate protein samples were used to evaluate the HPLC approaches. The first protein sample was a Waters® SEC200 protein standard mix consisting of five components intended for determining the total inclusion and exclusion volumes of SEC columns capable of separating proteins between approximately 10 kDa and 500 kDa. The second protein was the commercial monoclonal antibody, infliximab, previously shown to contain a minor amount of aggregate formation.[1]

For accurate delivery of a desired pH, an empirical table was generated that accounted for the effect of increasing salt concentration on mobile phase pH. Instrument methods for both conventional SEC-HPLC and Auto•Blend Plus SEC-HPLC were created using Empower 3 Software (Figure 1). For conventional SEC-HPLC, all relevant instrument details were outlined as depicted in Figure 1A. The Auto•Blend Plus SEC-HPLC method was created by selecting Auto•Blend Plus from the QSM option in the instrument method and itemizing the desired pH and salt concentration, as depicted in Figure 1B. Addition of empirical data was accessed by selecting Buffer System and then selecting the Empirical Data option on the right side of the new window (Figure 2). It is recommended that labs generate their own Auto•Blend Plus tables as suppliers of raw chemicals and standard operating procedures may yield different pH values than those listed in the figure.

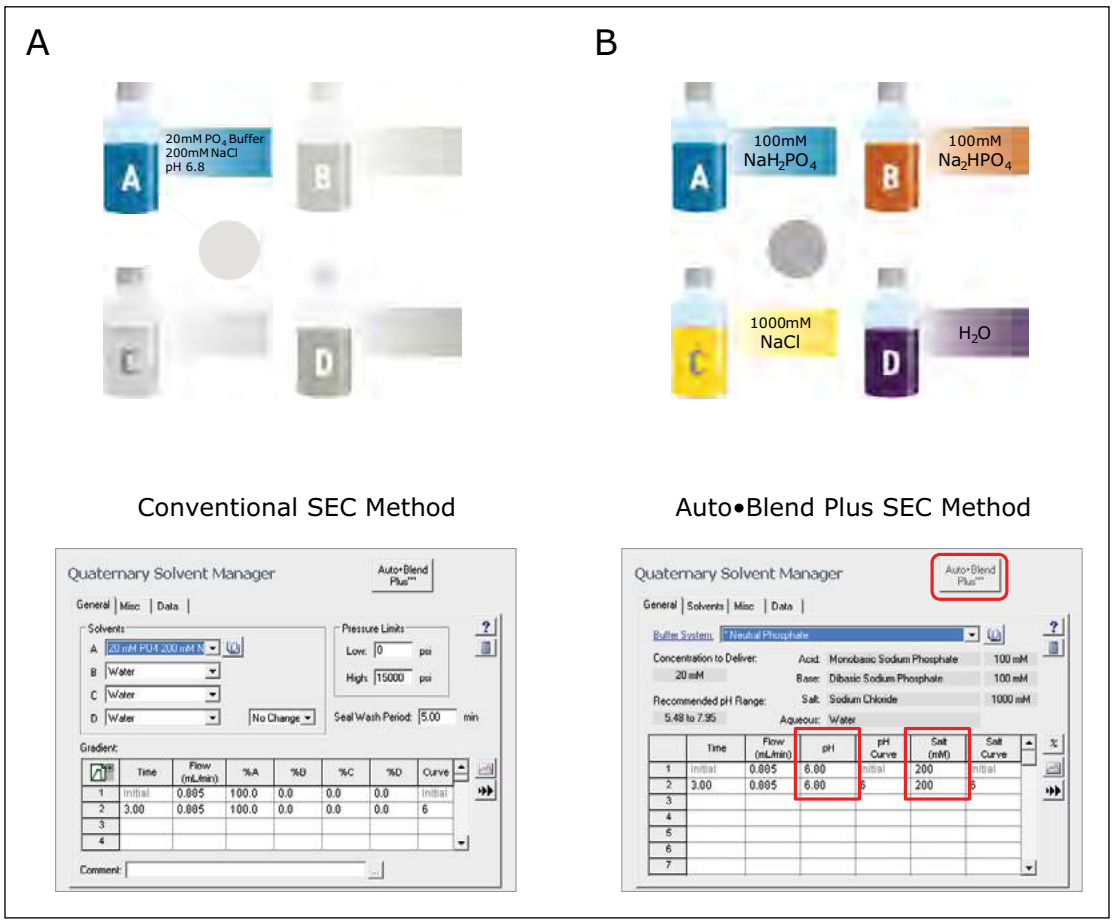


Figure 1. Conventional SEC and Auto•Blend Plus assisted SEC arrangements. Presented instrument method windows relate to the SEC-UPLC method. (A) Conventional SEC arrangement typically has a single prepared mobile phase on one solvent line, which is itemized in the instrument method as a 100% solvent A. (B) In Auto•Blend Plus SEC arrangements, mobile phases corresponding to acid (NaH_2PO_4), base (Na_2HPO_4), salt (NaCl), and water are configured on 4 solvent lines. The instrument method is modified to request the desired pH and salt composition rather than a percent mobile phase, as illustrated by the red boxed items. Similar windows exist for SEC-HPLC with appropriate changes to flow rate and method duration.

SEC-HPLC with Auto•Blend Plus generates identical results to conventional SEC-HPLC

To determine the comparability of Auto•Blend Plus for SEC-HPLC, a benchmark separation was first established using the conventional SEC-HPLC method with the BioSuite SEC 10-µm column. In the first instance, the SEC200 protein standard mix was chromatographically separated and all peaks were shown to elute within the method run as expected (Figure 3A). All relevant chromatographic data is recorded in Table 1. With migration times established for each protein standard component, the ACQUITY UPLC H-Class Bio was configured to run Auto•Blend Plus methods by exchanging the conventional SEC mobile phase arrangement (Figure 1A) with the Auto•Blend Plus mobile phase arrangement (Figure 1B). The same column and SEC200 protein standard mix were used. Each component of the standard was shown to exhibit near identical migration times when compared to the conventional SEC-HPLC method results (Figure 3B and Table 1). Relative peak areas associated with each component were also shown to be highly comparable, indicating the ability of Auto•Blend Plus to generate identical chromatography when compared to conventional HPLC.

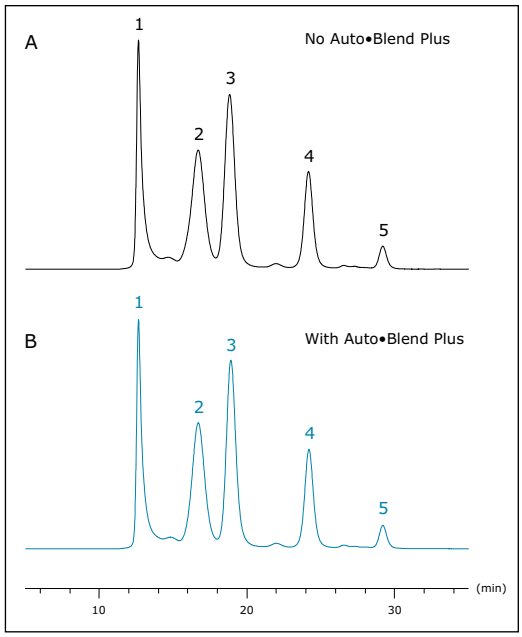


Figure 3. Auto•Blend Plus assisted SEC-HPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-HPLC using conventional mobile phase preparation; (B) SEC-HPLC using Auto•Blend Plus Technology for mobile phase delivery.

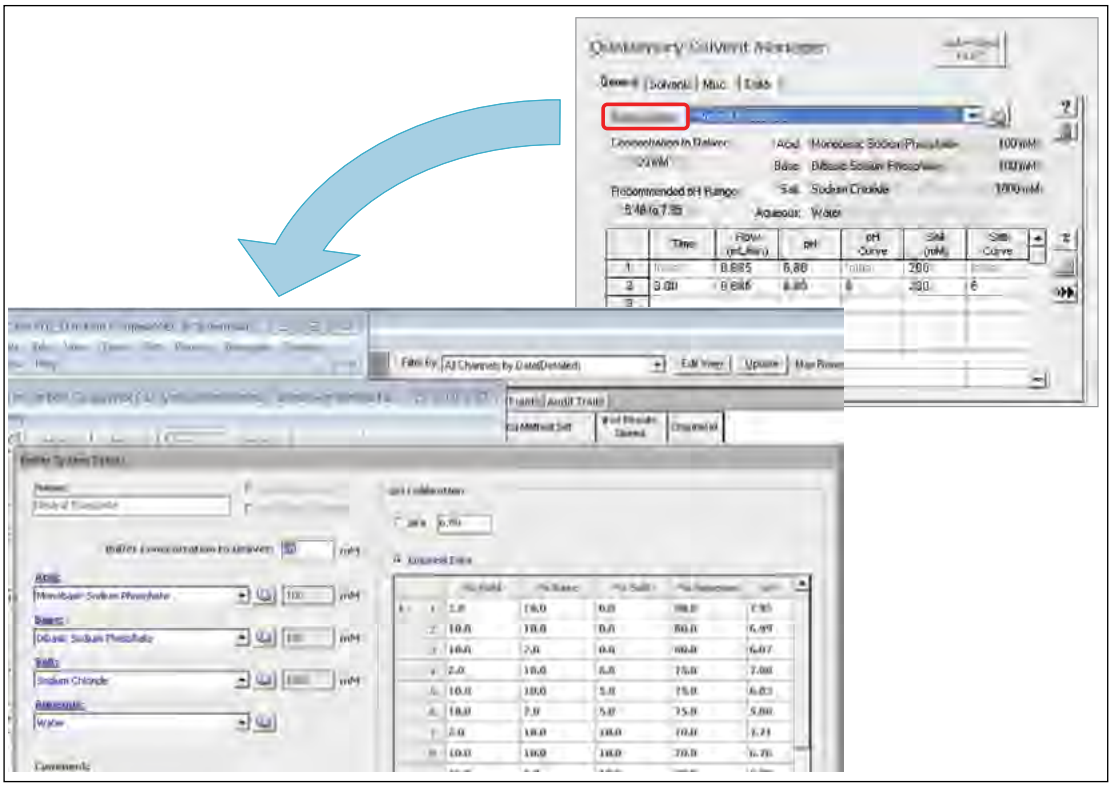


Figure 2. Recording empirical data in Auto•Blend Plus methods. Correcting pH due to salt concentration can be added to Auto•Blend Plus methods by first selecting the "Buffer System" in the QSM tab of the Empower instrument method window. In the new window, the option of "pKa" or "Empirical Data" is available. Selecting "Empirical Data" activates the table where pH values corresponding to the composition itemized in each row can be entered.

To investigate the comparison with a true commercial large molecule protein therapeutic, we used each SEC approach to measure the extent of aggregation in infliximab. As can be seen in Figure 4, the migration time for both the infliximab dimer and monomer were highly comparable, indicating Auto•Blend Plus as a suitable replacement for conventional mobile phase delivery.

SEC-UPLC with Auto•Blend Plus generates identical results to conventional mobile phase preparation

Moving from SEC-HPLC to SEC-UPLC offers a number of improvements to chromatography previously described.[1] In addition to increasing chromatographic resolution and sensitivity by moving to SEC-UPLC, method robustness can also be improved by incorporating Auto•Blend Plus into the instrument method. Transferring the SEC-HPLC method to SEC-UPLC results in an increase in flow rate from 0.4 mL min^{-1} to 0.885 mL min^{-1} with a corresponding reduction in run time from 35 min to just 3 min.

To determine if Auto•Blend Plus could generate comparable results as those observed with SEC-HPLC, we ran both the SEC200 protein mix standard and infliximab using either conventional SEC-UPLC or Auto•Blend Plus-assisted SEC-UPLC. An ACQUITY UPLC Protein BEH SEC 200 Å Column (1.7-µm, 4.6 x 150 mm) was used with the ACQUITY UPLC H-Class Bio System for the assay. Benchmark SEC-UPLC using the SEC200 protein standard mix was generated as illustrated in Figure 5A. Auto•Blend Plus-assisted SEC-UPLC was then run and compared to the conventional SEC-UPLC, with results indicating no difference in individual component migration times (Figure 5B and Table 1). The same comparison was performed using infliximab, where similar results were obtained (Figure 6 and Table 1).

Results of SEC-UPLC unequivocally illustrate that using Auto•Blend Plus Technology for SEC-UPLC can replace conventional SEC-UPLC with no impact on component migration time or relative peak area.

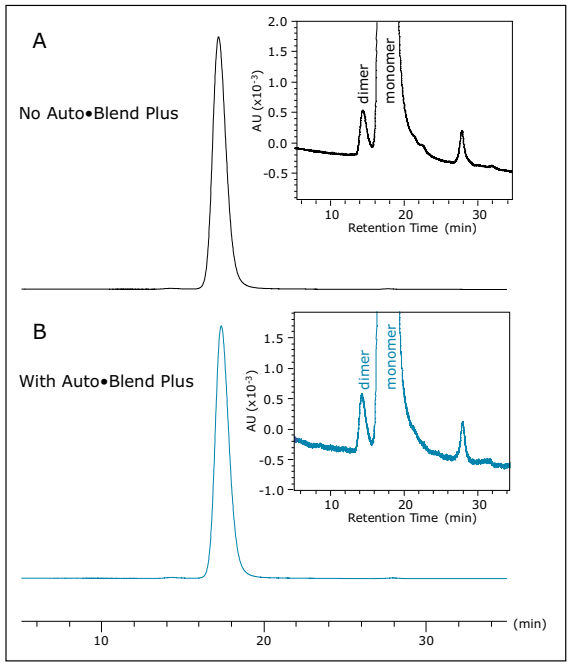


Figure 4. Auto•Blend Plus assisted SEC-HPLC of infliximab generates equivalent separation compared to conventional SEC-HPLC. (A) Infliximab separated using conventional SEC-HPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-HPLC.

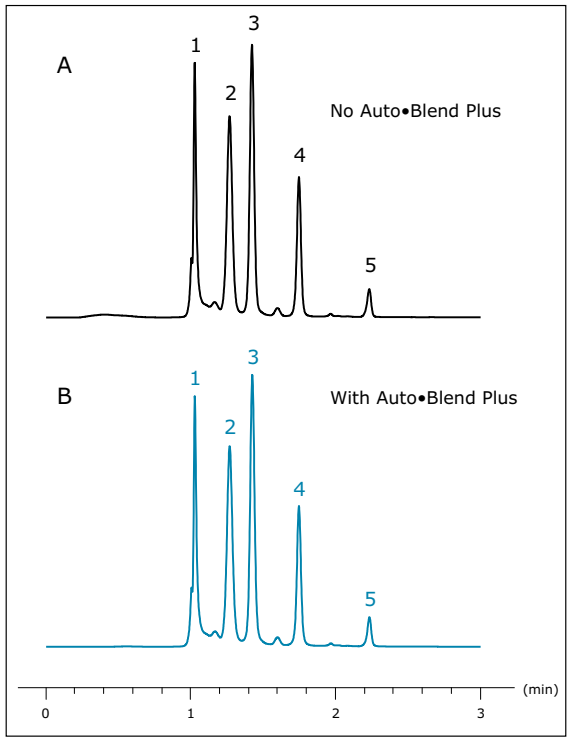


Figure 5. Auto•Blend Plus assisted SEC-UPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-UPLC using conventional mobile phase preparation; (B) SEC-UPLC using Auto•Blend Plus Technology for mobile phase delivery.

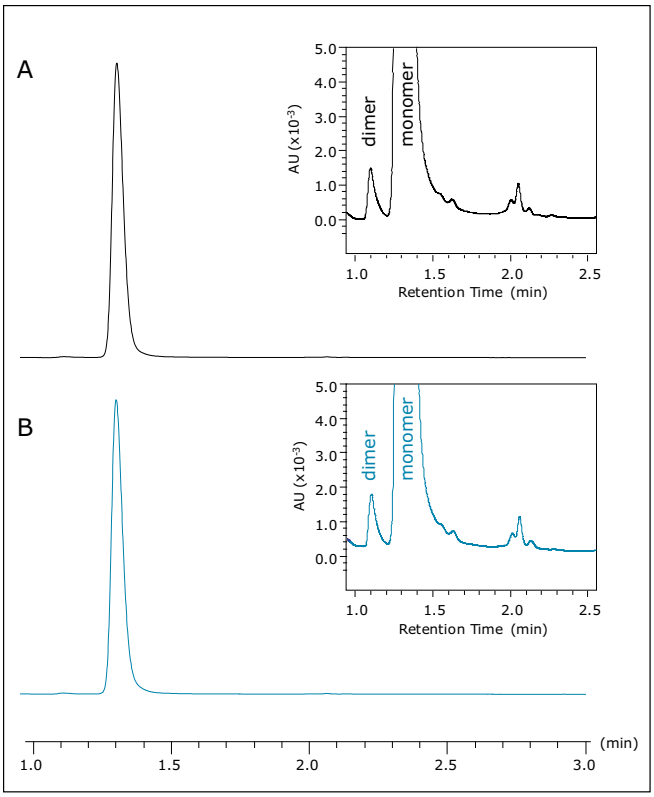


Figure 6. Auto•Blend Plus assisted SEC-UPLC of infliximab generates equivalent separation compared to conventional SEC-UPLC. (A) Infliximab separated using conventional SEC-UPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-UPLC.

Peak	SEC component	Retention time (min)				Peak area (%)			
		Auto•Blend Plus				Auto•Blend Plus			
		-		+		-		+	
		$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	Thyroglobulin	12.65	0.001	12.66	0.002	23.79	0.638	23.64	0.015
2	IgG	16.64	0.002	16.70	0.002	27.64	0.260	27.83	0.012
3	BSA	18.86	0.002	18.91	0.005	31.38	0.240	31.15	0.021
4	Myoglobin	24.07	0.004	24.19	0.003	14.45	0.107	14.60	0.015
5	Uracil	29.07	0.002	29.19	0.002	2.74	0.032	2.78	0.012
1	Mab dimer	14.19	0.022	14.29	0.003	0.47	0.000	0.53	0.010
2	Mab monomer	17.18	0.001	17.53	0.004	99.53	0.000	99.47	0.010
1	Thyroglobulin	1.03	0.001	1.03	0.001	25.19	0.151	25.34	0.200
2	IgG	1.27	0.001	1.27	0.001	27.14	0.217	27.17	0.209
3	BSA	1.42	0.000	1.43	0.001	30.52	0.482	30.19	0.511
4	Myoglobin	1.7	0.001	1.75	0.002	14.30	0.084	14.43	0.087
5	Uracil	2.23	0.001	2.23	0.001	2.85	0.032	2.87	0.021
1	Mab dimer	1.14	0.000	1.11	0.001	0.47	0.030	0.46	0.020
2	Mab monomer	1.32	0.001	1.30	0.000	99.53	0.040	99.54	0.020

Table 1. Quantitative comparison conventional SEC versus Auto•Blend Plus assisted SEC Retention time and peak area data represent the averaged data of triplicate analyses.

CONCLUSION

Conventional SEC relies on the accurate preparation of pH dependent mobile phases where subtle variation in pH can lead to significant changes in chromatographic retention times. As a means for reducing variability in the preparation of buffered mobile phase, Auto•Blend Plus Technology available through the Waters ACQUITY UPLC H-Class Bio System can prepare buffered mobile phase across a range of pH and NaCl concentrations from 4 standard stock solvents. In this application note, we have demonstrated the steps required to convert conventional SEC methods to Auto•Blend Plus methods. The benefits of Auto•Blend Plus span both SEC-HPLC and SEC-UPLC, where equivalent chromatography can be achieved with a more robust and reproducible solvent delivery system for pH dependent mobile phases.

Reference

1. Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio to Run SEC HPLC and SEC UPLC. Waters Application Note. 2014: 720005057en.

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Successful Transfer of Size-Exclusion Separations Between HPLC and UPLC

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Seamless scalability and transfer of protein SEC methods between UPLC and HPLC instrumentation
- Simplifying method transfer between laboratories with different instrumentation
- Enabling UPLC method scale-up for peak characterization
- 200Å and 450Å pore sizes provide a broad protein size separation range

WATERS SOLUTIONS

XBridge® Protein BEH SEC 200Å and 450Å, 3.5 µm Columns

ACQUITY UPLC® Protein BEH SEC, 200Å, 1.7 µm, and 450Å, 2.5 µm Columns

Alliance® HPLC System

ACQUITY UPLC H-Class Bio System

Empower® 3 Software

Waters BEH200 and BEH450 SEC Protein Standard Mixture and mAb Standard

KEY WORDS

Size-exclusion chromatography, UPLC, HPLC, method transfer, aggregates

INTRODUCTION

Over the last several years Waters has produced the highest resolving and highest sample throughput size-exclusion chromatography (SEC) columns available for protein and peptide analysis.[1,2] These size-exclusion UPLC® (SE-UPLC) columns consist of 1.7 µm diameter ethylene bridged hybrid (BEH) particles with 200Å or 125Å pore sizes, or a 2.5 µm diameter BEH particle with a 450Å pore size. Based on observations, SE-UPLC technology has been adopted in many biopharmaceutical analytical laboratories as an important tool to monitor protein aggregation during product development due to the high sample throughput that it provides. However, often times SE-UPLC methods cannot be transferred to other laboratories due to the lack of available UPLC instrumentation. Therefore, in an effort to provide the capability to transfer SEC methods between UPLC and HPLC instruments, Waters has introduced 3.5 µm particle diameter BEH-based size-exclusion HPLC (SE-HPLC) columns specifically for use on traditional HPLC instrumentation. These columns provide the analyst with the unique capability to use consistent particle chemistries that can be directly scaled between HPLC and UPLC instrumentation. This application note will highlight the considerations that must be made when transferring an SEC method between UPLC and HPLC columns and systems.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters® and Sigma-Aldrich®). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

LC conditions

LC system:	Waters Alliance HPLC or ACQUITY UPLC H-Class Bio System with 30 cm Column Heater
Columns:	XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 x 300 mm (p/n: 176003596); XBridge Protein BEH SEC, 450Å, 3.5 µm, 7.8 x 300 mm (p/n: 176003599); ACQUITY UPLC Protein BEH SEC, 200Å, 1.7 µm, 4.6 x 300 mm (p/n: 186005226); ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300 mm (p/n: 176002997)
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.84 mL/min
Mobile phases:	5 mM sodium phosphate, 150 mm sodium chloride, pH 7.2 (prepared using Auto•Blend Plus™ Technology or 5.5% A: 100 mM NaH_2PO_4, 14.5% B: 100 mM Na_2HPO_4, 15.0% C: NaCl, 65% D:H_2O)
Gradient:	Isocratic
Standard:	BEH200 SEC Protein Standard Mix (p/n: 186006518); BEH450 SEC Protein Standard Mix (p/n: 186006842); Intact mAb Mass Check Standard (p/n: 186006552)
Sample Vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and pre-slit PTFE/Silicone Septa, 1 mL (p/n: 186000385DV)
Detection:	Alliance HPLC TUV Detector; ACQUITY UPLC TUV Detector with 5 mm Titanium flow cell
Wavelength:	280 or 214 nm
Chromatography Software:	Waters Empower Pro (v2 and v3)

RESULTS AND DISCUSSION

LC system considerations

The performance of the LC system in an SEC separation can have a significant effect on the quality of the chromatographic resolution that can be achieved. Fundamentally, this can be represented by the equation highlighted in Figure 1, which has been adapted from Gritti and Guiochon.[3] From this equation, the total peak variance σ^2_{Total} can be derived from the sum of the peak variances that occur prior to the analyte reaching the column ($\sigma^2_{Pre\text{-}Column}$), on the column ($\sigma^2_{Column}$), and after the analyte elutes off of the column ($\sigma^2_{Post\text{-}Column}$). Using this relationship, it can be readily derived that as the peak variance contributions from extra-column dispersion ($\sigma^2_{Pre\text{-}Column}$ and $\sigma^2_{Post\text{-}Column}$) increase and become significant relative to the variance that occurs on the column (σ^2_{Column}), that the SEC separation efficiency will be measurably reduced. Another important concept illustrated by Figure 1 is the additive characteristic of both the pre-column and post-column variances in SEC. This is due to the absence of significant binding interactions between the analyte and the particle surface in SEC. Conversely, in other bind-and-elute based protein separations such as reversed-phase or ion-exchange chromatography, the contributions of $\sigma^2_{Pre\text{-}Column}$ become far less significant due to the strong binding-driven, analyte refocusing that occurs at the head of the column.

$$\sigma^2_{Total} = \sigma^2_{Pre\text{-}Column} + \sigma^2_{Column} + \sigma^2_{Post\text{-}Column}$$

Figure 1. Equation highlighting sources of peak dispersion in SEC. Dispersion in blue (pre-column and post-column) results in reduced resolution when levels are significant relative to dispersion in green (column). Consult text for further explanation.

The practical effect of these considerations is demonstrated in Figure 2, which shows a comparison of the separation of protein standards on two columns packed with the same batch of 3.5 µm diameter, 200Å pore-size BEH particles on an Alliance HPLC System. One of the columns was an experimental column with an internal diameter (I.D.) of 4.6 mm, and the other the commercially available 7.8 mm I.D. column. The flow rates were adjusted to provide equivalent linear velocities for each column. It is readily observed that the resolution for the 4.6 mm I.D. column is significantly lower than that observed for the 7.8 mm I.D. column on the same instrument. As an example, the resolution observed between IgG and BSA is 30% higher for the 7.8 mm I.D. column in comparison to the 4.6 mm I.D. column. To understand why the performance of the 7.8 mm I.D. is markedly better, we can reconsider the relationships of the equation presented in Figure 1. In this equation, the peak dispersion that occurs within the column (σ^2_{Column}) increases with column I.D., however, this is offset by a proportional increase in pore volume ,which increases separation efficiency, with the end result being that the resolution of an SEC column is fundamentally independent of column I.D.. Therefore, as the I.D. of an SEC column increases, the separation efficiency remains constant , assuming packing efficiencies are maintained, while σ^2_{Column} increases. This results in the contribution that $\sigma^2_{Pre\text{-}Column}$ and $\sigma^2_{Post\text{-}Column}$ have to σ^2_{Total} becoming less significant, which practically results in an improved chromatographic separation.

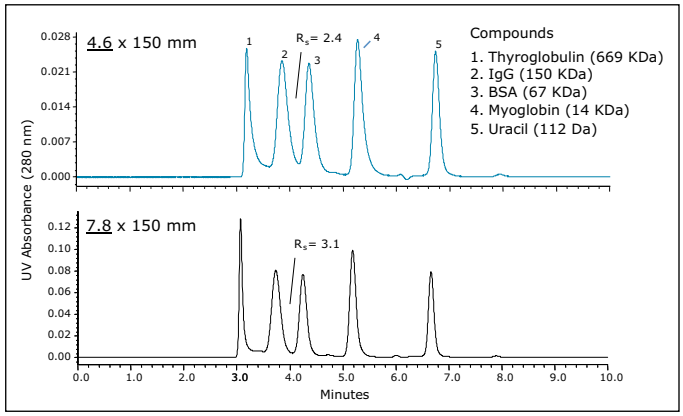

Figure 2. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n 186006518) link: on two XBridge Protein BEH SEC, 200Å, 3.5 µm Columns. The top frame is an experimental column with a 4.6 mm I.D. and the bottom frame is a commercially available column with a 7.8 mm I.D.. Both columns were run using an Alliance HPLC and the flow rates were scaled based on column I.D. to 0.3 mL/minute for the 4.6 mm I.D. column and 0.86 mL/minute for the 7.8 mm I.D. column. Samples loads were also adjusted for column volume.

Transfer of SEC methods between UPLC and HPLC columns

There are two primary considerations to be made when transferring an SEC method from one column to another. Most importantly the surface chemistry of the particles must be comparable. In addition to matching pore size, size-exclusion particles can potentially have both ionic and hydrophobic interactions with biomolecules, and the nature of these interactions must be comparable if the separations are going to be performed using the same mobile phase and temperature. Secondly, the separation must be appropriately scaled with respect to particle size. The first step in scaling relative to particle size is to match as best as possible the ratio between length and particle diameter for the two columns using Equation 1:

$$L_{HPLC} = \frac{L_{UPLC} \times d_{p,HPLC}}{d_{p,UPLC}}$$

Equation 1: Where: L_{HPLC} and L_{UPLC} are the lengths of the HPLC and UPLC columns (mm), and $d_{p,HPLC}$ and $d_{p,UPLC}$ are the particle diameters of the HPLC and UPLC columns (µm).

Following this, a flow rate can be calculated by running both columns at the same reduced linear velocity, which is proportional to the product of the linear velocity and the particle diameter for a given analyte. Since the flow rate is proportional to the product of the linear velocity and square of the column I.D., the correct scaled flow rate can be readily calculated using Equation 2:

$$F_{HPLC} = \frac{F_{UPLC} \times d_{p,UPLC} \times D^2_{HPLC}}{d_{p,HPLC} \times D^2_{UPLC}}$$

Equation 2: Where: F_{HPLC} and F_{UPLC} are the flow rates (mL/minute) of the HPLC and UPLC columns, and D_{HPLC} and D_{UPLC} are the internal diameters of the HPLC and UPLC columns (mm).

Finally, the injection volume can be scaled for column volume, which is proportional to the product of the square of the column and the column length. This can be represented by Equation 3:

$$V_{HPLC} = \frac{V_{UPLC} \times L_{HPLC} \times D^2_{HPLC}}{L_{UPLC} \times D^2_{UPLC}}$$

Equation 3: Where: V_{HPLC} and V_{UPLC} are the injection volumes of the HPLC and UPLC columns (µL).

We will first demonstrate the successful scaling between the ACQUITY UPLC Protein SEC 200Å, 1.7 µm, 4.6 X 300 mm column and the XBridge Protein SEC, 200Å, 3.5 µm, 7.8 mm I.D. HPLC column format. Given that there is an approximately 2-fold increase in particle size (based on Equation 1) the HPLC column length will need to be twice that of the UPLC Column (60 cm) to yield comparable resolution. This can be accomplished by connecting two 30 cm length HPLC columns in series. It should be noted that if a 15 cm, 1.7 µm particle size UPLC Column had been used, then the separation should be properly transferred to a single 30 cm length, 3.5 µm particle size HPLC column. Given the column I.D. and particle diameter values for each column, and using Equations 2 and 3, the flow rate should be 1.4 times greater and the injection volume should be 5.75 times greater for the HPLC analysis than for the UPLC analysis.

The results of this method scaling are shown in Figure 3 and Figure 4. Figure 3 is a comparison of the separation of the BEH200 SEC Protein Standard Mix, and Figure 4 is a comparison of the separation of the biotherapeutic monoclonal antibody infliximab. The UPLC separations were performed on an ACQUITY UPLC H-Class Bio System and the HPLC separations were performed on an Alliance HPLC System. The mobile phase used for both separations was a phosphate buffered saline (PBS) solution with low ionic strength and a slightly basic pH (25 mM sodium phosphate, 150 mm sodium chloride, pH 7.2). This buffer was selected due to its common use for SEC separations. Additionally, the pH and low ionic strength of this buffer will mask subtle variations in particle surface chemistry that can lead to undesired protein-particle interactions to a much lower extent as compared to higher ionic strength buffers.[4] In both comparisons, the time axis has been normalized. Both pairs of chromatograms show comparable profiles with the primary difference being the analysis time, which is four to five times lower for the UPLC separation. While the HPLC analysis time is significantly longer, it has the same selectivity (profile) as the UPLC separation, thus allowing methods to be developed more rapidly and used on UPLC and then subsequently transferred to HPLC when the receiving lab does not have UPLC instrumentation.

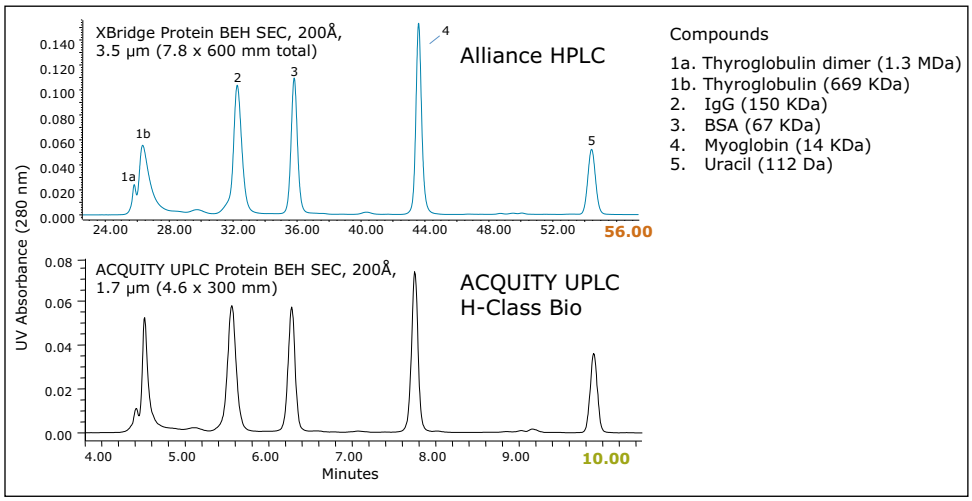

Figure 3. Shown is a comparison of separations of BEH200 SEC Protein Standard Mix (p/n 186006518) on two, XBridge Protein BEH SEC 200Å, 3.5 µm, 7.8 x 300 mm Columns run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 200Å, 1.7 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.42 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC Column. Samples loads were also adjusted for column volume.

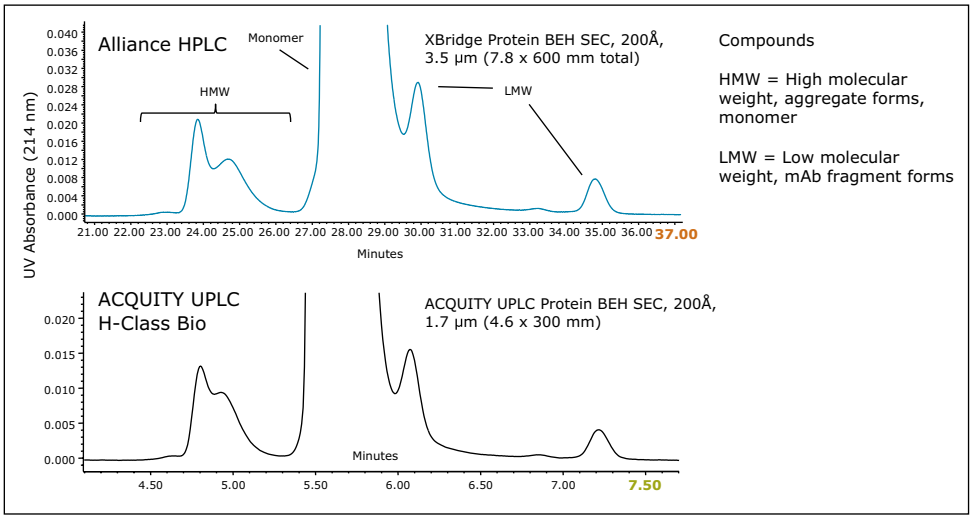

Figure 4. Shown is a comparison of separations of the biotherapeutic monoclonal antibody Infliximab on two, XBridge Protein BEH SEC 200Å, 3.5 µm columns (300 x 7.8 mm I.D.) run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 200Å, 1.7 µm column (300 x 4.6 mm I.D.) using an ACQUITY H-Class Bio UPLC (bottom frame). The flow rates were approximately scaled based on particle diameter and column I.D. to 0.5 mL/minute for the two HPLC columns run in series and 0.4 mL/minute for the UPLC Column. Samples loads were also adjusted for column volume. Peak identities for chromatograms are: HMW (high molecular weight, aggregate forms), monomer, and LMW (low molecular weight, mAb fragment forms).

Next, we present the transfer between an ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300mm, column and the XBridge Protein BEH SEC, 450Å, 3.5 µm particle size, 7.8 mm I.D. HPLC column format. Using Equation 1, the length of the HPLC column should be 42 cm, which can be approximated by connecting a 30 cm and a 15 cm XBridge SEC, 3.5 µm Column in series. In the event that the method transfer is from a 15 cm length, 2.5 µm UPLC Column, the appropriate length HPLC column would be 21 cm, which is not an available configuration. In this case, a 30 cm HPLC column could be used. However, if matching reduced linear velocities were used, the resolution for the HPLC column should be approximately 20% greater, as the resolution difference is approximately proportional to the square root of compared column lengths.

Figure 5 and Figure 6 demonstrate the effectiveness of scaling from a 30 cm, 2.5 µm, 450Å, 4.6 mm I.D., SE-UPLC column to a total 45 cm length 3.5 µm particle size, 450Å, 7.8 mm I.D., HPLC column. Figure 5 is a comparison of the separation of the BEH450 SEC Protein Standard Mix, and Figure 6 shows the separations observed for IgM in its pentameric and dipentameric forms with molecular weights of approximately 900 KDa and 1.8 MDa, respectively. As in the previous example, the UPLC separations were performed on an ACQUITY UPLC H-Class Bio System and the HPLC separations were performed on an Alliance HPLC System, and in both comparisons, the time axis has been normalized. Both pairs of chromatograms show comparable profiles over the molecular weight range thereby demonstrating transferability between the UPLC and HPLC formats. In this example, UPLC is approximately 2-fold faster than HPLC.

Overall, these data demonstrate the seamless and effective method transfer from BEH-based, SE-UPLC columns operated on UPLC systems to SE-HPLC columns operated on HPLC systems. The chromatographic profiles observed underscore the chemical comparability of the particle surfaces, as well as their pore characteristics.

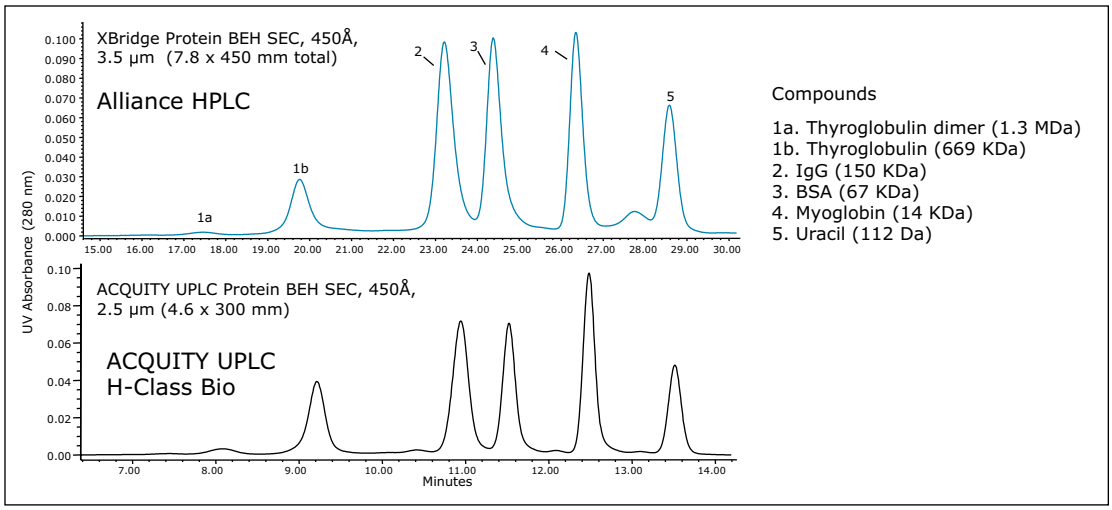

Figure 5. Shown is a comparison of separations of BEH450 SEC Protein Standard Mix (p/n 186006518) on two XBridge Protein BEH SEC, 450Å, 3.5 µm Columns (7.8 x 150 + 300 mm) run in series using an Alliance HPLC (top frame) and on an ACQUITY UPLC Protein BEH 450Å, 2.5 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.62 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC column. Samples loads were also adjusted for column volume.

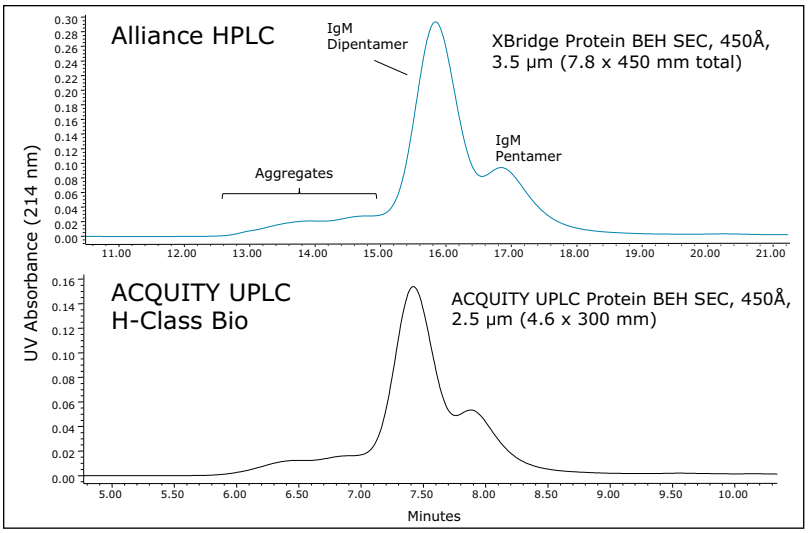

Figure 6. Shown is a comparison of separations of IgM (1 mg/mL) on two XBridge Protein BEH SEC 450Å, 3.5 µm, 7.8 x 150 + 300 mm Columns run in series using an Alliance HPLC System (top frame) and on an ACQUITY UPLC Protein BEH SEC, 450Å, 2.5 µm, 4.6 x 300 mm Column using an ACQUITY UPLC H-Class Bio (bottom frame). The flow rates were scaled based on particle diameter and column I.D. to 0.62 mL/minute for the two HPLC columns run in series and 0.3 mL/minute for the UPLC column. Samples loads were also adjusted for column volume.

CONCLUSIONS

Successful implementation of SE-UPLC separation technology requires high efficiency columns and low dispersion LC systems capable of operating at high back pressures. The advantage in doing so is the capability to dramatically improve sample throughput while decreasing sample requirements and mobile phase use. However, there are several situations where it is beneficial to be able to transfer between SE-UPLC and SE-HPLC separations, including instrumentation limitations in a method transfer, or in the event that a UPLC separation needs to be scaled up in order to facilitate the structural or functional characterization of low abundance species. However, in some cases, a direct method transfer using the same mobile phase and temperature conditions is not possible between SE-UPLC Columns packed with BEH-based particles and SE-HPLC columns packed with traditional silica-based particles due to the differences in the surface characteristics of the two particle types. These differences can necessitate the re-optimization of the method in order to get comparable results. Waters' recent development of HPLC compatible 200Å and 450Å, 3.5 µm particles based on the same diol-coated BEH-particle chemistry as that used in ACQUITY UPLC Protein BEH SEC Columns provides the capability to readily transfer between SE-UPLC and SE-HPLC for the first time. This application note details and provides examples of the methodology that can successfully employed for this method transfer.

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Advances in Size-Exclusion Chromatography for the Analysis of Macromolecular Proteins

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution of macromolecular proteins by SE-UPLC®
- Outstanding column stability and reliable column-to-column reproducibility
- Increased size separation range when the new 450Å BEH450 SEC, 2.5 µm Column is used in series with the Waters® ACQUITY UPLC® BEH200, 1.7 µm, SEC Column

WATERS SOLUTIONS

ACQUITY UPLC BEH450 SEC, 2.5 µm Column

ACQUITY UPLC H-Class Bio System

Auto•Blend Plus™ Technology

BEH450 SEC Protein Standard Mix

KEY WORDS

Size-exclusion chromatography, SEC, peptides, proteins, SE-UPLC, gel filtration chromatography, calibration curves, macromolecules, IgM

INTRODUCTION

The separation of macromolecular proteins by size-exclusion chromatography has been an area of significant interest since the introduction of cross-linked dextran based soft-gels by Porath and Flodin in 1959.[1] This mode of separation was further improved by the introduction of particles comprised of cross-linked polystyrene (µ-Styragel™) and ultimately porous-silica particles. With that perspective, the newly introduced sub-3-µm 450Å BEH SE-UPLC particle presented in this note represents the latest step in the technological evolution of the size-based separation of biological macromolecules. This note will highlight the performance characteristics of this column with respect to column-to-column reproducibility and column stability. Additionally, the distinct advantages that sub-3-µm packing material offers over a larger (8 µm) particle size for the separation of large proteins will also be shown. Finally, we will demonstrate where the size-separation range of this 450Å pore-size SE-UPLC Column complements that of the 200Å ACQUITY® BEH200 SEC Column and how the two columns can be used together to increase the molecular weight range of a protein separation.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters and Sigma-Aldrich). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

(unless noted otherwise)

LC conditions

System:	Waters ACQUITY UPLC H-Class Bio System with 30-cm Column Heater
Detection:	ACQUITY UPLC TUV with 5-mm titanium flow cell Wyatt miniDAWN TREOS light scattering detector
Wavelength:	280 or 214 nm
Columns:	Waters ACQUITY UPLC BEH450 SEC Column, 450Å, 2.5 µm, 4.6 x 150 mm (p/n 176002996) and 4.6 x 300 mm (p/n 176002997) Waters ACQUITY UPLC BEH200 SEC Column, 200Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225) and 4.6 x 300 mm (p/n 186005226)
HPLC Column:	450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.35 mL/min
Mobile phases:	25 mM sodium phosphate, 250 mm sodium chloride, pH 6.8 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH450 SEC Protein Standard Mix (p/n 186006842)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL (p/n 186000385DV)

Data management

Chromatography software:	Waters Empower® Pro (*v*2, FR 5) Waters UNIFI® (v1.6) Wyatt Astra (v5.3.4.16)

RESULTS AND DISCUSSION

The benefits of UltraPerformance Liquid Chromatography (UPLC) combined with 125Å and 200Å pore-size, sub-2-µm size-exclusion UPLC (SE-UPLC) packing materials for the analysis of peptides and proteins have been previously described.[2,3] The newly introduced BEH 450Å pore size, sub-3-µm packing material, designed to expand the molecular weight range of SE-UPLC separations to include biological macromolecules with large radii of hydration (Rh), such as IgM and multimeric self-associated proteins, will be evaluated. As part of this evaluation, the critical performance characteristics of column-to-column reproducibility, lifetime stability, and the separation efficiency advantages of this packing material with respect to larger particle-size (8 µm) HPLC packing materials will be demonstrated. In addition, this note will demonstrate where the size-separation range of this 450Å pore-size SE-UPLC Column complements that of the 200Å ACQUITY BEH200 SEC Column and how the two columns can be used together to increase the molecular weight range of a protein separation.

ACQUITY UPLC BEH450 Column reproducibility and stability

In addition to analyte resolution and sensitivity, the two major concerns that an analyst has when selecting an SEC column for method development are column-to-column reproducibility and column lifetime. An overlay of the chromatograms for a series of molecular weight standards is shown in Figure 1. These chromatograms demonstrate the reproducibility of five 300-mm length ACQUITY BEH450 SEC Columns packed from three different production lots of packing material. For these standards at a flow rate of 0.35 mL/min, the retention time standard deviation ranged from a minimum of 0.005 min for the earliest eluting component (IgM pentadimer) to 0.022 min for the total permeation component (uracil) with an average standard deviation of 0.017 min for all components labeled in Figure 1.

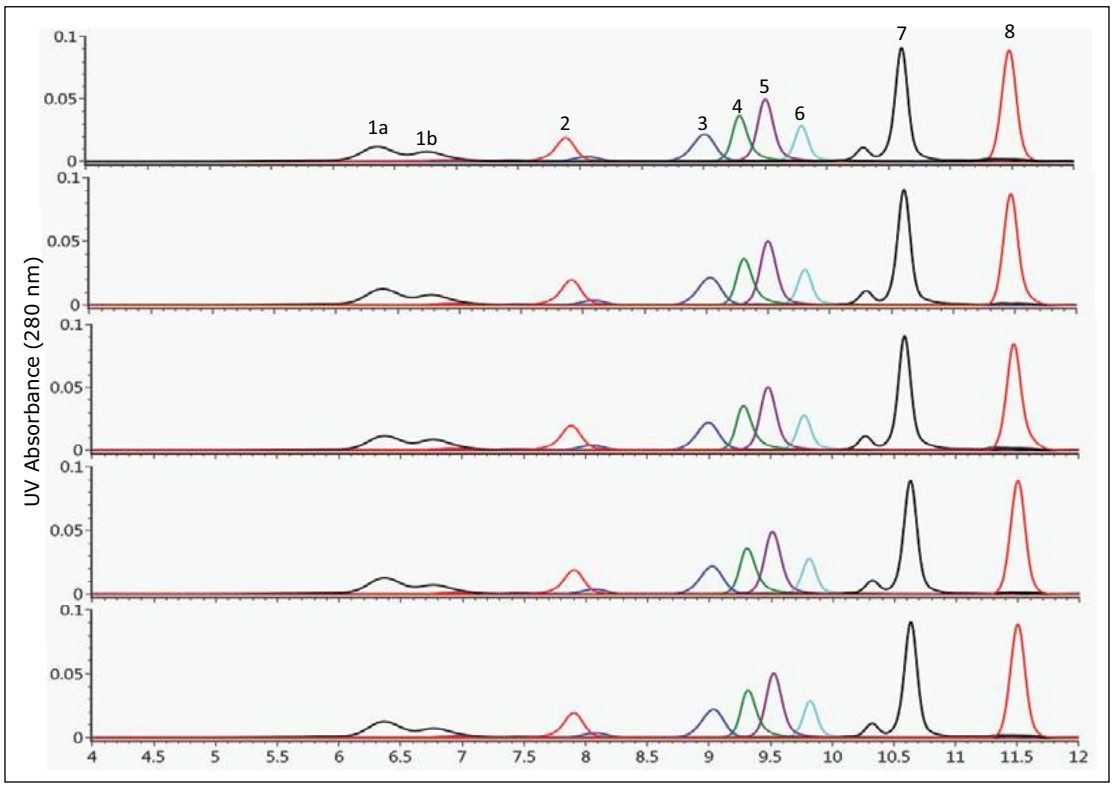

Figure 1. Column-to-column reproducibility of the 300 mm ACQUITY UPLC BEH450 SEC Column. The chromatograms for five columns packed from three different production batches are shown. Compounds: 1a. IgM Dipentamer (1.8MDa), 1b. IgM Pentamer (900 Kda), 2. Thyroglobulin (667 KDa), 3. Apoferritin (443KDa), 4. β-Amylase (200 Kda), 5. IgG (150 KDa), 6. BSA (66 KDa), 7. Myoglobin (17 KDa), and 8. Uracil (112 Da).

The stability of the ACQUITY BEH450 SEC Column (300 m) was evaluated by injecting a series of standards over the course of over 800 total injections. The retention time stability for the largest protein evaluated in this study (thyroglobulin), which is used to probe the chemical stability of the particle surface, is shown in Figure 2. The peak asymmetry of the uracil standard is also shown in Figure 2. This compound, which elutes in the total permeation volume, is used to test the mechanical integrity of the column. These data demonstrate that the ACQUITY BEH450 SEC Column can provide the reproducibility and stability needed to develop reliable assays for use in a quality control environment.

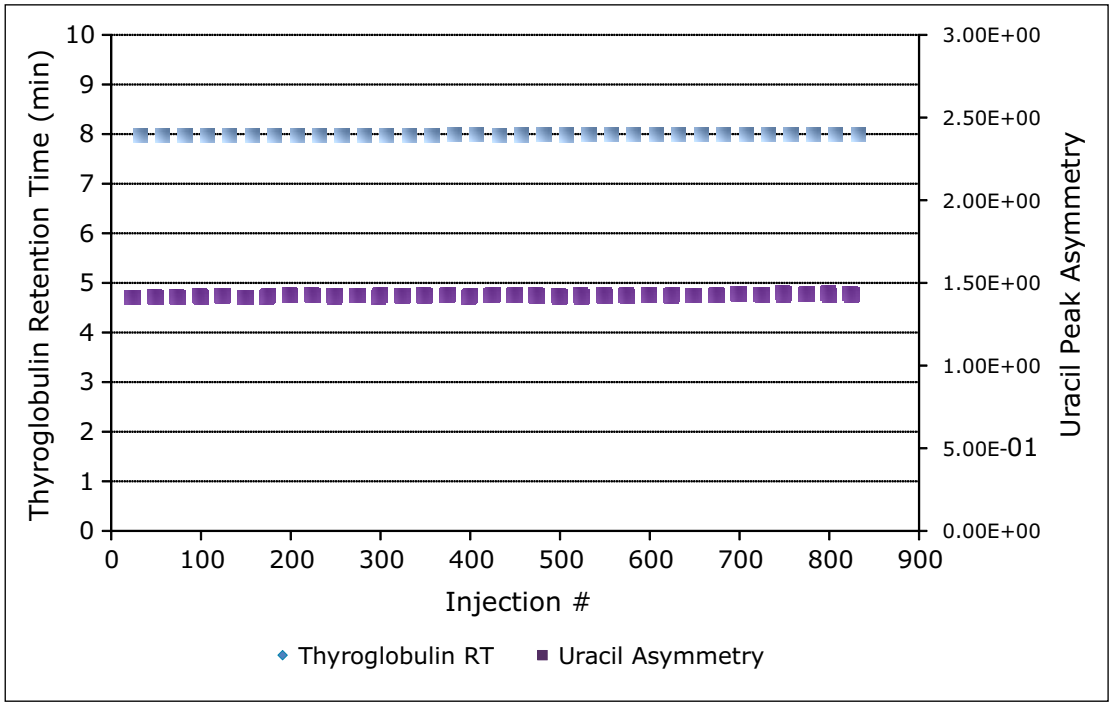

Figure 2. Performance stability of the 300 mm ACQUITY UPLC BEH450 SEC Column over 800 injections. The retention time of thyroglobulin and the asymmetry of the uracil standard (at 4.4% peak height) are shown.

Effect of particle size

The benefits of smaller particles for size-exclusion chromatography have been well documented with improvements in efficiency and resolution.[4] A set of proteins was analyzed individually on a 450Å pore-size UPLC-based BEH SEC column (2.5 µm) and a 450Å pore-size HPLC-based silica SEC column (8 µm) using the same ACQUITY UPLC H-Class Bio System and aqueous mobile phase conditions, shown in Figure 3. The flow rates and injection volumes used were proportional to the size of the column tested. Improved sensitivity and narrower peak widths were observed on the 2.5 µm packing material across the separation range. USP resolution values (half-height measurement) calculated for the separation between the apoferritin monomer (MW=443 KDa) and dimer (MW=886 KDa) forms, shown in Figure 4, were base-line resolved with a resolution of 2.49. This resolution is 1.8 times greater than that observed using the 8 µm particle-sized column (R_s=1.42).

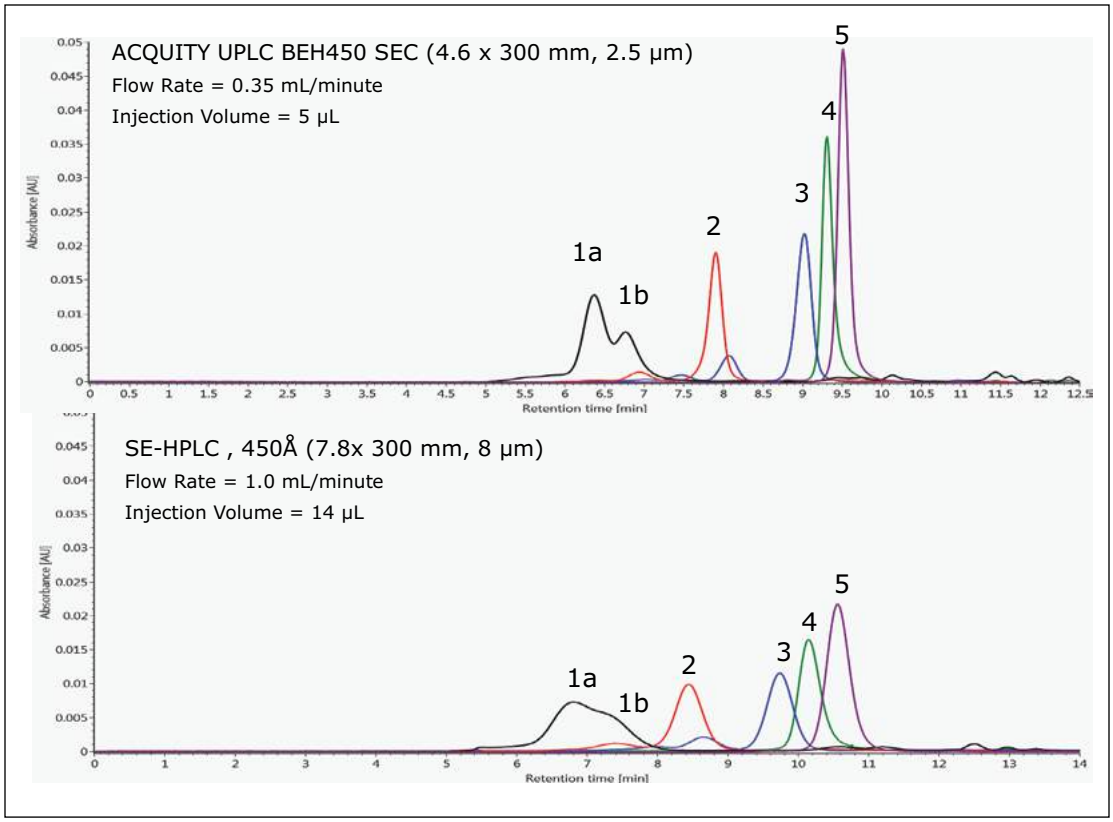

Figure 3. Comparison of the ACQUITY UPLC BEH450 SEC Column (300-mm length) to a Biosuite 450 HR Column (300-mm length). Compounds: 1a. IgM Dipentamer (1.8MDa), 1b. IgM Pentamer (900 Kda), 2. Thyroglobulin (667 KDa), 3. Apoferritin (443KDa), 4. β-Amylase (200 KDa), 5. IgG (150 KDa). Sample injection volumes and flow rate were normalized for column geometry. UV absorbance (280 nm) shown at same scale for both columns. The identities of the peaks observed for the IgM sample were confirmed by SEC-MALLS analysis.

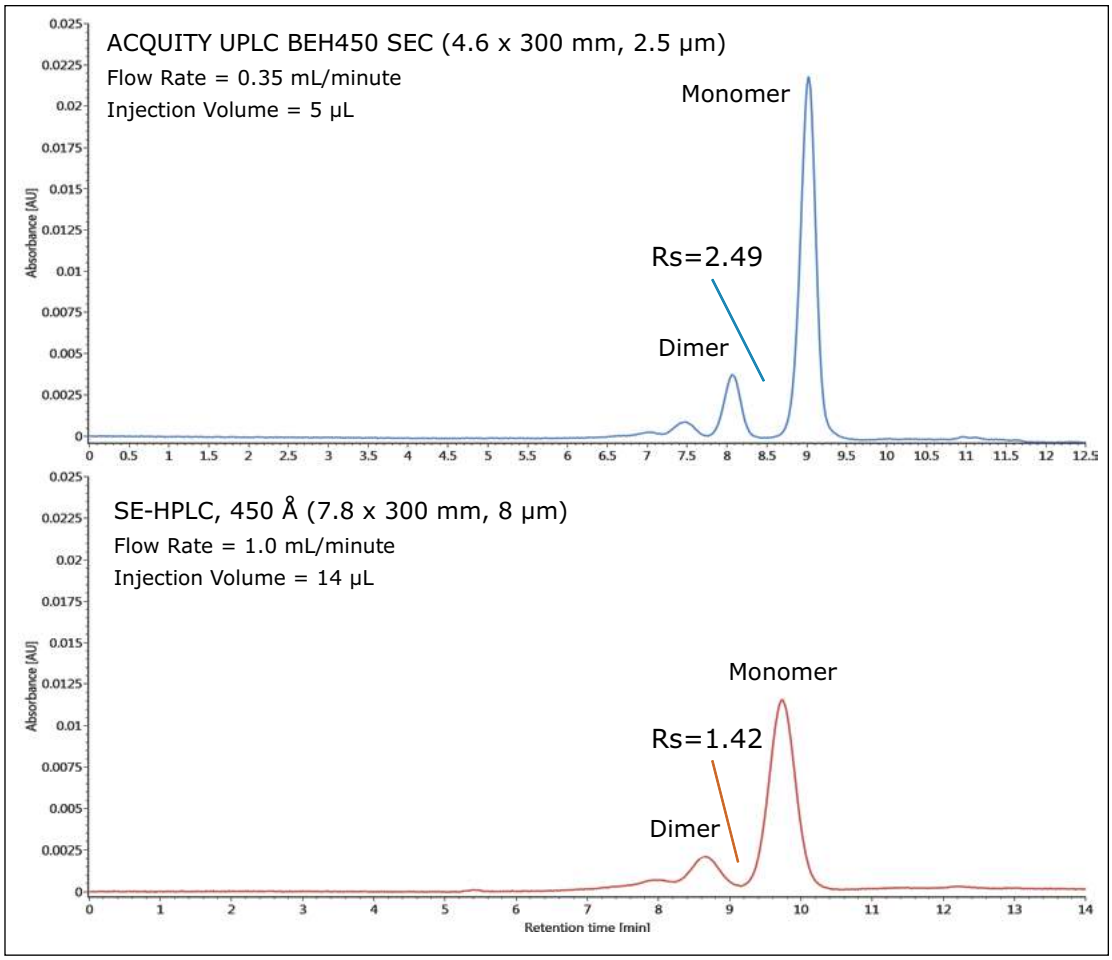

Figure 4. Comparison of the ACQUITY UPLC BEH450 SEC Column (300-mm length) to a Biosuite 450 HR Column (300-mm length) for the separation of apoferritin (443 KDa). Sample injection volumes and flow rate were normalized for column geometry. UV absorbance (280 nm) shown at same scale for both columns.

Expanding molecular weight range: combination with ACQUITY BEH200 SEC (200Å)

Comparisons were made between the 450Å and 200Å pore-size particles for the separation of proteins. Additionally, both columns used in the series were evaluated. The comparison of the separation achieved on the 200Å and 450Å SE-UPLC columns (300-mm length) and the 150-mm length version of both columns connected in series (BEH200 followed by BEH450) for the Waters BEH200 SEC Protein Standard Mix (p/n 186006518) is shown in Figure 5. For this standard mix, it is clear that the resolution of the separation for the standard components with molecular weights up to that of the IgG standard (150 KDa) is higher with the 200Å column as noted by the separation between IgG and BSA (66 KDa). For higher molecular weight components like thyroglobulin (667 KDa) and its dimeric form (1340 KDa or IgM pentamer (900 KDa) and IgM dipentamer (1800 KDa), however, the 450Å can provide improved separations, as shown in Figure 4. This higher molecular weight range may be of use when analyzing multimeric protein aggregates or proteins conjugated to compounds that have relatively large Rh values, such as long chain polyethylene glycols.

A third option available to the analyst in need of analyzing a sample containing both low molecular weight and multimeric high molecular weight forms in a sample, is to use the two columns in series. As the back pressure generated by the 200Å column is greater than that of the 450Å column due to the smaller particle size of 200Å column, the 200Å column was placed first in the series for this study. The result of this two-column configuration is shown in the center panel of Figure 5. By using both columns, the functional upper molecular weight range of the separation is increased as noted by the improved separation of thyroglobulin and its dimer compared to that same separation using the 200Å column alone. Additionally, for the lower molecular weight forms, there is an improvement in resolution compared to the use of the 450Å column alone, as evidenced by the improved separation between IgG and BSA. These observations can be confirmed by evaluating the elution volumes of these components in the calibration curves generated from these data, as shown in Figure 6. The calibration curve for the ACQUITY UPLC BEH200 SEC Column showed greater linearity, and elution volume differences up to at least the IgG (150 KDa) standard compared to the ACQUITY UPLC BEH450 SEC Column. Conversely, using the same criteria, the BEH450 column is better suited for proteins with a molecular weight of near that of thyroglobulin (667 KDa) and greater. The BEH200 and BEH450 columns in series produced an intermediate calibration curve with the broadest pseudo-linear range. The compromise of this two-column configuration is that the resolutions achieved for proteins within the optimal range of each individual column are diminished, however.

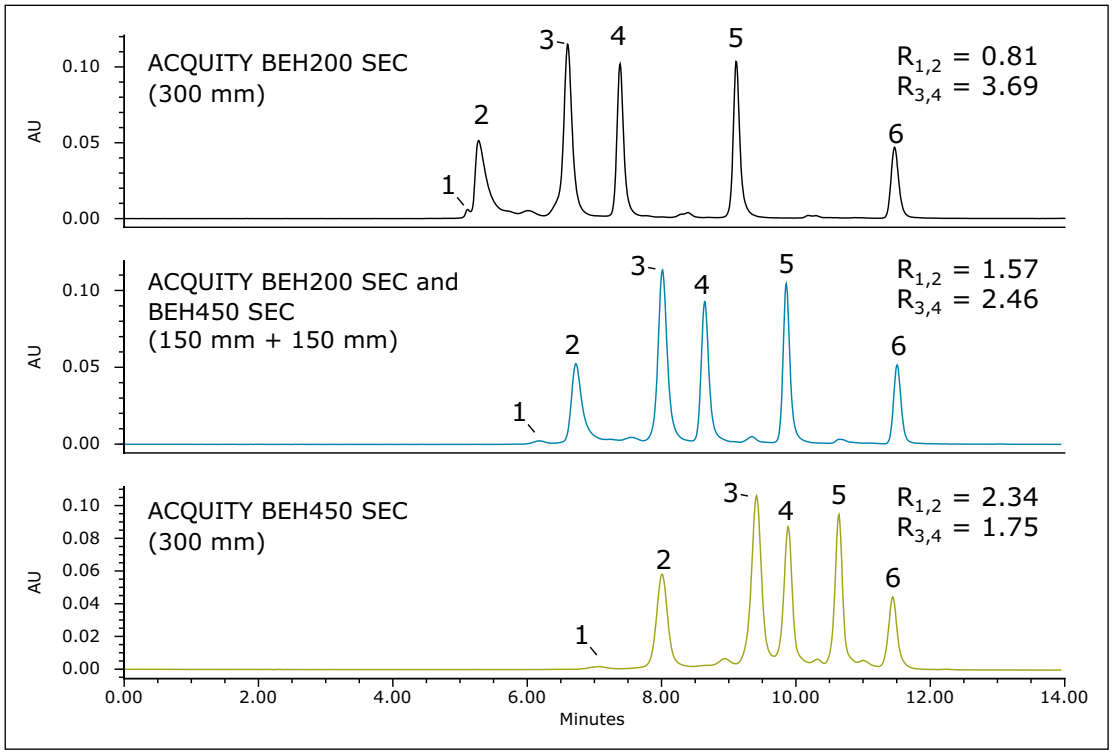

Figure 5. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (both 300-mm length). The center panel was generated using both a BEH200 and BEH450 column in series. Compounds: 1. Thyroglobulin dimer (1,340 KDa), 2. Thyroglobulin (667 KDa), 3. IgG (150 KDa), 4. BSA (66 KDa), 5. Myoglobin (17 KDa), 6. Uracil (112 Da). Resolution values were calculated based on peak width at half maximum.

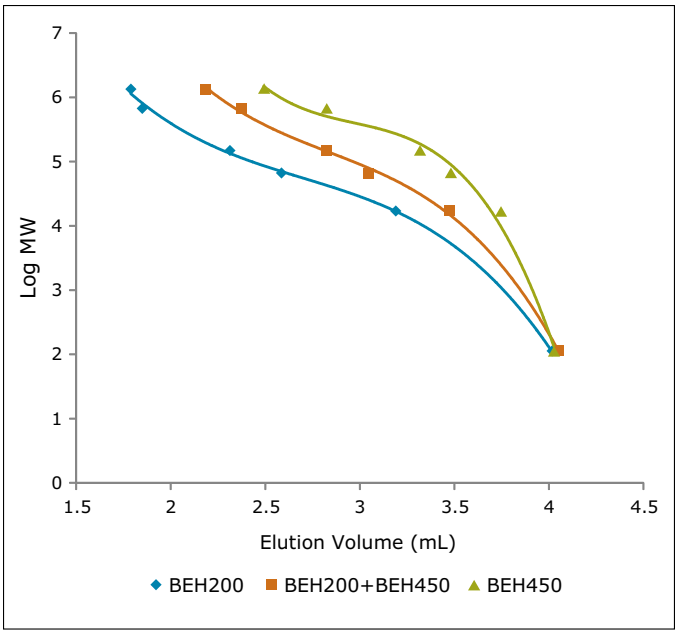

Figure 6. Comparison of the ACQUITY UPLC BEH450 SEC and ACQUITY UPLC BEH200 SEC Column calibration curves. Retention times of compounds tested in Figure 5 are represented.

CONCLUSIONS

Size-exclusion chromatography has been the preferred method for the analyses of proteins based on size. By combining 450Å sub-3-µm packing materials with a low dispersion ACQUITY UPLC H-Class System, separations with improved resolution and high-throughput of SE-UPLC can be realized for macromolecular proteins and highly aggregated proteins with molecular weights of up to approximately 2000 KDa. Additionally, the use of the BEH450 and BEH200 columns in series can provide a broader molecular weight range than can be obtained by using the columns individually.

The ACQUITY UPLC BEH450, 2.5 µm SEC Column in combination with the ACQUITY UPLC H-Class Bio System provides the following benefits:

- Outstanding column-to-column reproducibility and stability
- Nearly a two-fold increase in resolution compared to traditional SE-HPLC packing materials
- Improved resolution for large proteins and aggregates with molecular weights above the linear separation range of the smaller pore-size SE-UPLC columns
- Expanded molecular weight range when used in series with a UPLC BEH200, 1.7 µm SEC Column

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Analysis of Proteins by Size-Exclusion Chromatography Coupled with Mass Spectrometry Under Non-Denaturing Conditions

Paula Hong, Stephan Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and sensitivity with SE-UPLC as compared to traditional SE-HPLC
- Non-denaturing SEC method for MS identification of unknown biotherapeutic components
- Exact molecular weight confirmation of intact bimolecules
- BEH particles provide columns with reduced secondary interactions that allow for mobile phases with reduced salt concentrations
- SEC column with minimal MS column bleed provides improved sensitivity

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC BEH200 SEC, 1.7 µm Column

Xevo® G2 Q-Tof Mass Spectrometer

MassLynx® Software

KEY WORDS

SE-UPLC, SEC-MS, QuanTof, monoclonal antibodies, biotherapeutics

INTRODUCTION

Ultra performance size-exclusion chromatography (SE-UPLC) provides a high throughput, robust method for separation of biomolecules based on size in solution.[1] SE-UPLC is typically performed under non-denaturing conditions, which are intended to preserve the state of self-association of the biomolecule, with a UV detector for quantification. Molecular weight estimates based on this technique require the use of an appropriate set of molecular weight standards for calibration. Other methods capable of providing molecular weight information under non-denaturing conditions include on-line multi-angle light scattering (MALS) and off-line analytical ultracentrifugation (AUC), both of which do not rely on molecular weight standards. These low resolution techniques cannot always resolve minor differences in molecular weight due to post-translational modifications or degradation. The combination of SEC using non-denaturing mobile phase and mass spectrometry (MS) provides accurate on-line mass determination for biomolecular species observed by SE-UPLC, however, the form of the non-covalent self-associated species is not provided by this method.

In this application, we describe SEC-MS under non-denaturing conditions. While a similar application has been evaluated for SE-HPLC,[2] UPLC® Technology in combination with sub-2-µm SEC column packing and a time-of-flight mass spectrometer, Xevo G2 Q-Tof, allows for direct analysis with improved chromatographic resolution and sensitivity. The resulting separations are comparable in retention time to those obtained using typical SEC mobile phases that are not MS compatible. By combining these conditions with a Xevo G2 Q-Tof, SE-UPLC-MS analysis can be used as an effective complementary characterization method to low-resolution, non-denaturing mass determination methods such as MALS or AUC, and low-resolution, denaturing size separations such as capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) to confirm the identification of biomolecular species observed by size-exclusion chromatography.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System with PDA detector
Flow cell:	Titanium 5 mm (part number 205000613)
Wavelength:	280 nm
Column:	ACQUITY UPLC BEH200, SEC 1.7 µm, 4.6 x 300 mm (part number 186005226)
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	2 µL
Flow rate:	0.15 mL/min or 0.2 mL/min
Mobile phase:	100 mM ammonium formate and 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8
Additive:	Acetonitrile, 0.8% formic acid, at 0.2 mL/min
External pump:	Waters 515 HPLC pump
Vials:	LCMS Certified Max Recovery vials (part number 600000755CV)

MS conditions

MS system:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	Sensitivity
Acquisition range:	500-5000
Capillary voltage:	3.00 kV
Cone voltage:	40.0 V
Source temp.:	150 °C
Desolvation temp.:	450 °C
Cone gas flow:	0.0 L/Hr
Desolvation gas flow:	800.0 L/Hr
Calibration:	NaI 2 µg/µL from 1000-4000 *m/z*

Data management

MassLynx Software

MaxEnt™1 Software

Sample description

The protein standard (obtained from Bio-Rad) containing bovine thyroglobulin (5 mg/mL), bovine γ–globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 µg/µL) and Vitamin B_{12} (0.5 µg/µL) in deionized water was analyzed. Horse heart myoglobin (Sigma) was prepared at 2 mg/mL in deionized water. A recombinant humanized monoclonal antibody, trastuzumab, was analyzed past expiry undiluted (21 µg/µL).

RESULTS AND DISCUSSION

The analysis of proteins by size exclusion chromatography (SEC) is typically performed under non-denaturing conditions which preserve the three dimensional structure and can be correlated with biological activity of the protein. Common mobile phases are 100% aqueous in a physiological pH range (6-8) and typically require non-volatile buffers and salts such as sodium phosphate and sodium chloride.[3] In order to obtain MS characterization of sample fractions separated under these conditions, the most common solution is to desalt the sample prior to analysis; however, this approach can result in sample speciation and can be cumbersome.

Another strategy is to perform SEC under denaturing conditions, so that species are efficiently ionized for detection by MS.[4, 5] These methods typically require the use of mobile phases containing acetonitrile, formic acid and trifluoroacetic acid (TFA) for direct coupling of SEC to MS. While TFA does cause ion suppression in MS, it is required to minimize secondary interactions between the column packing material and the biomolecule. This application provides a useful tool for desalting of a sample without the need for column re-equilibration and has been used for the analysis of reduced and alkylated monoclonal antibodies as well as other smaller proteins.[5, 6] This method does not typically preserve the self-associated state of the protein.

An alternative approach to SEC-MS has been the use of aqueous mobile phases that are MS compatible such as ammonium formate and ammonium acetate at low concentrations (<100 mM). While these mobile phases may not completely preserve the native structures for biomolecules,[3] they have been found to provide MS sensitivity while best preserving protein self-association and size-based chromatographic separation.

METHOD DEVELOPMENT

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column was evaluated at varying ammonium formate concentrations (5-200 mM) for resolution and MS sensitivity. Initial screening by UV evaluated the effect of salt concentration on both peak shape and resolution. A protein standard (Bio-Rad Laboratories) was used for the analysis. At low ammonium formate concentrations (<100 mM), secondary interactions result in poor peak shape and increased tailing for most of the proteins compared to phosphate buffers. These interactions can be due to either an “ion-exchange” or ”ion-exclusion” effect between the free silanols on the packing material and the biomolecules.[7] While peak shape and resolution improved at higher ammonium formate concentrations, ion suppression in the ESI process was also observed with lower intsenity counts. The final mobile-phase conditions were selected to balance resolution and ion suppression. At 100 mM ammonium formate, no tailing significant was observed and the MS signal was adequate for peak identification.

Comparison of the UV chromatograms with 100 mM ammonium formate and PBS (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8) mobile phases show similar retention and peak shape (Figure 1). For this example, ammonium formate provides an adequate SEC separation. However, not all biomoelcules exhibit the same degree of secondary interactions. In instances in which there are greater secondary interactions, the ammonium formate concentration can be altered to improve peak shape.

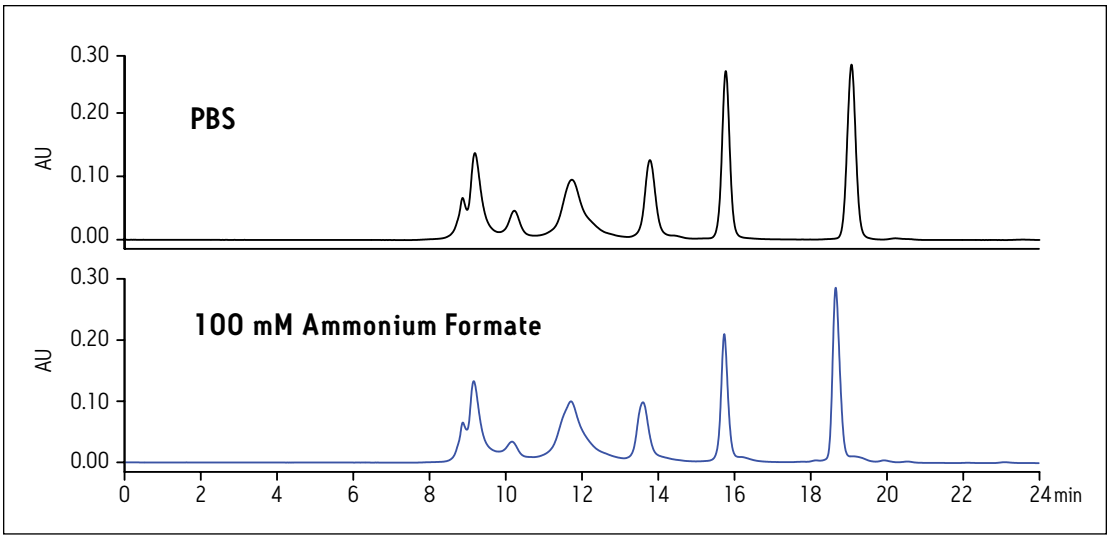
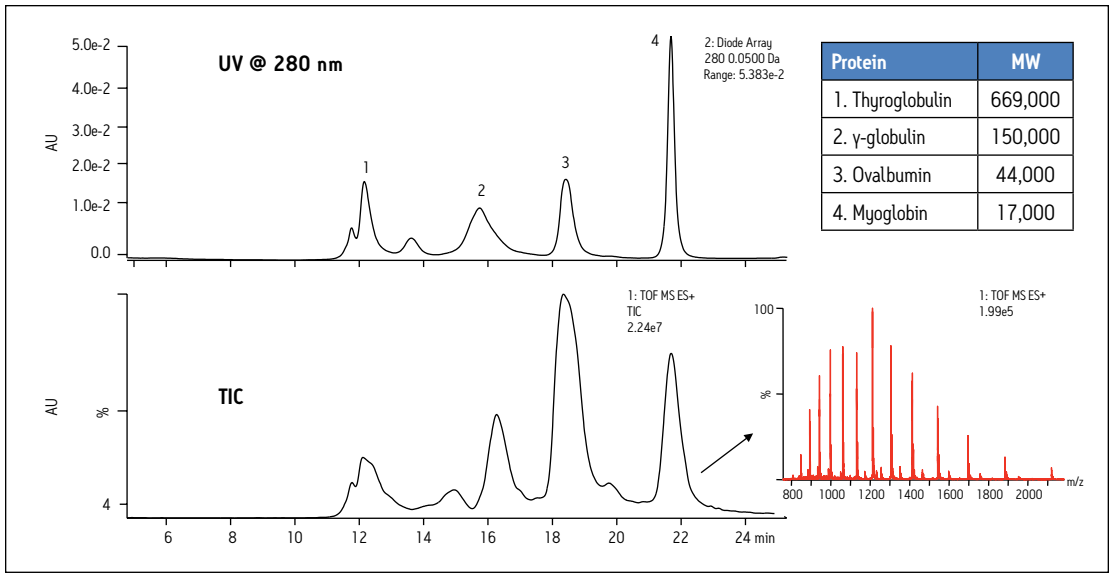


Figure 1. Influence of mobile-phase composition on the SEC separation of a protein standard.

As described above, ammonium formate was selected because of its volatility and MS compatibility. Since the use of non-denaturing mobile phases such as ammonium formate can reduce MS signal by a factor of 10 or greater,[8] a denaturing modifier (formic acid in acetonitrile) was added to the eluent post-column. The post-detector tubing and external pump were connected with a tee just prior to the MS inlet valve. Differences in resolution between the UV and TIC were minimal (Figure 2). As expected, there were significant differences in relative peak area ratios of the proteins in the TIC and UV chromatograms due to differential ionization efficiencies of the protein species. In these experiments the ESI-MS TIC was used solely for identification purposes, and the UV traces for quantification, where relevant.



Protein	MW
1. Thyroglobulin	669,000
2. γ-globulin	150,000
3. Ovalbumin	44,000
4. Myoglobin	17,000

Figure 2. SEC-UV-MS analysis of a protein standard.

Analysis of myoglobin aggregates

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column provides adequate resolution and MS sensitivity of the myoglobin size variants, including the monomer (peak 1), dimer (peak 2) and higher order aggregates (peak 3) (Figure 3). The ESI mass spectrum of the myoglobin monomer and dimer show multiple charged ion signals (Figure 4). The spectrum for the monomer reveals multiple-charge states from *m/z* approximately 800 to 2000 corresponding to charge states from $[M+8H]^{+8}$ to $[M+21H]^{+21}$. The deconvoluted spectrum of the monomer mass spectrum confirms the intact mass of myoglobin at 16,951. The MS signal for the dimer is a factor of 10 weaker than that of the monomer. The ESI mass spectrum of the dimer shows multiple charge states from $[M+20H]^{+20}$ to $[M+40H]^{+40}$. The deconvoluted spectrum shows the presence of both myoglobin monomer and the dimer (*m/z* 16,951 and 33,886).

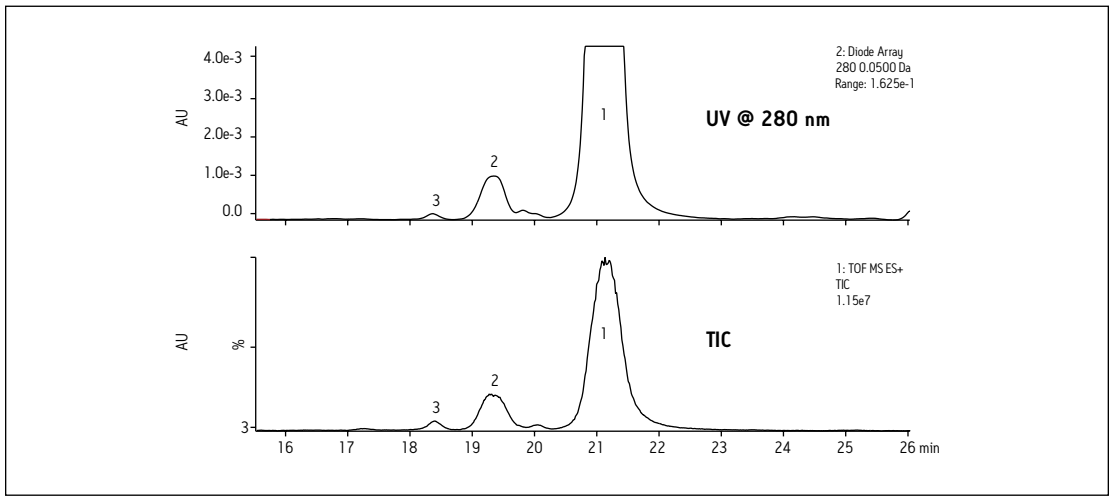


Figure 3. SEC-UV-MS anslysis of myoglobin monomer and aggregates.

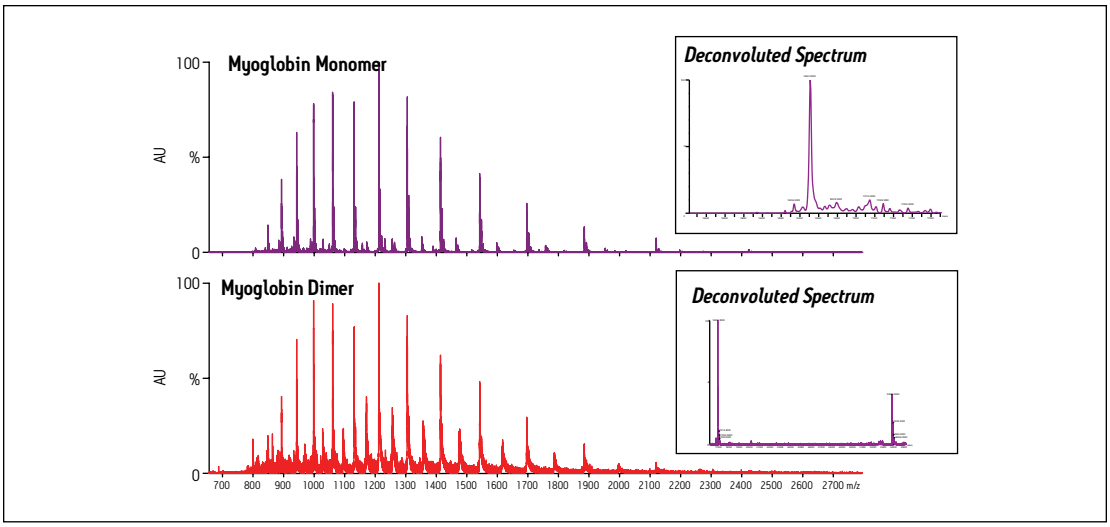


Figure 4. ESI mass spectrum and deconvoluted spectrum (inset) of myoglobin monomer and dimmer.

The simultaneous presence of monomer and dimer in the deconvoluted spectrum may be due to a variety of factors including dissociation of the non-covalent dimer in source, and/or presence of additional size variants. As described above, an acidic organic modifier is required post-column to provide adequate ionization of the proteins. These sample conditions can cause the proteins to denature, thus disrupting protein-protein interactions including non-covalent interactions.[2] An additional factor may be due to the presence of misfolded forms of myoglobin. While separation of the myoglobin monomer and dimer is achieved, a minor peak is present between the two peaks, possibly due to misfolded proteins or other size variants. These forms may be one factor for the appearance of the monomer mass in the deconvoluted spectrum of the dimer. Nevertheless, the presence of only myoglobin monomer and dimer indicates that the aggregation is primarily related to self-association of myoglobin.

Identification of unknown components in a biotherapeutic

An intact monoclonal antibody biotherapeutic, which was past expiry, was analyzed by SEC (Figure 5) using MS-friendly, non-denaturing conditions. In the UV chromatogram, not only are the mAb aggregate and monomer observed, but a low molecular weight (LMW) peak eluting after the intact mAb is partially resolved as well. In addition to these peaks, the UV chromatogram reveals two other LMW species.

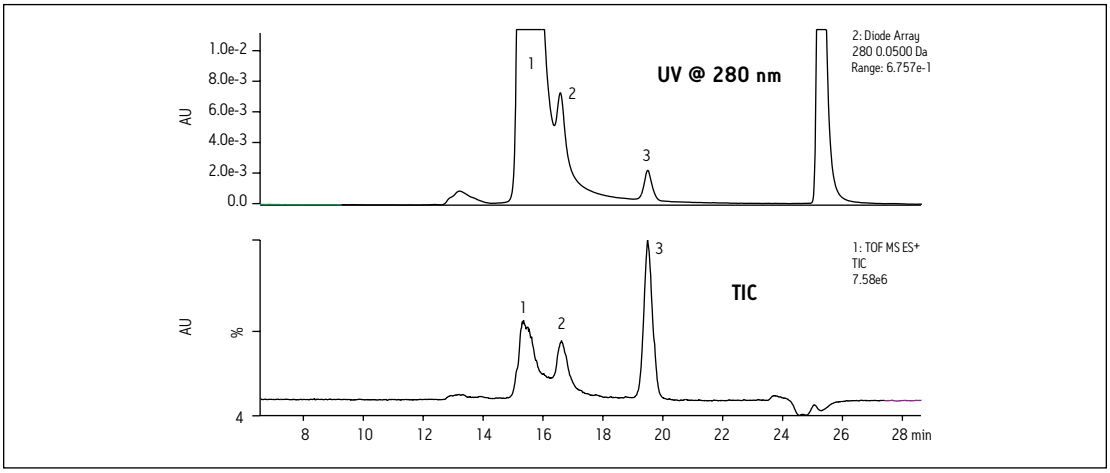


Figure 5. SEC-UV-MS of a recombinant humanized monoclonal antibody.

The ESI-mass spectrum of the monoclonal antibody (1) shows charge-states from $[M+34H]^{+34}$ to $[M+70H]^{+70}$ (Figure 6). The sensitivity of the method is illustrated by the high TIC satellite peaks of the $[M+39H]^{+39}$ and $[M+40H]^{+40}$ charge-states of the monomer . The deconvoluted spectrum of the monomer peak confirms the presence of the major glycosylated forms of the intact antibody with values corresponding to previously published results.[9] The exact masses can be assigned to G0F/G0F (148,058 *m/z*), G0F/G1F (148,219 *m/z*) and (G01F)2 or G0F/G2F (142,379 *m/z*).

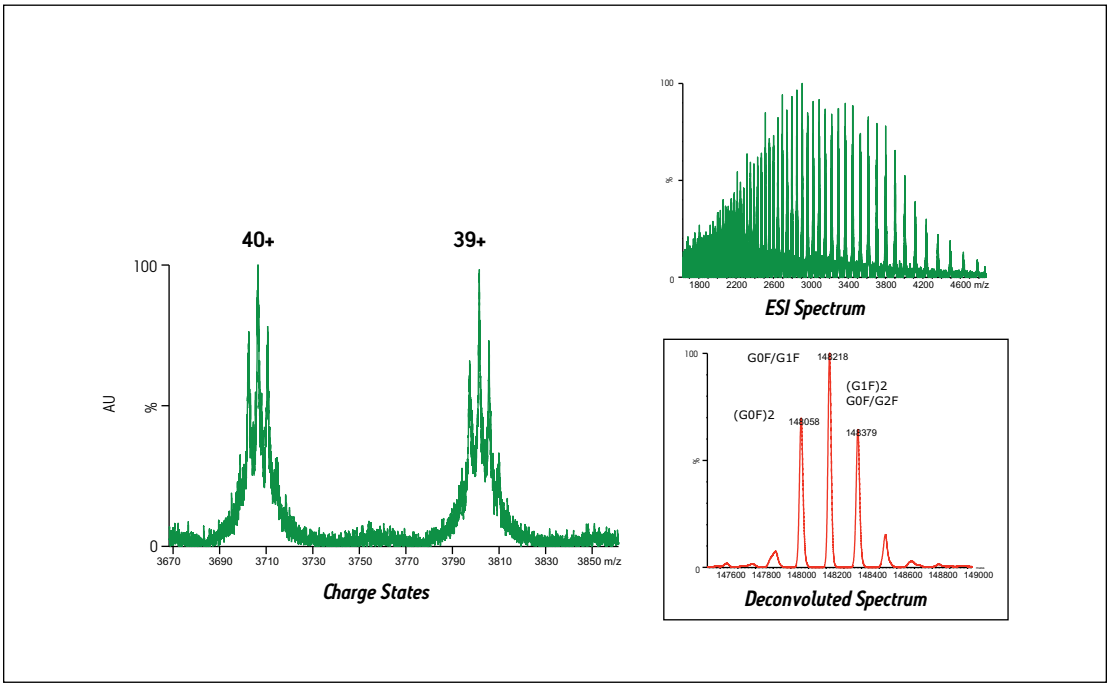


Figure 6. ESI mass spectrum of an intact monoclonal antibody. Deconvoluted spectrum (inset) shows intact mAb as well as gylcosylated forms.

The LMW peak (peak 3) eluting at 19 minutes also provides an adequate MS signal for molecular weight confirmation. Analysis of the ESI spectrum shows the presence of two different charge envelopes from 1100-2400 *m/z* (Figure 7). This is evident in the magnified view in which the satellite peaks for both sets of charge-states are resolved. The deconvoluted spectrum shows multiple peaks (Figure 8 inset), with 47,269 *m/z* (F1) and 47,636 *m/z* (F2) having the highest intensities. These intact masses correspond to the two prominent multiply charged ion states in the ESI mass spectrum: the charge states from $[M+19H]^{+19}$ to $[M+31H]^{+31}$ are shown in the zoomed spectrum. Based on the sequence of the protein, the main peaks in the deconvoluted spectrum can be assigned to Fab fragments resulting from hydrolytic cleavage of the heavy chain: the mass of F1 (47,270 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Asp^{224} while the mass of F2 (47,637 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Thr^{228}.

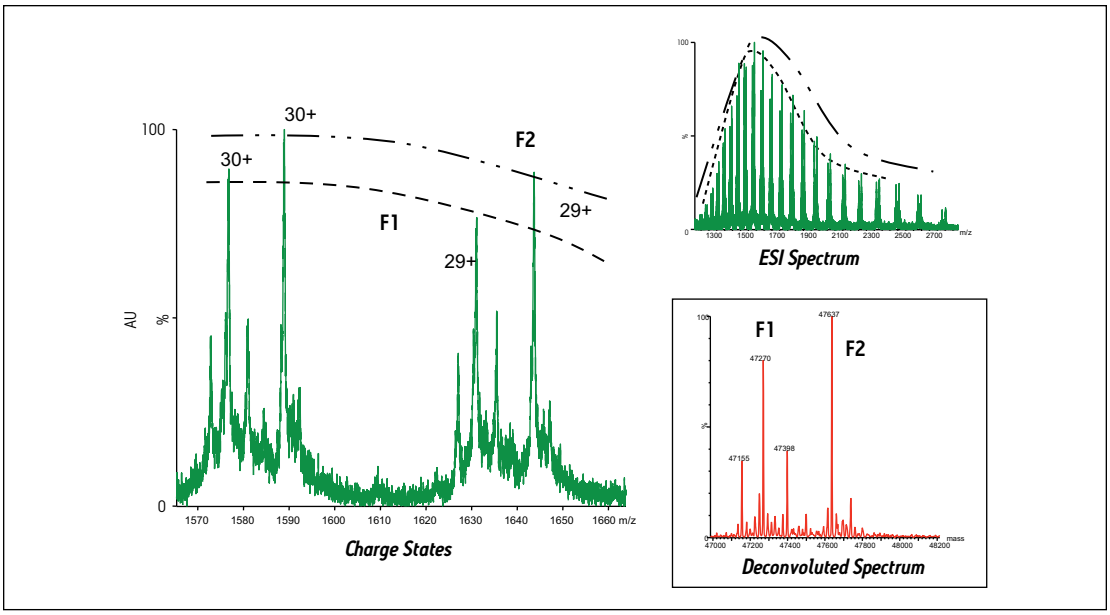


Figure 7. ESI mass spectrum of low molecular weight species (peak 3) in a recombinant humanized monoclonal antibody.

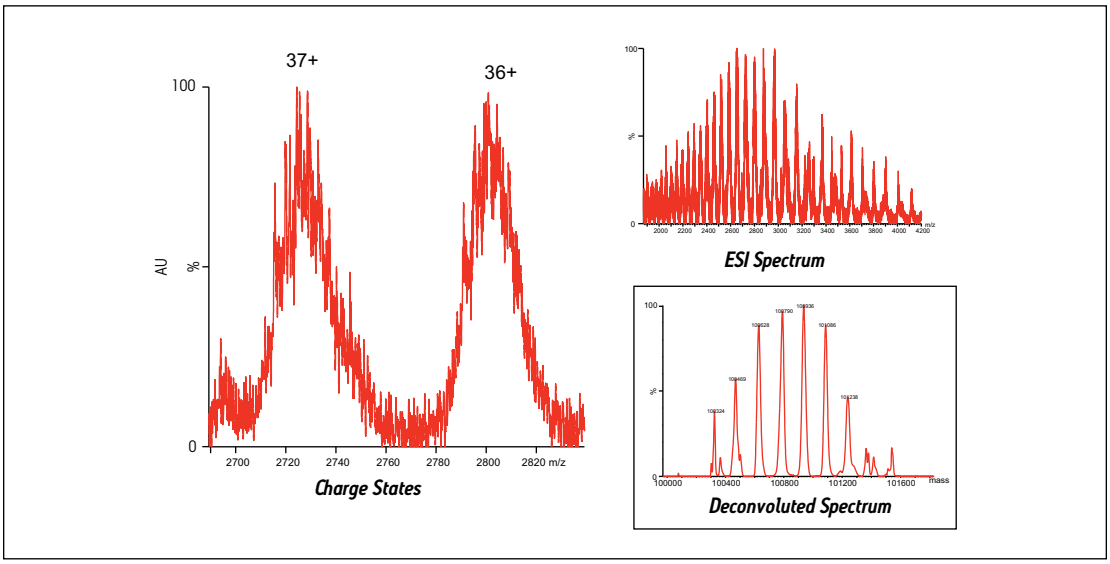


Figure 8. ESI mass spectrum of fragment (peak 2) in a recombinant humanized monoclonal antibody.

A similar analysis can be performed for the partially resolved low molecular weight species (peak 2 in Figure 5). The ESI mass spectrum of the fragment shows charge states from $[M+32H]^{+32}$ to $[M+48H]^{+48}$ at 2200-4000 *m/z*. While the satellite peaks are not well-resolved for the lower molecular weight species (Figure 7), the charge states are evident. The deconvoluted spectrum (Figure 8) shows molecular weights consistent with antibodies that have a missing Fab arm with fragments ranging from 100,468 to 101,237 *m/z* (Figure 8 inset). The species observed at 100,468 is consistent with an antibody without one of the Fab arms cleaved at the N-terminal side His^{229}. The confirmation of other, minor fragments that appear to be present is beyond the scope of this application note.

The SEC-MS analysis of the recombinant humanized monoclonal antibody allows for identification of not only the intact monoclonal antibody, but also the lower molecular weight fragments. Deconvolution of the ESI mass spectrum provides intact molecular weight information for the monomer and fragment species.

CONCLUSIONS

Size exclusion chromatography under non-denaturing conditions is a standard method for testing biomolecules and their aggregates. MALS and AUC are established detectors but cannot provide exact mass for unknown species with a sufficient accuracy. The presence of an unexpected peak requires further investigation and/or confirmation of molecular weight, and SE-UPLC-MS under aqueous, non-denaturing conditions can provide valuable information that would more rapidly solve an organization's issues with characterization or quality.

While SEC-MS does not typically preserve protein self association, it can assist in identification. The analysis of myoglobin illustrates the utility of an SEC-MS approach by confirming that the HMW forms observed in the myoglobin sample are related to the protein. The SEC-MS analysis of a humanized monoclonal antibody under non-denaturing conditions provides exact masses for LMW antibody fragments. By efficiently combining the ACQUITY UPLC BEH200 SEC, 1.7 µm Column and the benchtop Xevo G2 Q-Tof with an extended *m/z* range, the intact antibody and its associated fragments can be identified, providing a rapid method for exact molecular weight determination of intact biomolecules.

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Method Development for Size-Exclusion Chromatography of Monoclonal Antibodies and Higher Order Aggregates

Paula Hong and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Robust analysis of mAb monomer and aggregates
- High throughput SEC separation
- Consistent purity profile
- Reproducible quantitation of higher order aggregates
- Easy SEC method development

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class Bio System
- ACQUITY UPLC BEH200 SEC 1.7 µm Column
- Auto•Blend Plus™ Technology
- Empower® 2 Software

KEY WORDS

Size-exclusion chromatography, UPLC, monoclonal antibody, method development, aggregates

INTRODUCTION

Since the early introduction of biologic based therapeutics, the presence of protein aggregates can compromise safety and efficacy.[1] Given these factors, protein aggregates are typically monitored throughout the production of a biotherapeutic. While a variety of analytical techniques have been used to analyze soluble aggregates, the dominant technique continues to be size-exclusion chromatography (SEC).[2]

While SEC has been performed with silica-diol coated columns and HPLC instrumentation, the introduction of UPLC® or low dispersion systems in combination with sub-2-µm particles has allowed for improvements in these isocratic separations, including improved resolution, higher throughput and sensitivity.[3] However, as in any SEC method, a variety of parameters can be adjusted to improve resolution and method robustness. In the following application, we will investigate the impact of some of these parameters, including mobile-phase composition, flow rate and column length on a SEC separation. Evaluation of the separation will be based on a variety of criteria such as column calibration, resolution, and aggregate quantitation.

EXPERIMENTAL

Sample description

The protein standard (BioRad) containing bovine thyroglobulin (5 mg/mL), bovine γ –globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 mg/mL) and Vitamin B12 (0.5 mg/mL) in de-ionized water was analyzed. A murine monoclonal antibody, purified by Protein A affinity chromatography, was analyzed. The sample concentration was 10 mg/mL in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3.

Samples were not controlled for inter-experiment conditions.

LC conditions

System:	ACQUITY UPLC H-Class Bio System with TUV and Titanium flow cell
Wavelength:	214 and 280 nm
Column:	ACQUITY UPLC BEH200 SEC 1.7 µm, 4.6 x 150 mm, PN: 186005225
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	2 µL (unless otherwise specified)
Flow rate:	0.4 mL/min (unless otherwise specified)
Mobile phase:	Prepared using Auto•Blend Plus technology
Final composition:	25 mM sodium phosphate, pH 6.8, 200 mM sodium chloride, (unless otherwise specified)

Data management

Software:	Empower 2

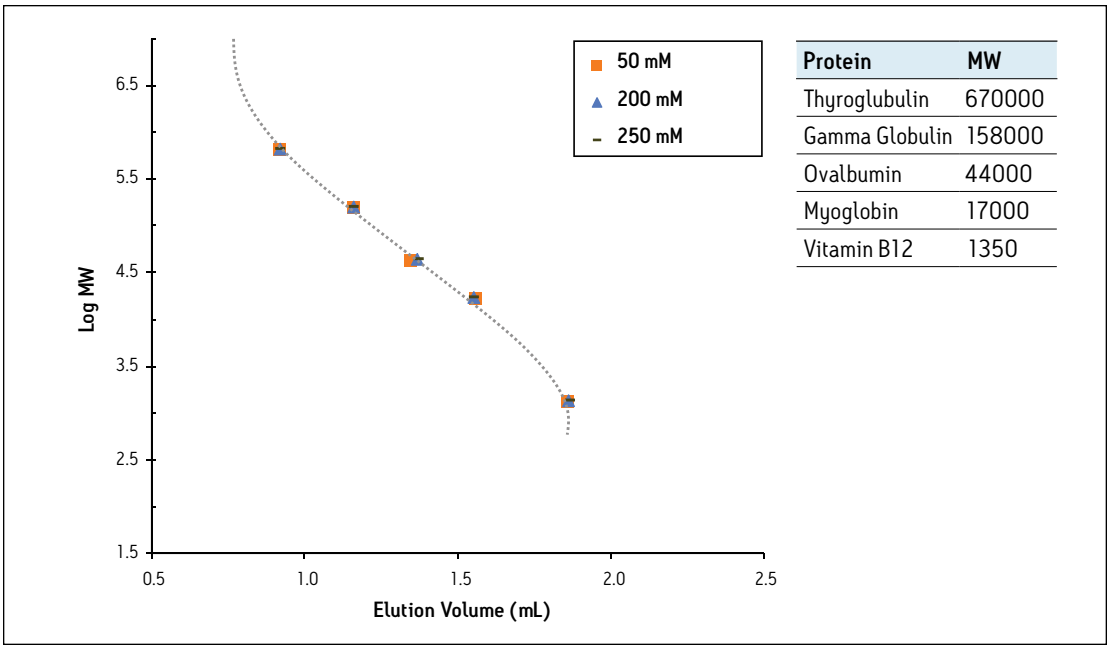
RESULTS AND DISCUSSION

A number of factors need to be evaluated in SEC method development. Ideally SEC separations are based on the size of the proteins in a solution. For this reason, size-exclusion chromatography of biomolecules is performed under aqueous, native conditions. However, the presence of mixed mode interactions can obscure size measurements.[4] More specifically, the charged sites on the packing material can interact with the proteins, resulting in an 'ion- exchange' effect. To determine the influence of these effects the mobile-phase conditions of the separation need to be evaluated. However, the conditions of the chromatographic separation can alter the protein structure and state. The concentration and identity of the salt and pH can affect the 3-D structure and the protein-protein interactions. For these reasons, evaluation of a SEC method must incorporate information of the biological activity of the biomolecule.

In the following discussion, we will outline considerations and parameters for developing a SEC method. While the SEC method development steps are illustrated on UP-SEC, the same principles apply to any HP-SEC separation. Methods will be evaluated based on peak shape, resolution, calibration accuracy, and quantitation. Optimization of the mobile-phase ionic strength and pH can easily be accomplished with a quaternary eluent management system in combination with software that can take advantage of this four eluent blending system.[5] This approach was used throughout the studies described.

Mobile-phase Ionic Strength

The ionic strength of the mobile-phase should be adjusted to minimize any secondary interactions between the packing material and proteins. To determine the effect of mobile-phase concentration on the calibration curve, a set of protein standards was analyzed at 50–250 mM sodium chloride. Sodium chloride was selected since it is the most common salt used in SEC separations. The buffer concentration (sodium phosphate) and pH were kept constant at 25 mM and pH 6.8, respectively. Over the concentration tested, the retention times for each protein were within 0.07 minutes with the greatest retention time variability observed for ovalbumin (Figure 1). These results indicate the calibration curves are not sensitive to salt concentration.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 1. Effect of sodium chloride on a SEC calibration curve.

Note: Calibration points deviate from a straight line because of protein shape in solution.

In addition to protein standards, the SEC separation of a murine monoclonal antibody (mAb) was evaluated at 50–250 mM sodium chloride (Figure 2). As is commonly observed with gel filtration packing materials,2 higher ionic-strength mobile phases lead to decreased peak tailing and narrower peaks for the mAb monomer. With increasing sodium chloride concentrations from 50–200 mM, the mAb peak height increases from 0.189–0.289. The USP tailing factor also decreases from 1.64 to 1.22. Changes are less pronounced as the ionic strength of the mobile phase is increased from 200 to 250 mM sodium chloride (USP Tailing = 1.20).

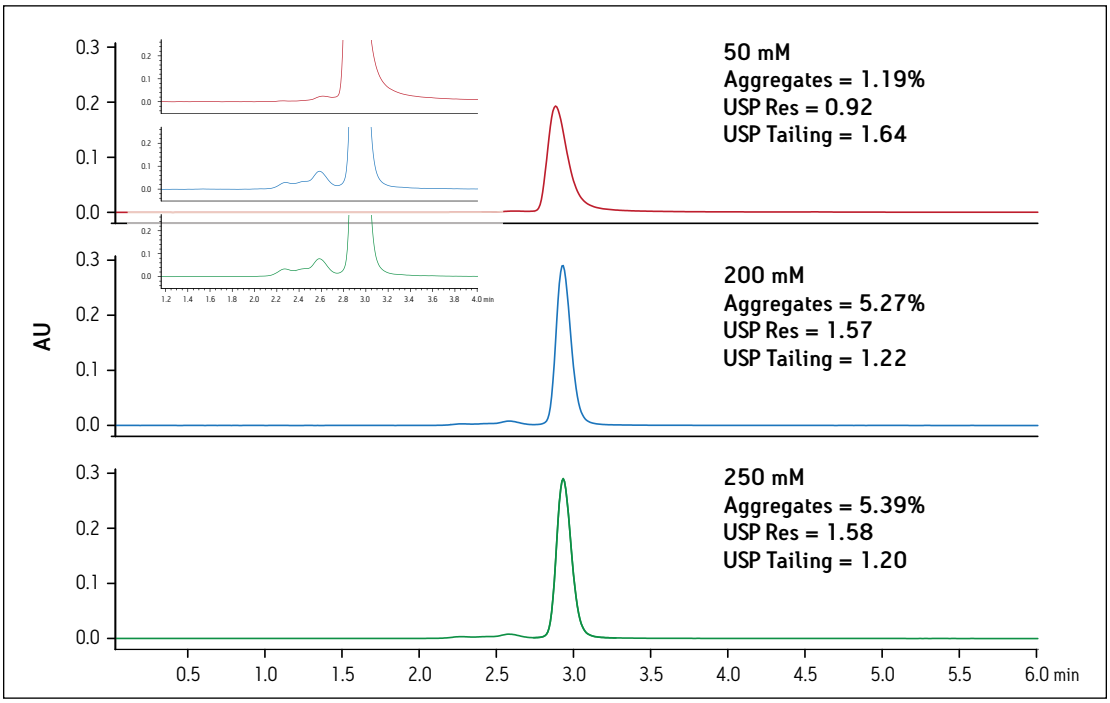


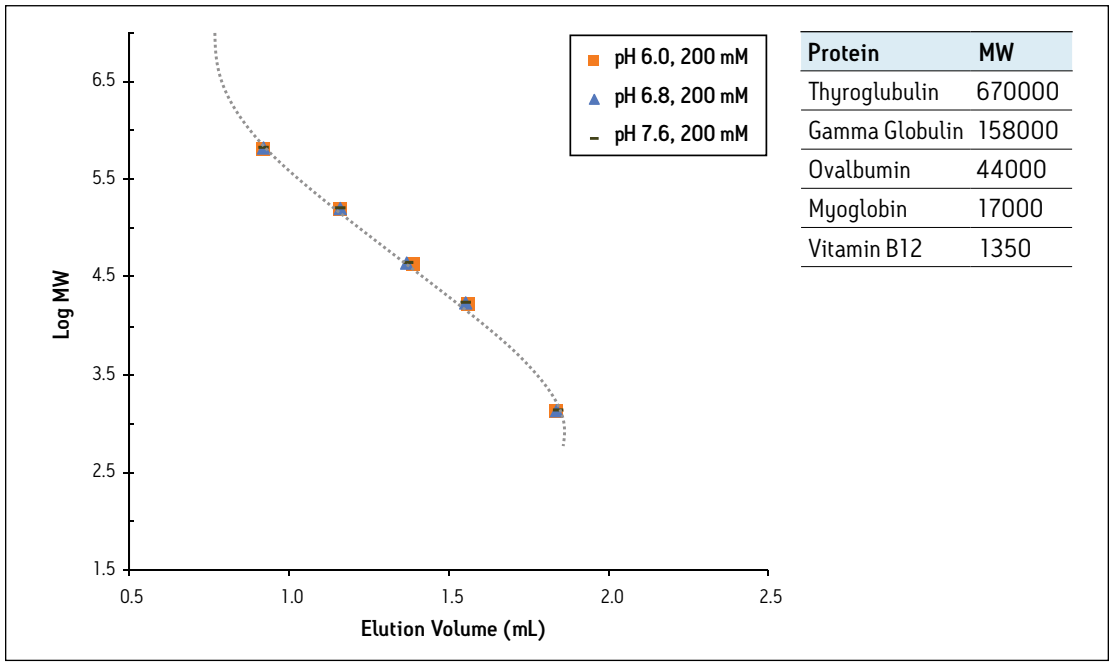
Figure 2. Effect of sodium chloride on the SEC separation of a murine mAb.

The effect of buffer ionic strength on the observed amount of aggregate was also analyzed. In the experiments previously described, increasing sodium chloride concentrations from 50–200 mM results in greater observed recovery of aggregates (see inset). The aggregate % area increased from 1.18% to 5.27%. However, at concentrations above 200 mM sodium chloride, aggregate quantitation did not change significantly. This suggests minimal secondary interactions above this concentration.

The variability in retention time and changes in peak shape indicate secondary interactions between the protein and the column packing material, as has been observed for the materials used to prepare SEC packings. These interactions, which can lead to increased retention and irregular peak shape, are easily minimized by increasing the ionic strength of the buffer.

Mobile-phase pH

Given the influence of pH on both secondary interactions and the structure of the protein, SEC method development should also evaluate pH and its influence, if any, on the separation and quantitation of the biomolecule. The BEH200 column was evaluated with the protein standard mix from pH 6.0–7.6. This analysis was performed to evaluate the effect of pH on the column calibration. The pH range was based on the buffering capacity range of the sodium phosphate buffer. The sodium chloride concentration was kept constant at 200 mM. The results show no significant shift in retention times were observed for the proteins. All of the retention times were within 0.02 minutes (Figure 3), suggesting pH has no significant affect on calibration under the conditions tested.



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 3. Effect of mobile-phase pH on a SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

To test the effect of pH on a typical biotherapeutic, the mAb was analyzed under the same conditions (pH 6.0 to 7.6, 200 mM sodium chloride) (Figure 4). As the pH increases from 6.0 to 7.6, the mAb monomer peak height decreases and shifts to earlier retention time (Figure 4). However, the aggregate quantitation over the pH range from pH 6.0–7.6 was within 0.4% (5.7–5.3%), indicating mobile phase pH has no effect on the measured proportion.

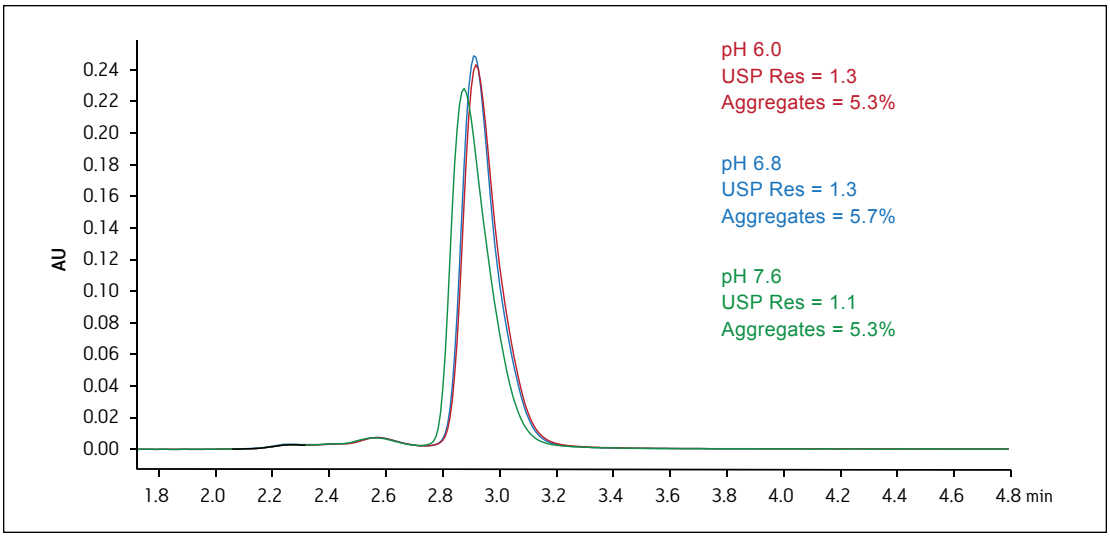


Figure 4. Effect of mobile-phase pH on a SEC separation of murine mAb. Mobile phase: 25 mM sodium phosphate, 200 mM sodium chloride pH 6.0–7.6.

The buffer pH can influence secondary interactions. In this case we observe changes for monomer elution profile but not for the dimer. This suggests a change in the hydrodynamic radius rather than a change in the secondary interactions.

Flow rate

Resolution in size based separations can be influenced by linear velocity. Although using lower flow rates results in longer run times, the increased resolution gives greater confidence in aggregate quantitation. In addition, the use of sub-2-µm particles for this application allows the use of shorter columns. Thus, the throughput achieved with UPLC-SEC is still greater than that of traditional HP-SEC.[3]

In order to test the reliability and robustness of the method, the effect of flow rate on the SEC separation of a mAb was analyzed. Triplicate injections of the mAb were analyzed at flow rates of 0.2 and 0.4 mL/min (Figure 5). Analysis of the separations shows no significant change in aggregate quantitation with flow rate. However, decreasing the flow rate did increase the monomer/dimer resolution by 15%. While the lower flow rates allow for increased resolution, higher flow rates allow for greater throughput and faster analyses times.

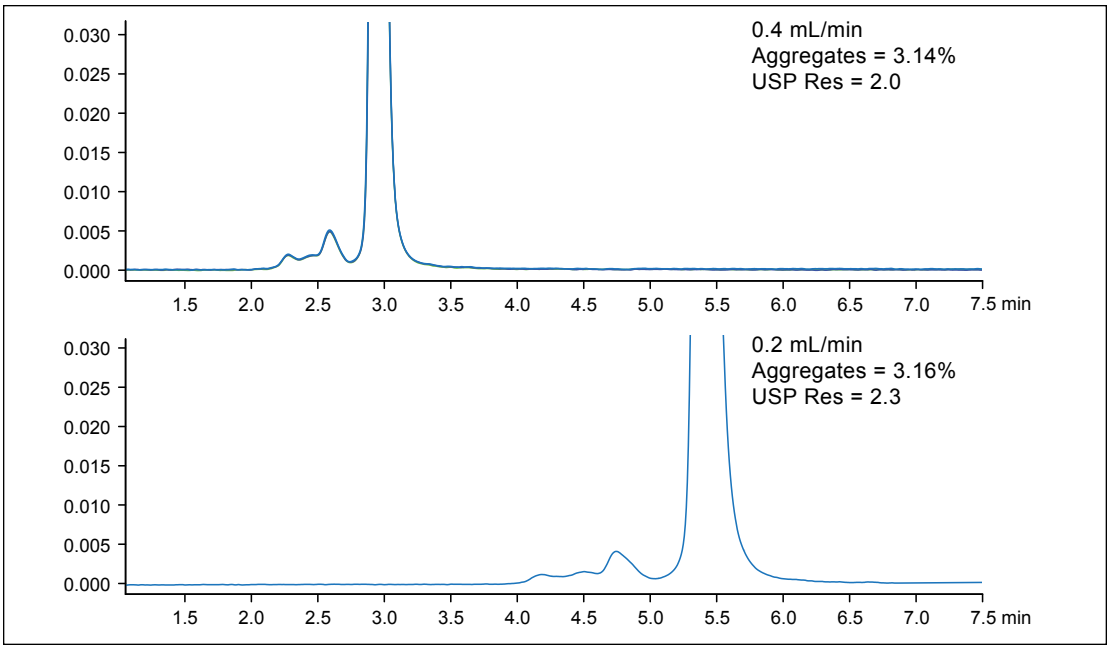
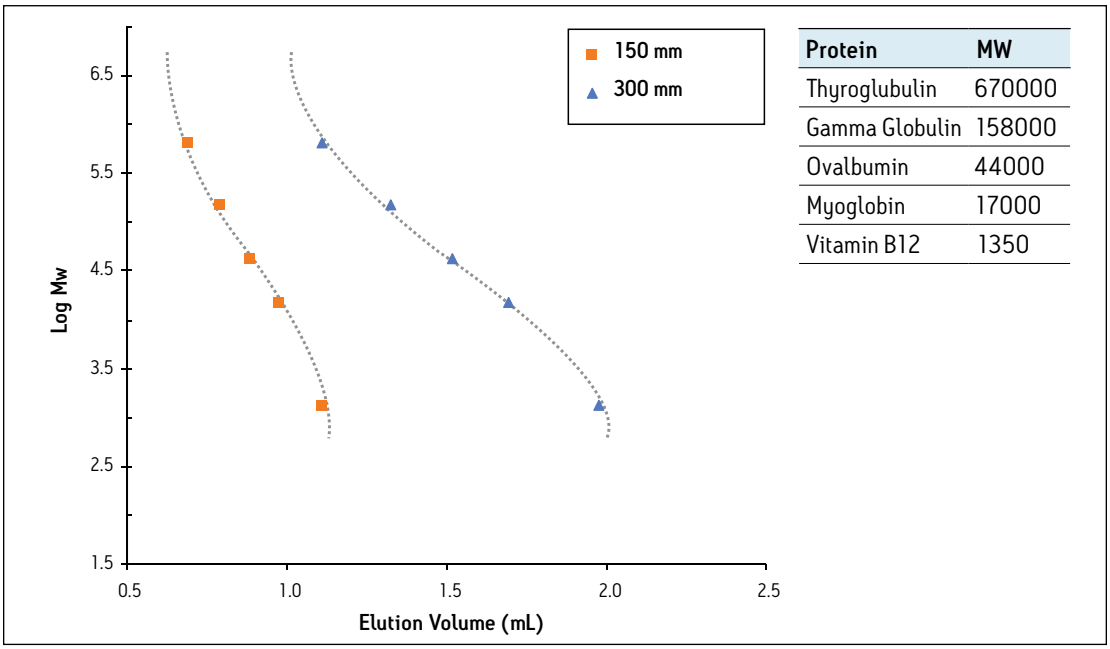


Figure 5. Effect of flow rate on a SEC separation of a murine mAb.

Column length

Improvements in SEC resolution can also be gained by increasing column length. SEC separations are based on diffusion into and out of the pores of the column's packing material. The larger proteins cannot access the pores and thus elute earlier. The smaller the protein, the longer the residence time within the pores, which results in longer retention times. These principles allow for greater resolution with longer column lengths.

To demonstrate these effects, a set of protein standards were run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Comparison of the calibration curves reveals a shallower slope for the 300 mm column as compared to the 150 mm, demonstrating the higher resolving power achievable on a longer column (Figure 6).



Protein	MW
Thyroglubulin	670000
Gamma Globulin	158000
Ovalbumin	44000
Myoglobin	17000
Vitamin B12	1350

Figure 6. Effect of column length on SEC calibration curve.
Note: Calibration points deviate from a straight line because of protein shape in solution.

The effect of column length was also tested for the SEC separation of a murine mAb run on both a 4.6 x 150 mm and 4.6 x 300 mm column. Under the same conditions, the longer column provided improved resolution for the monomer/dimer (2.07 to 2.80) (Figure 7) with comparable aggregate quantitation. The improved resolution is also apparent in the monomer peak tail, in which a small, lower molecular weight peak is partially resolved on the 300 mm but not on the 150 mm column. However, the improved resolution is accompanied by an increase in retention time (from 3.0 to 6.0 minutes).

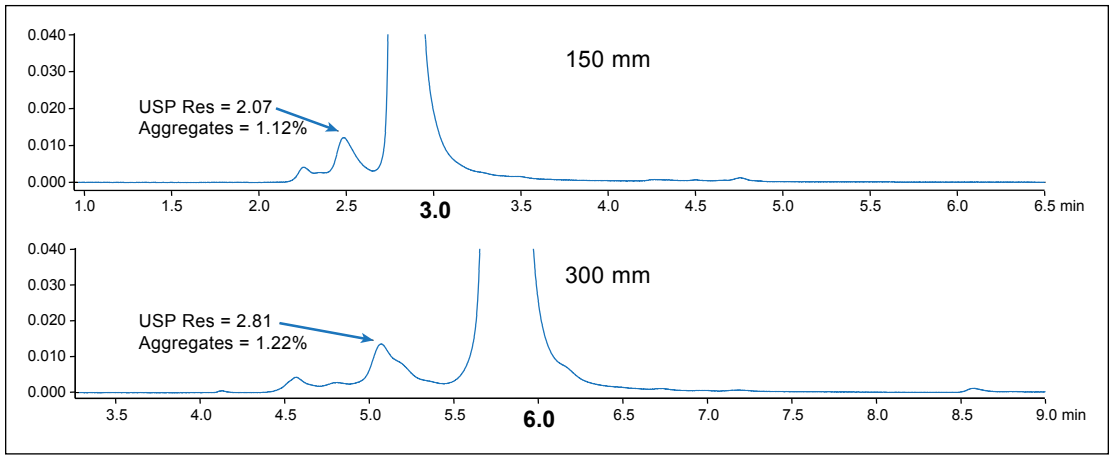


Figure 7. Effect of column length on a SEC separation of murine mAb.

These results indicate that column length can be a useful tool in method development. Depending on the method requirements, column length can be chosen to either provide improved resolution or higher throughput. For example, in a manufacturing environment a longer column allows for improved resolution. While in discovery or development, a shorter column allows for faster analysis time and high throughput.

CONCLUSIONS

Size-exclusion chromatography continues to be a standard technique for the analysis of monoclonal antibodies and their aggregates. However, as in any SEC method, a thorough evaluation needs to be performed to develop an optimum separation. While HP- SEC can be time consuming, the use of UP-SEC allows method optimization to be predicted in less time with a high level of efficiency and higher degree of confidence. In addition, the use of Auto•Blend Plus Technology makes it easier and less labor intensive to systematically examine the effects of mobile phase on protein structure and on secondary interactions.

As described, optimization should evaluate a number of conditions, including mobile phase (pH and ionic strength), flow rate, and column length. In addition – although not described in detail – injection volume, mass load and temperature can also affect SEC separations. Therefore, a suggested set of experiments should evaluate:

1. Ionic strength
2. pH
3. Column length
4. Flow rate
5. Other variables (mass load, injection volume, temperature, etc.)

These experiments should incorporate information on the biological activity of the protein. If factors affecting the proteins biological activity are limited, PBS is the recommended starting mobile phase.

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Guidelines for Routine Use and Maintenance of Ultra-Performance Size-Exclusion and Ion-Exchange Chromatography Systems

GOAL

To outline good practices for the routine maintenance and use of UltraPerformance LC® Systems in conjunction with high ionic strength, 100% aqueous mobile phases, typical eluents used in size-exclusion and ion-exchange chromatography.

BACKGROUND

Given the complexity of biotherapeutics, full characterization typically requires a variety of orthogonal methods. While many chromatographic techniques are conducted under reversed-phase conditions, others are conducted under native separation conditions, requiring high ionic strength, 100% aqueous eluents. For high performance liquid chromatography systems, these conditions can be problematic: in the absence of bactericides, lack of proper maintenance can lead to bacterial contamination within hours. The presence of high salt concentrations increases the potential of particulates in the mobile phases. The presence of bacteria and particulates in the LC system can affect chromatography quality and column lifetime.

The components of the chromatographic system are equally important. If the chromatographic system is not inert or bio-compatible, metal-protein adducts or undesired protein interactions can occur. Long-term use of high ionic strength, 100% aqueous mobile phases can also lead to rust formation if the chromatographic system contains steel components in the wetted path. However, with proper set-up and care of a chromatographic system, robust and reproducible chromatography can be achieved with minimal down time.[1,2]

Proper Set-Up and Maintenance of an ACQUITY UPLC System Allows for Robust and Reliable SEC and IEX Separations.

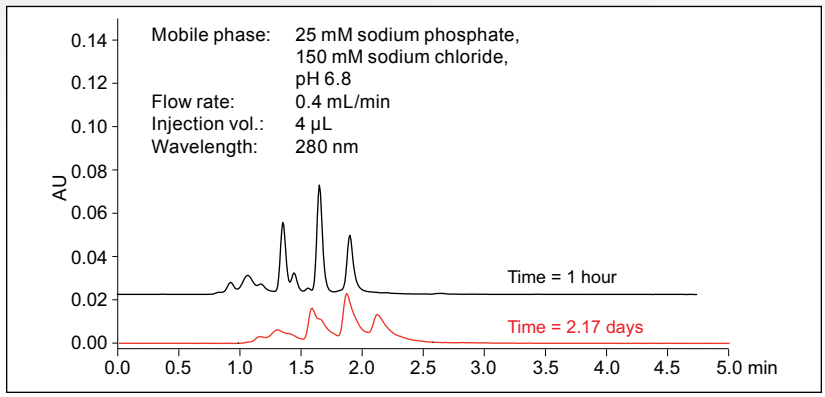

Figure 1. Effect of microbial growth on a SEC chromatogram of protein mix. Contamination confirmed by analytical analysis of column frit.

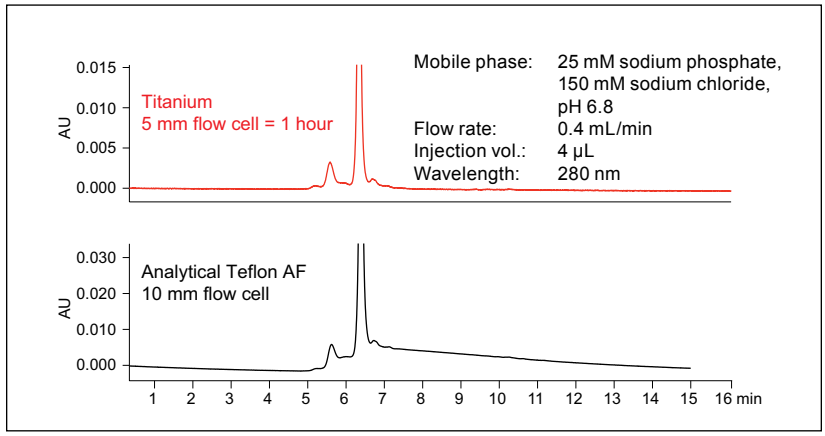

Figure 2. SEC-PDA chromatogram of bovine serum album (BSA) (5 mg/mL in water) shows the effect of flow cell material on peak shape. BSA monomer exhibits extensive peak tailing.

THE SOLUTION

The care and use of a size-exclusion and/or ion-exchange chromatographic system requires many of the same standard practices as any other system. However, there are some additional protocols that are required for high salt, aqueous mobile phases. While the practices outlined in this document are described for ACQUITY UPLC® Systems, the principles apply to any chromatographic system. Overall system recommendations include:

- If using a steel system, modify according to manufacturer's recommendations. For use with a Waters UPLC® System, detailed guidelines are available.
- Clean laboratory glassware properly.
- If chronic loss of prime, check valve problems, reproducibility of retention time, pressure or quantitative reproducibility are observed, clean the system following a standard protocol.[3]
- If possible, use mobile phases containing a bacteriostat (i.e., 0.02% sodium azide) to prevent microbial growth.
- Retention time or pressure fluctuation can be indicative of problems with the pump.
- Area or peak variability without retention time variation can be indicative of an injector problem.

Additional recommendations are listed below by component. These considerations are for microbial growth, system suitability and/or protein stability.

Solvent Delivery System

The buffers used in SEC and IEX can favor microbial growth leading to contamination of the column and system (Figure 1). Recommendations include:

- Always filter aqueous mobile phase through compatible 0.22 µm or smaller membrane filters. The use of sterile filters and containers is also recommended.
- Use only high purity water (18.2 MΩ cm). Bottled water should be opened the day of use.
- Never 'top-off' mobile-phase bottles. Always change bottles when replacing mobile phase.
- High-ionic-strength eluents (> 100 mM) should be replaced every two weeks.
- Low-ionic-strength eluents (< 100 mM) should be replaced every 2–3 days.
- Water (100%) bottles should be replaced daily.
- Higher salt concentrations, which inhibit microbial growth, would reduce the frequency of solvent replacement. While it is not always practical to change mobile phases, a quaternary solvent mixing system can accomplish the same effect by combining high concentration buffers (> 100 mM) to produce SEC and IEX mobile phases.[5]
- All eluent bottles should be visually inspected daily for microbial growth and/or particulates. Microbial growth can be a film on the bottle surface or may be observed by swirling the bottle.
- If microbial growth has occurred in the eluent bottle, replace the mobile-phase filter or flush it with a 70% isopropanol solution. Microbes can contaminate mobile-phase filters.
- Use compatible mobile-phase filters, such as titanium. Clean mobile-phase filters weekly to reduce microbial contamination. Sonicate or flush with 70% isopropanol solution.
- Recommended seal wash cycle time of 0.10 minutes (6 seconds).
- Seal wash recommendation of 90/10 water/methanol.
- The solvent manager should never be left idle in either high salt mobile phases or 100% water.
  - For short-term storage, maintain 0.1 mL/min of drawing an equal percentage of mobile phase from all lines in use.
  - If system will be idle for more than 2 days, prime each line for 10 minutes with high purity water. Thoroughly flush system. Repeat steps with 90/10 water/methanol.

Sample Manager

SEC and IEX conditions often require high-ionic-strength solutions in the sample manager wash lines (wash/purge, strong and weak needle). However, these eluents may have a detrimental affect on the sample syringe and/or needle. For variable flow through needle systems, remove salt deposit on a regular basis to minimize maintenance and repairs. Other recommendations include:

- Ensure the sample is soluble in the mobile-phase and sample manager washes.
- Follow the manufacturer's recommendations for wash solvents. For example, washes containing less than 500 mM salt(s) are recommended for the ACQUITY UPLC H-Class Bio System.
- If visible salt deposits appear, clean the surfaces. If salt deposits reappear, check connections and system for problems.[4]
- If the sample manager is idle for more than 2 days, purge needle and/or wash lines with high purity water (minimum of 20 cycles or 200 seconds). Repeat steps with 90/10 water/methanol. The sample manager should never be left idle for longer than 2 days in lines containing high salt washes (>100 mM).

UV Detectors

Waters recommends titanium or stainless steel optical flow cells when performing SEC or IEX under aqueous conditions. The standard ACQUITY® optical flow cell contains Teflon AF in the fluidic path. Some proteins, under native conditions, may interact with the flow cell surface, resulting in peak tailing and sloped baseline (Figure 2). Recommendations for detectors include:

- Use titanium or stainless steel flow cells to reduce protein-surface interactions. Other flow cell material (i.e., Teflon) may cause peak tailing.
- Never leave the detector idle in high salt eluents. Flush thoroughly with water (60 minutes at 0.2 mL/min) followed by 90/10 water/methanol or higher organic eluent.

Column Storage

To maintain long column lifetimes and minimize the risk of microbial contamination, the following recommendations should be followed:

- Columns should never be stored in high salt, aqueous mobile phase or 100% water.
- Before switching to recommended storage conditions, flush columns with 10-20 column volumes of water.
- Flush and store columns following the manufacturer's recommendations. Typical recommendations are 10-20% methanol or with a bactericide (i.e., 0.1% sodium azide).
- Consider the use of guard columns to extend column lifetimes. Regular replacement may be required. Frequency of guard column replacement may be dependent on sample cleanliness.
- Size-exclusion columns can typically be stored at 4-8 °C to reduce microbial growth. Ion-exchange columns are usually stored at room temperature. Check the manufacturer's recommendations for details.

SUMMARY

SEC and IEX chromatography are performed under native conditions, requiring high-ionic strength, 100% aqueous eluents. To minimize protein-surface interactions these conditions may require the use of a bio-compatible chromatographic system specifically designed for these applications. Precautions must be taken to prevent and minimize bacterial contamination. Signs of such contamination, which can occur within hours include: deteriorating peak shape, resolution and column lifetime. Unfortunately, once the column has been contaminated, regeneration is difficult. To decrease the frequency of system repairs and contamination, a series of steps have been outlined for maintenance and care of a chromatographic system and columns used for the analysis of biomolecules. These recommendations include maintenance for the solvent manager, sample manager, detector and column. Using these procedures in combination with good laboratory practices ensures a robust, reproducible system for ultra-performance-size-exclusion and ion-exchange chromatography.

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PEGylated Protein Analysis by Size-Exclusion and Reversed-Phase UPLC

Stephan Koza and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Two UPLC® alternatives, SE-UPLC and RP-UPLC, are presented for the analysis of PEG-Protein conjugate, non-PEGylated protein, and free active PEG levels in PEGylated protein preparations.
- SE-UPLC provides a rapid and high resolution separation of an unmodified protein from its PEGylated form. Successful application of SE-UPLC for this analysis can be predicted based on theoretical calculations of the hydrodynamic viscosity radii of the analytes.
- Separation of PEG-Protein conjugate, non-PEGylated protein, and free aPEG based on their differences in their hydrophobicities is provided by RP-UPLC for this application.

WATERS SOLUTIONS

ACQUITY UPLC® Protein Separation Technology (PrST) Columns

ACQUITY UPLC H-Class Bio System with ACQUITY UPLC TUV and ACQUITY UPLC ELSD

KEY WORDS

Size-exclusion chromatography, SEC, monoclonal, proteins, SE-UPLC, Gel filtration chromatography, polyethylene glycol, PEG, PEGylated protein, reversed-phase chromatography, RP-UPLC

INTRODUCTION

The first PEGylated biotherapeutic, pegademase, which is a bioconjugate of the bovine derived enzyme adenosine deaminase and 5 KDa molecular weight (MW) polyethylene glycol (PEG), was introduced in 1990. Pegademase is used for the treatment of individuals with severe combined immunodeficiency disease (SCID). As of 2012, there were ten approved PEGylated bioconjugates on the market and other candidates in clinical studies.[1] Among other benefits, PEGylation can improve the pharmacokinetics and stability of a biotherapeutic. Interestingly, however, it has been reported that approximately 25% of the normal healthy population has a titer of antibodies against PEG which may be a result of the prevalent use of these compounds in personal care products. The development of anti-PEG antibodies has also been observed in the clinic for PEG conjugates.[2, 3] Since both the efficacy and potentially the safety of PEGylated bioconjugates can depend on the extent of their PEGylation it is a critical quality attribute that should be monitored.

PEGylated proteins can be separated by a number of different methods including ion-exchange (IEC), size-exclusion (SEC), and reversed-phase (RPC) chromatography.[4] For this application, the separation of three species, a 50 KDa molecular weight protein, a 40 KDa activated-PEG (aPEG) and the conjugate, were evaluated using UPLC configurations of both SEC (SE-UPLC) and RPC (RP-UPLC), as these methods can be readily developed to be compatible with an evaporative light scattering detector (ELSD). While the use of SEC for this type of analysis has been reported,[5] the extent of success for the SEC mode of separation for this application type will ultimately be dependent upon the hydrodynamic viscosity radii of the three components as well as their polydispersity. Alternatively, the success of a RPC separation for this application is dependent on the differences in the hydrophobicities of the three components.

EXPERIMENTAL

Sample description

All samples were provided by a collaborator and stated concentrations are nominal values.

Method conditions

(unless noted otherwise):

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System with 30 cm Column Heater
Detection:	ACQUITY UPLC TUV Detector with 5mm Titanium flow cell Settings: 280 nm, 1 Hz sampling rate ACQUITY® ELSD Detector
Settings:	Gain = 500, Data Rate = 20 pps, Time Cont. = Fast, Gas Pressure = 40.0 psi, Nebulizer Heating at 10% Power Level, Drift Tube Temp. 50 °C
Columns:	Waters ACQUITY UPLC PrST SEC Column, 450Å, 2.5 µm, 4.6 x 150 mm (p/n: 176002996) Waters ACQUITY UPLC PrST SEC Column, 200Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225) Waters ACQUITY UPLC PrST C_4 Column, 300Å, 1.7 µm, 2.1 x 50 mm (p/n: 186004495)
Column temp.:	SEC=40 °C; RPC=90 °C
Sample temp.:	10 °C
Injection volume:	SEC = 10 µL; C_4 = 5 µL (unless otherwise specified)
Flow rate:	SEC = 0.4 mL/min, C_4 = 0.5 mL/min
Mobile phases:	SEC = 200 mM ammonium formate, 5% ACN; C_4 = Water (A)/ACN(B), 0.1% (v/v) TFA
Gradient:	SEC=Isocratic C_4=Gradient

Time	%A	%B
Initial	95	5
1	95	5
16	5	95
17	5	95
20	95	5
25	95	5

Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/ Silicone Septa, 1 mL (p/n: 186000385DV)

Data Management

Chromatography software:	Waters Empower® Pro (v2, FR 5)

RESULTS AND DISCUSSION

SE-UPLC

The use of both BEH200 (200Å pore-size) and BEH450 (450Å pore-size) SE-UPLC columns (150 mm lengths) in series was selected for this evaluation due to the extended MW weight range that this combination of columns can provide.[6] Proprietary samples were obtained from a collaborator and consisted of a 50 KDa molecular weight protein, a 40 KDa aPEG and the PEG-Protein conjugate. A volatile mobile phase comprised of 200 mM ammonium formate and 5% (v/v) acetonitrile was selected for these analyses. This buffer composition provided optimal separation of the active-PEG and conjugate critical pair and this volatile buffer could also be used if an evaporative light scattering detector (ELSD), which would provide improved sensitivity for the aPEG component in contrast to UV absorbance, was to be used. The 40 KDa aPEG used in this study has a broad and weak UV absorbance with a maximum at approximately 300 nm; therefore, for this study the UV absorbance at 280 nm provided adequate sensitivity for the high aPEG sample loads that were evaluated. The full-scale chromatograms of the conjugate, the 50 KDa protein, and the 40 KDa activated PEG are shown in Figure 1. Additionally, shown in Figure 2 is an overlay of the chromatograms of the aPEG and conjugate. Based on the chromatograms of these three samples, the SEC method provides useful resolution between the conjugated and the unconjugated protein, however, the separation between the conjugate and the aPEG is clearly not adequate for quantitation of a low level aPEG species in the presence of the predominant conjugate species.

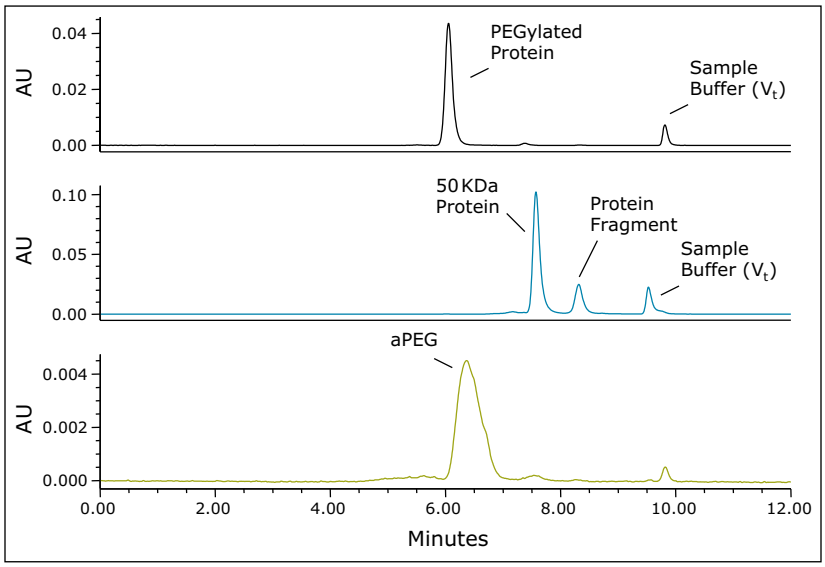

Figure 1. SE-UPLC UV traces (280 nm) for the 50 KDa PEGylated protein (black), the 50 KDa protein (blue), and the activated 40 KDa PEG (green).

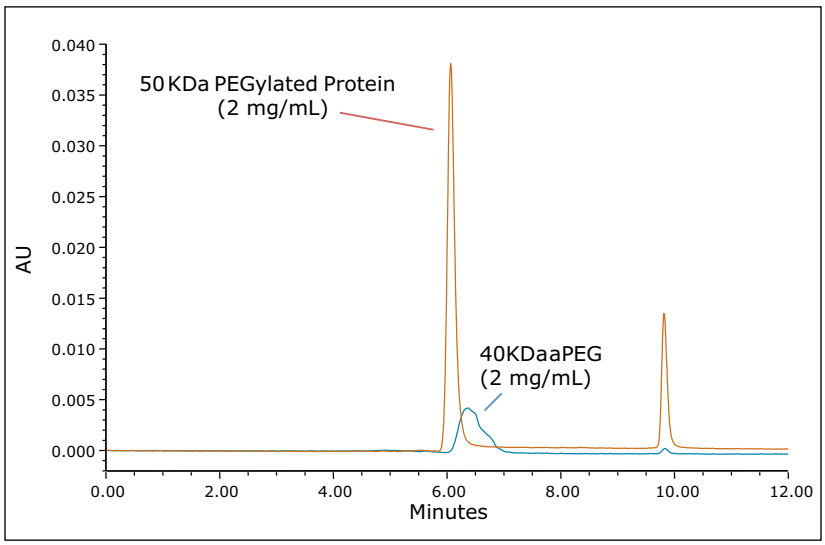

Figure 2. Overlaid SE-UPLC UV traces (280 nm) for the 50 KDa PEGylated protein (brown) and the activated PEG (blue).

These results demonstrate that achieving an SEC-based separation for the quantitation of PEGylated protein and the free aPEG forms may not be achievable in some cases. This can be due to a number of factors including the polydispersity of the aPEG, which will broaden its elution profile as well as that of the PEG-protein conjugate. Additionally, the nature of the interaction between the bound PEG and the surface of the protein may greatly limit the utility of a size-based separation. Ultimately, the critical factors that dictate the success of an SEC separation are the hydrodynamic viscosity radii (R_h) distributions of the aPEG, protein, and the conjugate. These R_h values can be empirically approximated using the relationships proposed in the work of Fee and Van Alstine.[7,8] Based on these relationships the R_h of PEG is typically much greater than that of a protein at a given molecular weight. Typically the ratio of the R_h values for two components should be approximately 1.26 or greater, or the inverse which is 0.79 or smaller, in order to resolve those components by SEC.[8] For globular proteins, this corresponds to a 2-fold increase in MW ($R_h \propto MW^{1/3}$). Using these theoretical relationships, it is it is clear to see that to develop a size-based separation that can resolve the non-PEGylated protein, the aPEG, and the conjugate from a mixture will be challenging. Shown in Table 1 are the predicted R_h ratios for various combinations of MW for these three components. Based on these predicted values covering a broad range of protein and PEG MW, there are only three combinations of components that would be predicted to have all three components resolve by SEC. The prediction for the 50 KDa protein and 40 KDa aPEG used in this study confirms what was observed experimentally where adequate resolution was achieved between the protein and the both the aPEG and the conjugate. However, the R_h ratio between the conjugate and the aPEG is well below 1.26 (1.07), which is in agreement with the insufficient resolution observed between those two species.

Protein MW (Da)	PEG MW (Da)	$R_{h,PEG}/R_{h,pro}$	$R_{h,pro+PEG}/R_{h,pro}$	$R_{h,pro+PEG}/R_{h,PEG}$
25000	5000	0.93	1.42	1.53
50000	5000	0.74	1.32	1.78
100000	5000	0.59	1.24	2.11
150000	5000	0.51	1.20	2.35
25000	10000	1.37	1.71	1.25
50000	10000	1.09	1.52	1.40
100000	10000	0.86	1.38	1.60
150000	10000	0.75	1.32	1.75
25000	20000	2.02	2.22	1.10
50000	20000	1.61	1.89	1.18
100000	20000	1.27	1.64	1.29
150000	20000	1.11	1.54	1.38
25000	40000	2.98	3.08	1.03
50000	40000	2.37	2.52	1.07
100000	40000	1.88	2.10	1.12
150000	40000	1.64	1.91	1.17

Table 1. Predicted ratios of the hydrodynamic viscosity radii for several PEG ($R_{h,PEG}$), proteins ($R_{h,pro}$), and their conjugate forms ($R_{h,pro+PEG}$). Ratio values of 1.26 or greater and 0.79 or less (green) indicate that adequate analytical separation between those species by SEC is predicted. R_h values between 0.79 and 1.26 (blue) predict that analytical resolution of the two compounds is not expected. The MW values of the protein and aPEG are highlighted in green for combinations for which resolution of all three components is predicted.

It should be noted that the predicted R_h ratios contained in Table 1 are approximations and that the possibility of successfully separating different species lessens as their R_h ratio approaches a value of 1.0. However, successful SEC separations could be obtained for species with borderline R_h ratios and such analyses may warrant experimental investigation. It is also worth noting that in cases where resolution of only two of the three components is required, such as in applications designed to quantitate the levels of the non-PEGylated protein and PEGylated protein a useful SEC separation is predicted (Column $R_{h,pro+PEG}$ / $R_{h,pro}$ in Table 1) for all but the largest proteins with the lowest MW 5 KDa PEGylation.

RP-UPLC

As an alternative to SEC, and with the understanding that PEGylation may likely have a profound effect on protein hydrophobicity, RP-UPLC using a C_4-bonded stationary phase was evaluated for the separation of the non-PEGylated protein, aPEG, and conjugate mixture. For this analysis, an ELSD was used in series after the UV detector to aid in the characterization of the observed peaks and to provide greater sensitivity for the unreacted PEG. A column temperature of 90 °C was selected for this separation to maximize sample recovery and peak shape quality. An overlay of the chromatograms obtained for the three components is presented in Figure 3. Under these conditions, the selectivity and peak widths obtained resulted in excellent resolution between the three analytes. Shown in Figure 4 is an overlay of the ELSD and TUV (A280) traces for the conjugated sample. In the ELSD trace (black), a low level (nominally 5%) aPEG peak is observed as well a low level unmodified protein peak (nominally 3.4%). The values determined by ELSD are relative as the response is not linear and is dependent on the nature of the analyte and the mobile phase composition. As a result, the level of unmodified protein based on the measured A280 peak areas is significantly higher (13.1%). However, the low level of free aPEG in the sample was below the limit of detection by UV absorbance. Consequently, the use of both detectors in series is essential in order to effectively monitor the levels of all three components in a single analysis.

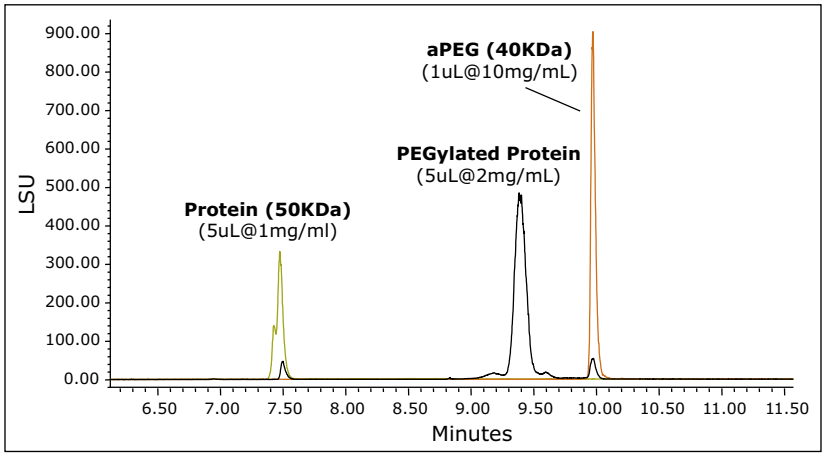

Figure 3. Overlay of the C_4 RP-UPLC ELSD traces for the 50 KDa PEGylated protein (black), the 50 KDa protein (green), and the activated 40KDa PEG (brown).

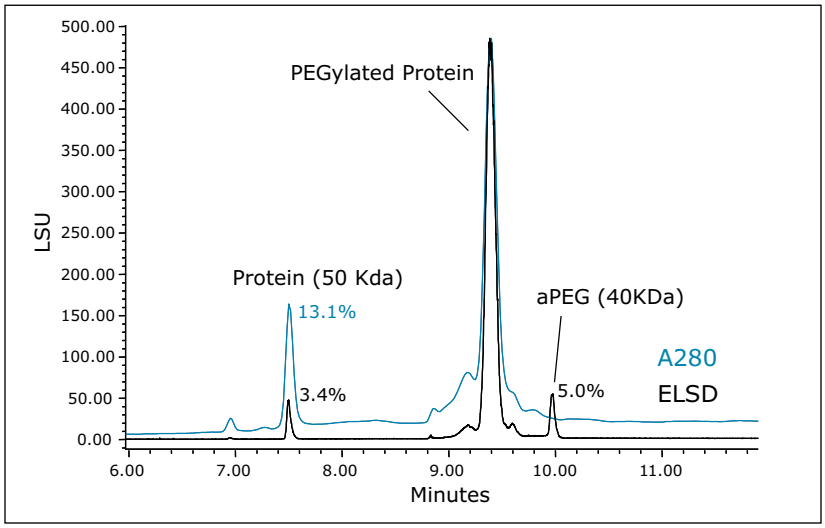

Figure 4. Normalized overlay of the ELSD (black) and 280 nm UV absorbance (blue) chromatograms for the 50 KDa PEGylated protein separated using a RP-UPLC BEH300 C_4 column.

CONCLUSIONS

SE-UPLC can provide rapid analysis of the products and unreacted components of a protein PEGylation reaction if the R_h values for the non-PEGylated protein, aPEG, and conjugate are sufficiently different. Based on predictions of the R_h values for combinations of protein and PEG molecular weights, in many circumstances SE-UPLC cannot provide the necessary analytical separation of all three components. This was indeed the case for this application where the model correctly predicted that the 40 KDa PEG and PEGylated 50 KDa protein R_h values were not significantly different to enable their separation by SE-UPLC. However, in many instances, SE-UPLC can be used to separate the modified and unmodified protein components of the sample, particularly for samples where large MW PEG (20 and 40 KDa) are being used.

By comparison, for this specific application it was found that all the three components were well separated based on differences in their hydrophobicities using a 300Å BEH column at high temperature (90 °C). Additionally, the use both a UV and an ELSD detector in series may be used to for their quantitation.

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Size-Exclusion Ultra Performance Liquid Chromatography Method Development for the Analysis of the Degradation Products of the Trastuzumab Antibody

Stephan Koza, Paula Hong, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution and sensitivity of antibody fragments and HMW forms
- High throughput SEC separation
- Robust method development to facilitate a successful SE-UPLC method transfer

WATERS SOLUTIONS

BEH200 SEC 1.7 µm Column

ACQUITY UPLC® H-Class Bio System

Auto•Blend Plus™ Technology

Empower® 2 Software

KEY WORDS

Size-exclusion chromatography, UPLC, monoclonal antibody, trastuzumab, method development, protein degradation

INTRODUCTION

Size-exclusion chromatography (SEC) has been the predominant technique for the analysis of biotherapeutic protein aggregation, or high molecular weight forms (HMW), an important degradation pathway that has been found to correlate with undesired immunogenic effects and/or decreased efficacy.[1,2] An additional degradation pathway for monoclonal antibodies (mAb) that can be observed by SEC is non-enzymatic peptide bond hydrolysis in the hinge region of these proteins resulting in antibodies that are missing one or both FAb arms.[3] Identification of these low molecular weight degradants (LMW) are provided in a previous Waters Application Note 720004254EN: "Analysis of Proteins by Size-Exclusion Chromatography Coupled with Mass Spectrometry Under Non-Denaturing Conditions".

The introduction of Ultra Performance Liquid Chromatography (UPLC®) or low dispersion systems in combination with sub-2-µm particles has allowed for improvements in these isocratic separations, including higher throughput, improved resolution, and enhanced sensitivity. The advantages of using size-exclusion UPLC (SE-UPLC) for monitoring the extent of mAb hinge region fragmentation in addition to aggregation using a non-denaturing mobile phase have been evaluated for trastuzumab, an anti-HER2 IgG_1 mAb. As in any SEC method, a variety of parameters can be adjusted to improve resolution and method robustness. In the following application, we will present the impact that mobile phase composition (pH and ionic strength) can have on this separation. Additionally, we will discuss some of the primary considerations necessary for the successful transfer of a developed SE-UPLC method.

EXPERIMENTAL

Sample description:	The IgG_1 mAb sample was trastuzumab that was analyzed past expiry (21 mg/mL).

LC conditions

System:	ACQUITY UPLC H-Class Bio System with TUV and Titanium Flow Cell
Wavelength:	280 nm
Column:	ACQUITY BEH200 SEC 1.7 µm, 4.6 x 300 mm (p/n 186005226)
Column temp.:	25 °C (without Active Pre-Heater Assembly)
Sample temp.:	4 °C
Injection volume:	5 µL (unless specified otherwise)
Flow rate:	0.4 mL/min
Mobile phase:	Prepared using Auto•Blend Plus Technology four solutions: A: 100 mM sodium phosphate monobasic B: 100 mM sodium phosphate dibasic C: 1.0 M sodium chloride D: water
Final target Composition:	25 mM sodium phosphate, pH 6.8, 200 mM sodium Chloride, (unless specified otherwise)

Data management

Software:	Empower 2 with Auto•Blend Plus

RESULTS AND DISCUSSION

In the following discussion, we will outline some of the parameters that should be evaluated when developing an SE-UPLC method. While these SEC method development steps are demonstrated on UPLC SEC, the same principles apply to any SEC separation. Method performance will be evaluated based on peak shape, resolution, and quantitative reliability.

Mobile phase optimization (Ionic strength and pH)

Following the selection of a column with appropriate characteristics such as pore size, particle composition and bonding, particle size, and column geometry, the next step in developing a SEC method typically involves evaluation of the mobile phase to ensure good peak shape and component resolution. Modifying the ionic strength and pH of the mobile phase can be easily accomplished with a quaternary solvent management system in combination with software that can take advantage of this four-solvent blending system.[4] This approach was used throughout the studies described.

The SE-UPLC separation of trastuzumab was evaluated at NaCl concentrations between 150 and 350 mM and pH levels between 6.0 and 7.5 (Figure 1). In this design space and based on a visual assessment the chromatographic profile appears to be optimal and stable at NaCl levels of 250 mM through and 350 mM, and at a pH of 6.0 to 6.5 (25 mM phosphate buffer). As is commonly observed with gel filtration packing materials, lower ionic strength mobile phases lead to increased peak tailing for the monomeric mAb[4]. This observed peak tailing effect was also less pronounced at pH values 6.0 and 6.5 (Figure 2), which in this case closely matches the pH of the trastuzumab formulation (pH 6.0). Pragmatically, the results of interest for this analysis are the integrated percent peak areas for the product-related impurities, the HMW and LMW forms. Within the studied mobile phase compositions, in which the HMW and the later eluting LMW2 (FAb arm) peaks are observed, the integrated percentage of these impurities are not significantly influenced by the peak shape (tailing factor) of the intact mAb monomer. However, increases in peak tailing of the monomer can result in significant increases in the integrated percent area of the earlier eluting LMW1 impurity (mAb minus one FAb arm) peak (Figure 3). Based on these data, an appropriate mobile phase that should result in reproducible chromatographic profiles would be at pH 6.25 (25 mM phosphate buffer) with 300 mM NaCl (Figure 4).

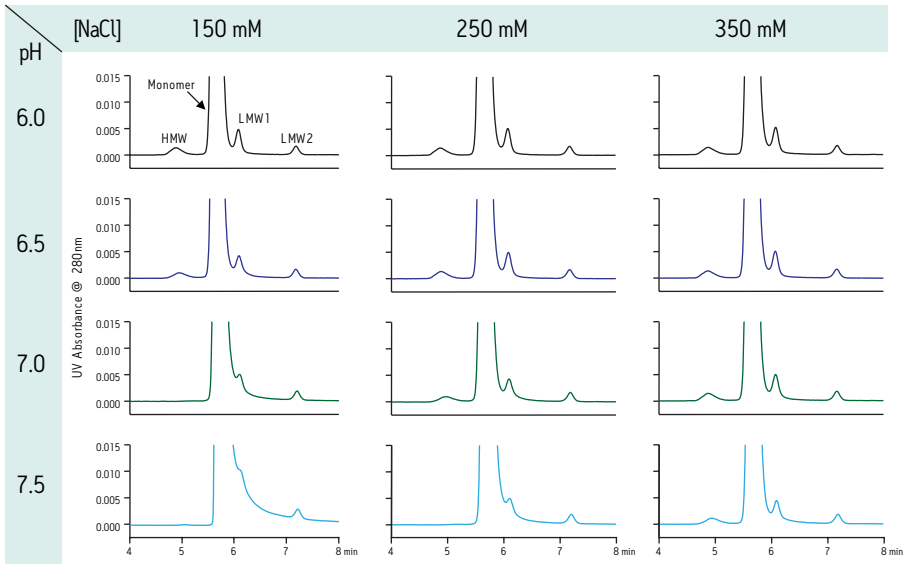


Figure 1. Expanded view chromatograms demonstrating the effect of pH and ionic strength on SEC separation of trastuzumab on an ACQUITY® BEH200 (4.6 mm x 300 mm) Column.

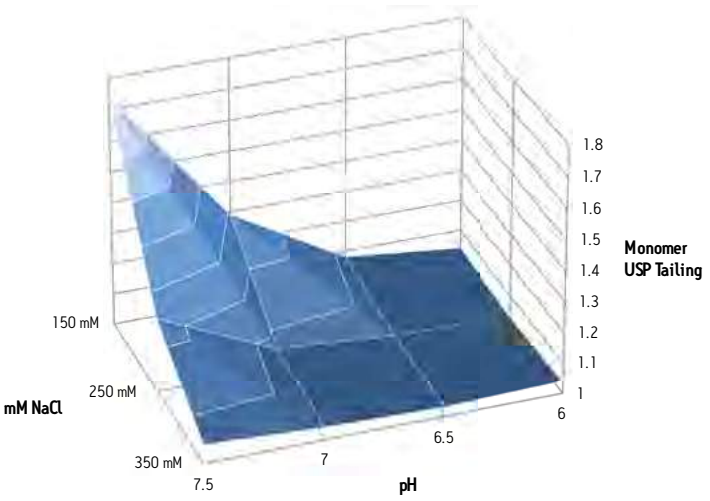


Figure 2. Response surface demonstrating the effect of pH and ionic strength on the USP peak tailing of trastuzumab monomer.

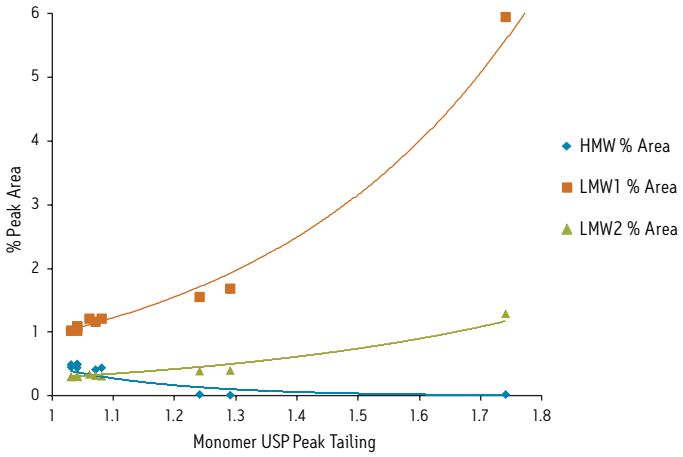
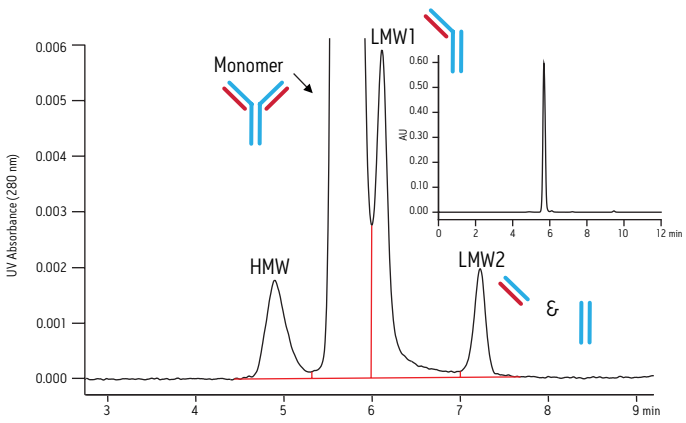


Figure 3. Accumulated data from the pH and ionic strength optimization studies showing the effect that monomer peak tailing has on the integrated peak areas of trastuzumab HMW and LMW forms. Peak identities are shown in Figures 1 and 4.



Evaluation (n=4)		HMW	Monomer	LMW1	LMW2
Average Peak	Area (%)	0.51	98.02	1.15	0.33
%RSD	Area (%)	0.98	0.03	1.31	3.08

Figure 4. Expanded and full scale (inset) views of the optimized SE-UPLC separation. Shown in the accompanying table is the quantitative evaluation of the trastuzumab monomer, HMW, and fragment (LMW) forms.

It should be noted that while this approach can provide a reproducible assessment of the HMW forms present in a protein sample, the accuracy of the analyses should be assessed by the use of an orthogonal method such as Analytical Ultracentrifugation or Asymmetric Field Flow Fractionation.

Extra-column dispersion

Most of the chromatographic separation modes commonly used for the analysis of biotherapeutic proteins rely on a gradient elution of analytes that have been pre-concentrated at the head of the column (e. g. cation exchange and reversed phase). In this format, pre-column dispersion does not significantly degrade chromatographic performance. SEC, however, is operated in an isocratic mode and as a result, in addition to post-column dispersion that is observed for all of these modes, the performance of the SEC separation is far more prone to degradation due to pre-column dispersion. One of the primary advantages of using sub-2-µm particle columns for SEC analysis is the significant decrease in peak dispersion that these columns can provide relative to the larger 5–10 µm particle sizes typically used for SE-HPLC. However, this benefit can be easily obscured by the effects of extra-column dispersion. In this respect, the chromatographic instrumentation used and its configuration play key roles in the dispersion of the analyte peak(s).

With respect to quantitative analysis, the effect of extra-column dispersion is most problematic in SE-UPLC separations in which there are partially resolved analytes. Separations where the peak area of the later eluting analyte of the critical pair is very low in comparison to that of the preceding peak are particularly sensitive to these effects. Once a low-dispersion chromatographic system has been selected and its performance optimized, selection of appropriate tubing and fittings must be made.

For a UV-absorbance based SE-UPLC method, the two system components that contribute to extra-column dispersion are the autosampler and the detector. Low dispersion systems specifically designed to operate at the high pressures needed to realize the full benefits of SE-UPLC are required for this methodology. In this regard, the performance of prospective instrumentation under the expected operating conditions should be evaluated thoroughly.

In addition to the UPLC system, significant extra-column dispersion can also be introduced by the tubing and fittings used to configure the SE-UPLC column into the chromatographic system. The extent of extra-column dispersion contributed by the capillary tubing used in a UPLC system may be estimated by a transition equation that was derived from the Taylor-Aris expression, which defines dispersion in long tubes and the equation defined by Attwood and Golay for dispersion in short tubes:

$$\sigma^2_{\upsilon,\text{tubing}} = \frac{(\pi \cdot r^2 \cdot L)^2}{3 + (24 \cdot \pi \cdot L \cdot \frac{D_m}{F})}$$

where $\sigma^2_{\upsilon,\text{tubing}}$ is the variance or band spreading contribution of the tubing, r and L are the radius and length of the capillary $\sigma^2_{\upsilon,\text{tubing}}$ is the diffusion coefficient of the analyte, and F is the flow rate.[5] However, for trastuzumab, which has a diffusion coefficient of approximately 4×10^{-7} cm^2/s,[6] and at the suggested operating flow rate of 0.4 mL/min, this equation may be simplified back to the Attwood-Golay expression for the limiting case of no diffusion of the analyte until tubing lengths begin to exceed several meters.

$$\sigma^2_{\upsilon,\text{tubing}} = \frac{(\pi \cdot r^2 \cdot L)^2}{3}$$

The above expression shows that the contribution to peak dispersion due to the tubing used to configure a UPLC system is proportional to the length of the tubing raised to the second power and to the inner-diameter raised to the fourth-power. To the chromatographer, this relationship suggests that SE-UPLC performance can be maximized by configuring a system with tubing having the shortest length and the smallest diameters insofar as is practical.

In addition to the capillary tubing used as part of the sample flow path, the selection and correct use of the fittings used to connect the system components and column are of critical importance in SE-UPLC. In this regard, it is important that the cut ends of the tubing be smooth and orthogonal relative to the walls of the tubing. The fittings used prior to the column must be able to withstand the pressures applied during an SE-UPLC analysis (~500 bar) without slipping and creating a void. If permanently swaged fittings are used, it is critical that the tubing and fittings be replaced if the column is replaced to ensure proper seating. An example of how an improper or failed tubing connection can lead to non-reproducible quantitative results is presented in Figure 5. In this example, the intentional generation of a 0.6 mm gap creating a void of approximately 1.2 µL at the connection to the head of the column was made. This alteration resulted in a resolution loss between the monomer peak and the LMW1 from a USP resolution of 1.50 down to 1.46. The loss of resolution between this critical pair resulted in a significant increase (~7%) in the integrated relative area of the LMW1 peak from 1.13% to 1.21%. However, the relative areas of the HMW and LMW2 peaks were not significantly different. This is due to how well the HMW and LMW2 peaks are resolved from the predominant monomer peak and the elution order of the HMW form relative to the monomer, which makes the HMW and LMW2 integration less susceptible to change due to variation in the peak tailing of the monomer. Albeit a predictable result, this example emphasizes the sensitivity that a quantitative SE-UPLC method can have to extra column dispersion.

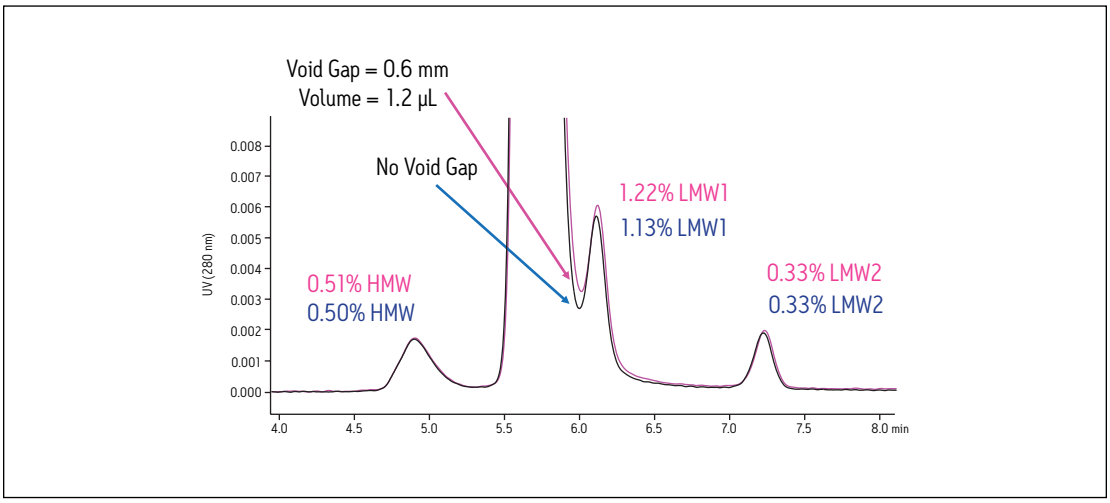


Figure 5. Expanded views of the optimized SE-UPLC separation showing the effect that the improper connections can have on integrated peak areas. In this example, a gap (0.6 mm) was created in the fitting connecting the capillary tubing to the head of the column.

Analytical method transfer

Once a reliable analytical SE-UPLC method has been developed it may be necessary to transfer that method to a different internal laboratory or external contracted research organization. As shown earlier, a systematic and thorough development process can result in a quantitative method that will not be sensitive to minor variations in mobile phase composition or pH as long as the quality of the columns used is appropriately controlled by the manufacturer.

The next major consideration in SE-UPLC method transfer is the extra-column dispersion of the chromatographic systems used in the two labs. In this regard, careful documentation of the system configuration, the critical components and connections (Figure 6), system performance, and the appropriate training of laboratory personnel are critical to the success of the method transfer exercise. In this regard, the use of an appropriate control sample to rigorously evaluate the performance with respect to the extra-column dispersion of the chromatographic system is recommended.

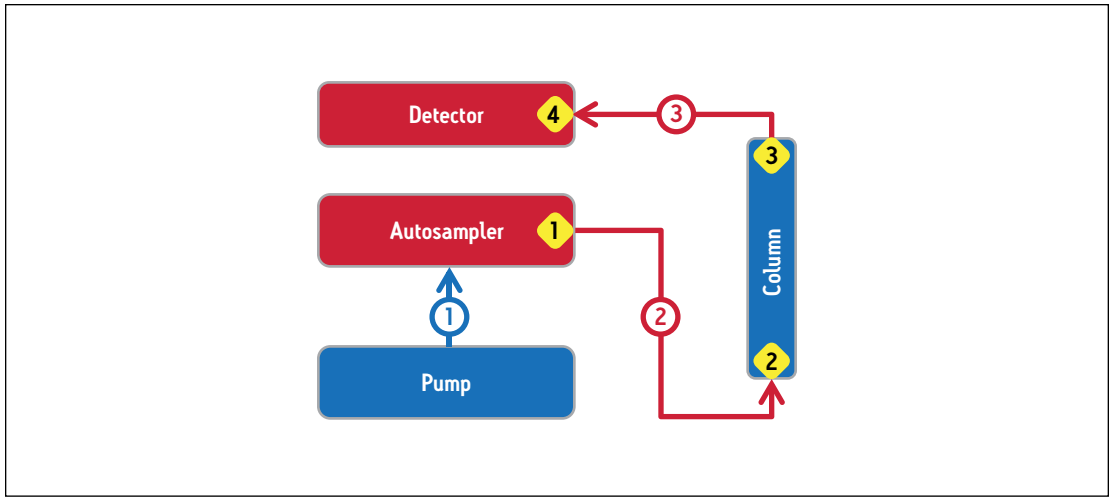


Figure 6. Shown in the figure are the critical components and tubing that must be considered when transferring an SE-UPLC method to another instrument (shown in red). The critical fittings that can result in extra-column dispersion are shown as yellow diamond. The high pressure fittings of particular concern discussed in the text are those connecting the autosampler and the column (Tubing 2). The Waters part numbers of critical connectors (yellow) and tubing (red) used for this application were: Connector 1 (p/n 700002645 and 700002635), Connector 2 (p/n 700003169, 700003114, and 700003115), Connector 3 and 4 (p/n 410001905), Tubing 2 and 3 (p/n 700008914).

CONCLUSION

Size-exclusion chromatography continues to be a standard technique for the analysis of monoclonal antibodies and their aggregates. Additionally, as a result of the improved resolutions observed by SE-UPLC, the extent of hinge region fragmentation in the native state of the molecule can also be determined. As in any SEC method, a thorough evaluation needs to be performed to develop an optimum SE-UPLC separation. Conditions that should be systematically evaluated include mobile phase (pH and ionic strength), flow rate, column length, the chromatographic system used and its configuration. The optimization of these parameters for an SEC separation may be evaluated based on critical performance criteria such as resolution, peak tailing, and quantitative reproducibility.

The analytical transfer of an optimized and robust SE-UPLC method may be made successfully. However, the analysts must take care to ensure that the chromatographic systems being used are configured comparably and set up correctly, both initially or when one of the components or columns is replaced, in order to maintain minimal extra-column dispersion.

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The Analysis of Multimeric Monoclonal Antibody Aggregates by Size-Exclusion UPLC

Stephan Koza, Matthew Lauber, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- ACQUITY UPLC® SEC Technology delivers increased high molecular weight protein (up to 1.8 MDa) component resolution compared to traditional, HPLC-based SEC separations.
- Size separation range increased with the BEH450 SE-UPLC® Column used in series with the BEH200 SE-UPLC Column for the analysis of dimer through multivalent mAb aggregates by SE-UPLC.

WATERS SOLUTIONS

ACQUITY UPLC BEH450 SEC, 2.5 µm Column

ACQUITY UPLC BEH200 SEC, 1.7 µm Column

ACQUITY UPLC H-Class Bio System

BEH450 SEC Protein Standard Mix

Auto•Blend Plus™ Technology

Empower® 3 Chromatography Data Software

KEY WORDS

Size-exclusion chromatography, SEC, monoclonal antibodies, mAb, proteins, SE-UPLC, gel-filtration chromatography, macromolecules, IgM, multimers

INTRODUCTION

Monoclonal antibodies (mAb) have become one of the predominant protein classes in the biotherapeutic landscape. Both the level and valency of soluble protein aggregation are critical quality attributes (CQA) that require monitoring for mAb preparations intended for human use. Protein aggregation, which may occur throughout the manufacturing process from cell culture through drug product shelf-life, may be indicative of partial denaturation or other perturbations of protein structure which can deleteriously effect the safety and efficacy of the protein biotherapeutic.[1] While it is important to quantitatively assess low valency (e.g. dimer) aggregate levels as a measure of process and product stability, as well as product safety, it is also critical to elucidate the distribution of high valency multimeric soluble aggregate forms in protein biotherapeutic preparations. These multimeric aggregate forms may be more effective in eliciting an immune response, due to their ability to trigger an immunological pathway independent of T-cell involvement.[2]

The recently introduced BEH 450Å pore size, sub-3-µm packing material has been designed to expand the molecular weight range of size-exclusion UPLC (SE-UPLC) separations to include biological macromolecules with large radii of hydration (R_h), such as IgM and multimeric self-associated proteins.[3] In this study, a 450Å pore sub-3-µm packing material (BEH450) was evaluated for the analysis of an mAb. The data demonstrate the advantages of a UPLC-based, size-exclusion separation compared to an HPLC-based, size-exclusion analysis for the separation of macromolecular protein complexes. In addition, data are presented showing the benefits of combined ACQUITY UPLC BEH SEC Columns of 200Å and 450Å pores for the analysis of an mAb sample that contains high valency multimeric mAb aggregates.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures. The IgG_1 mAb sample was biotherapeutic trastuzumab that was analyzed past expiry. Sample concentrations were 1.0 mg/mL (nominal) unless otherwise noted.

Method conditions (unless otherwise noted)

LC conditions

System:	ACQUITY UPLC H-Class Bio with 30 cm Column Heater
Detection:	Waters® ACQUITY UPLC TUV Detector with 5-mm Titanium flow cell Wyatt miniDAWN TREOS light scattering detector
Wavelength:	280 or 214 nm
Columns:	ACQUITY UPLC PrST SEC, 450Å, 2.5 µm, 4.6 x 150 mm (p/n 176002996) and 4.6 x 300 mm (p/n 176002997) ACQUITY UPLC PrST SEC, 200Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225) and 4.6 x 300 mm (p/n 186005226) HPLC column: Silica-based, diol bonded 450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.35 mL/min
Mobile phases:	5 mM sodium phosphate, 250 mM sodium chloride, pH 6.8 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH450 SEC Protein Standard Mix (p/n 186006842)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 1 mL (p/n 186000385DV)

Data management

Waters Empower® 3 Software

Waters UNIFI® Information System

Wyatt Astra Software

Cross-linking experimental detail

Covalent high molecular weight IgG aggregates were prepared using the Waters Intact mAb Standard (p/n 186006552), and the lysine-specific cross-linking agent, BS3 (Pierce, Rockford, IL). Reactions were performed with the antibody at a final concentration of 10 mg/mL and reagent-to-protein molar ratio of approximately 5:1 for 30 minutes.

RESULTS AND DISCUSSION

Comparison of SE-UPLC and SE-HPLC of IgM and IgM dimer

The BEH450 UPLC Column was compared to a silica-based 8 µm particle size HPLC column for the separation of IgM, a pentameric immunoglobulin with a molecular weight of 900 KDa, and the di-pentamer form of IgM with a molecular weight of 1.8 MDa (Figure 1). The sample loads and flow rates were adjusted for the column geometries used, and both analyses were performed on the same ACQUITY UPLC H-Class Bio System. The BEH450 column produced significantly better separation between the di-pentamer and pentamer forms, and improved sensitivity with peak height greater than 50% compared to the HPLC column. This remarkable improvement in separation efficiency is principally due to decreased particle size. Additionally, it can be observed that the molecular weight range of the BEH450 column extends above that of di-pentamer based on the observation of multimeric dimer forms eluting earliest in the chromatogram.

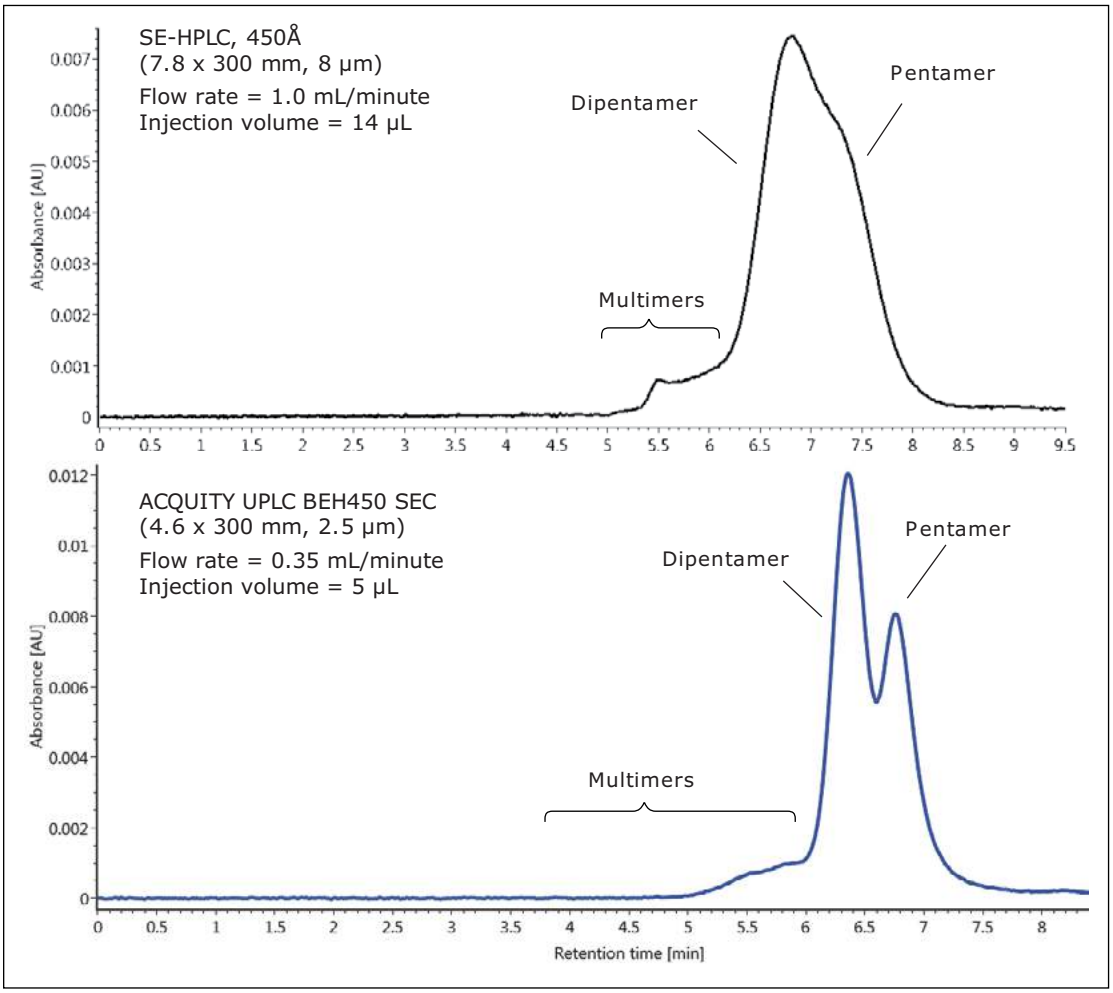

Figure 1. Comparison of an HPLC silica-based, 8-µm particle-size, 450Å SEC column (300-mm length) to an ACQUITY UPLC BEH450 SEC Column (300-mm length) for the separation of IgM pentamer (900 Kda) and IgM dipentamer (1.8 MDa). Sample injection volumes and flow rates were normalized for column geometry. The identities of the peaks were confirmed by SEC-MALS analysis.

Expanding the molecular weight range of mAb aggregation analysis

The outstanding efficiency provided by the BEH450 column for the separation of proteins above the upper molecular weight range of the BEH200 (approximately 450 KDa) suggests that using the two columns in series can provide some advantages for SE-UPLC separations over a broad molecular weight range. A comparison of the separation achieved on the BEH200 and BEH450 columns alone (each 300 mm in length) and the 150-mm length version of both columns connected in series (BEH200 followed by BEH450) for the Waters BEH200 SEC Protein Standard Mix (p/n 186006518) is shown in Figure 2. As the back pressure generated by the 1.7-µm particle size BEH200 column is greater than that of the 2.6-µm particle size BEH450 column, the BEH200 column was placed first in the series for this study. The result of this two-column configuration is shown in the center panel of Figure 2. By using both columns in series, the functional upper molecular weight range of the separation is increased as noted by the improved separation of thyroglobulin and its dimer compared to the separation using the 200Å column alone. Additionally, for the lower molecular weight forms, there is an improvement in resolution compared to the use of the 450Å column alone, as proven by the improved separation between IgG and BSA.

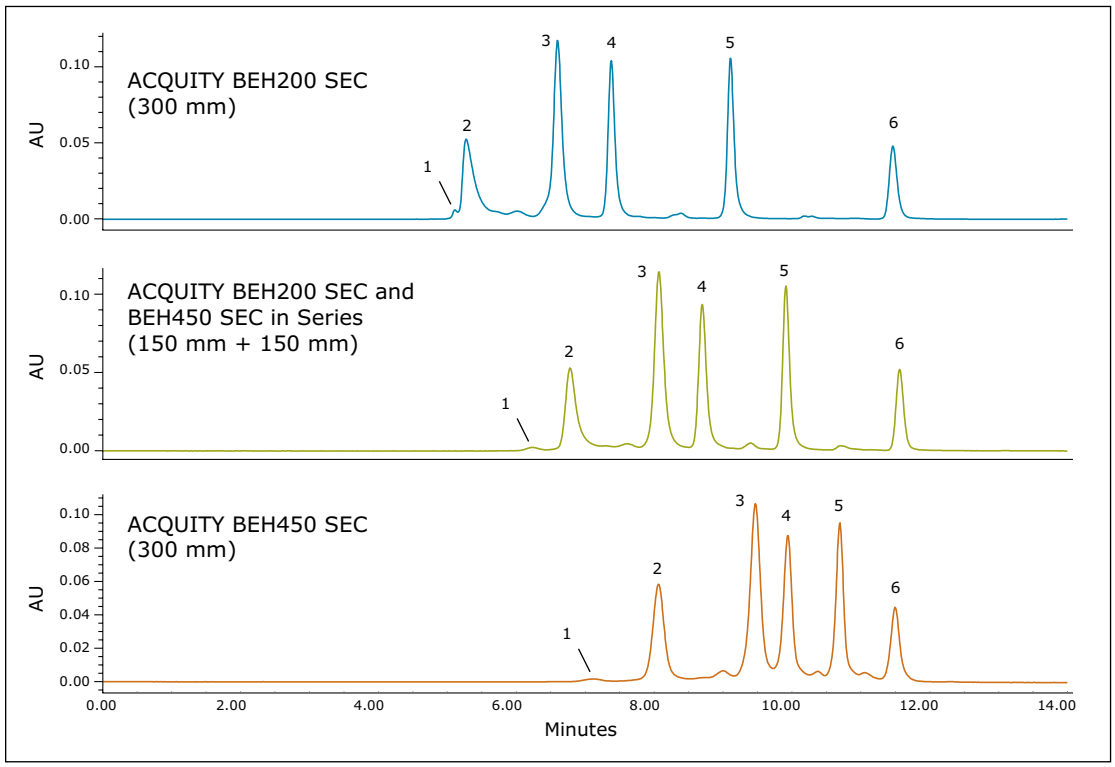

Figure 2. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (300-mm lengths). The center panel was generated using both a BEH200 and BEH450 column in series. Columns were connected using Waters part number 186006613. Compounds included the following: 1. Thyroglobulin dimer (1340 KDa), 2. Thyroglobulin (667 KDa), 3. IgG (150 KDa), 4. BSA (66 KDa), 5. Myoglobin (17 KDa), and 6. Uracil (112 Da).

The use of two SEC columns of different pore size can provide separations over a broader molecular weight range. One example of such a separation is the multivalent aggregate, trimer, dimer, and monomeric forms of an mAb. To demonstrate this, a sample of IgG (p/n 186006552) was then cross-linked to generate covalent dimeric and multimeric forms in order to generate a stable sample with an abundant level of mAb multimeric species. This sample was then used to define the molecular weight range of aggregated mAb species that can be separated by the BEH200 and BEH450 columns. The cross-linking chemistry produced high levels of multimeric species that were easily characterized by multi-angle laser light scattering (MALS) measurements; however, the polydispersity of the peaks increased due to the nature of the cross-linker reaction, also resulting in non-crossed linked additions of the reagent to the proteins. These chromatograms, shown in Figure 3, along with the peak assignments based on the MALS data, demonstrate the advantages of using the BEH200 and BEH450 columns in series. For the separation using only the BEH200 column, excellent resolution is obtained between the monomer and dimer forms. However, when compared to the separation observed on the BEH450 column, the distribution of aggregate forms larger than trimer elute near the total exclusion volume of the BEH200 column. By using the two columns in series (middle chromatogram in Figure 3), the distribution of higher aggregate forms can be observed while better resolution between the monomer and dimer is achieved compared to using the BEH450 column alone.

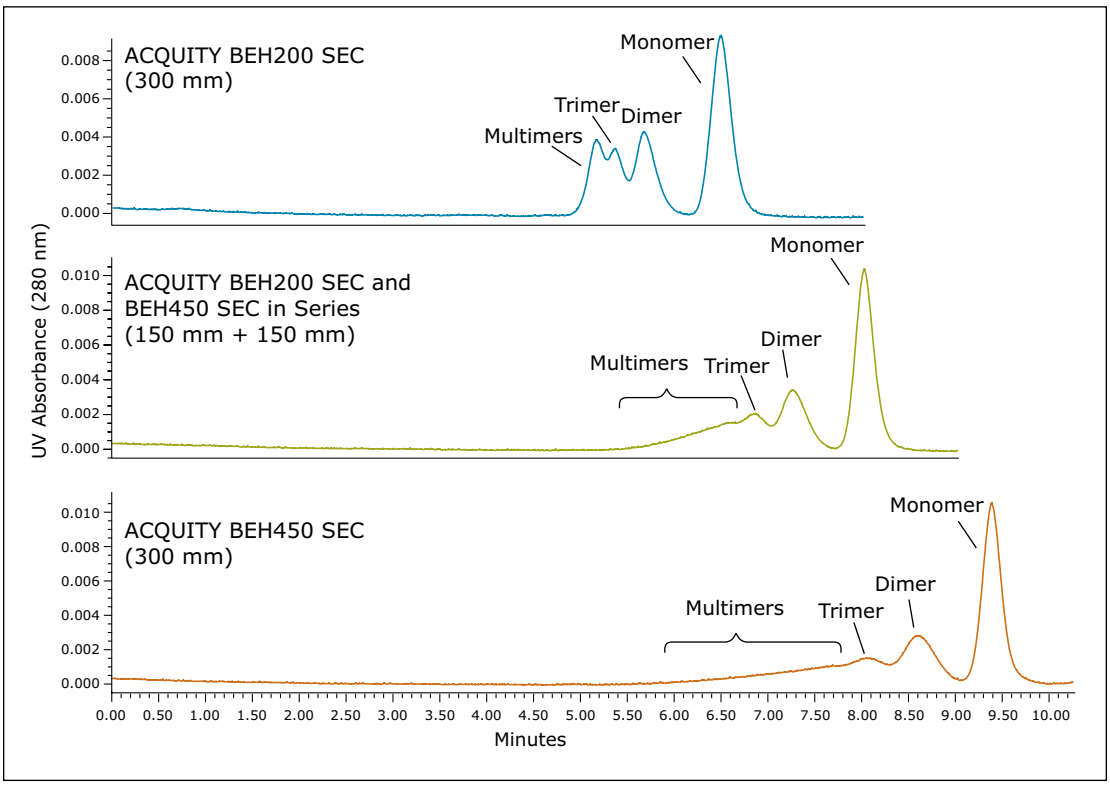

Figure 3. Comparison of the ACQUITY UPLC BEH450 SEC Column to the ACQUITY UPLC BEH200 SEC Column (300-mm lengths), and both a BEH200 and BEH450 column (150-mm lengths) in series for the separation of covalently cross-linked antibody sample. Columns were connected using Waters part number 186006613. The identities of the peaks were confirmed by SEC-MALS analysis.

Application of the BEH200 and BEH450 columns in series for the SE-UPLC analysis of a biotherapeutic IgG_1 mAb (trastuzumab) was then investigated. In order to generate a more relevant sample for this study, the trastuzumab sample was subjected to a series of freeze-thaw events to increase the levels of non-covalent aggregates in the sample. The aggregate levels were then evaluated using the BEH200 column (300-mm length) or the BEH200 and BEH450 columns in series (each 150-mm length). These results (Figure 4) show that the use of the two columns in series provides a separation in which the distribution of multimeric aggregate forms can be observed along with an improved separation between the dimeric and trimeric aggregate species when compared to the BEH200 column alone. Conversely, use of the BEH200 column alone provides a better separation of the mAb fragments that result from cleavage in the hinge region of the mAb.[4]

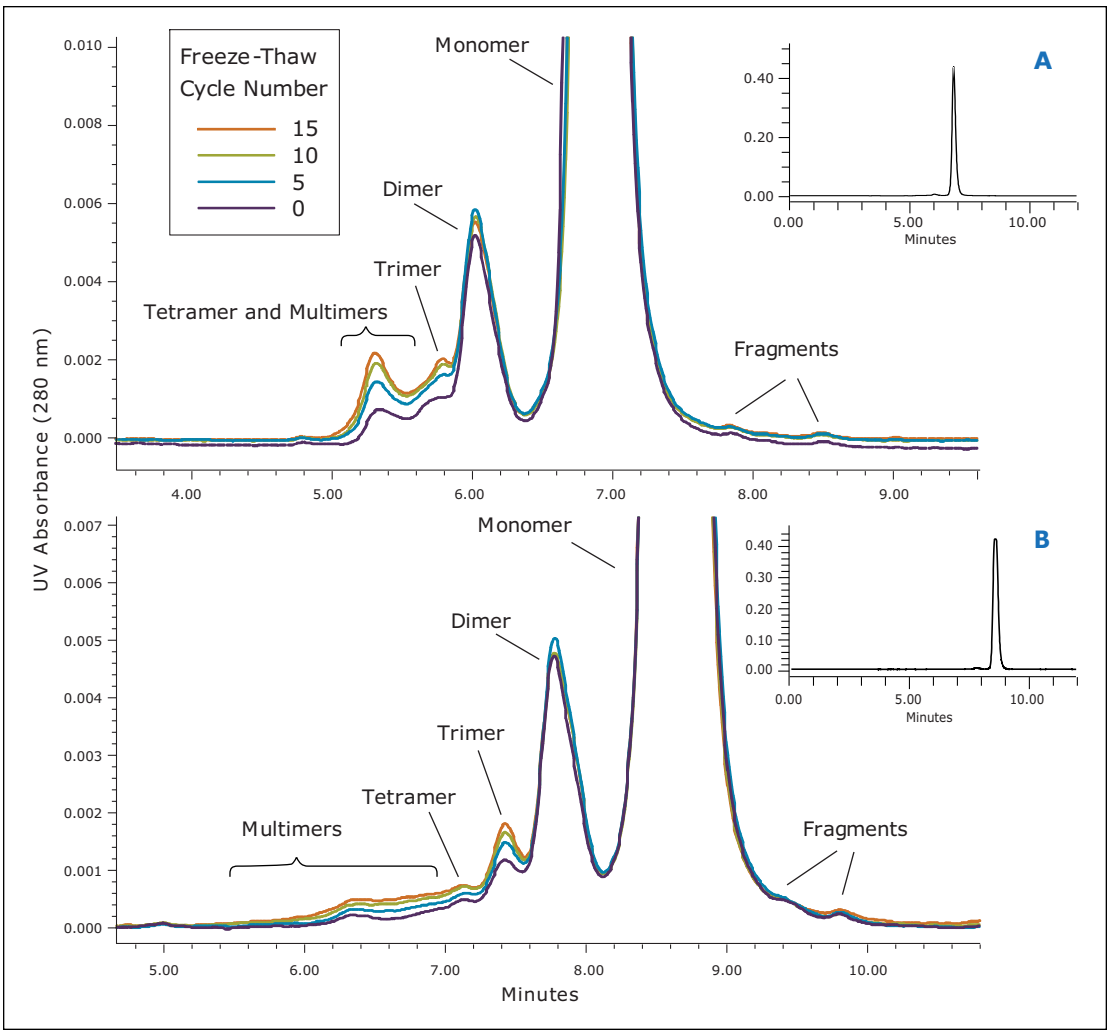

Figure 4. Comparison of a 300-mm length BEH200 column (A) versus a BEH200 and a BEH450 column (150-mm each) in series (B) for the separation of trastuzumab (IgG1) aggregation generated by increasing freeze-thaw cycles (0, 5, 10, and 15). Low-level peak identities are predicted based on elution position and previous monoclonal antibody assignments from Figure 3.

By evaluating the change in this profile over the course of the freeze-thaw study, it can be visually observed for both column configurations that the overall level of soluble trimer and multimeric aggregate is increasing. Both column configurations also provided comparable results for the determination of the relative levels of dimer, as well as the pooled trimer and multimer aggregate (Figure 5). This quantitative comparison required pooling the trimer and multimeric peak areas, as the resolution between the trimer and larger multimeric forms using the BEH200 column alone did not allow for accurate integration. However, the use of the BEH200 and BEH450 columns in series provides a significant benefit for this application in that the trimer and tetramer forms are more resolved compared to use of the BEH200 column alone. Additionally, the distribution of aggregate forms greater in valency than trimer and tetramer can be monitored better. These results are consistent with those presented previously (Figure 2), which demonstrate that the upper molecular weight range for the BEH200 column for a globular protein is approximately that of thyroglobulin (667 KDa), nearly the molecular weight of IgG tetramer (600 KDa). By comparison, the upper molecular weight range for the BEH450 column is approximately that of IgM dipentamer (1.8 MDa) which is the molecular weight of an IgG 12-mer. This additional information provided by the larger pore-size BEH450 column may be beneficial in characterizing a biotherapeutic protein, since in addition to the level of protein aggregation, the valency of that aggregation may potentially alter immunogenicity.

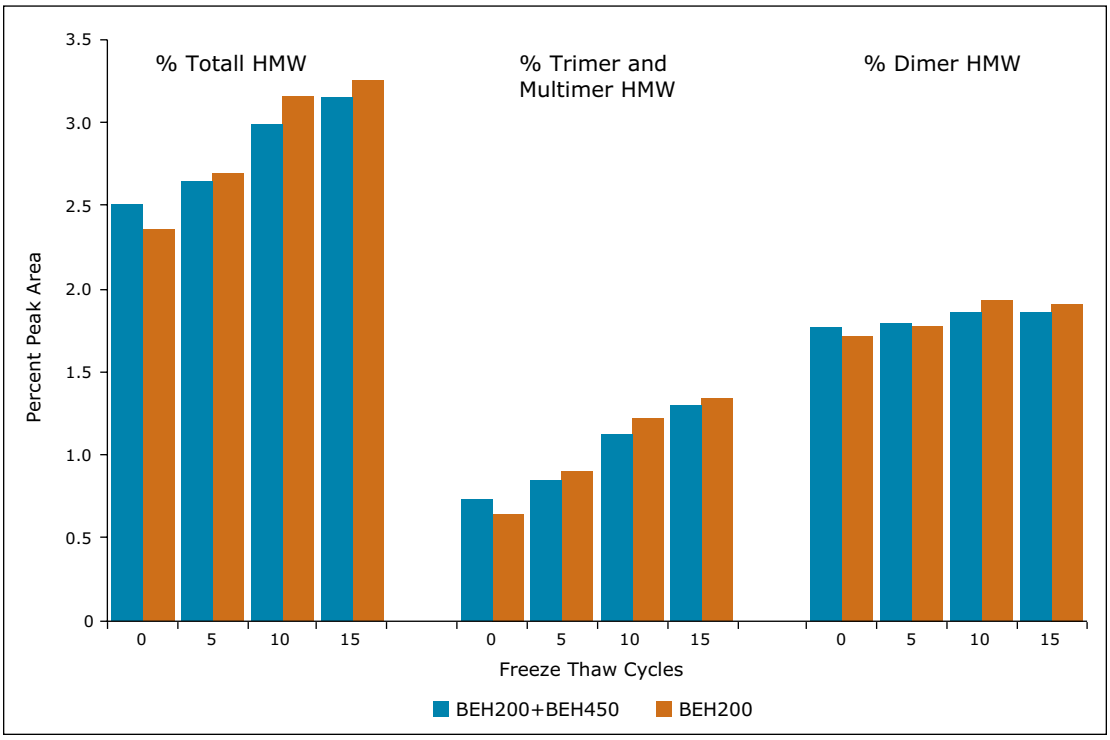

Figure 5. Comparison of relative peak areas observed in trastuzumab (IgG_1) as a result of aggregation generated by freeze-thaw cycles (0, 5, 10, and 15). Integrated results were determined from chromatograms presented in Figure 4.

CONCLUSIONS

Both the levels and the nature of soluble aggregates are important CQA for biotherapeutic protein preparations. The introduction of the BEH450 SEC column provides an extended upper molecular weight range for SE-UPLC analyses, and significantly improves resolution compared to a 450Å pore-size HPLC-based size-exclusion column. The use of the BEH450 SE-UPLC Column in series with the 200Å pore-size BEH200 column provides an expanded molecular weight range that can be used for the analysis of both dimeric and multimeric aggregates of an mAb, while taking advantage of the sensitivity, resolution, and throughput of SE-UPLC.

The ACQUITY UPLC BEH450, 2.5 µm SEC Column in combination with the ACQUITY UPLC BEH200, 1.7 µm SEC Column, and the ACQUITY UPLC H-Class Bio System provide the following benefits:

- Greater resolution and sample throughput compared to traditional SE-HPLC packing materials
- An extended useful SE-UPLC molecular weight range (approximately 10 KDa to 1800 KDa)
- Ability to observe the distribution of multivalent mAb aggregate forms while maintaining excellent resolution between the dimeric aggregate and monomeric mAb species

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Advanced HPLC Size-Exclusion Chromatography for the Analysis of Macromolecular Proteins Using 3.5 µm Ethylene Bridged Hybrid (BEH) Particles

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved resolution of macromolecular proteins by SE-HPLC
- Outstanding column stability and reliable column-to-column reproducibility
- Both 200Å and 450Å pore sizes provide a broad protein size separation range
- 2-fold increased sample throughput with minimal compromise in resolution compared to traditional HPLC separation

WATERS SOLUTIONS

XBridge® Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns

Alliance® HPLC System

Auto•Blend Plus™ Technology

BEH200 and BEH450 SEC Protein Standard Mix

KEY WORDS

Size-Exclusion Chromatography, SEC, HPLC, proteins, SE-HPLC, Gel Filtration Chromatography, IgG, IgM

INTRODUCTION

In 2010 Waters first introduced a 200Å pore-size size-exclusion chromatography (SEC) based on UPLC® Technology.[1] These size-exclusion UPLC (SE-UPLC) columns consist of sub-2-µm diameter ethylene bridged hybrid (BEH) particles, which are more structurally and chemically stable than pure silica-based particles. It is the enhanced structural stability of these particle that has indeed enabled the advent of SE-UPLC. However, the small particle-size and narrow 4.6 mm internal diameter of SE-UPLC columns are not optimal for use with an HPLC system. As a result, Waters has introduced HPLC-compatible, 3.5 µm particle diameter and 7.8 mm internal diameter size-exclusion HPLC columns (SE-HPLC) based on the robust BEH chemistry. This provides laboratories with HPLC instrumentation a means to take advantage of the benefits provided by this unique particle technology including its capability to withstand higher back pressures as compared to silica-based SEC particles. This note will highlight the performance characteristics of both the 200Å and 450Å pore-size versions of these columns, designed for the separation of macromolecular proteins, with respect to resolution, column-to-column reproducibility, and column stability. Additionally, the distinct advantages in terms of resolution and sample-throughput that these sub-4-µm packing material offers over larger (5 and 8 µm) standard HPLC particle sizes for the separation of large proteins will also be shown.

EXPERIMENTAL

Sample description

All samples were diluted in mobile phase unless otherwise noted. Proteins were purchased as individual standards or as mixtures (Waters and Sigma-Aldrich). Sample concentrations were 1.0 mg/mL (nominal) unless noted otherwise.

Method conditions

LC conditions

LC system:	Alliance HPLC or ACQUITY UPLC® H-Class Bio System with 30 cm Column Heater
Detection:	Alliance HPLC TUV Detector ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 or 214 nm
Columns:	Waters XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 x 150 mm (p/n 176003595) and 7.8 x 300 mm (p/n 176003596) XBridge Protein BEH SEC, 450Å, 3.5 µm, 7.8 x 150 mm (p/n 176003598) and 7.8 x 300 mm (p/n 176006599)

Comparator

Columns:	250Å, 5 µm, Silica-DIOL SEC, 7.8 x 300 mm Silica-DIOL SEC, 450Å, 8 µm, 7.8 x 300 mm
Column temp.:	Ambient
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.84 mL/min
Mobile phases:	25 mM sodium phosphate, 150 mm sodium chloride, pH 7.2 (prepared using Auto•Blend Plus Technology)
Gradient:	Isocratic
Standard:	BEH200 SEC Protein Standard Mix (p/n: 186006518) BEH450 SEC Protein Standard Mix (p/n: 186006842) Intact mAb Mass Check Standard (p/n: 186006552)
Sample vials:	Deactivated Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with cap and preslit PTFE/Silicone Septa, 1 mL (p/n: 186000385DV)

Data management

Chromatography software:	Empower® Pro (v2 and v3)

RESULTS AND DISCUSSION

The benefits provided by BEH Technology™ when used in the manufacturing of size-exclusion UPLC (SE-UPLC) packing materials for the analysis of peptides and proteins; have been previously described.[2,3] However, the diameter of these UPLC particles precluded their use in column dimensions applicable to HPLC instrumentation. In order to take advantage of the chemical and structural capabilities of BEH particle technology for the SEC separation of proteins and other macromolecules on HPLC instrumentation 7.8 mm ID columns packed with 3.5 µm BEH particles with pore sizes of either 200Å or 450Å have been introduced. These two column types provide a broad molecular weight range of SE-HPLC separations to include biological macromolecules with large radii of hydration (Rh), ranging from approximately 10 KDa to nearly 2 MDa. As part of this evaluation, the separation efficiency advantages of this packing material with respect to larger particle-size (5 and 8 µm) HPLC packing materials, and the critical performance characteristics of column-to-column reproducibility and lifetime stability will be demonstrated. In addition, this note will define the protein size-separation range of these two columns.

Advantages of reduced BEH particle size

Due to the significantly higher extra-column dispersion volumes and lower pressure limits of HPLC systems relative to UPLC Systems the resolution benefits provided by UPLC size-exclusion particles have not been available to laboratories that currently use HPLC instrumentation. In an effort to provide optimal resolutions for the SE-HPLC separation of proteins, a series of columns have been introduced based on BEH particle technology. To demonstrate their performance, protein molecular weight standards and a monoclonal IgG standard were separated on 250Å pore-size silica-based SEC column (5 µm, 7.8 x 300 mm) and on a 200Å pore-size BEH-based SEC column (3.5 µm, 7.8 x 300 mm) using the same Alliance HPLC System and aqueous mobile phase conditions (Figure 1).

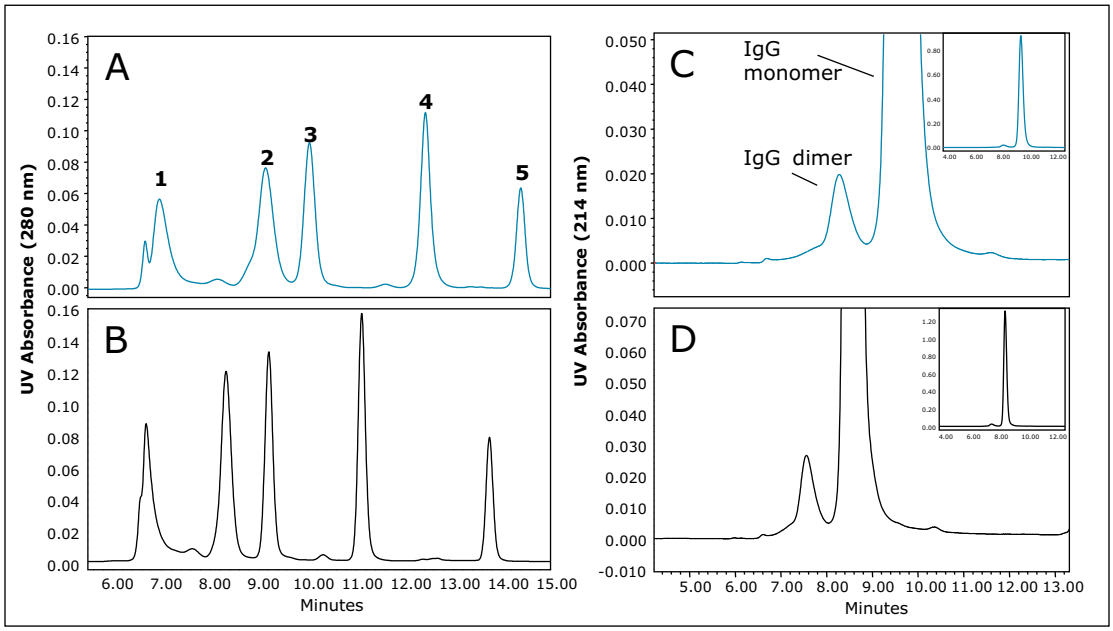

Figure 1. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 250Å, silica-based 5 µm (Frames A and C) and 200Å, BEH 3.5 µm (Frames B and D) SEC columns. Both columns were the same dimensions (7.8 x 300 mm) and separations were performed with the same flow rate (0.84 mL/minute) and the same sample loads. Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms C and D the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively.

The flow rates and injection volumes used were equivalent. Improved sensitivity and narrower peak widths were observed on the 3.5 µm packing material across the separation range of the molecular weight standards. USP resolution values (half-height measurement) calculated for the separation between the IgG monomer (MW=150 KDa) and dimer (MW=300 KDa) forms demonstrated an improvement of over 40% for the 3.5 µm particle over the resolution observed for the 5 µm particle size column. This improvement in resolution approaches the improvement that would be predicted by doubling the column length (Rs ∝ √L). Similar results comparing the chromatograms generated for the 450Å pore-size, silica-based, SEC column (8 µm, 7.8 x 300 mm) to the 450Å pore-size BEH-based SEC column (3.5 µm, (7.8 x 300 mm)) were observed (Figure 2). However, in this comparison the relative improvement observed for the separation between the IgG monomer and dimer is approximately 75%. This is due to the greater decrease in particle size between these two columns as compared to the smaller pore size 250Å silica-based and BEH 200Å particles. As a general observation, it should be noted from these data that the BEH 450Å SEC column provides an outstanding separation of both the dimeric and multimeric aggregate forms in this IgG sample.

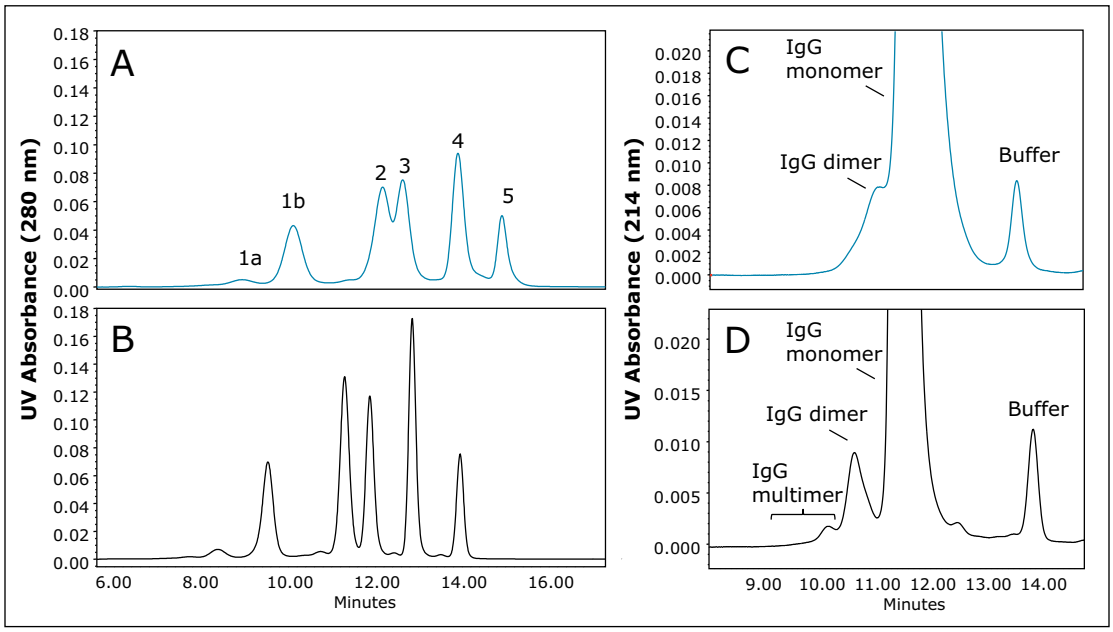

Figure 2. Shown is a comparison of separations of Waters BEH450 SEC Protein Standard Mix (p/n: 186006842) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 450Å, silica-based 5 µm (Frames A and C) and 450Å, BEH 3.5 µm (Frames B and D) SEC columns. Both columns were the same dimensions (7.8 x 300 mm) and separations were performed with the same flow rate (0.84 mL/minute) and with the same sample loads. Peak identities for chromatograms A and B are: 1a) thyroglobulin dimer (1.3 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms in frames C and D the molecular weights of the IgG monomer, dimer, and multimer are approximately 150 KDa ,300 KDa, and ≥450 KDa, respectively.

Advantages of BEH Particle Strength

BEH SEC particles have improved mechanical strength in comparison to silica-based particles. An opportunity presented to the analyst due to this characteristic is the ability to run at higher flow rates and pressures than can be tolerated by traditional SE-HPLC columns. By increasing the flow rate, the analysis time can be reduced proportionally in SEC, however, it should be noted that SEC resolution decreases as a function of flow rate. Taking these characteristics under consideration, if higher SE-HPLC sample throughput is an essential requirement the 3.5 µm BEH SE-HPLC can accommodate this demand. In this study a comparison (Figure 3) was made between a traditional 250Å, 5 µm silica based SE-HPLC column (7.8 x 300 mm). and a 3.5 µm BEH-based SE-HPLC column (7.8 x 300 mm). The 5 µm silica-based SE-HPLC column flow rate was set to 1.0 mL/minute (maximum flow rate: 1.2 mL/minute) and the 3.5 µm BEH SE-HPLC column was set to 2.0 mL/minute (maximum flow rate: 2.7 mL/minute). Comparable molecular weight standard profiles are observed, with the exception that the larger pore-size of the 250Å, 5 µm silica-based particle provides improved resolution of the thyroglobulin dimer peak (1.3 MDa) than what is observed on the 200Å, 3.5 µm BEH-based particle. While increasing the flow rate by a factor of two decreases the analysis time proportionally there will be a concomitant loss of resolution. As an example, the resolution observed between IgG and BSA was 2.5 on the 3.5 µm BEH based column as compared to 2.0 on the 250Å, 5 µm silica-based column (data not shown) at a flow rate of 1.0 mL/minute. However, at a flow rate of 2.0 mL/minute, the resolution on the 3.5 µm BEH-based column decreased approximately 25% to a resolution of 1.9.

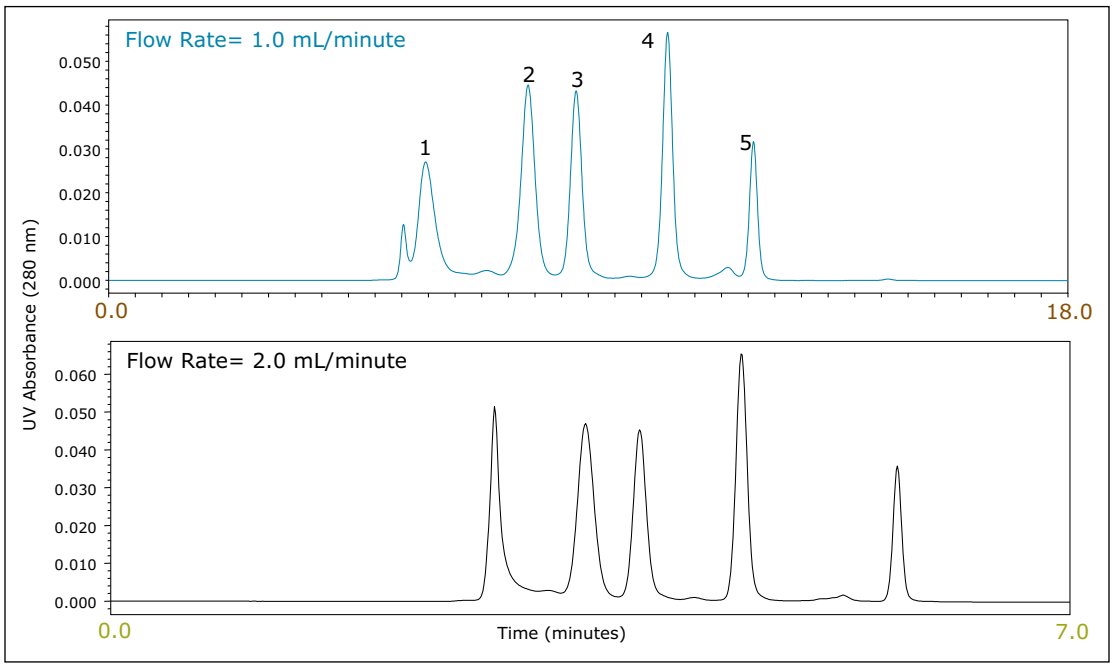

Figure 3. Shown is a comparison of separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) on 250Å, silica-based 5 µm SEC column, separated at 1.0 mL/minute (Frame A) and on a 200Å, BEH 3.5 µm (Frame B) SEC column separated at 2.0 mL/minute. Both columns were the same dimensions (7.8 x 300 mm) and the same sample loads were used. The time axis for the main chromatograms have been normalize, the actual times of the separations are provided in the inset. Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da).

Note: Comparable molecular weight standard profiles are observed, with theexception that the larger pore-size of the 250Å, 5 µm silica-based particles provide improved resolution of the thyroglobulin dimer peak (1.3 MDa) than what is observed on the 200Å, 3.5 µm BEH-based particle. Use of Waters XBridge Protein BEH SEC, 450Å, 3.5 µm is recommended for the analysis of proteins, such as thyroglobulin and its dimer, whose molecular weights exceed those recommended be analyzed on the XBridge Protein BEH SEC, 200Å, 3.5 µm Column.

XBridge Protein BEH SEC 200Å, and 450Å, 3.5 µm Columns: reproducibility and stability

Major concerns that an analyst has when selecting an SEC column for method development or use in a validated method are column-to-column and batch-to-batch reproducibility as well as obtained column lifetime when used in methods. Shown in Figure 4 is an overlay of the chromatograms for a series of molecular weight standards for both the 200Å and 450Å, 3.5 µm SEC columns in a (7.8 x 300 mm). These chromatograms demonstrate the reproducibility of 6 SEC columns packed from 3 different production lots of packing material. For these standards, and at a flow rate of 0.84 mL/minute, the retention time standard deviations for the 200Å pore size, SEC column ranged from a minimum of 0.037 minutes to 0.084 minutes with an average standard deviation of 0.064 minutes for all components labeled in Figure 1. For the 450Å pore size, SEC column the retention time standard deviations ranged from a minimum of 0.045 minutes to 0.068 minutes with an average standard deviation of 0.060 minutes.

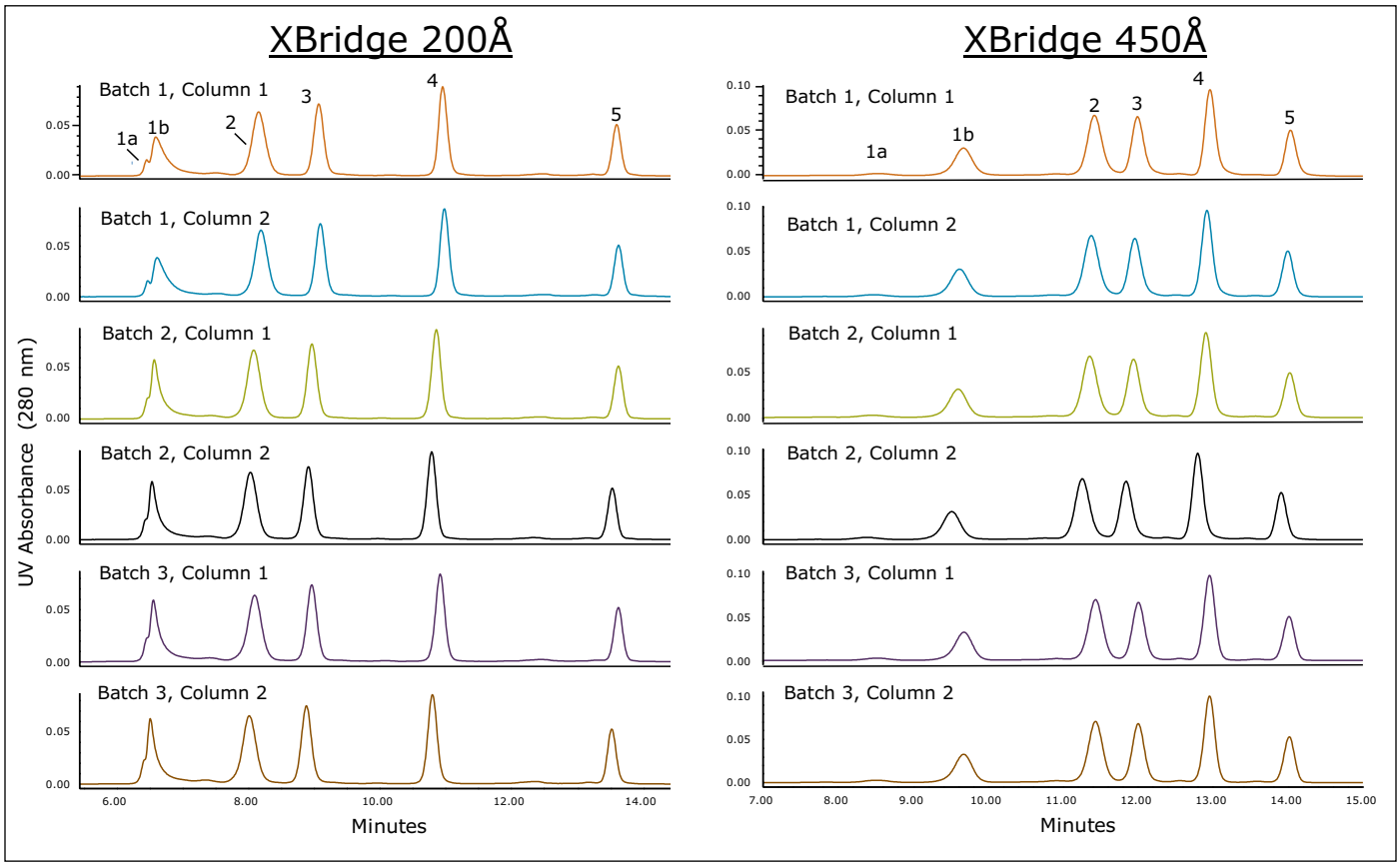

Figure 4. Shown are overlays of the separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and BEH450 SEC Protein Standard Mix (p/n: 186006842) on 200Å and 450Å BEH 3.5 µm SEC columns. Two columns (7.8 x 300 mm) were packed from 3 individual manufacturing batch of particles to evaluate both column-to-column and batch-to-batch reproducibility. Peak identities are: 1a) thyroglobulin dimer (1.34 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). Separations were performed on an ACQUITY UPLC H-Class Bio System.

The stability of the 200Å and 450Å, 3.5 µm SEC columns (7.8 x 300 mm) was evaluated by injecting a series of standards over the course of over 600 total injections. Given that the stability of silica-based SEC columns can be deleteriously altered by mildly basic pH levels, the pH of the mobile phase was set to 7.2, equivalent to that of phosphate buffered saline (PBS) buffer. Shown in Figures 5 and 6 are comparisons of the profiles obtained for the molecular weight standards and the IgG standard from the start to the finish of the study for both columns.

The resolution between two of the critical peak pairs, IgG and BSA, and IgG Dimer and IgG monomer were determined for each column. Both columns demonstrated remarkable stability with only modest depreciation of the calculated resolutions as highlighted in the Figure caption. These data demonstrate that XBridge Protein BEH SEC columns containing 3.5 µm particles can provide the reproducibility and stability needed to develop reliable assays and run them routinely in a quality control environment.

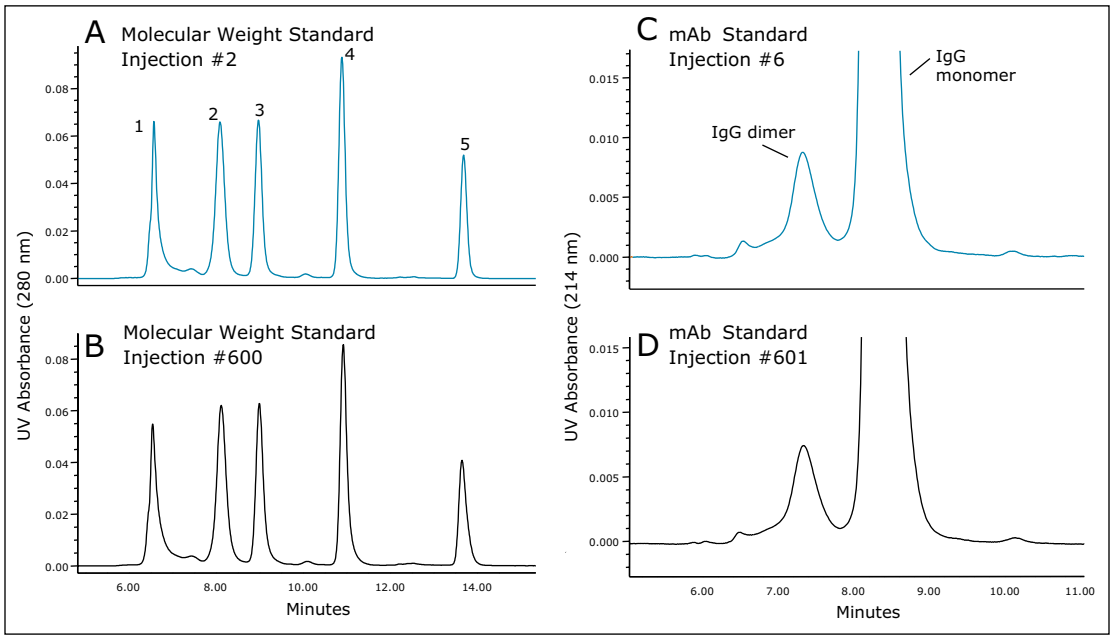

Figure 5. Shown are comparisons of column lifetime study separations of Waters BEH200 SEC Protein Standard Mix (p/n: 186006518) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 200Å BEH 3.5 µm SEC column (7.8 x 300 mm). Peak identities for chromatograms A and B are: 1) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms of the mAb standard the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively. Separations were performed on ACQUITY UPLC H-Class Bio System.

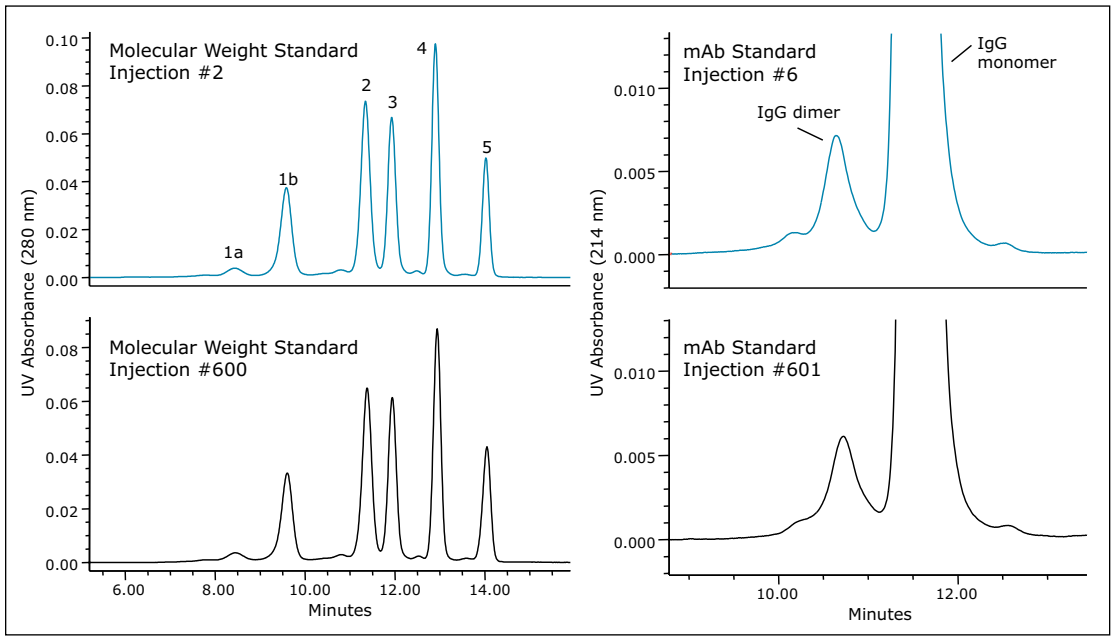

Figure 6. Shown are comparisons of column lifetime study separations of Waters BEH450 SEC Protein Standard Mix (p/n: 186006842) and Intact mAb Mass Check Standard (p/n: 186006552, diluted to 1 mg/mL) on 450Å BEH 3.5 µm SEC column (7.8 x 300 mm). Peak identities are: 1a) thyroglobulin dimer (1.34 MDa), 1b) thyroglobulin (669 KDa), 2) IgG (150 KDa), 3) BSA (67 KDa), 4) myoglobin (14 KDa), and uracil (112 Da). For the chromatograms of the mAb standard the molecular weights of the IgG monomer and dimer are approximately 150 KDa and 300 KDa, respectively. Separations were performed on an ACQUITY UPLC H-Class Bio System.

Molecular weight range

Comparisons were made between the XBridge Protein BEH SEC 450Å and 200Å, 3.5 µm columns for their ability to resolve a series of defined standards. The protein molecular weight calibration curves are shown in Figure 7. For proteins, the linear molecular weight range for the 200Å pore-size column is estimated to be from approximately 10 KDa to 450 KDa, whereas the 450Å pore-size column is estimated to be from approximately 50 KDa to over 1.3 MDa. This upper limit is based on the chromatographic separation observed (Figure 2) for thyroglobulin (669 KDa) and it dimer (1.3 MDa). The 450Å column separation of IgM pentamer (900 KDa) and IgM dipentamer (1.8 MDa) as shown in Figure 8 shows partial resolution between these two forms, which is indicative that the pore volume accessible to the dipentamer is limited, thereby demonstrating that 1.8 MDa is beyond the linear molecular weight range of this column and close to practical upper molecular weight limit for this column. This higher molecular weight range may be of use when analyzing multimeric protein aggregates or proteins conjugated to compounds that have relatively large radius of hydration values such as long chain polyethylene glycols or when running proteins under denaturing SEC conditions.

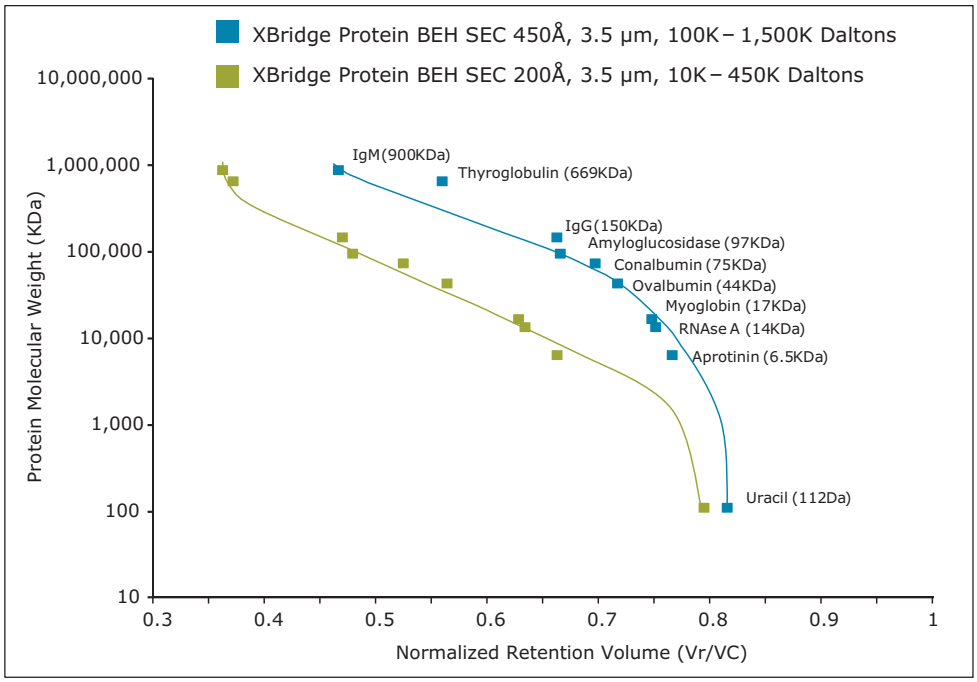

Figure 7. Shown are calibration curves of various proteins, peptides, and uracil generated for both the BEH 200Å and 450Å, 3.5 µm SEC columns.

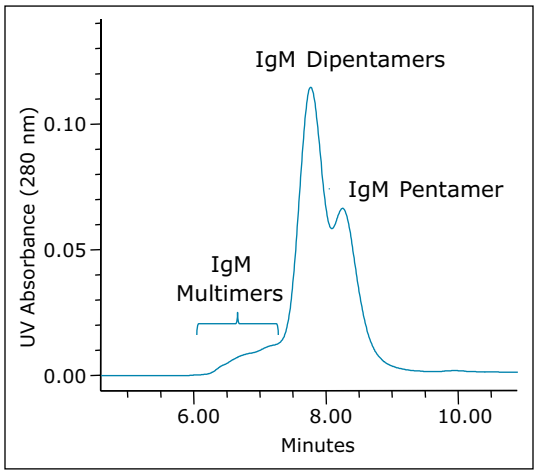

Figure 8. Shown is the separation of IgM pentamer, IgM dipentamer, and the multimeric forms of pentameric IgM separated on a BEH, 450Å, 3.5 µm SEC column. The molecular weight of the proteins are: IgM pentamer (900 KDa), IgM dipentamer (1.8 MDa), and IgM multimers (≥2.7 MDa). Separation was performed on an ACQUITY UPLC H-Class Bio System.

CONCLUSIONS

A reliable, high resolving, size-exclusion method is often an integral part of the quality assessment of a protein biopharmaceutical and also has a key role in the evaluation of protein samples in other areas of research. The introduction of HPLC-compatible, XBridge Protein BEH SEC 200Å and 450Å, columns containing 3.5 µm particles provide improved component resolution in LC-based SEC separations compared to use of traditional silica-based SEC columns containing 5 µm particles. In addition, higher throughput analyses are possible due to the structural strength of the BEH particle. This critical particle strength characteristic in combination with use of stable diol-bonded particles work to deliver outstanding column lifetimes. As part of the Waters' quality manufacturing guidelines, these columns are produced to rigorous tolerances and quality tested with relevant analytes. Although not presented within this report, these HPLC separations are also directly scalable to SE-UPLC separations using ACQUITY UPLC Protein BEH SEC Columns containing 1.7 µm or 2.5 µm diameter particles and narrower column internal diameters (4.6 mm I.D.) which can proved even greater resolution and sample-throughput when coupled with UPLC capable chromatographic systems.[4]

The XBridge Protein BEH SEC, 200Å and 450Å, 3.5 µm Columns provide:

- HPLC-based SEC resolution of proteins from 10–1,500K Daltons with higher throughput capability
- Outstanding SEC column life
- Less non-desired, protein/column interactions than silica-based SEC columns
- Comprehensive testing to provide unmatched column consistency and increased confidence in validated methods
- Complement ACQUITY UPLC-based SEC columns for seamless method transfer based on application needs

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Future-Proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System to Run SEC-HPLC and SEC-UPLC

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Transfer size exclusion chromatography (SEC) applications from HPLC to UPLC®
- Future-proof laboratories with a flexible system that is capable of both HPLC and UPLC to perform aggregation assays in protein characterization

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ACQUITY UPLC® H-Class Bio System

Biosuite™ SEC Column

ACQUITY UPLC Protein BEH SEC Column

BEH200 SEC Protein Standard Mix

Empower® 3 Chromatography Data Software

KEY WORDS

Size exclusion chromatography (SEC), HPLC, UPLC, method transfer, higher peak resolution, faster separation time, improved sensitivity, infliximab

INTRODUCTION

Aggregation represents a serious concern for companies manufacturing large-molecule therapeutics. Numerous assays aid in establishing the extent of aggregation for a given product, one of which is size exclusion chromatography (SEC). SEC is a straightforward assay requiring minimal sample preparation that exploits the size, or more specifically the hydrodynamic radius, of a given molecule as the mechanism of separation. SEC is unique from many other large-molecule chromatographic approaches in the sense that separation occurs under isocratic conditions. Because separation is influenced exclusively by an analyte's hydrodynamic radius and its ability to penetrate the pores of the stationary phase particle, there is no requirement for gradient conditions as adsorption is ideally non-existent and therefore does not influence migration times.

As observed with other large-molecule assays, significant benefits can be achieved in chromatographic quality when transitioning a method from HPLC to UPLC. The most obvious benefit is an increase in chromatographic resolution, driven principally by the increased chromatographic efficiency obtained through reduced column-particle sizes coupled with the use of low-dispersion instrumentation. This is particularly noticeable in SEC of large molecules as several higher-order aggregates can potentially be identified and quantified due to the increased resolution gained with SEC-UPLC. The added increase in sensitivity obtained through improved efficiency in SEC-UPLC also facilitates the identification of low abundant, higher-order aggregates that may have otherwise been undetected in SEC-HPLC.

Transferring SEC methods from HPLC to UPLC can be considered one of the more straightforward tasks as only a limited number of parameters need to be considered. With this in mind, there are significant opportunities for analysts to move legacy SEC-HPLC methods to UPLC technology.

To demonstrate the applicability of the ACQUITY UPLC H-Class Bio System for performing both SEC-HPLC and SEC-UPLC, we present here the transfer of an SEC-HPLC assay for monoclonal antibody aggregation from legacy HPLC instrumentation to the ACQUITY UPLC H-Class Bio. Following transfer of the HPLC method, we demonstrate a simplified approach to migrate the SEC-HPLC method to UPLC column chemistry.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLCH-Class Bio comprised of: ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
	ACQUITY UPLC H-Class Bio Sample Manager (SM)
Extension loop:	100 µL (p/n 430002625)
Detector:	ACQUITY UPLC Tunable UV Detector with Ti flow cell
Absorption	Wavelength: 214 nm
Column temp.:	Ambient
Mobile phase:	20 mM Phosphate, 200 mM NaCl, pH 6.8
Sample:	Waters BEH200 SEC Protein Standard Mix (p/n 186006518)

HPLC method conditions

HPLC column:	Biosuite SEC Column, 250 Å, 10 µm, 7.5 mm x 300 mm (p/n 186002170)
Injection vol.:	20 µL
Flow rate:	0.400 mL min^{-1}
Method length:	35 min

UPLC method conditions

UPLC column:	ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 mm x 150 mm (P/N 18605225)
Injection vol.:	4 µL
Flow rate:	0.885 ml min^{-1}
Method length:	3 min

Results of this study illustrate that the ACQUITY UPLC H-Class Bio System is capable of producing highly similar SEC-HPLC data compared to legacy HPLC instrumentation. Moreover, improvement in chromatographic resolution is obtained by moving from HPLC to UPLC. A reduction in run time is also observed without compromise to monoclonal antibody aggregate quantification. The experiment also illustrates the flexibility of using the ACQUITY UPLC H-Class Bio System for both SEC-HPLC and SEC-UPLC and the benefits of moving to smaller particle sizes for SEC-based assays.

RESULTS AND DISCUSSION

Legacy SEC-HPLC methods prove highly comparable when run on the ACQUITY UPLC H-Class Bio System

To determine the ability of the ACQUITY UPLC H-Class Bio to perform legacy SEC-HPLC assays, we established a benchmark SEC-HPLC method using a quaternary pump HPLC instrument coupled with the BioSuite SEC 250 Å 10-µm Column (7.5 mm x 300 mm).

Two samples were used to evaluate the separation. The first sample was the Waters SEC200 protein standard mix, used to determine the total inclusion and exclusion volume of each column. The second sample was a therapeutic monoclonal antibody, infliximab, which was selected to measure aggregation, if any, and therefore a useful sample for evaluating the accuracy of method transfer between SEC run on the HPLC instrument and the ACQUITY UPLC H-Class Bio System. With respect to HPLC method parameters, separation in SEC is isocratic and, as such, the basic requirement of the method is that it run long enough to deliver a minimum of one column volume. For the selected HPLC column, a volume of approximately 13 mL was required and therefore a run time of 35 min was selected based on a delivered flow rate of 0.4 mL $min.^{-1}$ A standard mobile phase of 20 mM phosphate buffer, 200 mM NaCl at pH 6.8 was selected for use and initial analyses.

To first establish a benchmark chromatogram, the SEC200 protein mix standard was separated using the quaternary pump HPLC instrument with the SEC-HPLC column. Results of this separation are presented in Figure 1A. The SEC-HPLC column was then transferred to the ACQUITY UPLC H-Class Bio System without any changes to the method parameters. The same protein mix standard was separated, with results presented in Figure 1B. As can be clearly seen from these figures, very little difference is observed in the chromatography performed on each instrument. Details of the retention times and relative peak areas provided in Table 1 further indicate the accuracy of method transfer across analytical instruments, as evidenced by minimal differences between data recorded from each instrument.

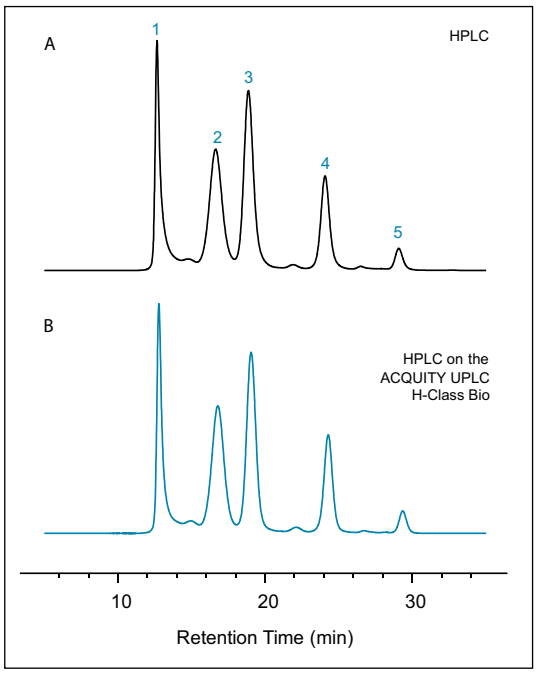

Figure 1. SEC transferred from HPLC to the ACQUITY UPLC H-Class Bio generates equivalent separation. (A) SEC on the HPLC instrument; (B) HPLC SEC on the H-Class Bio.

Peak	SEC Component	Retention Time (min)			Peak Area (%)			
		HPLC	H-Class	Δ	HPLC	H-Class	Δ	
1	Thyroglobulin	12.78	12.65	-0.13	23.24	23.79	-0.55	Figure 1.
2	IgG	16.78	16.70	-0.08	28.31	27.64	0.67	
3	BSA	19.04	18.82	-0.22	30.42	31.38	-0.96	
4	Myoglobin	24.28	24.16	-0.12	15.13	14.45	0.68	
5	Uracil	29.34	29.19	-0.15	2.90	2.74	0.16	
1	Mab Dimer	14.49	14.19	-0.30	0.48	0.47	0.01	Figure 2.
2	Mab Monomer	17.69	17.18	-0.51	99.52	99.53	0.01	

Table 1. Quantitative comparison of HPLC SEC run on a traditional HPLC versus the ACQUITY UPLC H-Class Bio. Retention time and peak area data represent the averaged data of triplicate analyses.

With conditions indicating a successful transfer of the SEC method to the ACQUITY UPLC H-Class Bio System, the monoclonal antibody infliximab was separated on both the quaternary pump HPLC instrument and the ACQUITY UPLC H-Class Bio System to determine the relative abundance of monomer and aggregate species within the sample. Similar to the protein standard results across instruments, very little difference was observed in chromatography (Figure 2) as well as relative peak areas in both the SEC200 protein standard mix and infliximab samples (Figure 3). Retention times, relative peak areas, and the resolution between the IgG monomer and dimer, also presented in Table 1, provide convincing evidence that SEC-HPLC assays can successfully be transferred to the ACQUITY UPLC H-Class Bio System without any compromise to the legacy analytical method criteria.

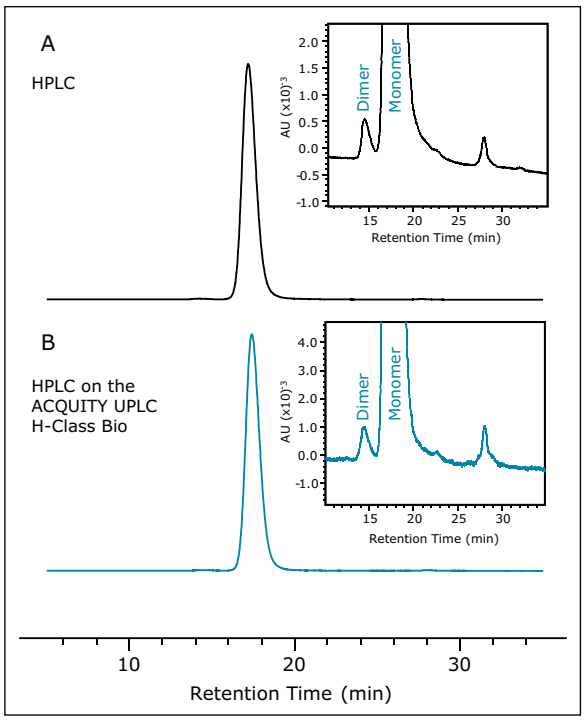

Figure 2. Separation of infliximab on SEC gives equivalent results between the HPLC instrument and the ACQUITY UPLC H-Class Bio. (A) SEC on the HPLC; (B) SEC on the H-Class Bio.

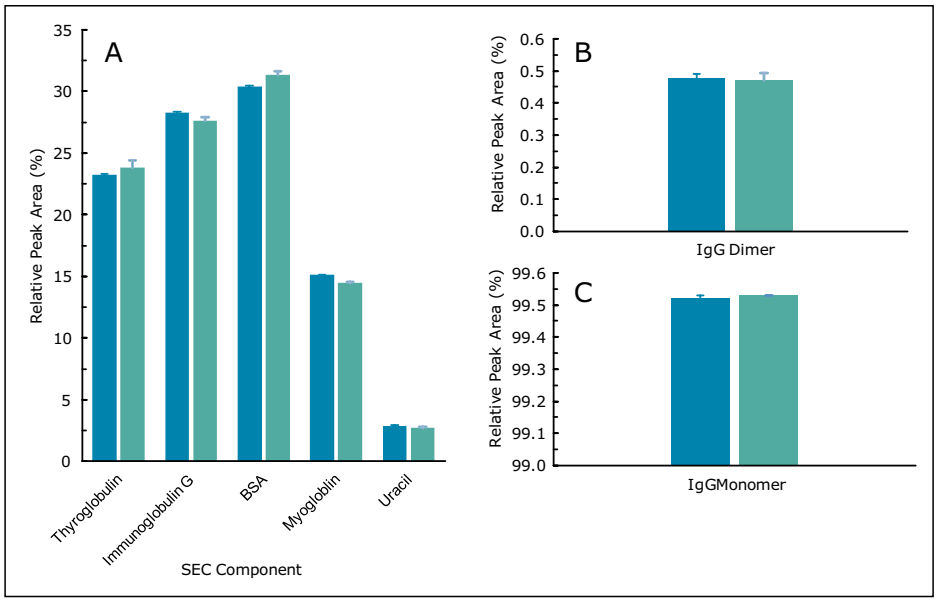

Figure 3. Evaluation of relative peak area between SEC performed on HPLC and the ACQUITY UPLC H-Class Bio System. In each figure, the dark blue columns represent values measured from the HPLC instrument while the light blue columns represent values measured from the ACQUITY UPLC H-Class Bio. (A) Peak areas measured from the SEC200 protein standard mix. (B) Measurement of the relative peak area of the infliximab dimer. (C) Measurement of the relative peak area of the infliximab monomer.

Migration from HPLC to UPLC improves resolution

Significant benefits can be obtained by transferring legacy SEC-HPLC methods to SEC-UPLC columns, which provide improved chromatographic resolution through reduced column particle size. To illustrate this benefit, the SEC-HPLC method used for method transfer between the quaternary pump HPLC and the ACQUITY UPLC H-Class Bio System was adapted to be run on SEC-UPLC using the Waters ACQUITY UPLC Protein BEH SEC Column (200 Å, 1.7 µm, 2.1 mm x 150 mm).

To demonstrate the improved resolution as a result of reduced particle size on SEC, we separated the SEC200 protein standard mix using the 10-µm Biosuite SEC Column and the 1.7-µm ACQUITY UPLC Protein SEC column and calculated the differences in chromatographic performance. Both HPLC and UPLC separations were performed using the Waters ACQUITY UPLC H-Class Bio System. To accommodate for differences in particle size and column dimensions between the HPLC and UPLC columns, the flow rate was adjusted based on the following formula:

$$F_2 = F_1 \left(\frac{d_2^2}{d_1^2} \right) X \frac{d_{p1}}{d_{p2}}$$

Where F refers to flow rate, d refers to column internal diameter, and dp refers to particle diameter. Based on the two columns used and an HPLC flow rate of 0.4 mL min,$^{-1}$ a new flow rate of 0.885 mL min^{-1} was calculated. A new run time of 3 minutes was also determined for the UPLC separation, given the reduced volume of the UPLC column and the increased flow rate.

To determine the improvement in UPLC-based SEC, the SEC200 protein standard mix was separated on the BEH SEC 200 Å 1.7-µm column using the updated run time and flow rate. Most noticeable from the separation was the significantly reduced run time from 35 min in HPLC to just 3 minutes in UPLC (Figure 4). This was not at the cost of chromatographic performance, where relative peak areas are equivalent between HPLC and UPLC (Figure 5A) but a significant improvement in resolution between all peak pairs is observed in UPLC (Figure 5B). Resolution between all peak pairs are tabulated for reference (Table 2).

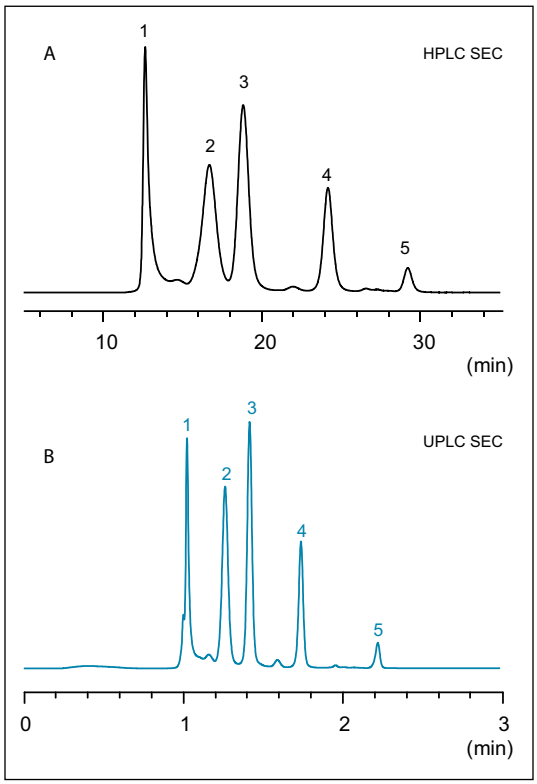

Figure 4. SEC transferred to UPLC generates higher peak resolution, faster separation time, and improved sensitivity. The HPLC SEC method was scaled for chromatography using the ACQUITY UPLC Protein BEH SEC, 200 Å column. A flow rate of 0.885 mL min^{-1} was performed over a 3-min time frame. Equivalent resolution was obtained. Each separation was performed in triplicate.

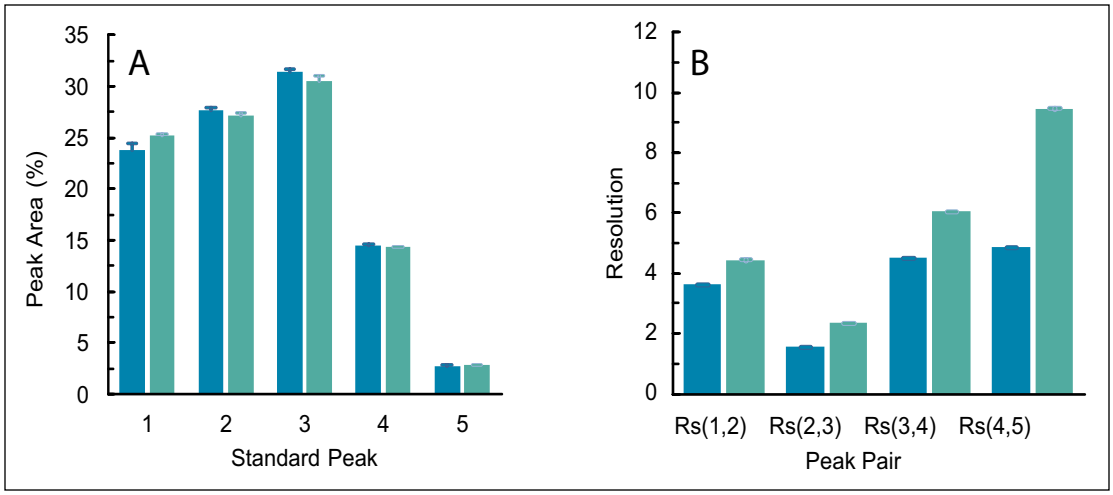

Figure 5. Comparative assessment of SEC-HPLC against SEC-UPLC. In each figure, dark blue columns refer to HPLC relative peak area measurements while light blue columns refer to UPLC measurements. (A) Measurement of the SEC200 protein standard mix where 1 represents thyroglobulin, 2 represents IgG, 3 refers to BSA, 4 represents myoglobin, and 5 represents uracil. (B) Measurement of the change in resolution between each peak pair of the SEC200 protein standard mix.

To determine what effect, if any, occurred on quantification in SEC, we separated the monoclonal antibody infliximab using the SEC-HPLC column and the SEC-UPLC column. In each SEC experiment, peaks corresponding to the monomer and dimer were integrated. As expected, a noticeable improvement in resolution was observed when separating infliximab in UPLC (Figure 6). Most importantly, the improvement in resolution did not affect the amount of aggregation quantified, where near identical relative peak areas for infliximab monomer and dimer were calculated on HPLC and UPLC. All chromatographic data has been reported in Table 2.

LC Mode	Peak	SEC component	Retention time			Peak area			Resolution		
			$\bar{x}$	σ	% RSD	$\bar{x}$	σ	% RSD	$\bar{x}$	σ	% RSD
HPLC	1	Thyroglobulin	12.65	0.001	0.008	23.79	0.638	2.683			
	2	IgG	16.64	0.002	0.010	27.64	0.260	0.940	3.63	0.003	0.072
	3	BSA	18.86	0.002	0.011	31.38	0.240	0.765	1.56	0.002	0.149
	4	Myoglobin	24.07	0.004	0.015	14.45	0.107	0.740	4.52	0.004	0.092
	5	Uracil	29.07	0.002	0.007	2.74	0.032	1.175	4.88	0.005	0.111
	1	Mab dimer	14.19	0.022	0.155	0.29	0.000	0.000			
	2	Mab monomer	17.18	0.001	0.006	99.71	0.000	0.000	1.91	0.014	0.733
UPLC®	1	Thyroglobulin	1.03	0.002	0.149	25.19	0.151	0.601			
	2	IgG	1.27	0.002	0.121	27.14	0.217	0.798	4.43	0.06	1.251
	3	BSA	1.42	0.001	0.081	30.52	0.482	1.580	2.36	0.02	0.757
	4	Myoglobin	1.75	0.001	0.066	14.30	0.084	0.586	6.06	0.00	0.038
	5	Uracil	2.23	0.002	0.078	2.85	0.032	1.127	9.43	0.04	0.452
	2	Mab dimer	1.10	0.002	0.188	0.48	0.006	1.57			
	3	Mab monomer	1.30	0.001	0.133	99.53	0.036	0.036	2.47	0.017	0.688

Table 2. Quantitative comparison of SEC-HPLC and SEC-UPLC performed on the ACQUITY UPLC H-Class Bio System. Retention time and peak area data represent the averaged data of triplicate analyses.

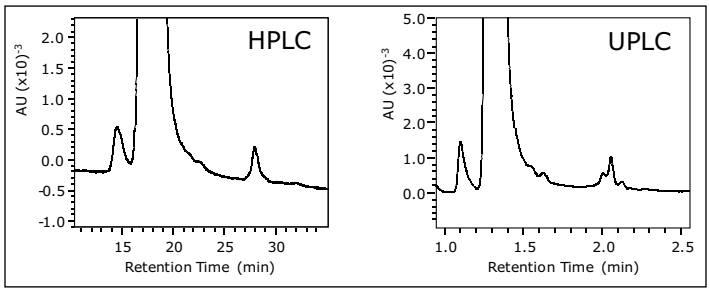

Figure 6. Improved resolution is observed when quantifying molecular species in UPLC. Comparison of peak resolution identified the presence of multiple species in UPLC that were unidentifiable in HPLC.

CONCLUSIONS

Size exclusion chromatography is a common method used for investigating the extent of aggregation in protein therapeutics. In this application note, we have demonstrated that legacy SEC-HPLC methods can be easily transferred to the ACQUITY UPLC H-Class Bio System without any modification to existing method details. With adjustments to flow rate and run time based on changes in particle size and column dimensions, SEC-UPLC can easily be performed with the same ACQUITY UPLC H-Class Bio System without any consequence to protein quantification requirements.

This permits QC labs to align LC technology and associated methods with development labs, while continuing to support legacy large-molecule assays currently deployed in the QC environment.

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Simultaneous Determination of Molecular Size, Concentration, and Impurity Composition of Biotherapeutics with SEC and the Biopharmaceutical Platform Solution with UNIFI

Robert Birdsall, Thomas E. Wheat, Henry Shion, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Efficient and integrated workflow for running batched samples to maximize analytical information content of biotherapeutics.
- Increased productivity through the automation of an analytical workflow including data acquisition, processing, and reporting.
- Increased productivity through automated assessment of protein aggregates.

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ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector with 5-mm titanium flow cell

Auto•Blend Plus™ Technology

ACQUITY UPLC Protein BEH SEC Column, 200Å, 1.7-µm

KEY WORDS

Size exclusion chromatography (SEC), monoclonal antibody (mAb), bioseparation, method development, quantification

INTRODUCTION

Size exclusion chromatography (SEC) is often used to assess the size distribution of molecular species for therapeutic proteins in a solution (*e.g.* protein clips, aggregates, etc.). The non-denaturing buffers commonly employed in SEC allow for the characterization of proteins in their native state. In addition to measuring molecular size, peak areas from SEC can be readily used in the relative and absolute quantitation of biological samples for increased productivity. As such, this technique has been particularly useful in the biotechnology industry for detecting and quantifying protein aggregation of biotherapeutics.

Protein aggregation in biotherapeutics have been linked to potential loss of therapeutic efficacy as well as unwanted immunogenic responses.[1,2] Controlling factors that contribute to aggregate formation, for example, protein misfolding during expression stages,[3] protein denaturation during purification processes,[1] and high protein concentration during formulation,[4] has been an area of continuing interest in the pharmaceutical industry.

Increasing demand from regulatory bodies to provide detailed information about the quantity and nature of aggregates in biotherapeutics, combined with rising development costs and a demanding work environment, require cost-effective solutions that have minimum impact on productivity. Efficient workflows that seamlessly combine characterization and quantitation information for biotherapeutics are highly desirable.

EXPERIMENTAL

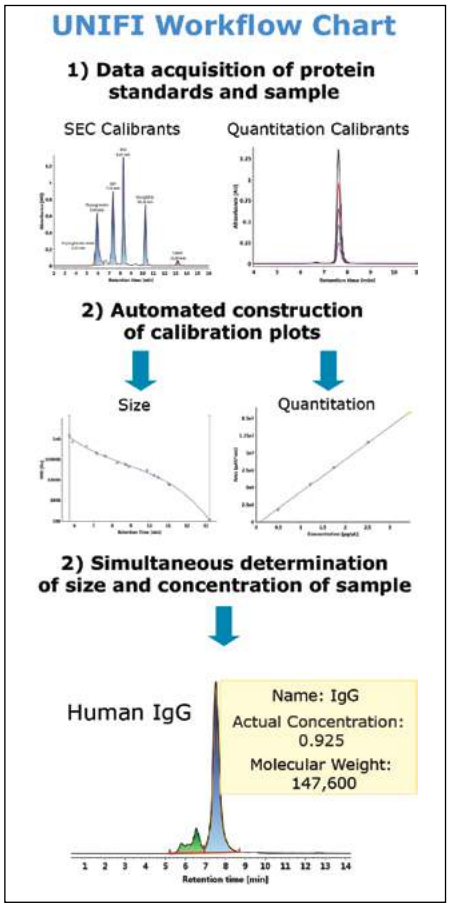
LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus Technology
Detector:	ACQUITY UPLC TUV
Absorption	Wavelength: 220 nm
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	ACQUITY UPLC Protein BEH SEC, 200Å, 1.7-µm, 4.6 x 150 mm (p/n 186005225)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	2 µL
Flow rate:	0.150 mL/min
Mobile phase A:	100 mM sodium phosphate monobasic monohydrate (NaH_2PO_4)
Mobile phase B:	100 mM sodium phosphate dibasic (Na_2HPO_4)
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Autoblend Plus Method:	Isocratic (150 mM NaCl in 20 mM phosphate buffer; pH 7.4)

Informatics for data collection and processing

UNIFI Scientific Information System, v1.6

The Waters Biopharmaceutical Platform solution with UNIFI is developed to streamline the analytical workflow to increase the productivity in the characterization of biotherapeutic samples. The ability to perform SEC using conditions that minimally perturb aggregate composition make it ideal in the assessment and communication of multiple attributes of biotherapeutics such as size, aggregate composition, and concentration. Through the use of calibrated standards, tools within the UNIFI Scientific Information System can simultaneously determine the molecular size (apparent molecular weight) and the concentration of chromatographically resolved species in a biotherapeutic sample in the same analysis.



The objective of this application note is to demonstrate the ability to determine molecular weight and amount of the constituents of an antibody sample using UNIFI informatics. A purified antibody from human serum was used as a model protein to test the application.

Protocol

A Waters ACQUITY UPLC Protein BEH SEC Column was conditioned as outlined by the manufacturer. Waters BEH200 SEC protein standards (p/n 186006518) and BEH125 SEC protein standards (p/n 186006519) were prepared in 1 mL and 0.2 mL of 18 MΩ water, respectively. Apoferritin (p/n A3660), β-amylase (p/n A8781), carbonic anhydrase (p/n C7025), insulin (p/n I0516), sodium phosphate monobasic monohydrate (p/n S3522), sodium phosphate dibasic (p/n S5136), and sodium chloride (S5886) were purchased from Sigma Aldrich. The Waters Glycoworks control standard (p/n 186007033; purified human IgG) was used as an "unknown" and prepared at a concentration of 1 µg/µL as per the labeled amount using 18 MΩ water. Apoferritin, β-amylase, carbonic anhydrase, and insulin were prepared at concentrations of 10 µg/µL, 2.9 µg/µL, 1.5 µg/µL, and 5.0 µg/µL, respectively. The Waters mAb mass check standard (p/n 186006552) used for quantification was prepared at concentrations of 0.49 µg/µL, 1.22 µg/µL, 1.74 µg/µL, 2.51 µg/µL, and 3.46 µg/µL in 18 MΩ water.

RESULTS AND DISCUSSION

Integrated informatics tools for the construction of calibration plots

Size exclusion chromatography is often employed by the pharmaceutical industry for the assessment of aggregate content in biotherapeutic samples. In principle, the elution time of a protein in a SEC separation is determined by how much of the intra-particle pore volume is accessible to the protein.[6] In practice, this separation mechanism prescribes that protein species will elute in order of decreasing hydrodynamic radius. This chromatographic behavior is illustrated by the lower left panel of Figure 1, where Waters SEC protein standards (calibrants) are separated using an ACQUITY UPLC Protein BEH SEC Column (200Å, 1.7-µm, 4.6 x 150 mm).

Using the known molecular weight of the calibrants defined in the component manager as shown in the top panel of Figure 1, the built-in UNIFI informatics tool automatically constructs a size calibration plot (log MW *vs.* RT) for the standards as shown in the lower right panel of Figure 1. Proteins used in this optimized size calibration plot include thyroglobulin dimer, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, ribonuclease A, insulin, and uracil. The calibration plot can be constructed using the logarithmic scale of MW of protein standards as y-axis plotted against either the elution time or the elution volume (x-axis). The data is automatically fitted with a linear or a higher-order polynomial equation to acquire a calibration curve as shown in Figure 1.

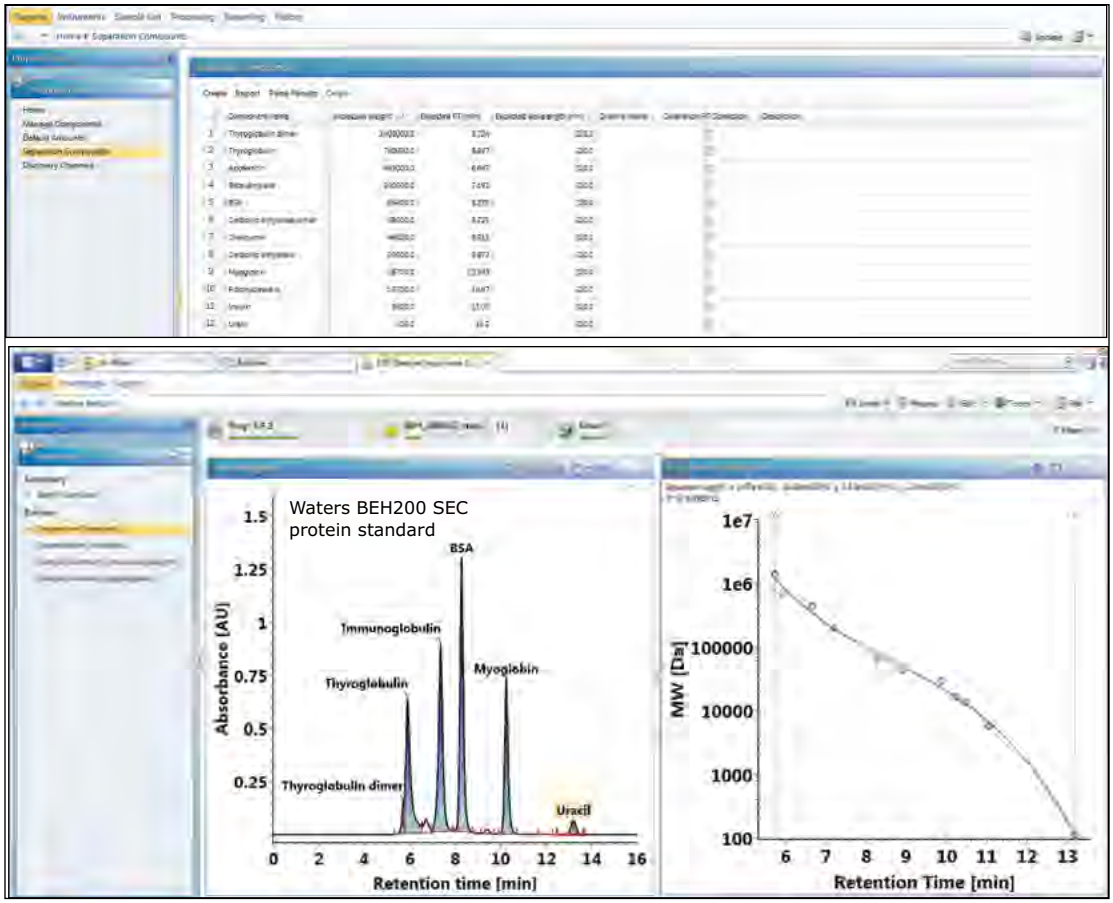


Figure 1. Waters BEH200 SEC protein standards (p/n 186006518) are separated (bottom left panel) using an ACQUITY UPLC Protein BEH SEC Column (p/n186005225). A separation calibration plot of log MW versus retention time (lower right panel) is automatically constructed using UNIFI informatics tools from a set of defined protein standards (top panel).

For disparate measurements such as apparent molecular weight (elution time vs. molecular weight) and concentration (area vs. amount), individual assays that generate targeted data sets are usually required. UNIFI informatics allows for the incorporation of multiple calibration plots that can be applied to a single data stream for the measurement of such dissimilar attributes as size and concentration.

Figure 2 illustrates how UNIFI constructs a concentration calibration curve in the same analysis as the apparent molecular weight measurement using a Waters mAb mass check standard as a calibrant for proof of principle. Using the mAb standard, 200 µL of 18MΩ water was added to the 1 mg of lyophilized protein to generate a stock calibrant solution with a concentration of 5.00 µg/µL. From the stock calibrant, five standard samples were prepared at concentrations of 0.493 µg/µL, 1.22 µg/µL, 1.74 µg/µL, 2.51 µg/µL, and 3.46 µg/µL. Three replicates of the five standard samples were performed in a serial fashion with a constant volume (2 µL) injected on an ACQUITY UPLC Protein BEH SEC Column (200Å, 1.7-µm, 4.6 x 150 mm). Using the defined concentrations as indicated by their concentration level in the component summary window of Figure 2, UNIFI automatically constructs the concentration calibration plot as shown in the bottom right panel of Figure 2.

The ability to automate the construction of multiple calibration plots and apply them in a single data stream to discern uniquely disparate critical quality attributes makes the Waters Biopharmaceutical Platform Solution with UNIFI a preferred system for increasing productivity during the method development process.

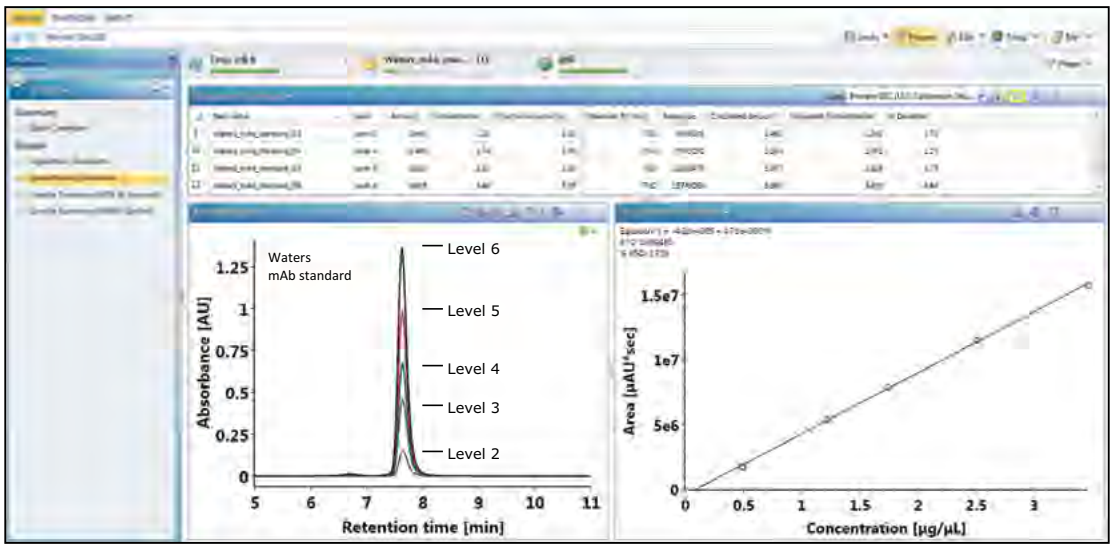


Figure 2. The Waters mAb mass check standard was injected at five different concentration levels (lower left panel). Using the supplied concentrations as indicated by their level in the component summary panel, UNFI informatics automatically constructs a calibration plot of peak area versus sample concentration (bottom right panel).

Simultaneous determination of apparent molecular weight and concentration of purified human IgG from a single injection

The experimental results for the calibration plots show that as an integrated platform, UNIFI is fully capable of determining the apparent molecular weight and concentration of biotherapeutics analyzed in the same sample set as the protein standards. A purified human IgG sample (p/n 186007033) was analyzed using the method to demonstrate the capability of the platform to generate such information for an "unknown" sample. The lyophilized sample was reconstituted in 100 µL of 18 MΩ water to a concentration of 1 µg/µL. Three replicate injections of the sample were analyzed within the same sample set of the calibration standards as shown in the bottom left pane of Figure 3. For each run, 2 µL of the sample was injected.

At the end of the analysis workflow, UNIFI automatically reports the calculated concentration (µg/µL), amount (µg), and molecular weight (Da) of the parent peak or monomer peak of the human IgG sample as shown in the upper component summary pane of Figure 3. Using the data from the component summary pane, the mean concentration and apparent molecular weight of the human IgG parent peak were calculated to be 0.93 µg/µL ± 0.01 µg/µL and 147,600 Da ± 100 Da, respectively. The ability to automatically determine apparent molecular weight and concentration of a sample within a single injection confirms that Waters Biopharmaceutical Platform with UNIFI is an integrated solution for increasing productivity and maximizing characterization content for the analysis of biotherapeutics.

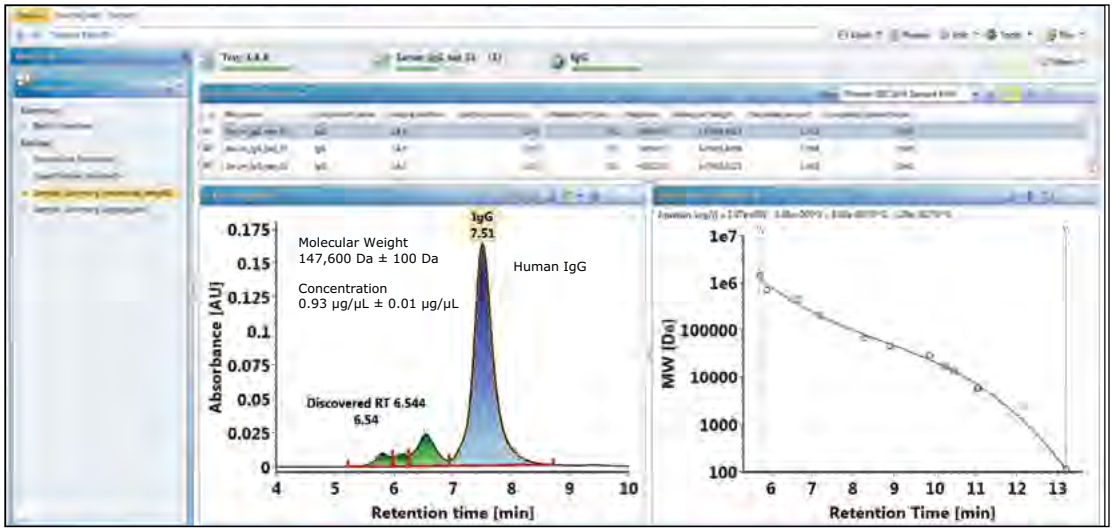
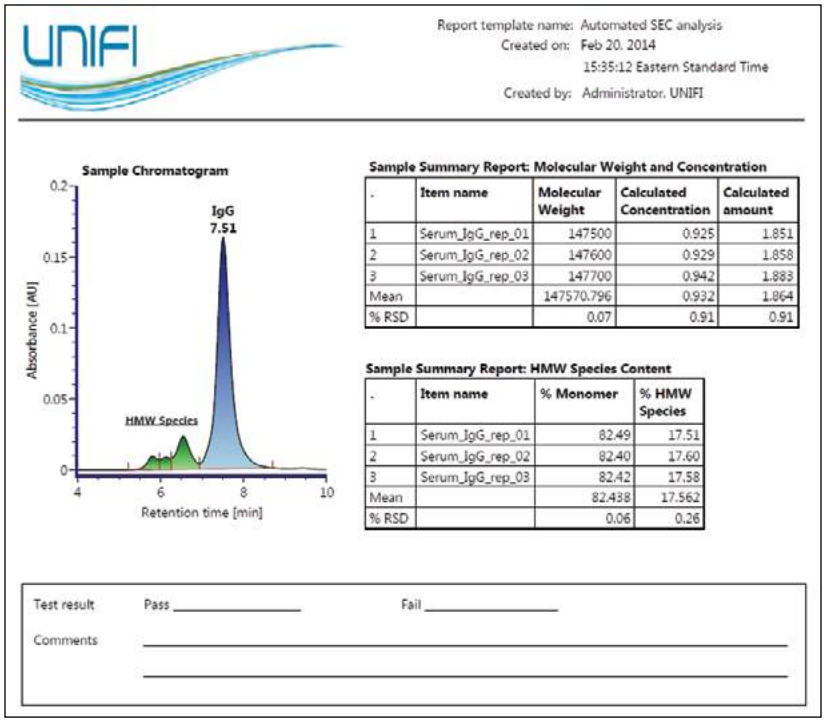


Figure 3. UNIFI reports the concentration, amount, and molecular weight (top pane) of a human IgG parent peak (lower left pane) using the calibration plots constructed from reference standards (lower right pane).

Bioinformatics tools for automated reporting of SEC characterization of biotherapeutics



.	Item name	Molecular Weight	Calculated Concentration	Calculated amount
1	Serum_IgG_rep_01	147500	0.925	1.851
2	Serum_IgG_rep_02	147600	0.929	1.858
3	Serum_IgG_rep_03	147700	0.942	1.883
Mean		147570.796	0.932	1.864
% RSD		0.07	0.91	0.91

.	Item name	% Monomer	% HMW Species
1	Serum_IgG_rep_01	82.49	17.51
2	Serum_IgG_rep_02	82.40	17.60
3	Serum_IgG_rep_03	82.42	17.58
Mean		82.438	17.562
% RSD		0.06	0.26

Figure 4. A report template example created by UNIFI. The calculated molecular weight concentration, amount, as well as the relative higher molecular weight species content is shown in the report for the IgG sample.

UNIFI integrates strong reporting functionalities with the ability to generate meaningful analytical measurements to form a seamless informatics workflow. These informatics tools allow for custom reports to be automatically generated for the efficient communication and cataloging of analytical results. Report templates can be readily constructed and customized for assessment of analysis results.

Figure 4 is an example of a report template designed for SEC characterization of biotherapeutics such as monoclonal antibodies. Using the results for the purified IgG sample from Figure 3, a summary report on the apparent molecular weight, concentration, and relative amount of the parent peak (monomer) and corresponding statistical evaluation (*e.g.* mean and % R.S.D) is generated after data acquisition and processing. In addition, pertinent biotherapeutic information on the level of relative aggregation in each sample is also assessed and reported as a percentage of monomer and the percentage of higher molecular weight (HMW) species (collectively).

The flexibility to design custom report templates based on analysis needs makes the Biopharmaceutical Platform Solution with UNIFI a powerful integrated system for the acquisition, processing, and reporting of analysis results.

CONCLUSIONS

Assessing and controlling aggregate content in therapeutic proteins is a critical component in the manufacturing process. Increasing sample complexity coupled with the rising research and development costs highlight the need for more efficient analytical methods that are readily deployable in therapeutic protein characterization. Waters' Biopharmaceutical Platform Solution with UNIFI offers answers to these challenging problems.

The efficient built-in workflow aided by the UNIFI's data acquisition, processing, and reporting capabilities enable simultaneous determination and reporting of the apparent molecular weight, concentration, and aggregate composition of biotherapeutic proteins. This process, which is fully automated, makes the Biopharmaceutical Platform Solution with UNIFI ideal for increasing productivity through efficient method deployment for biotherapeutic characterization.

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IEX for Charge Variants

The characterization of proteins frequently requires use of orthogonal analytical techniques so that the structure of a specific biotherapeutic can be more confidently defined and reported.

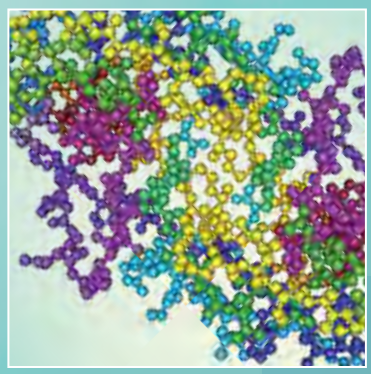
Ion-exchange chromatography (IEX) is often utilized to analyze biotherapeutic proteins because of its ability to separate protein charge variants formed by post-translational modifications. The charge heterogeneity of a monoclonal antibody may be caused by several structural changes, such as deamidation of asparagine or glutamine residues and partial removal of C-terminal lysine residues. These modifications are monitored throughout manufacturing to ensure process stability and ensure quality control.

IEX is based on the reversible interaction between a charged protein and an oppositely charged ligand contained on the surface of the chromatography medium. Biomolecules with even small differences in net charge can be resolved by choosing the optimal ion-exchange stationary phase and separation conditions, notably pH, ionic strength, and temperature.

Solution highlights

Since each protein has a unique charge distribution, chromatographic selectivity can only be obtained through method optimization. For IEX analyses, mobile phase pH is the most useful parameter for method development. Such experiments are, however, time-consuming and cumbersome, making it sometimes difficult to develop a robust, adequately resolving method.

The **ACQUITY UPLC H-Class Bio System** is an inert system that provides wetted surfaces that are chemically stable in the aqueous, high ionic strength buffers common to IEX separations. In addition, **Auto•Blend Plus Technology**, enabled in the instrument control software, makes it possible to screen multiple IEX buffer system by allowing for simplified pH, ionic strength, or simultaneous pH and ionic strength gradient control.



Waters Protein-Pak Hi Res IEX Columns can assist in the UPLC characterization of recombinant protein therapeutics. With these columns, an analyst can use the Waters **IEX Cation and Anion Test Standards** to perform column benchmarking and to monitor system performance.

These tools translate to savings in both time and reagent costs, increasing overall productivity.

Charge Variant Analysis of Therapeutic Monoclonal Antibodies Using a pH Gradient Generated by Auto•Blend Plus

Robert Birdsall and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increased productivity through the automation of analytical method parameter evaluations
- Thorough method development for charge separation using pH gradients for confirmation and quantification of biotherapeutic charge variants
- Efficient pH or ionic strength screening using concentrated stocks

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP SCX Column

KEY WORDS

Auto•Blend Plus,™ cation exchange, antibody, IEX, SCX, bioseparation, therapeutic protein, method development, pH gradient, UPLC

INTRODUCTION

Charge-based separation methods play an important role in characterization studies and quality control strategies for biotherapeutics.[1-4] For the analysis of charged species of antibodies, ion exchange chromatography (IEX) has a widespread use in the biopharmaceutical industry for its ability to resolve species related to protein conformation, size, sequence variants, glycosylation, and post-translational modifications. The capability to perform protein characterization under non-denaturing conditions combined with the ability to isolate charge variants easily has both contributed to the popularity of IEX in charge variant analysis of biotherapeutics.

Protein separations by IEX methods routinely utilize salt (ionic strength) or pH gradients to elute the protein from the IEX column. Although somewhat different in the separation mechanisms of the two eluting methods, method parameters such as column types, mobile phase composition, and pH (or salt concentration) gradients often need to be evaluated to yield the optimal separation for each individual antibody.[5] However, the evaluation of the selected method parameters often requires a time consuming, iterative process that involves preparing and testing discrete buffers of varying compositions. This requirement imposes a great challenge to the method development process, and calls for an intelligent setup/process that decreases time spent on method development, and improves the efficiency of the workflow.

Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class System's quaternary solvent manager to blend individual pure solutions and concentrated stocks from the reservoirs to deliver pH gradients for the separations of charge variants in therapeutic proteins. The technology allows the analyst to evaluate multiple buffer compositions from the concentrated stocks; enabling the evaluation process to be easily automated to increase the productivity of charge variant analysis. Auto•Blend Plus Technology allows for the use of mixed buffers with different pka values to increase the buffering capacity and extend the effective working pH range that a single buffering species cannot deliver.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class System with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm (p/n 186004930)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	3 µL
Flow rate:	0.50 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM sodium phosphate dibasic
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Buffer concentration:	20 mM
Gradient:	pH 5.2 to 7.9 in 30 minutes (Figure 2); pH 6.5 to 7.2 in 15 minutes (Figure 6)

Informatics for data collection & processing

UNIFI Scientific Information System, v 1.6

In addition to these benefits, the unique design of Auto•Blend Plus Technology provides the flexibility to allow analysts to switch between pH or salt gradients during the method development process for the determination of optimal separation parameters. The objective of this application note is to demonstrate the performance of Auto•Blend Plus for optimizing IEX methods for charge variant separations using pH gradients. A therapeutic monoclonal antibody, infliximab, was used as a model protein to evaluate the functionality.

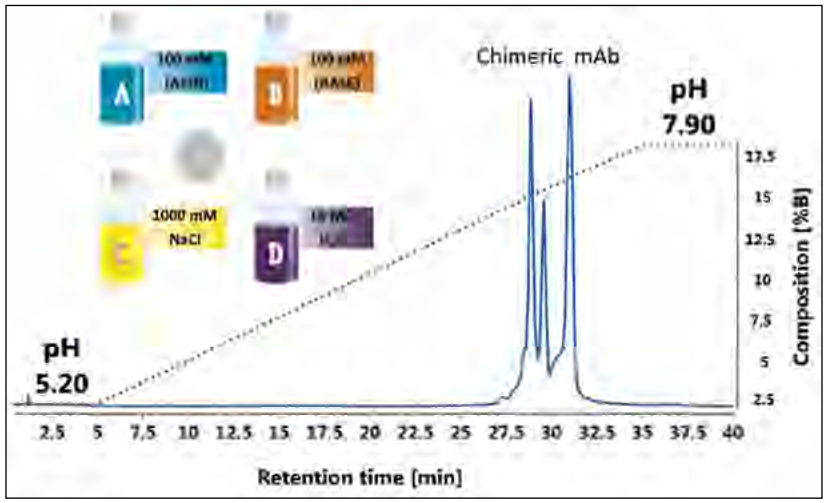


Figure 1. Automated delivery of an extended pH gradient range by the Auto•Blend Plus Technology. The chromatogram shows a gradient spanning from pH 5.20 to 7.90 is formed for the separation of lysine truncation charge variants of a chimeric monoclonal antibody (infliximab).

Sample Preparation

A Waters Protein-Pak Hi Res SP, strong cation exchange column (7 µm, 4.6 x 100 mm, p/n 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (p/n A69892), sodium phosphate dibasic (p/n S5136), and sodium chloride (p/n S1679) were purchased from Sigma Aldrich. The pH gradients generated by Auto•Blend Plus were monitored on-line using a GE Healthcare Monitor pH/C-900 similar to previous work (p/n 720004149en). Calibration was performed at flow rates of 1 mL/min with the column off-line using the reference pH values from the empirical table data. The mAb samples evaluated in this study were used as received for all experiments at a concentration of 20 µg/µL.

RESULTS AND DISCUSSION

Flexible method development with the Auto•Blend Plus

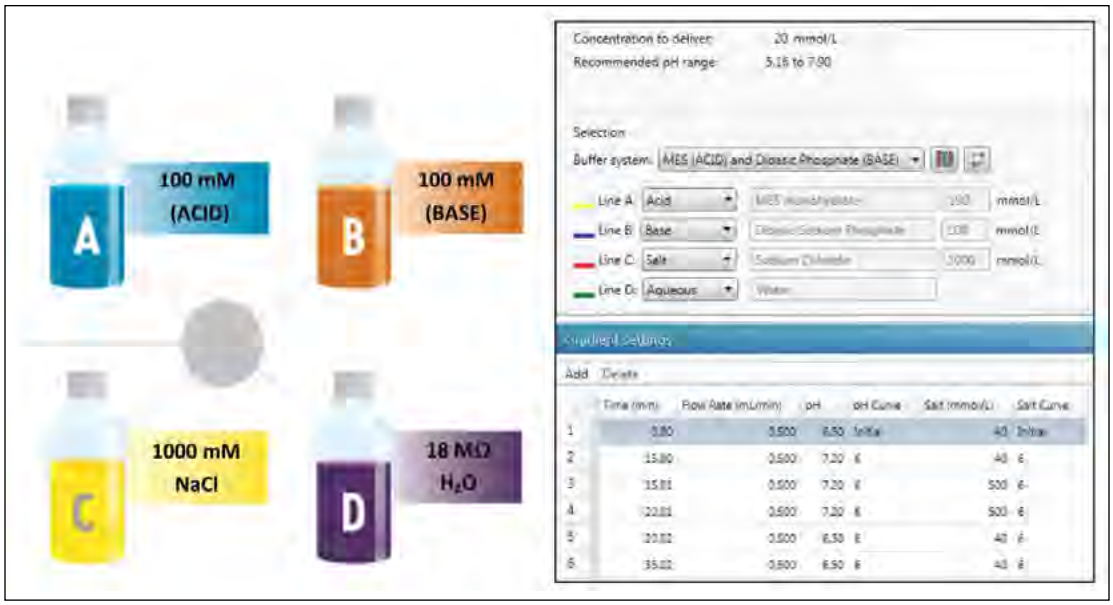


Figure 2. An example of the intuitive interface for programming pH or salt gradients rendered by the Auto•Blend Plus Technology. The software algorithm takes in the specified values and automatically calculates the percentage of acid and base required to deliver the desired pH gradient range and ionic strength.

Figure 2 shows a pH gradient table that Auto•Blend Plus Technology is programmed to generate using pure solutions and concentrated stocks on a quaternary solvent management system. The gradient table presents an easy-to-use interface to the end user, where the gradient is expressed directly in terms of pH and ionic strength. The software algorithm can independently control pH or ionic strength enabling the analyst to generate a variety of gradient conditions including constant pH with varying ionic strength, vice versa, or change pH and ionic strength simultaneously. With Auto•Blend Plus, the software automatically calculates the percentage of acid and base required for each pump stroke to deliver the specified pH using the chosen buffer system. Auto•Blend Plus allows for multiple buffer compositions to be mixed and evaluated from a single set of pure components, reducing cost and time in method development.

Increasing productivity with custom buffer systems

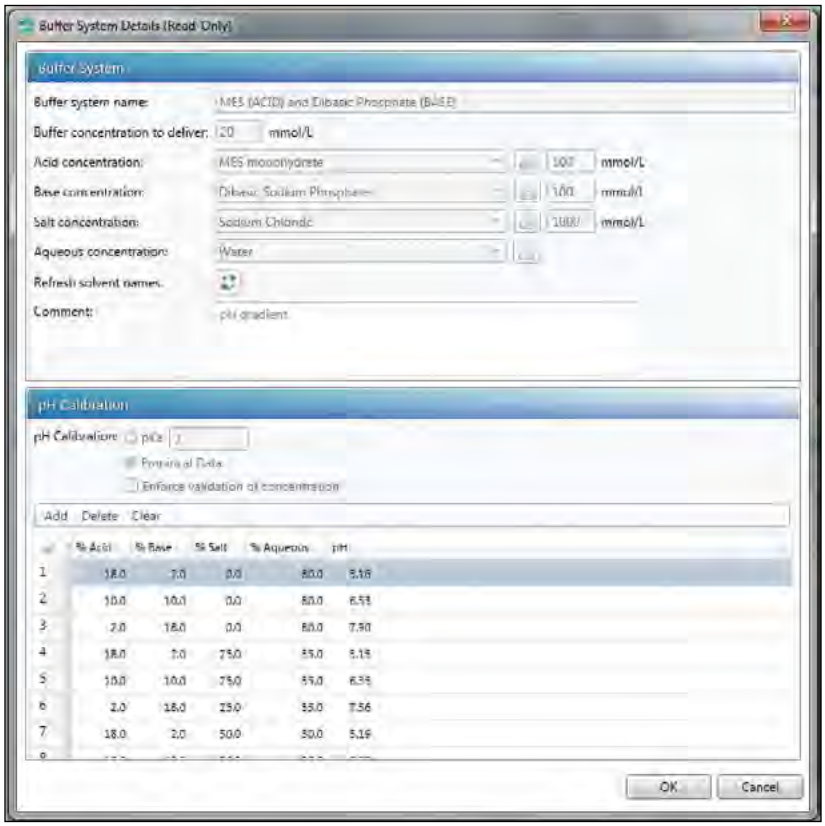


Figure 3. An example of the empirical table used by Auto•Blend Plus. Nine buffer mixture standards were used to create the exemplary reference table from which Auto•Blend Plus is based to automatically deliver a pH range over 5.2 to 7.9.

Traditional ion exchange chromatography employs buffers comprised of the same molecular species such as MES buffer (pH 5.5 to 6.7), phosphate buffer (pH 6.7 to 7.6), and HEPES buffer (pH 7.6 to 8.2). The limited working pH ranges of these individual buffer systems prolong the method development process for a separation based on pH gradients since multiple buffers need to be prepared and tested to optimize the separation performance over the entire pH range.

Auto•Blend Plus Technology allows for the preparation of custom buffers with an extended working pH range through the use of the empirical calibration table as shown in Figure 3. For this work, a 100 mM solution of 2-ethanesulfonic acid monohydrate (MES monohydrate) was prepared as the acidic reservoir and a 100 mM solution of sodium phosphate dibasic was prepared as the basic reservoir as illustrated in Figure 2. The empirical table was constructed from nine buffer mixture standards prepared from the concentrated stocks. The pH value was measured with a pH meter and entered into the table as shown in Figure 3. The use of MES monohydrate and sodium phosphate dibasic as an ion exchange buffer system gives an extended working pH range of 5.2 to 7.9, allowing for a larger set of experimental parameters to be tested from a single set of buffers.

The ability to generate extended pH ranges from concentrated stock buffers via Auto•Blend Plus without the need for multiple buffer systems makes it ideal for increasing productivity and reducing method development costs.

Linear pH gradients with Auto•Blend Plus

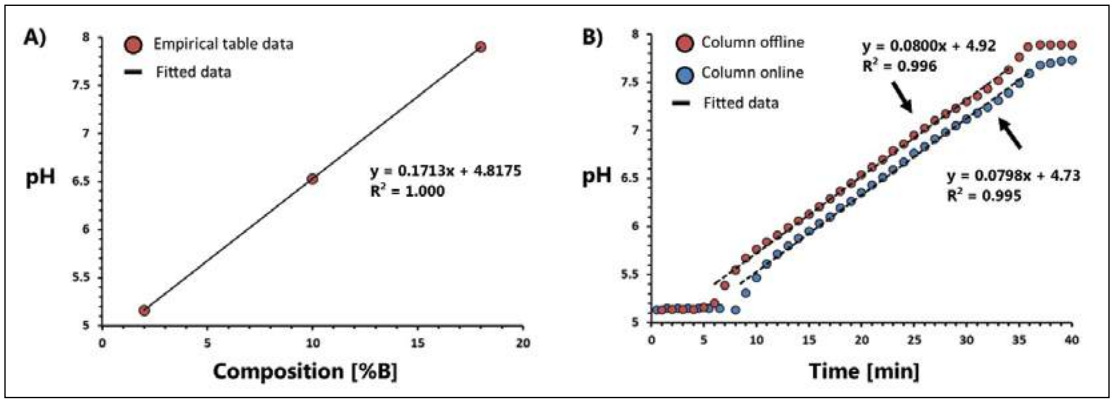


Figure 4. Using the A) empirical table data, Auto•Blend Plus was programmed to generate a 30 minute gradient from 5 to 35 minutes with a pH value ranging from 5.20 to 7.9. B) The generated gradient was evaluated for pH linearity in the presence or absence of the IEX column in the LC system. Each point in the figure was acquired using an on-line pH meter for the buffer composition (gradient) generated by Auto•Blend Plus.

The fidelity of a pH gradient generated by Auto•Blend Plus was assessed for its ability to produce a designed linear pH gradient over an extended pH range using a selection of buffer systems. For illustrative purposes, a plot of pH versus composition %B (% Base) was constructed from the empirical table data shown in Figure 3 to assess pH response linearity for the selected buffer system. From Figure 4A it can be seen that the empirical data for the chosen buffer system has a linear response over a pH range of 5.2 to 7.9. Auto•Blend Plus was programmed to generate a 30-minute gradient over a pH range of 5.2 to 7.9 starting at the 5-minute mark using the empirical table data from Figure 3. The programmed gradient was evaluated with or without the Waters Protein-Pak Hi Res SP, a strong cation exchange column, online so the impact of column effects on the variation of pH linearity could also be assessed.

Similar to a method from previous work[6] the mobile phase pH was monitored in-line using a GE Healthcare monitor pH/C-900 with pH being manually recorded in 1 minute intervals. From Figure 4B it can be seen that Auto•Blend Plus is capable of delivering a highly linear pH gradient over the gradient time window using the extended pH buffer system. The close agreement of the slopes of the fitted data on both plots indicates the column had no significant effect on the linearity of the pH gradient other than a small time delay due to the additional volume introduced by the column. The ability to deliver an extended linear pH gradient using custom buffer selections makes Auto•Blend Plus well suited for flexible method development.

Automation for pre-screening experimental method parameters

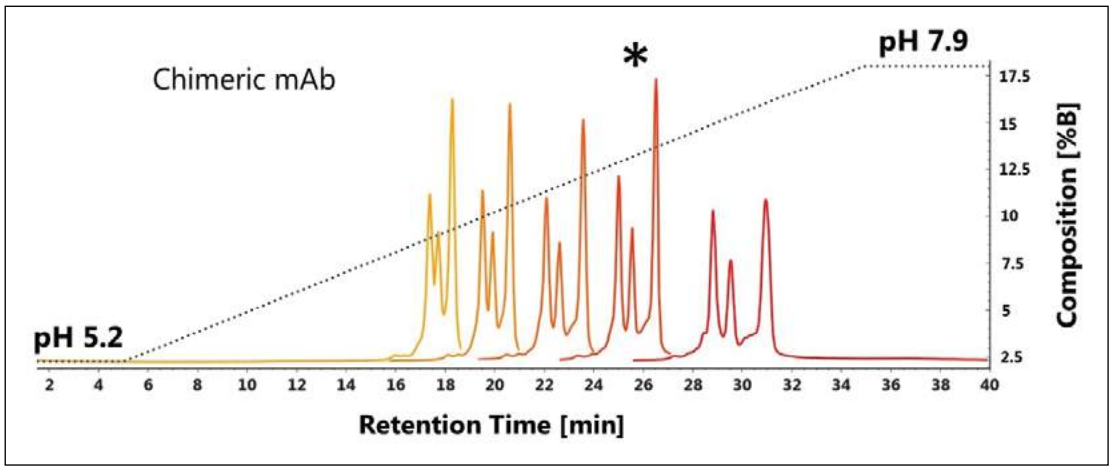


Figure 5. Optimization of ionic strength in the separation of lysine charge variants of a chimeric monoclonal antibody. From right to left, the ionic strength tested for each chromatogram was 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM NaCl. The ionic strength of 40 mM (denoted by the asterisks) provides the highest resolution between the main lysine variant peaks.

Method development of IEX separations often involves a time-consuming trial and error procedure because a successful separation cannot be readily predicted. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing each buffer system for separation performance until an adequate separation is achieved. The ability of Auto•Blend Plus Technology to blend multiple buffer compositions from a single set of concentrated stocks allows for evaluation of many experimental parameters in a highly efficient manner.

For example, it is well known that ionic strength affects separation performance, and that it should be evaluated in the optimization process. Figure 5 shows how Auto•Blend Plus Technology was used to evaluate the impact of ionic strength on a mAb charge separation with the extended pH buffer range prepared earlier. From the concentrated stocks the ionic strength (line C) was increased in 20 mM intervals for each chromatographic trace shown in Figure 5, starting with 20 mM on the right side of the plots.

Using the chromatographic peak that represents the charge variant of the monoclonal antibody containing two C-terminal Lysine residues as the investigative target (see Figure 6), we systematically evaluated the impact of ionic strength on the charge separation performance during our experiments. Resolution for the +2 Lys peak was reported as 2.66, 2.70, 2.33, 1.77, and 1.28 corresponding to the ionic strengths of 20 mM, 40 mM, 60 mM, 80 mM, and 100 mM, respectively. This automated evaluation process renders an efficient and consistent way to find the optimized ionic strength for the pH gradient slope, and demonstrates that Auto•Blend Plus is an integrated software solution designed to streamline method development.

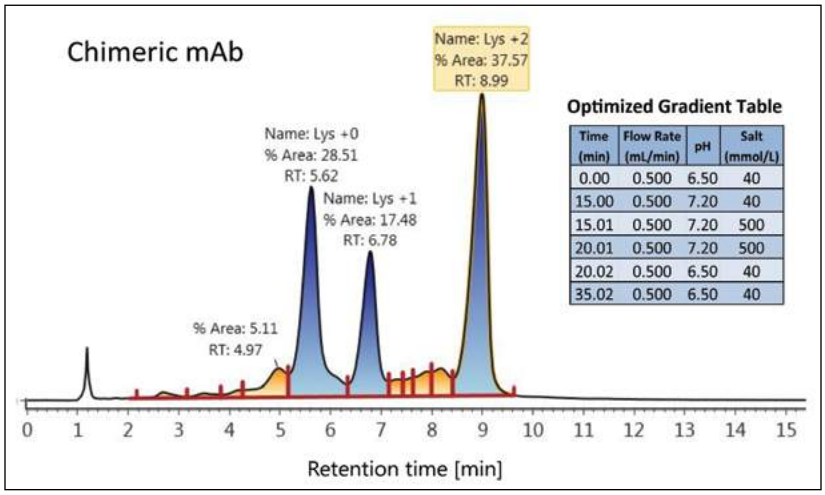


Figure 6. An optimized high throughput pH gradient separation of a chimeric monoclonal antibody in under 10 minutes. The pH gradient was optimized from pH 6.50 to 7.20 with ionic strength constant at 40 mM NaCl. Identification and integration of the C-terminal lysine variants were performed using tools within UNIFI Software and automatically displayed in the chromatogram window.

Integration with advanced informatics for automated data acquisition, processing, and reporting

The Waters Biopharmaceutical Platform Solution with UNIFI provides a highly efficient analytical platform solution for routine characterization of biotherapeutics. Auto•Blend Plus Technology combines with other informatics tools within UNIFI Software to deliver an integrated workflow to perform charge-based separation and optimization using pH gradients. This workflow possesses the capability to automatically acquire data, process the data and provide a report, further enhancing and streamlining the method development process. Utilizing the informatics tools from UNIFI, further optimization of the pH gradient was performed.

Examination of the separation results from Figure 5 suggests that the pH gradient range that would effectively deliver the same separation performance observed at 40 mM NaCl would only require a portion of the 40-minute run time. Since the pH gradient curve generated by Auto•Blend Plus Technology follows a predictable mathematical equation, the pH value at any point along the gradient can be readily calculated and changed so equivalent separation performance can be achieved with shorter analysis time.

Using this methodology, the separation achieved in Figure 5 at an ionic strength of 40 mM was further optimized with the elution of the full charge variant profile of the chimeric monoclonal antibody within 10 minutes as shown in Figure 6. Relative peak area, retention time, and component name of the chromatographic peaks of interest were automatically calculated and labeled by the UNIFI Software. Quick delivery of the information by UNIFI to evaluate the separation performance is time-saving and promotes the method development process.

CONCLUSIONS

The development of methods for the analysis of biopharmaceutical charge heterogeneity profiles is a time-consuming process that requires methods that can be automated, quickly adapted, and readily deployed to meet the demands of the biopharmaceutical industry. The combination of Auto•Blend Plus Technology with the ACQUITY UPLC H-Class System improves workflow efficiency by allowing multiple buffer compositions to be tested from a single set of pure components. The flexibility to work with pH or salt gradients, combined with the ability to automate, makes Auto•Blend Plus Technology a powerful tool for increasing productivity and reducing development costs.

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Developing Robust and Efficient IEX Methods for Charge Variant Analysis of Biotherapeutics Using ACQUITY UPLC H-Class System and Auto•Blend Plus

Robert Birdsall, Thomas Wheat, and Weibin Chen
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APPLICATION BENEFITS

- Increased productivity through the automation of analytical techniques
- Robust method development that delivers consistent and reproducible results for confirmation and quantification of biotherapeutic charge variants
- Reproducible and simplified method development without the need to prepare additional buffers

WATERS SOLUTIONS

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP SCX Column

UNIFI Scientific Information System

KEY WORDS

Auto•Blend Plus™ Technology, cation exchange, antibody, IEX, SCX, chromatography, bioseparation, protein, method development, robustness

INTRODUCTION

Charge variant analysis is critical for characterizing and monitoring quality attributes of therapeutic proteins. Protein modification such as deamidation, N-terminal pyroglutamation, isomerization, sialylated glycans, and C-terminal lysine clipping all contribute to charge variant formation.[1] In some cases, such changes affect binding, biological activity, patient safety, and shelf lifetime of therapeutic proteins.

The biopharmaceutical industry relies on tools such as ion exchange chromatography (IEX) and isoelectric focusing (IEF) gel electrophoresis to characterize charge variants. Ion exchange chromatography has been particularly useful in the development of biotherapeutics due to its ease of use, wide applicability, and high resolution.

In-depth characterization of charge heterogeneity of therapeutic proteins from the biopharmaceutical development process requires robust and efficient IEX methods. Method development involves a thorough evaluation of all possible experimental parameters such as buffer/ionic strength, buffer pH, salt gradient, flow rate, and column temperature. However, systematic evaluation on the impact of individual experimental parameters on the separation performance often requires a time-consuming and iterative process that involves preparing and testing discreet buffers of varying composition.

Variation in buffer preparation can lead to inconsistent results, consequently increasing method development time. Waters Auto•Blend Plus Technology takes advantage of the ACQUITY UPLC H-Class System's quaternary solvent management capabilities, and uses pure solutions and concentrated stocks to address these challenges. Calculation of the percentage of each stock to blend to achieve the desired pH is performed by the Auto•Blend Plus Technology, reducing error, consumable use, and development time.

With such integrated features, the Biopharmaceutical Platform Solution with UNIFI is well suited for robust method development and can be easily automated for increased productivity. The objective of this application note is to demonstrate the efficiency and robustness of Auto•Blend Plus Technology for optimization of an IEX method for charge variant separations. A chimeric monoclonal antibody, infliximab, was used as a model therapeutic protein to showcase the application.

EXPERIMENTAL

Sample description

A Waters Protein-Pak Hi Res SP, strong cation exchange column (4.6 x 100 mm, 7 µm, P/N 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (P/N AC327761000), MES sodium salt (P/N AC397351000), sodium chloride (P/N S640-500) were purchased from Fisher Scientific. The chimeric mAb evaluated in this study was used as received for all experiments at a concentration of 20 µg/µL.

LC conditions

LC system:	ACQUITY UPLC H-Class with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit
Column:	Protein-Pak Hi Res SP, 4.6 x 100 mm, 7 µm
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	3 µL
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Buffer conditions:	20 mM MES, pH 6.8
Gradient:	25 mM to 65 mM NaCl in 25 minutes (see Figure 2)

Informatics for data collection & processing

UNIFI Scientific Information System, v 1.6

RESULTS AND DISCUSSION

Auto•Blend Plus Technology

Method development of ion exchange chromatography (IEX) techniques often involves a time-consuming trial and error methodology. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing of each buffer system until an adequate separation is achieved.

The Auto•Blend Plus Technology system is integrated software that comes standard with an ACQUITY UPLC H-Class System. It is designed to take the guesswork out of method development and increase productivity in the analysis of charge variants. Auto•Blend Plus helps analysts configure the quaternary solvent management system to blend pure solutions and concentrated stocks to achieve a desired gradient (Figure 1). The end user is presented with an easy-to-use gradient table interface, where the gradient is expressed directly in terms of pH and ionic strength. The software automatically calculates the percentage of acid and base required for the specified pH using the known pK_a value of the chosen buffer system or an empirical calibration table (Figure 2).

Auto•Blend Plus Technology allows for multiple buffer compositions to be tested from a single set of pure components and can be easily automated to improve productivity.

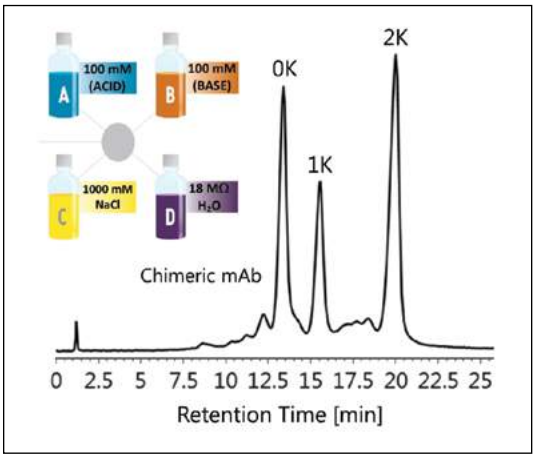
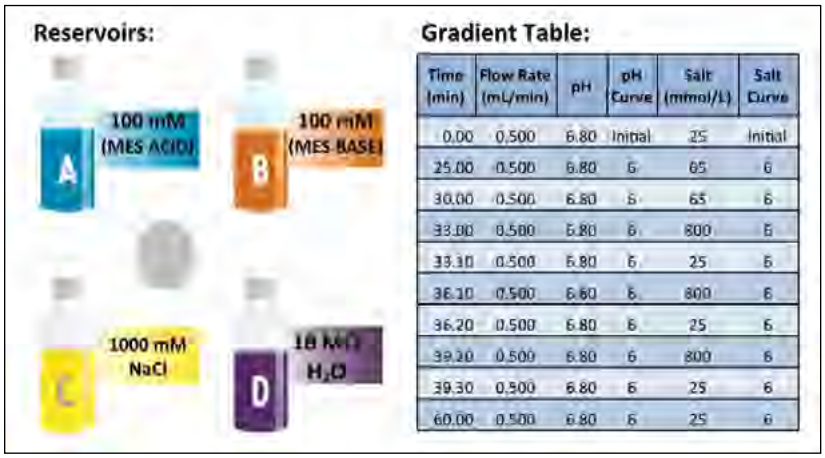


Figure 1. Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class quaternary solvent manager to blend individual pure buffers from the reservoirs to deliver robust separations of charge variants in therapeutic proteins. Here, it is used in the separation of C-terminal lysine truncation variants in a chimeric monoclonal antibody.



Time (min)	Flow Rate (mL/min)	pH	pH Curve	Salt (mmol/L)	Salt Curve
0.00	0.500	6.80	Initial	25	Initial
25.00	0.500	6.80	6	65	6
30.00	0.500	6.80	6	65	6
33.00	0.500	6.80	6	800	6
33.10	0.500	6.80	6	25	6
36.10	0.500	6.80	6	800	6
36.20	0.500	6.80	6	25	6
39.20	0.500	6.80	6	800	6
39.30	0.500	6.80	6	25	6
60.00	0.500	6.80	6	25	6

Figure 2. Illustration of a typical Auto•Blend Plus Technology reservoir setup with accompanying gradient table for separation of a chimeric monoclonal antibody.

Robust method development

Robustness is a measure of the ability of a separation method to maintain reproducible results with the introduction of small changes in the system. For ion exchange chromatography, these parameters can include pH, protein mass load, and reproducibility. For pharmaceutical companies a robust method can increase productivity with less time spent on method validation. These parameters were explored to evaluate the robustness of method development using the Auto•Blend Plus Technology.

Protocol for validating and qualifying Auto•Blend Plus

Auto•Blend Plus Technology enables easy system validation and qualification when transferring methods between instruments, analysts, and labs.

Three separate MES buffer systems were prepared and tested using the outlined protocol, below. From Table 1, it can be readily seen that the experimental pH from each buffer system is in good agreement with the desired test pH. The precision among the three separate buffer systems results in reproducible chromatograms as shown in Figure 3. Auto•Blend Plus Technology can readily be adapted to qualification protocols, minimizing time spent on system validation.

Install solutions

- A: 100 mL of 1.0 M MES monohydrate in 900 mL HPLC grade H_2O
- B: 100 mL of 1.0 M MES sodium salt in 900 mL HPLC grade H_2O
- C and D: HPLC grade H_2O

Cross-calibrate pH meter

- Low pH reference: Mix 1.8 mL from A, 0.2 mL from B, 8 mL from C
- Medium pH reference: Mix 1 mL from A, 1 mL from B, 8 mL from C
- High pH reference: Mix 0.2 mL from A, 1.8 mL from B, 8 mL from C
- Record pH

Test solutions

- Low: 0.5 mL/min at low pH reference (pH 5.13); Salt concentration: 0
- Medium: 0.5 mL/min at medium pH reference (pH 6.12); Salt concentration: 0
- High: 0.5 mL/min at high pH reference (pH 7.10); Salt concentration: 0

Collect samples

- Run to waste for 10 minutes
- Collect effluent in scintillation vial for 20 minutes
- Repeat for all three test solutions

Measure pH

- Confirm pH meter calibration
- Measure and record pH for each test solution

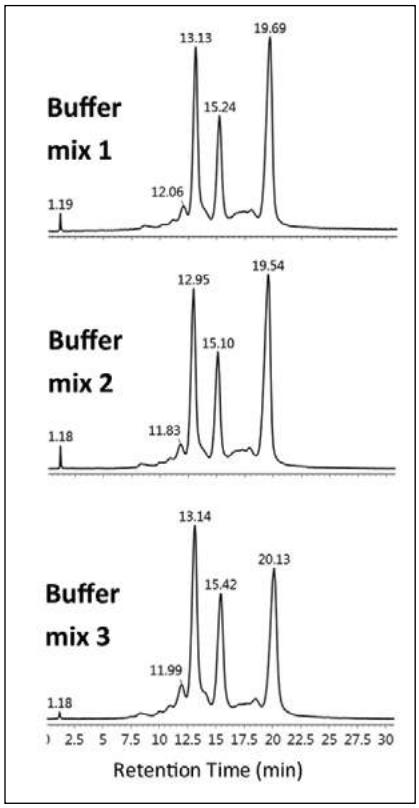


Figure 3. Separation of C-terminal lysine truncation variants with three different preparations of MES buffer over a two-week time period using Auto•Blend Plus Technology.

Test pH	Buffer mix 1	Buffer mix 2	Buffer mix 3	Mean	Std. Dev.	%RSD
5.13	5.10	5.02	5.10	5.07	0.05	0.91
6.12	6.19	6.05	6.19	6.14	0.08	1.32
7.10	7.23	7.06	7.23	7.17	0.10	1.37

Table 1. Experimental pH results for three MES buffer preparations.

Maintaining consistent separation performance with increasing sample concentration

Retention time and column performance can be affected by the amount of protein being injected onto the IEX column.

The effects of protein mass load on column performance were tested by injecting between 1-10 µL of the chimeric mAb stock solution in 1 µL intervals. Total peak area was integrated from 5-30 minutes for each injection. Reproducible retention times were observed over a 9-fold increase in mass load ranging from 20-180 µg of protein as shown in Figure 4. Coupled with the Auto•Blend Plus Technology, the ACQUITY UPLC H-Class System provides a high degree of fidelity for accurate quantification and characterization of charge variants in biotherapeutics.

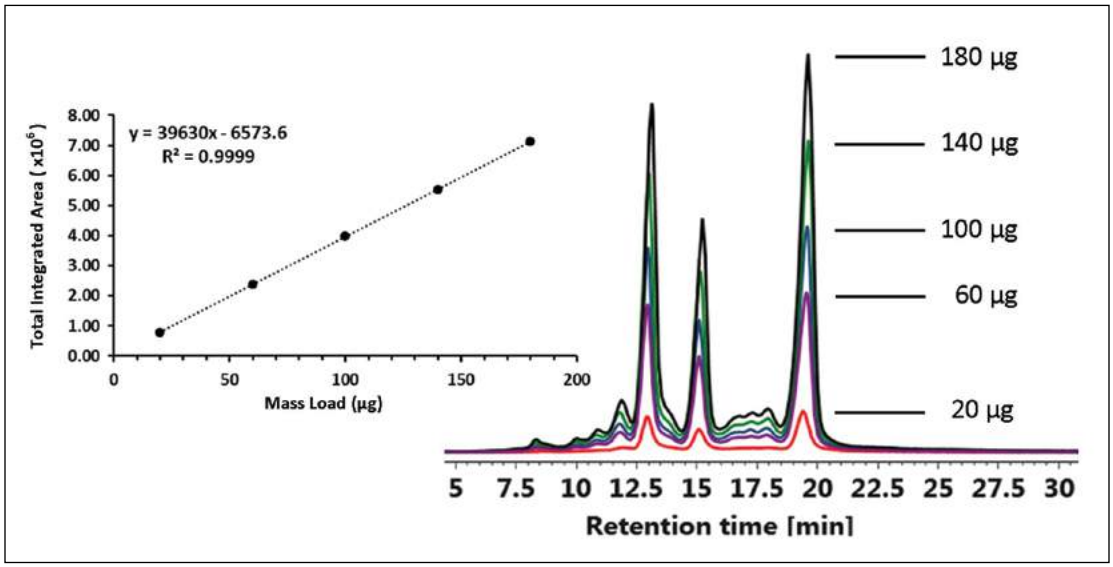


Figure 4. Chromatogram overlays of a chimeric monoclonal antibody separation with increasing protein concentration. The total peak area was integrated as a measure of precision as shown in the plot of integrated area vs. mass load.

Achieving highly reproducible separations across analysis replicates

Automation of analytical techniques can minimizes error in method development as well as increase productivity.

Auto•Blend Plus Technology was evaluated over 40 injections to simulate an unattended analysis over three days. Chromatograms are shown at injectiond 1, 20, and 40. The 60-minute separations as outlined in Figure 2 are comprised of a 30-minute separation gradient and a 30-minute cleaning and reconditioning phase. Integration intervals of five peak areas including the three main C-terminal lysine truncation variants are represented by the vertical drop lines in each chromatogram. Calculated areas of each peak area and total area are listed in Table 2.

It can be seen that Auto•Blend Plus Technology offers reproducible results well within U.S. FDA guidelines[2] with covariance of the individual peaks below 12% and the total peak area covariance below 9%. The ability to automate Auto•Blend Plus Technology, combined with its reproducibility, offers a reliable approach to robust method development for the characterization of charge variants in biotherapeutics.

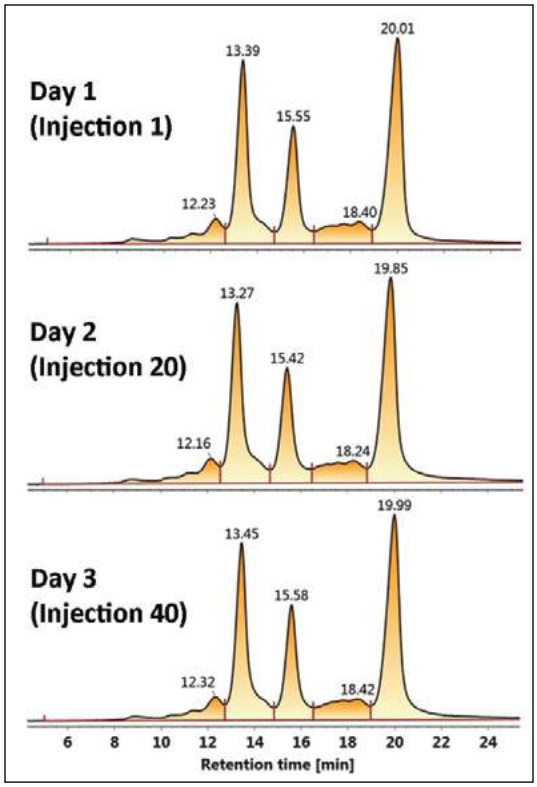


Figure 5. Separation of charge variants of a chimeric monoclonal antibody at three different time intervals over three days. Integration intervals represent the five peak areas calculated in Table 2.

Injection #	Peak 1 Area	Peak 2 Area	Peak 3 Area	Peak 4 Area	Peak 5 Area	Total
1	252260.0	788195.0	504001.0	296130.0	1052614.0	2893200.0
20	203498.0	660040.0	427519.0	237894.0	940898.0	2469849.0
40	214836.0	686459.0	437254.0	255744.0	974813.0	2569106.0
Avg	223531.3	711564.7	456258.0	263256.0	989441.7	2644051.7
SD	25517.4	67665.7	41632.2	29835.9	57276.6	221402.7
% RSD	11.42	9.51	9.12	11.33	5.79	8.37

Table 2. Integrated peak area results.

CONCLUSIONS

Analysis of charge heterogeneity profiles that arise during the development process of biopharmaceuticals require robust methods that can be automated, readily deployed, and quickly adapted to meet the demand of fast paced biopharmaceutical industry.

The combination of Auto•Blend Plus Technology with the ACQUITY UPLC H-Class System, which represent a UV-based option of the Biopharmaceutical Platform Solution with UNIFI, simplifies method development by allowing multiple buffer compositions to be tested from a single set of pure components. These features, combined with the ability to automate the process, makes Auto•Blend Plus a powerful tool for robust method development and increasing productivity.

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IEX Method Development of a Monoclonal Antibody and Its Charge Variants

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APPLICATION BENEFITS

- Robust method for confirmation of monoclonal antibody charge variants
- Reproducible and simplified method development without the need to prepare additional buffers
- Ideal for monitoring biopharmaceutical charge variants throughout manufacturing process

WATERS SOLUTIONS

Protein-Pak™ Hi Res IEX Columns

ACQUITY UPLC® H-Class Bio System

Auto•Blend Plus™ Technology

KEY WORDS

IEX, monoclonal antibody,
Protein-Pak Hi Res, lysine variants

INTRODUCTION

The use of biologic-based therapeutics, including monoclonal antibodies, has grown rapidly over the past twenty years. The complexity of these macromolecules requires the use of orthogonal analytical techniques for complete analysis and characterization. One technique, ion exchange chromatography (IEX), is used for analysis of charge heterogeneity biotherapeutics. Since each protein has a unique charge distribution, chromatographic selectivity can be adjusted by pH; however, these separations often lack resolution and consistency making method development more difficult. To address these issues, a solvent management system using pure solutions and concentrated stocks was developed. This system, capable of four solvent blending to prepare and adjust chromatographic mobile phases, can be combined with a high-resolution ion-exchange column to develop a separation for the lysine truncation variants of a chimeric monoclonal antibody. The weak-cation exchange column allows for method development over a range of pH's and with multiple buffers to obtain the optimum separation of these charge variants. This will be illustrated with a specific method for a chimeric antibody.

EXPERIMENTAL

Sample description

A chimeric monoclonal antibody sample containing C-terminal lysine truncation variants was prepared at 1.25 mg/mL in 20 mM MES buffer, pH 6.

C-terminal lysine cleavage was performed using Carboxypeptidase B (CpB) (Worthington Biochemical Corp., p/n LS005304) prepared at 1 mg/mL. The monoclonal antibody (1000 µL, 1.25 mg/mL) and CpB (12.2 µL, 1 mg/mL) were combined. At predetermined time intervals (t= 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 min), a 100 µL aliquot of the mixture was removed and combined with glacial acetic acid (1.7 µL) to halt the reaction.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System with Auto•Blend Plus Technology
Detector:	PDA Detection with Titanium Flow Cell
Wavelength:	280 nm
Sampling rate:	20 pts/sec
Filter time constant:	Normal
Column:	Protein-Pak Hi Res IEX CM, 4.6 x 100 mm, 7 µm (P/N 186004929)
Column temp.:	30 °C
Sample temp.:	4 °C
Injection volume:	10 µL
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM Sodium Phosphate, monobasic, or 100 mM MES monohydrate
Mobile phase B:	100 mM Sodium Phosphate, dibasic, or 100 mM MES sodium salt
Mobile phase C:	1000 mM Sodium Chloride (NaCl)
Mobile phase D:	Water
Purge and wash solvents:	20mM Sodium Phosphate, pH 6.0 or 20 mM MES, pH 6.0
Gradient:	0-10% C in 60 min, (pH specified in figures)

Data Management

Software:	Empower® 2 with Auto•Blend Plus Technology

RESULTS AND DISCUSSION

Charge heterogeneity of a monoclonal antibody may be caused by several structural changes including C-terminal lysine processing.[1] When present in biopharmaceuticals, these charge variants are often monitored throughout manufacturing to ensure control of the process. In the following study, an IEX method was developed to confirm and quantify the presence of C-terminal lysine truncation variants in a chimeric monoclonal antibody therapeutic. Method development was performed on a weak-cation exchange column (Protein-Pak Hi Res CM, 4.6 x 100 mm, 7 µm) by manipulating pH and ionic strength. A four solution blending system was used to make pH buffer adjustments by using a weak acid (line A) and the cognate base (line B). Sodium chloride (NaCl, line C) and water (line D) were used to adjust the ionic strength of the buffer. These adjustments were performed using Auto•Blend Plus Technology, which allowed the gradient to be expressed directly in terms of pH and ionic strength. The operating software automatically calculates the percentage of acid and base required for the specified pH from the known or measured pKa of the selected buffer system.

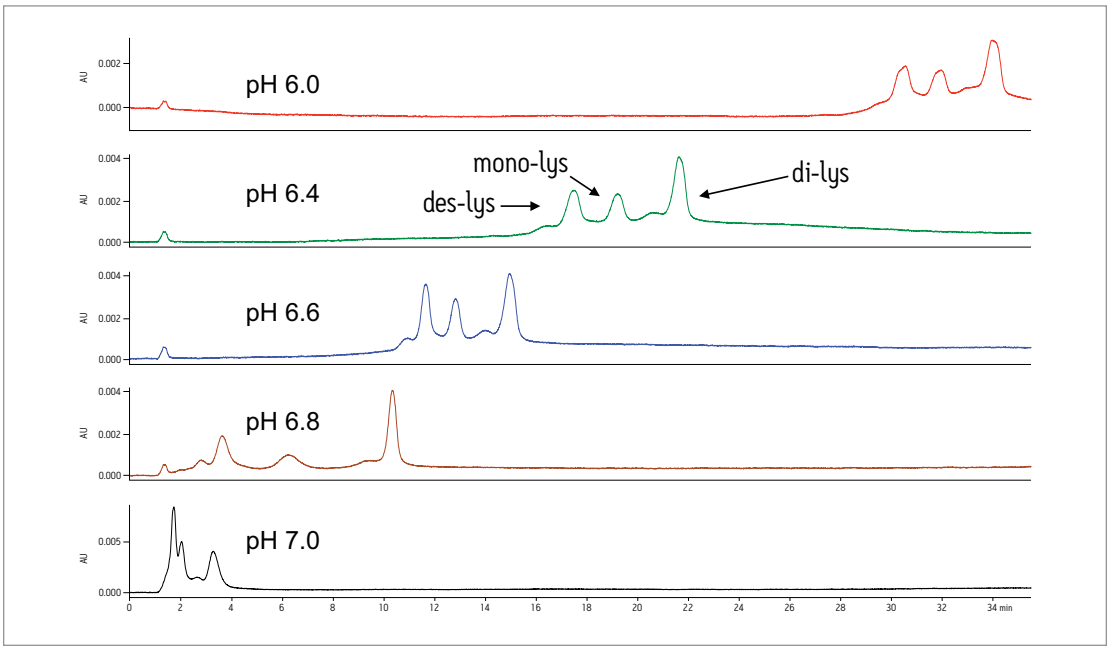

Figure 1. Analysis of a chimeric antibody and its truncated C-terminal lysine variants on a Protein-Pak Hi Res CM Column with sodium phosphate buffer. Separations were performed over a pH range of 6.0–7.0 using Auto•Blend Plus Technology.

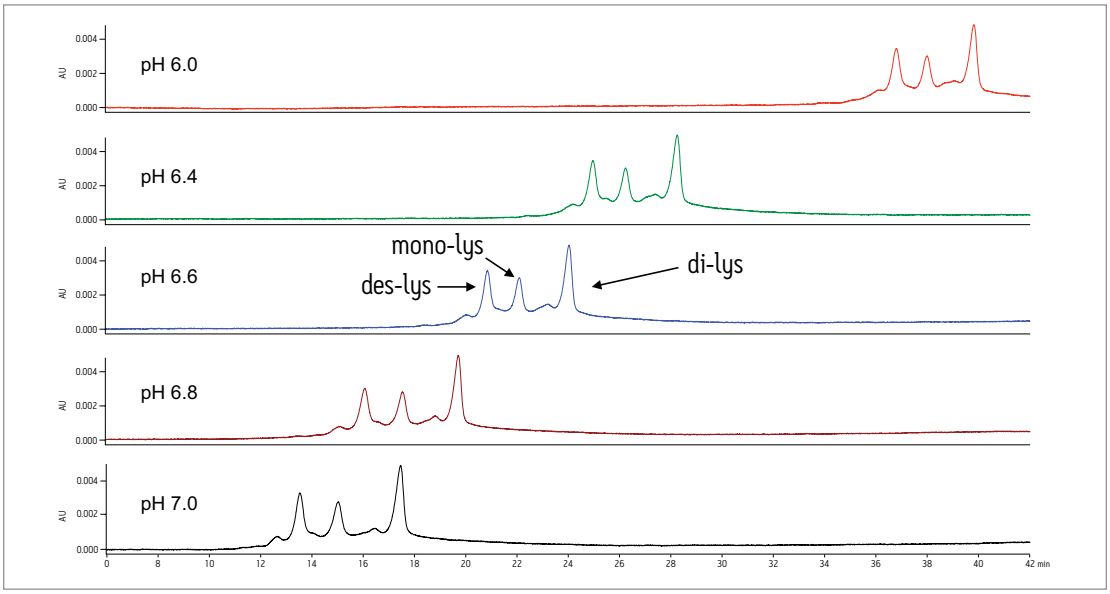

Figure 2. Analysis of a chimeric antibody and its truncated C-terminal lysine variants on a Protein-Pak Hi Res CM Column with MES buffer. Separations were performed over a pH range of 6.0–7.0 using Auto•Blend Plus Technology.

Two common cation-exchange buffers, sodium phosphate and MES ((N-Morpholino) ethanesulfonic acid), were compared. For each buffer system, the effect of pH was studied (Figures 1 and 2). A single salt gradient (0–100 mM NaCl in 60 min.) was tested over a pH range of 6.0–7.0.

With a sodium phosphate buffer system, the resolution of the monoclonal antibody (des-lys) and the C-terminal lysine truncation variants varies with pH. Higher pH corresponds to earlier elution of the chimeric antibody and the lysine truncation variants. This behavior is typical of cation-exchange chromatography since the overall positive charge of a protein decreases as pH increases, thereby resulting in elution of the analytes at a lower ionic strength. The non-truncation species (des-lys) elutes at retention times ranging from 3.4 minutes at pH 6.8 to 29.0 minutes at pH 6.0 (Table 1). This corresponds to NaCl concentrations ranging from 4 mM at pH 6.8 to 68 mM at pH 6.0 (Table 2). A similar trend is observed for the antibody with a single C-terminal lysine truncation (mono-lys). However, under the same conditions, the doubly C-terminal lysine truncation variant (di-lys) exhibits a smaller shift in both retention time and NaCl concentration. This difference in retentivity of the variants as pH is increased yields higher resolution between the mono-lys and di-lys variants at pH 6.8 (Figure 1) as compared to lower pH. A mobile phase pH of 7 results in almost no retention for the antibody and the charged variants.

The same study with an MES buffer system demonstrates the effect of buffer composition on an IEX separation. As similar to the sodium phosphate buffer system, a higher pH results in lower retention for the antibody and the charge variants. However, resolution is not significantly affected (Figure 2). Overall, the MES buffer results in later elution of the analytes when compared to the sodium phosphate buffer system. Varying pH with the MES buffer produces changes in retention. Selectivity is not significantly altered.

Retention Time (min)

	MES Buffer			Phosphate Buffer		
pH	des-lys	mono-lys	di-lys	des-lys	mono-lys	di-lys
6.0	36.6	37.8	39.6	29.0	30.3	32.1
6.4	25.0	26.3	28.3	16.3	17.9	20.2
6.6	21.0	22.2	24.1	10.9	12.0	14.0
6.8	16.3	17.8	19.9	3.4	5.8	9.7
7.0	13.8	15.3	17.7	1.6	1.9	3.1

Table 1. Retention times of a chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) at pH 6.0–7.0 with MES and sodium phopsphate buffer systems.

Concentration (mM)

	MES Buffer			Phosphate Buffer		
pH	des-lys	mono-lys	di-lys	des-lys	mono-lys	di-lys
6.0	87	90	95	68	71	76
6.4	58	61	66	37	41	46
6.6	48	51	56	23	26	31
6.8	37	40	45	4	10	20
7.0	30	34	40	0	1	3

Table 2. NaCl concentration in mM corresponding to the retention times of a chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) at pH 6.0–7.0 with MES and sodium phosphate buffer systems. Calculations are based on a 1 minute gradient hold and gradient delay volume of 0.35 mL.

Differences in the retention observed between the two buffers can be attributed to the different ionic strengths of each buffer system. The ionic strength of a buffer is based on the total number of ions contributed by both the sodium chloride and the buffering agent. For the two buffering agents used in this screening, this difference is largely due to the different number of sodium ions present. The sodium phosphate buffer system combines the mono- and di-basic forms of phosphate. Therefore, when the weak acid and cognate base are in equal proportion, three sodium ions are contributed by the buffer. In contrast, the MES buffer system is comprised of the weak acid form and the cognate sodium base. When both acid and base are in equal proportion in the MES buffer system, an amount of sodium equimolar to the cognate base (10 mM) is contributed by the buffer. Thus, when the ionic strength of NaCl is held constant and both buffering agents are at the same concentration and pH, the phosphate buffer system will have a greater ionic strength when compared to the MES buffer due to the additional sodium ions present. In quantitative terms, at a pH of 6.0 the sodium phosphate buffer system contributes an additional 22.7 mM of sodium ions to the mobile phase while the MES buffer system adds an additional 8.9 mM of sodium ions.

This difference in contributing ions results in earlier elution of the antibody and the C-terminal lysine truncation variants with a sodium phosphate buffer (Table 1). As pH is increased, sodium phosphate contributes even more sodium ions in the form of the base (32.3 mM at pH 7) as compared to MES buffer system (17.8 mM sodium ions), resulting in a greater ionic strength at a constant NaCl concentration, and thus a greater retention time shift with pH as compared to MES buffer system (Table 1).

The separation method was used to confirm the identity of the peaks. To confirm the C-terminal lysine truncation variants, the antibody biotherapeutic was treated with carboxypeptidase B following previously published protocols.[2,3] The separation was performed with MES buffer system at a pH of 6.6. These buffer conditions allow for separation of the antibody and the C-terminal lysine truncation variants in addition to the analysis of smaller eluting acidic or basic variants. The reaction was monitored over a period of 20 minutes. At predetermined time points, an aliquot of the sample was removed and combined with acetic acid to halt the reaction.

The time course study of the reaction demonstrates both the reproducibility of the IEX method and the conversion of the di-lys and mono-lys forms to the des-lys form. The retention time reproducibility was less than 0.1% RSD for all of the major components over the time course study (Table 3). The conversion of the mono-lys and di-lys variants to the des-lys antibody is also confirmed by analysis of % peak area. Within the first 2.5 minutes of the reaction, the latest eluting peak (di-lys) shows the greatest decrease in % peak area (Figure 3). In that same time period, the des-lys and mono-lys both show an increase in % peak area (Figures 3 and 4). Subsequent time point analyses show a continual increase in % peak area for des-lys, while both mono-lys and di-lys variants continue to exhibit a decrease in % peak area and are almost undetectable at 20 minutes (Figure 3). These results are consistent with conversion of the di-lys and mono-lys variants to the des-lys antibody.

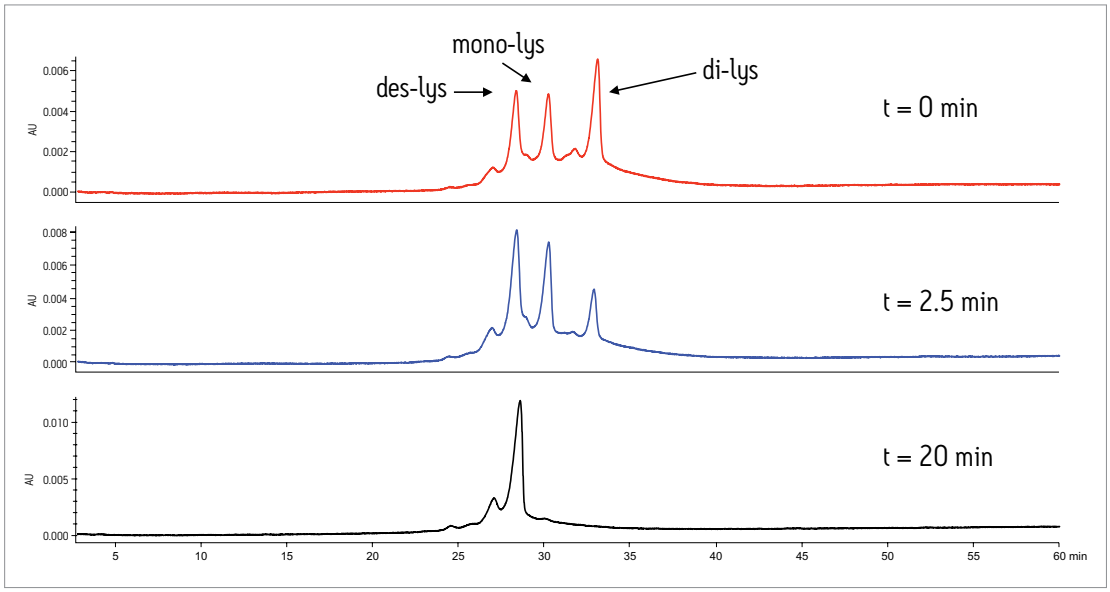

Figure 3. Treatment of a chimeric antibody with carboxypeptidase B over 20 minutes in MES buffer at pH 6.6. Analysis at intervals of 0, 2.5, and 20 minutes confirms the presence of truncated C-terminal lysine variants.

	Phosphate Buffer		
Retention Time (min)	des-lys	mono-lys	di-lys
0.0	27.77	29.63	32.50
1.0	27.74	29.65	32.45
2.5	27.79	29.65	32.28
5.0	27.82	29.55	32.12
7.5	27.88	29.49	32.08
10.0	27.95	29.48	31.81
12.5	27.93	29.44	N/A
15.0	27.96	29.40	N/A
20.0	27.98	29.42	N/A
Mean	27.87	29.53	32.21
Std Deviation	0.09	0.10	0.26
%RSD	0.33	0.32	0.80

Table 3. Retention time reproducibility for the IEX separation of a chimeric antibody and its truncated C-terminal lysine variants. Samples were treated with carboxypeptidase B and tested at set time intervals.

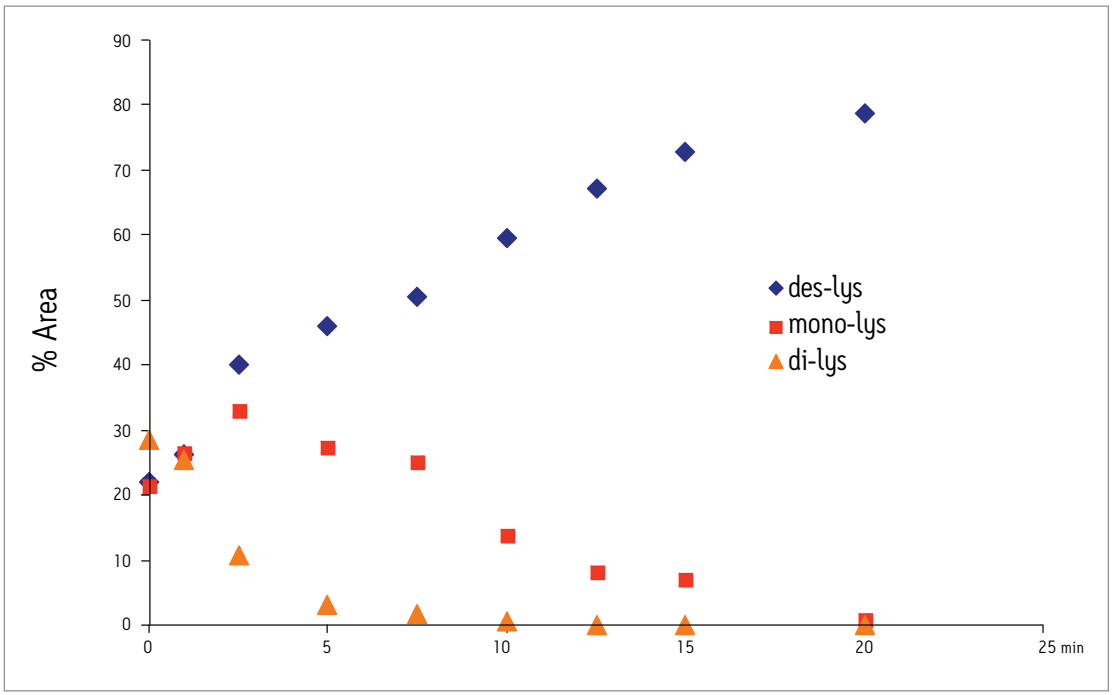

Figure 4. Analysis of the chimeric antibody (des-lys) and its truncated C-terminal lysine variants (mono-lys and di-lys) over 20 min after treatment with carboxypeptidase B. Total % peak area is not equal to 100 due the presence of additional variants.

CONCLUSIONS

The heterogeneity of a biopharmaceutical monoclonal antibody from C-terminal lysine truncation is typically monitored throughout manufacturing to ensure process stability and insure quality control. For these charge variants, the Protein-Pak Hi Res CM column provides a tool for the analysis and confirmation of a chimeric antibody and its C-terminal lysine truncation variants. The column, in combination with the ACQUITY H-Class Bio System and Auto•Blend Plus Technology, allows for simplified pH screening and evaluation of multiple buffer systems. Method development studies for the chimeric antibody demonstrate the dramatic affect of pH with a sodium phosphate buffer, which is partially due to the ionic strength of the buffering agent. In contrast, minimal resolution effects are observed with varying pH in a MES buffer system. All of the screening studies performed are simplified with the use of a four-solvent blending system and Auto•Blend Plus Technology. The resulting separation provides a robust method for analysis and confirmation of a monoclonal antibody and its C-terminal lysine truncation variants.

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Solutions in Practice

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Ion Exchange Chromatography Method Development on ACQUITY UPLC H-Class Bio System

GOAL

To simplify ion exchange chromatography (IEX) methods development for the analysis and characterization of proteins using the quaternary-based ACQUITY UPLC® H-Class Bio System in combination with Auto•Blend Plus™ Technology.

BACKGROUND

The complete analysis and characterization of proteins requires orthogonal analytical techniques focusing on different physical and chemical properties. Ion exchange chromatography is often utilized to assess the distribution of proteins or the presence of protein variants formed by post-transitional modifications (*e.g.*, deamidation) that can be recognized by charge differences. For these analyses, adjustments in mobile phase pH are the most useful parameter for method development. Such experiments are, however, time-consuming and cumbersome. The ACQUITY UPLC H-Class Bio System and its quaternary solvent manager takes advantage of UPLC® Technology and Auto•Blend Plus Technology to simplify IEX method development.

Auto•Blend Plus Technology allows users to manipulate pH and ionic strength by calculating and delivering the proportions of buffer stocks required for the desired conditions. The introduction of this new system provides users a robust, efficient tool for method development for IEX separations of proteins.

The ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology together help IEX users streamline the development of methods for the analysis of proteins and their charge variants.

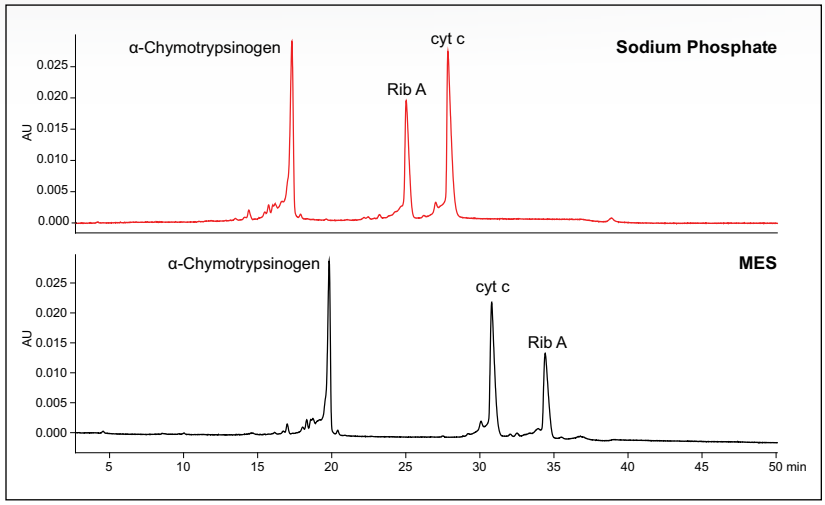

Figure 1. Effect of buffer composition on the IEX separation of proteins. Sample: Bovine, α-Chymotryspinogen, Bovine Ribonucelase A, Equine cyctochrome c. Column: Protein-Pak Hi Res CM 7-µm, 4.6 x 100 mm. Conditions: 20 mM buffer (MES or Sodium Phosphate) pH 6, 1 mL/min, 0 to 0.2 M NaCl in 34 min at 30 °C.

THE SOLUTION

Ion exchange chromatography of proteins combines the ACQUITY UPLC H-Class Bio System with Auto•Blend Plus Technology and Protein-Pak™ Hi Res Columns for simplified method development. The ACQUITY UPLC H-Class Bio System is an inert system that provides stability in the aqueous, high ionic strength buffers used for IEX separations, while also giving the highest recovery of the sample.

Auto•Blend Plus Technology takes advantage of the system's four-solvent blending capabilities to prepare and adjust chromatographic mobile phases using pure solvents and concentrated stocks of acid, base, salt, and water. In the newest implementation, its user interface allows for expressing the chromatographic method in parameters that are most familiar to the biochemist, specifically pH and ionic strength.

Using Auto•Blend Plus Technology, a series of experiments were performed to demonstrate the effect of buffer composition and pH on IEX separations of proteins. To illustrate the effect of buffer composition, a mixture of proteins was separated using weak cation-exchange chromatography. Two common cation-exchange buffers, Sodium phosphate and MES ((N-Morpholino) ethanesulfonic acid), were compared. At a pH of 6, different selectivity was observed for the most basic proteins, as shown in Figure 1.

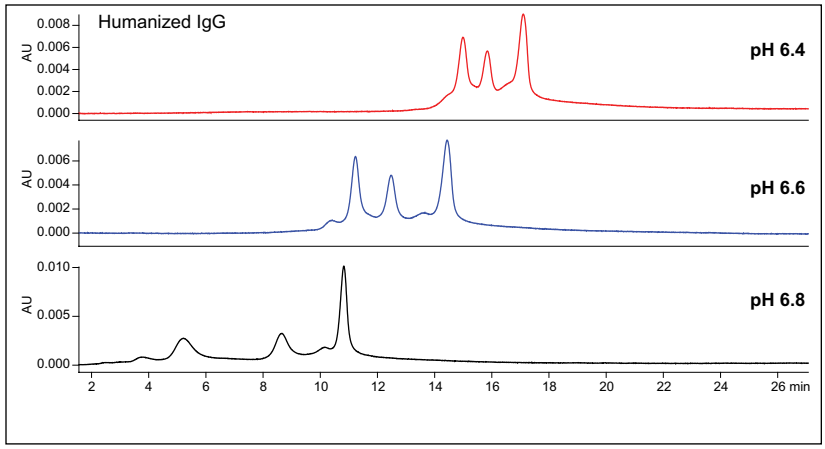

Figure 2. Effect of pH on IEX separation of humanized IgG. Sample: Humanized IgG, 1.5 mg/mL. Column: Protein-Pak Hi Res CM 7-µm, 4.6 x 100 mm. Conditions: 20 mM Sodium Phosphate, 0.5 mL/min, 0 to 0.1 M NaCl in 40 min at 30 °C.

The buffer system also influenced overall retention: MES buffers resulted in longer retention times for the proteins. In a second experiment, a monoclonal antibody containing lysine variants was separated with phosphate buffer at different pHs. The monoclonal antibody separations (Figure 2) demonstrate the influence of pH on retention time and selectivity for a humanized IgG and its variants. In these experiments, the use of Auto•Blend Plus Technology and a four-buffer blending system allowed for simple, fast method development for IEX separation of proteins.

SUMMARY

The ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology together provide IEX users with a fast and easy method development system for the analysis of proteins and their charge variants. Adjusting pH is simplified with the use of a four-solvent blending system (acid, base, salt, and water) in combination with Auto•Blend Plus Technology software. These improved protocols translate to savings in both time and reagent costs, increasing overall productivity.

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Reversed-Phase for Protein Separations

Reversed-phase liquid chromatography (RP-LC) involves the separation of molecules on the basis of relative difference in biomolecule hydrophobicity. It is one of the most common techniques used for the analysis of proteins, peptides and amino acids owing to its excellent resolution, high recoveries, and direct compatibility with mass spectrometry. RP-LC is widely used for assaying impurities and is an efficient tool for quantitation of desired product against process variants.

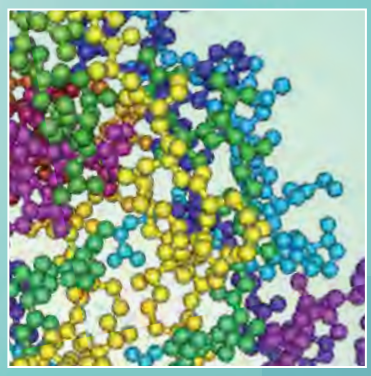
The resolving power of protein RP-LC separations is limited due to the high cooperativity and diversity of physical interactions between proteins and the column phase that result in peak broadening. In addition, large analytes have disadvantageously small diffusion coefficients affecting overall kinetics of separation potentially limiting both capacity and resolution. Nonetheless, there are many parameters that can be used to optimize the separation of a particular sample.

Solution highlights

The requirements for a reversed-phase analysis of intact proteins are satisfied by the system solution that combines ACQUITY UPLC H-Class Bio instrumentation and ACQUITY UPLC Protein BEH C_4 300Å Columns.

As with other LC-based, protein separation methods, eluent composition must be carefully determined in order to obtain a desired RP separation of a protein sample. Two critical components are the choice of organic solvent and ion-pair reagent used in these gradient based separations. The **ACQUITY UPLC H-Class Bio System featuring Auto•Blend Plus Technology** delivers the ability to determine, in an automated fashion, the optimal organic solvent type and ion-pair combination necessary to develop a validated method with a high degree of accuracy and precision over a repeated series of analyses.

The ACQUITY UPLC Protein BEH C_4 300Å, 1.7 µm Column is well suited to delivering reproducible, high-resolution RP separations of intact proteins due to it being based on a stationary phase that uniquely combines large pores and a short chain bonded phase with novel hybrid particle technology.



The MassPrep Protein Standard Mix contains proteins with a wide range of isoelectric points, molecular weights, and hydrophobicities, making it a perfect choice for verifying the performance of a UPLC instrument as well as evaluating the performance of Protein BEH C_4 300Å Columns.

Method Development Considerations for Reversed-Phase Protein Separations

Hillary B. Hewitson, Thomas E. Wheat, Paula Hong, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The BEH300 C_4 chemistry is available in sub-2-µm particles for maximum resolution
- Changes in operating conditions alter selectivity and resolution to meet the requirements for a particular sample
- The stability of the BEH300 C_4 chemistry allows use at high temperature for improved recovery and for selectivity modification
- The physical strength allows use of a range of organic solvents
- The inert surface allows use of different acids and concentrations
- The range of operating conditions permits automated, unattended method development
- The flexibility in creating conditions reduces the need for screening multiple columns

WATERS SOLUTIONS

- ACQUITY UPLC® System, fitted with peptide needle and peptide mixer, or ACQUITY® H-Class System, TUV detection at 220 nm
- ACQUITY UPLC BEH300 C_4, 2.1 x 50 mm, 1.7 µm Column, PN: 186004495;
  ACQUITY UPLC BEH300 C_4, 2.1 x 150 mm, 1.7 µm Column, PN: 186004497;
  ACQUITY UPLC BEH300 C_4, 4.6 x 150 mm, 3.5 µm Column, PN: 186004504
- MassPREP™ Protein Standard Mix, PN: 186004900

KEY WORDS

Monoclonal antibodies, reduced monoclonal antibody, gradient slope, protein, BEH300 C_4, MassPREP protein standard mix, method development

INTRODUCTION

Due to the resolving power that reversed-phase chromatography provides, it has long been a preferred analytical technique to characterize and quantify various products. With an ever increasing emphasis on protein biopharmaceuticals, there is a need to develop reversed-phase separations of these macromolecules. Reversed-phase separations for proteins are not as powerful as they are for small molecules. Changes to the protein are often small in proportion to the structure of the large molecule. Variant forms, therefore, have similar chromatographic properties. There are still many factors that can be used to optimize the separation of a particular sample. The requirements of this specific application will dictate the best approach for method development. This paper will consider each of these factors, including particle size, column length, flow rate, modifier concentration, organic solvent, column temperature, and gradient slope. The evaluation of each of these method variables will be demonstrated on a variety of proteins, including monoclonal antibodies, covering a wide range of properties. These include different isoelectric points, hydrophobicities, and molecular weights.

EXPERIMENTAL

Sample description

Protein Mixture: Prepared in 5% acetonitrile with 0.1% CF_3COOH

Protein	mg/mL
Ribonuclease A, bovine pancreas	0.08
Cytochrome C, horse heart	0.11
Albumin, bovine serum	0.40
Myoglobin, horse heart	0.25
Enolase, baker's yeast	0.43
Phosphorylase B, rabbit muscle	1.18

Intact Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL. Reduced/Alkylated Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL. Intact IgG Mixture: Humanized IgG_4, Chimeric IgG1, and Murine IgG1, prepared in 0.1% CF_3COOH, 0.5 µg/µL each.

Vials: Waters Certified Total Recovery, PN: 186000384c

LC conditions

(unless otherwise specified in figure captions)

System: ACQUITY UPLC (fitted with peptide needle and peptide mixer) with ACQUITY TUV Detector, at 220 nm

Column: BEH300 C_4, 2.1 x 50 mm, 1.7 µm, PN: 186004495

Column temp.: 40, 60, 80, or 90 °C (as indicated in figures)

Sample temp.: 10 °C

Injection volume: 3.3 µL

Flow rate: 0.2 mL/min

Mobile phase A: 0.1% CF_3COOH in water

Mobile phase B: 0.1% CF_3COOH in acetonitrile

Weak needle wash: 0.1% CF_3COOH in 5% acetonitrile

Strong needle wash: 0.1% CF_3COOH in 75% acetonitrile

Seal wash: 50/50 acetonitrile/water

Gradient: 20–71.4% in 29.6 min; 1 min regeneration at 90%; 17 min re-equilibration at initial conditions

Data management

Empower® 2 CDS

RESULTS AND DISCUSSION

Column chemistry and particle size

The advent of sub-2-µm particles along with UPLC® Technology has shown benefits for samples of all types. This technology was applied for the separation of biological macromolecules with the development of BEH300 C_4, a column chemistry that combines appropriate pore volume and chain length on a hybrid particle.[1] This stationary phase is available in 3.5 µm and 1.7 µm particle sizes, so methods can be directly transferred between HPLC and UPLC with the same chromatographic selectivity. Figure 1 shows the comparison of the effect of particle size, using a reduced and partially alkylated IgG sample. The relative positions of the peaks are exactly the same for both particle sizes. Since both separations were tested on the same UPLC system with the same mobile phases and conditions, the improvement in resolution observed for the 1.7 µm separation is directly attributable to the smaller particle size. The rigorous manufacturing control in the particle synthesis ensures scalability and constant selectivity across particle sizes. The improvement in resolution can only be fully realized with use of a system designed to minimize band-broadening during the separation.

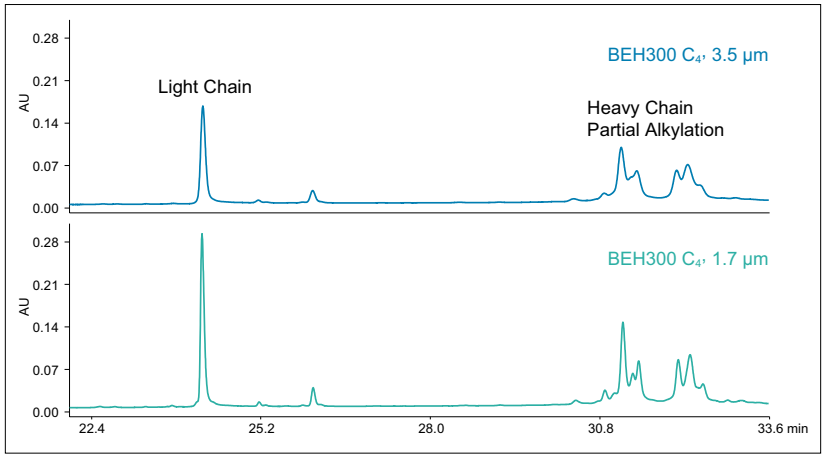


Figure 1. Comparison of a reduced and alkylated monoclonal antibody with 3.5 µm and 1.7 µm particles at 40 °C. While the relative position of the peaks are exactly the same in both separations, all of the peaks are narrower, and the improvement is particularly apparent for the multiple molecular forms of the heavy chain.

Gradient slope

In gradient separations, chromatographers will often change the gradient slope as a primary tool in method development. Gradient slope, defined by the percent increase in organic per column volume, can be adjusted to optimize a separation for resolution of components or speed of analysis. Typical protein separations use fairly shallow gradients of about 3% or less. Reducing the gradient slope does offer an increase in resolution. Sensitivity, however, is reduced as the gradient is made more shallow. The resolution improvement in protein separations is usually at a slower rate than the loss of sensitivity or increase in peak volume. This phenomenon can be seen readily in the separation of a mixture of IgG, shown in Figure 2. By reducing the gradient slope from 3% to 0.5%, there is only a marginal increase in resolution between the humanized and chimeric IgG peaks, while there is over a 3-fold loss of sensitivity and a 4-fold increase in run time. While gradient slope is a viable tool in method development, it is preferred to reserve that option until other techniques have been examined.

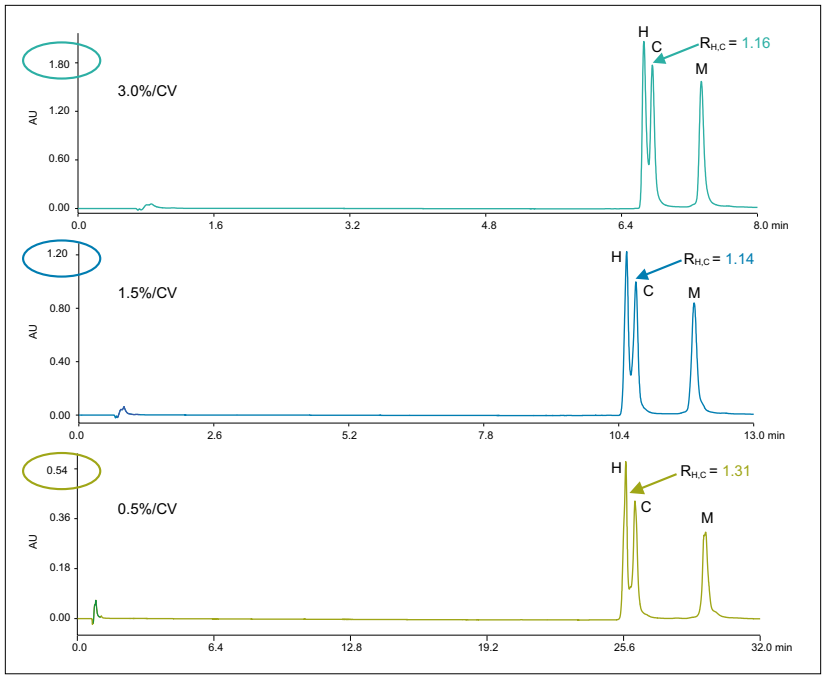


Figure 2. The separation at 80 °C of a mixture of humanized (H), chimeric (C), and murine (M) monoclonal antibodies with varying gradient slopes. There is more than a 3-fold loss in sensitivity, a 4X increase in run time, and a 5X increase in peak volume by decreasing the gradient slope from 3% down to 0.5% with only a marginal improvement in the resolution.

Organic solvent and mobile-phase modifier concentration

Alternative organic solvents can alter selectivity of a separation. Historically, acetonitrile has been the solvent of choice in protein separations. The use of isopropanol (IPA) has been common. Gradients of increasing IPA were seldom used because of the high pressure associated with the viscosity of such solvent mixtures. Therefore, an acetonitrile/isopropanol blend (3:7) was a preferred replacement. The higher pressure capability of ACQUITY UPLC instruments allow for use of 100% isopropanol, as shown in Figure 3. All of the proteins elute earlier with IPA, and for this sample, improved resolution of some minor components is observed.

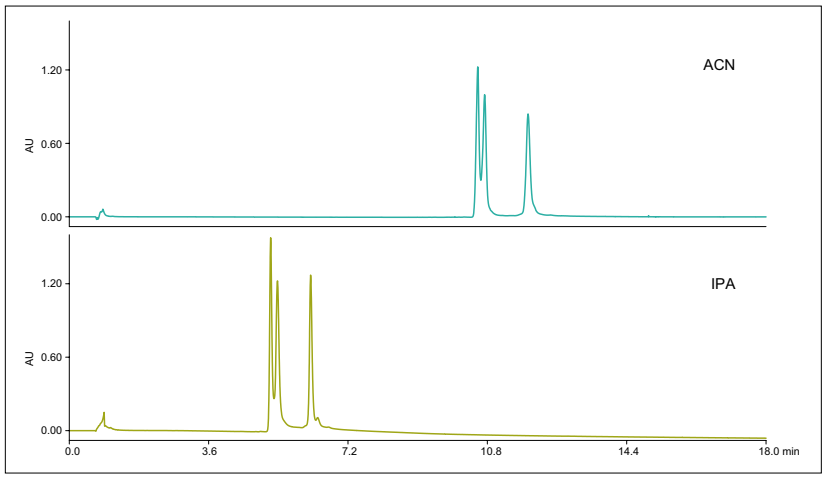


Figure 3. The separation of a mixture of monoclonal antibodies is shown at 80 °C with acetonitrile (ACN) and isopropanol (IPA) as the elution solvent. Reduced retention and improved resolution of trace components is observed with IPA.

Type and concentration of the acid modifier can also influence the separation. Formic acid is the preferred modifier in mass spectrometry applications, and trifluoroacetic acid (TFA) gives better chromatographic peak shape. Altering the acid concentration can change the selectivity of the separation. In general, protein peaks elute earlier with lower trifluroacetic acid (TFA) concentration, reflecting the reduced ion pairing.

There are a large number of organic solvent and acid concentration combinations possible in the development of a separation. This process can be streamlined with the application of Auto•Blend™ Technology, as embodied on the four solvent ACQUITY UPLC H-Class system. Figure 4 shows the preferred configuration of the system for testing the effect of organic solvent and mobile-phase modifier concentration on a protein separation. The conditions to be tested are programmed in the method as percentage flow from each of the four solvent lines. For example, different TFA concentrations are tested by blending a concentrated acid modifier at a series of percentages. This approach was used for the protein separation shown in Figure 5. While all of the peaks elute earlier at lower concentrations of TFA, myoglobin elutes earlier relative to the other proteins. It should also be noted that lower TFA concentration also results in generally wider peaks, which can lead to lower resolution, as can be seen with the different forms of phosphorylase b. Altering modifier concentration can be a very useful tool in method development, particularly where changes in selectivity are needed. The same approach can be used to compare different organic solvents.

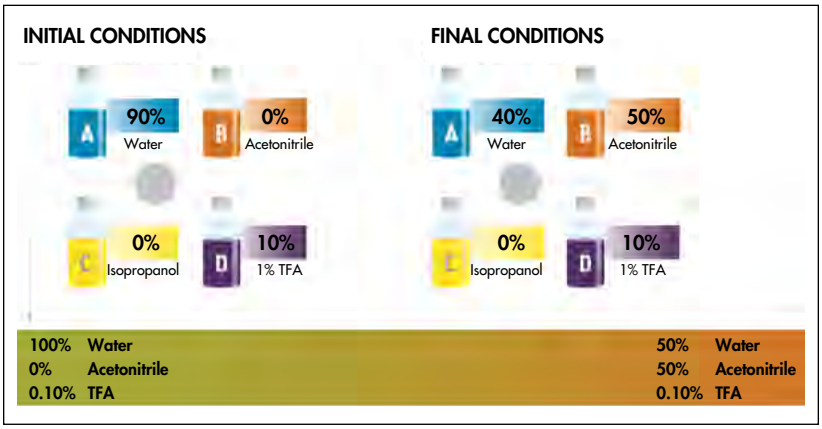


Figure 4. An example showing a possible configuration of four solvent lines of an ACQUITY H-Class System. Auto•Blend Technology enables a user to blend in varying amounts of a concentrated modifier and use different organic solvents in a single set of analyses when developing a method.

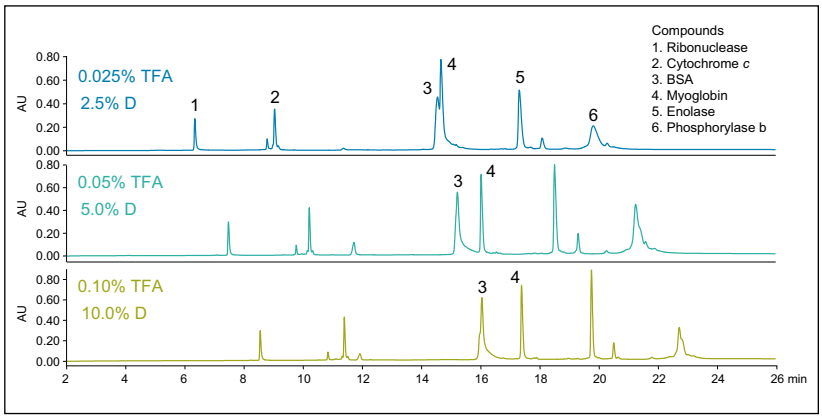


Figure 5. The protein test mixture was separated at 40 °C in the presence of various concentrations of TFA with the other conditions held constant.

Column temperature

Column temperature has a large effect on reversed-phase separation of molecules. Changes in recovery and selectivity are not uncommon with small molecule separations. While increasing the temperature for proteins can significantly improve recovery, particularly for intact monoclonal antibodies (Figure 6), it doesn't generally affect the selectivity of the separation.[2] However, not all proteins require higher temperatures for improved recovery. In fact, some protein separations have more desirable results with lower separation temperatures. Therefore, it is recommended that an evaluation of temperature be included in any method development strategy for new samples.

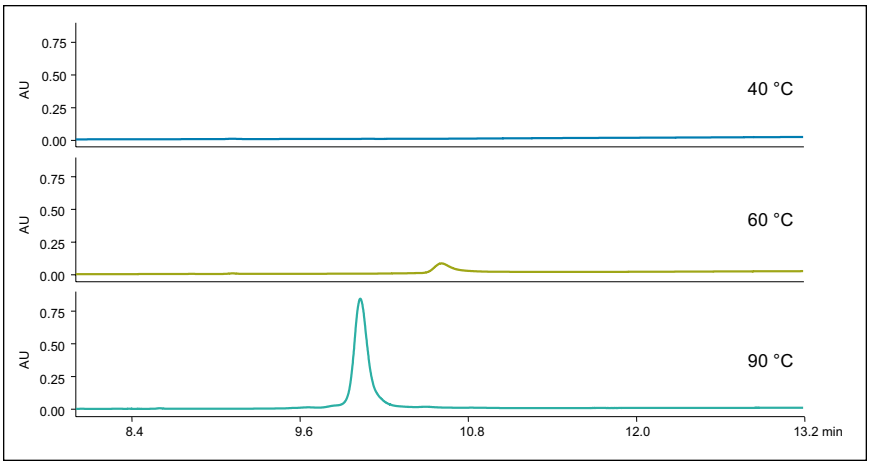


Figure 6. The intact IgG sample gave no observable peak at 40 °C, but recovery for the IgG sample improves with increasing temperature. There is not a measurable increase in recovery or improvement in peak shape above 80 ° C.

Column length

Increasing the length of the column will increase the resolving power for a separation. This is shown in Figure 7 with the separation of the protein mixture. The additional small peaks surrounding the Phosphorylase b can be seen more readily on the longer column, as seen in the inset, but it comes at the cost of a 3-fold increase in run time and ~40% loss of sensitivity. Depending on the application objective, this may be a useful parameter to improve resolution.

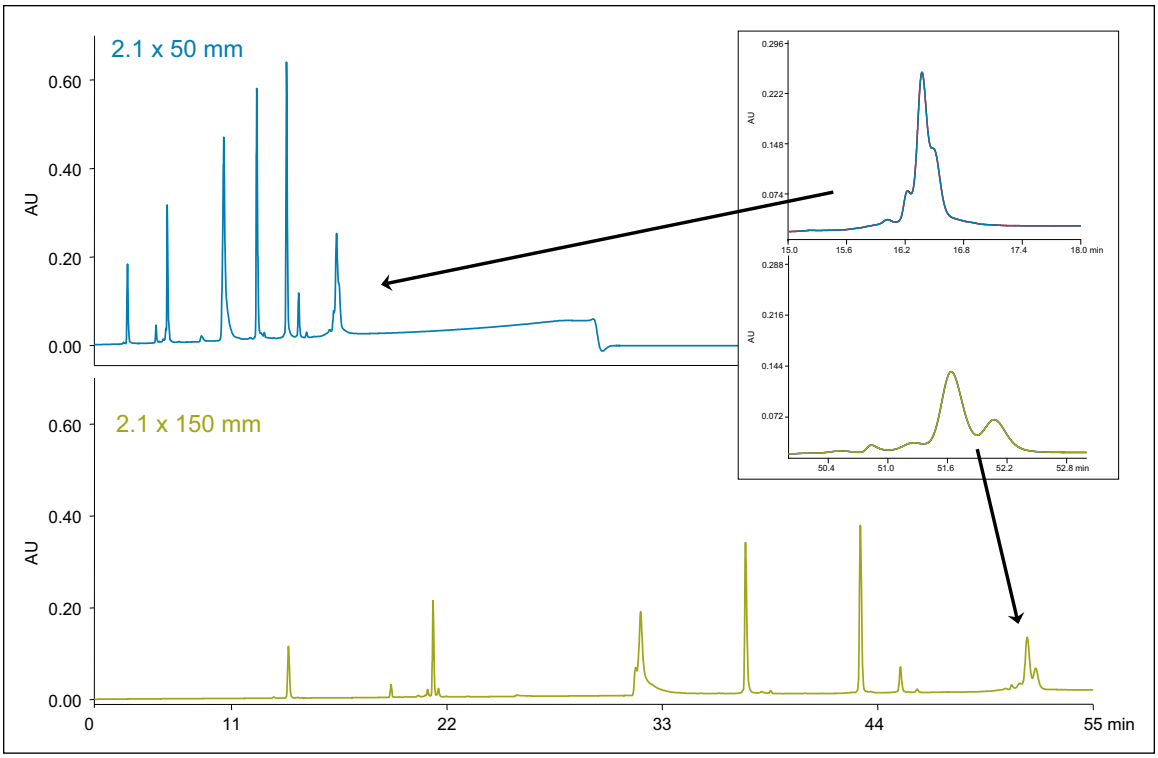


Figure 7. In the separation of the protein mixture at 40 °C, improvement in resolution of the additional small peaks surrounding Phosphorylase b (inset) can be readily seen on the longer column. The gain in resolution comes at the cost of a 3-fold increase in runtime and ~40% loss of sensitivity.

Flow rate

Flow rate is seldom treated as an important parameter in method development except as an indirect modification of gradient slope. The impact of this variable is, however, more significant for larger molecules. Figure 8 shows the comparison of a protein mix separation at 200 µL/min and 75 µL/min. The inset shows improved resolution with an increase in sensitivity of phosphorylase b at the lower flow rate.

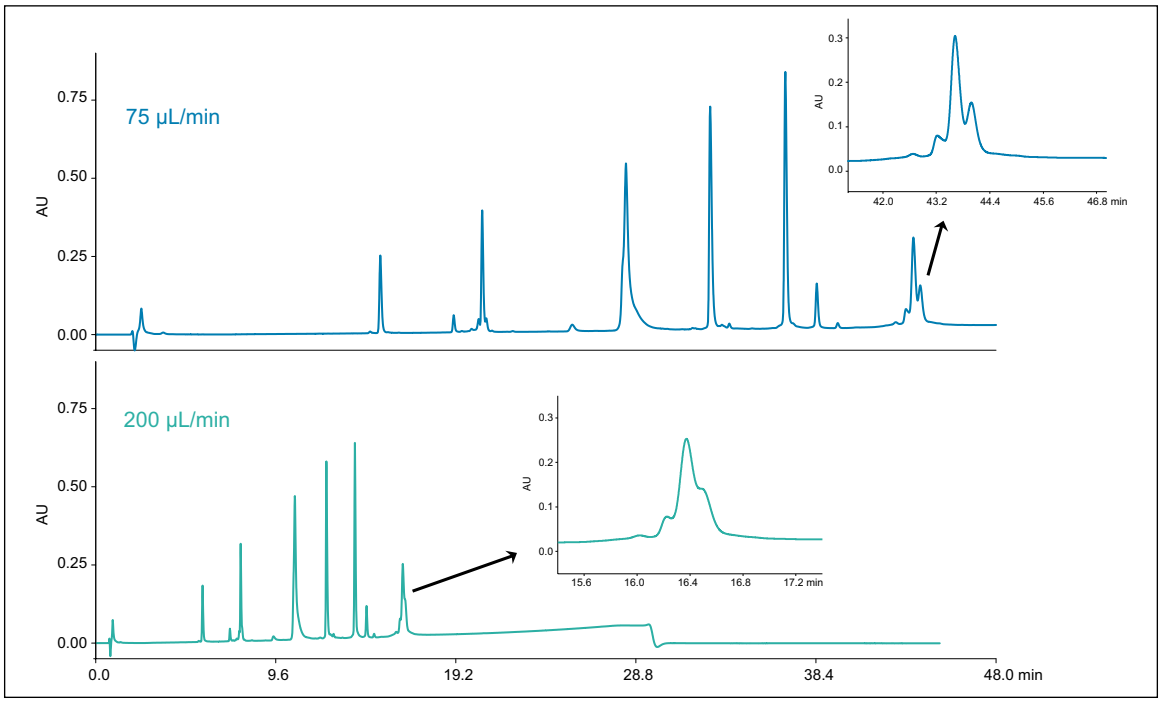


Figure 8. Decreasing the flow rate provides increased resolution without a compromise in the sensitivity, as seen in this separation of the protein mixture at 40 °C. The improved separation of the Phosphorylase b sub-units can be seen (inset) at the lower flow rate. The run time of the analysis is increased proportionally to preserve the same gradient slope in both separations.

CONCLUSIONS

There are many parameters available to the chromatographer in developing methods for separation of proteins. The approach to making adjustments to a method must take into consideration the objective of the analysis.

Protein separations do not tend to show the same dramatic resolution effects seen for small molecules. Therefore, most of the variables discussed here yield small improvements, often at the expense of sensitivity and run time. Smaller particle columns do, however, offer resolution improvements without loss of sensitivity or increased run time. Flow rate, column length, gradient slope, and modifier concentration can then be manipulated to further improve resolution.

Modifier concentration can be a useful tool in developing methods. It can provide resolution improvements by possible selectivity changes. Furthermore, changing the concentration can affect peak shape and detection.

Auto•Blend Technology is a convenient and efficient way to optimize systematically the effects of modifier concentration and organic solvent selection on the separation.

Adjustment in column temperature does not usually provide much selectivity change, but it can have a significant impact on the peak shape and recovery of proteins. It is not always possible to predict the ideal temperature for a protein sample. Therefore, it is good practice to include multiple temperatures in evaluation of appropriate conditions for a protein separation.

Both increased column length and decreased flow rate give increased resolution, both at increased run time. However, decreasing flow rate does not compromise sensitivity, as is the case with the longer column. The longer column, however, permits the injection of a larger sample, which may be valuable in the analysis of trace components.

Benefits in sensitivity, resolution, and run time can be achieved with smaller particles. But these improvements are best realized when applied with the holistic design of the UPLC system.

References

1. Developing Protein Separation Method on a Reversed-Phase UPLC Column, Waters poster 720002974EN
2. Protein Separation Technology ACQUITY BEH300 C_4, 1.7 µm, Waters care and use manual 715001870EN

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Quantitation of Monoclonal Antibodies with Reversed-Phase Liquid Chromatography Using the BEH300 C_4 Column Chemistry

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APPLICATION BENEFITS

- Reproducible reversed-phase chromatographic separations of monoclonal antibodies.
- Minimal carry over and memory effects.
- Linear quantitation over four orders of magnitude dynamic ranges.
- Quantitation of traces of antibody in mixtures of 100-fold ratio.

WATERS SOLUTIONS

ACQUITY UPLC with TUV, fitted with the Peptide Needle (P/N: 205000507) and 425 µL Peptide Mixer (P/N: 205000403)

BEH300 C_4, 1.7 µm, 2.1 x 50 mm (P/N: 186004495)

KEY WORDS

protein quantitation, peptide needle, peptide mixer, trace impurity quantitation, protein variants, monoclonal antibodies

INTRODUCTION

The quantitative analysis of biopharmaceutical proteins is required at various stages in the development process. Depending on the phase and specific focus of a biotherapeutic lifecycle, quantitation can vary from an estimation of the target protein (+/- 10%) to quantitation of impurities at levels <1% of the target protein. To accommodate these varying requirements, it is not only necessary to determine the best range for the assay, but to assure that the required dynamic range can be readily and reliably achieved. The BEH300 C_4 column chemistry was designed specifically so that the high resolution characteristic of UPLC® would be available for protein analyses. Using the BEH300 C_4 column chemistry, quantitation of a commercially-available monoclonal antibody is shown in this application note, demonstrating the linear range. Also shown is an example for detection and quantitation of trace-level impurities.

EXPERIMENTAL

Sample description

Linearity samples: Fully humanized IgG4 prepared in 0.1% CF_3COOH, for mass load targets of 0.05, 0.1, 0.5, 1, 5, 7.5, 10, 20, 30, 40, 50 µg on column (3.3 µL injection).

Trace impurity quantitation samples: 0.1%, 0.2% 0.5%, 1%, 2.5%, and 5% murine IgG1 in the presence of a constant 50 µg Humanized IgG4 on column (3 µL injection).

LC conditions

LC system:	ACQUITY UPLC® System (fitted with the Peptide needle and 425 µL peptide mixer)
Detector:	ACQUITY® TUV Detector
Sanple vial:	Waters Certified Total Recovery vials (P/N: 186000384c)
Column:	ACQUITY UPLC BEH300 C_4, 2.1 x 50 mm, 1.7 µm (P/N: 186004495)
Column temp.:	80 °C
Sample temp.:	10 °C
Injection volume:	3 µL or 3.3 µL
Injection mode:	Partial loop
Flow rate:	0.2 mL/min
Mobile Phase A:	0.1% CF_3COOH in water
Mobile Phase B:	0.1% CF_3COOH in IPA
Weak needle wash:	0.1% CF_3COOH in 5% acetonitrile
Strong needle wash:	0.1% CF3COOH in 75% acetonitrile
Seal wash:	50/50 acetonitrile/water
Gradient:	20 – 37% B in 14.7 min; 1 min regeneration at 90%; 13 min reequilibration at 20% B, UV detection at 220 and 280 nm

Data management

Software: Empower® 2 CDS

RESULTS AND DISCUSSION

UV detection at low wavelengths (e.g. 220 nm) is often used for protein analysis because of its high sensitivity. At low wavelength, low levels of a protein can be detected. However, detector saturation limits the dynamic range to a 50-fold range of concentrations (Figure 1). Saturation of the detector at 220 nm occurs between 5 and 7.5 µg of protein injected on a 2.1 x 50 mm column The analysis of the same mass load and injection volume of humanized IgG4 showed almost a 10-fold difference in peak height between the chromatograms at 220 nm and 280 nm (Figure 2). The reduced peak height at 280 nm was also accompanied by changes in baseline characteristics. The low end of the linear range, that is the lower limit of quantitation, is dependent upon both drift and short term detector noise. The noise is similar at the two wavelengths, on the order of 0.00002 AU. The drift, however, is more than ten-fold greater at 220 nm. Such anomalies directly affect the accurate integration of peaks. The increased saturation limit and reduced baseline anomalies combine to give a wider usable dynamic range at 280 nm.

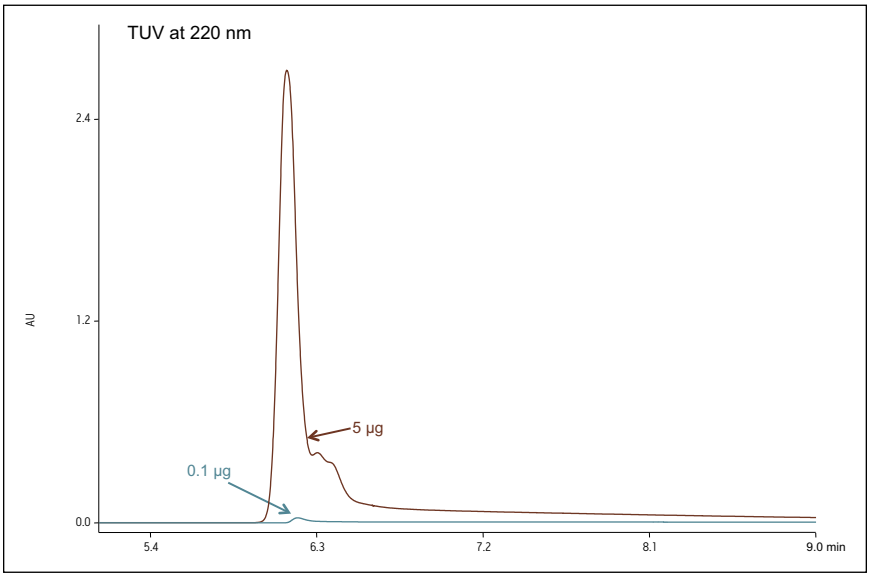

Figure 1. Chromatogram of humanized IgG4 at the low and high end of the linear dynamic range, with detection at 220 nm. Detector saturation is observed at loads above 5 µg on column.

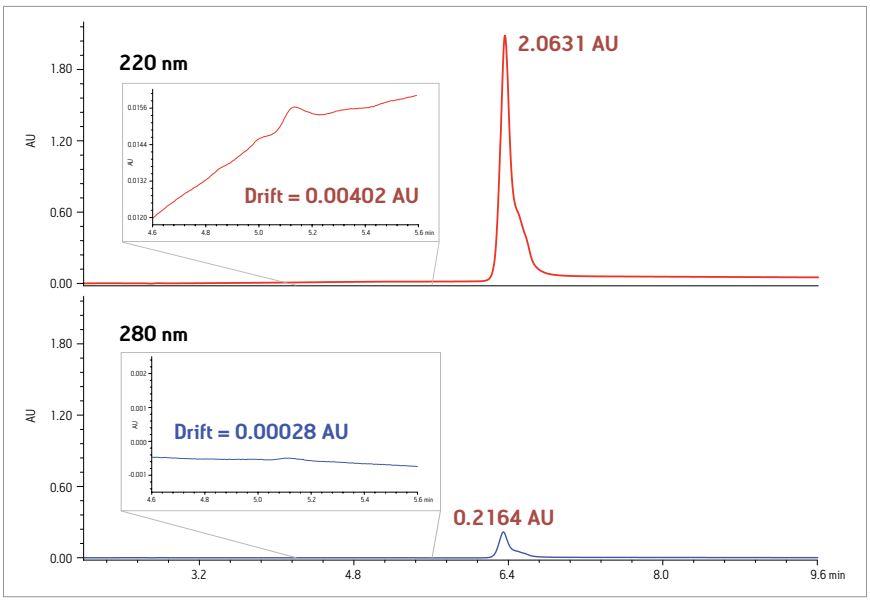

Figure 2. A comparison of the same 5 µg load of a monoclonal antibody is shown at both 220 nm and 280 nm. There is close to a 10-fold difference in peak height. The inset shows a one-minute segment of baseline prior to the protein peak eluting. There is close to a 14-fold increase in drift observed at 220 nm.

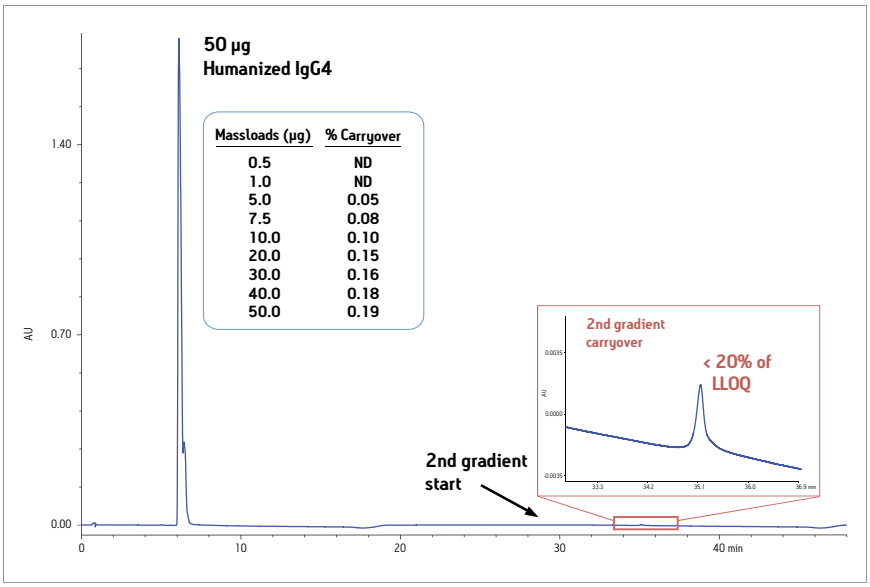

Figure 3. Effect of mass load on carryover. The chromatogram shows a 50 µg load of humanized IgG4 with a second internal gradient. Material eluting near 35 minutes represents a memory effect. The inset table shows the carryover measured for all levels tested, displayed as a percentage of area observed in the first gradient.

The humanized IgG4 was used to determine the linear range with UV detection at 280 nm. Ten different mass loads (0.1–50 µg) of protein were analyzed in triplicate at a constant injection volume of 3 µL. At 280 nm, the highest protein load of 50 µg on column was below the detector saturation limit, and the same variant forms of the protein were still detected. The method was linear across all levels tested, with an R_2 value of 0.994. The variability of multiple injections was also less that 2% across the full set (Table 1).

Validation of any quantitative assay must demonstrate the absence of carryover. Observation of carryover has been particularly problematic in protein assays. In developing an application, two sources of carryover are recognized, the fluid path of the instrument and the chromatographic column. Column-related carryover, or memory effect, can be measured by running a second gradient immediately following the sample analysis without making another injection. This internal gradient would show memory effects as a peak at the corresponding retention time in the second gradient.[1] This procedure was used for the same set of samples shown in Table 1 (Figure 3). All levels tested showed <0.2% carryover for the column, which was less than 20% of the lower limit of quantitation. The ability to quantitate proteins from 0.1–50 µg with minimal carryover makes this a good method for accurate and consistent quantitation of low level impurities.

The above experiments show the utility of quantitating a single protein peak using reversed-phase chromatography. At various stages in the biopharmaceutical development process, it is necessary to quantitate individual proteins in a mixture, most often proteins that are low abundance impurities in the presence of a larger amount of another protein. To demonstrate this, a series of spiked IgG samples was analyzed. These samples contained 50 µg of a humanized IgG with spiked levels of a murine IgG at levels of 1–5% of the larger IgG protein peak. As shown in Table 2, the measured impurity amount matched the expected values with good reproducibility.

Amount on column (µg)	Average area	Area % RSD
0.1	39848	1.91
0.5	285091	1.66
1.0	566568	1.47
5.0	2634778	0.52
7.5	3945844	0.08
10.0	5526662	0.03
20.0	9954028	0.21
30.0	14643136	0.88
40.0	22532273	0.79
50.0	27894201	1.10

Table 1. Average values for triplicate injections of humanized IgG4, from 0.1 µg to 50 µg mass load on column (3 µL constant injection volume). The data set was linear across all levels tested with an R2 value of 0.994. Variability for triplicate injections was <2%.

	Impurity		API
Nominal % of API	Measured % of API	Area % RSD	Area % RSD
1.00	0.94	1.61	0.83
2.50	2.48	1.05	0.36
5.00	4.88	2.43	2.78

%RSD values based on 5 replicates

Table 2. Quantitation of trace amounts of a different protein in an IgG formulation. Five replicate injections were made for each level. The measured % area of the active pharmaceutical ingredient (API) was compared to the nominal values. The area reproducibility for both API and trace impurity is shown for all levels tested.

CONCLUSIONS

The sensitivity and resolution offered by reversed-phase chromatography make it a good investigative tool for the characterization of proteins. Consistent analyses are observed over a wide range of protein concentrations without any distortion of the chromatographic retention or peak shape. Reliable and reproducible quantitative results are readily obtained for monoclonal antibodies using the BEH300 C_4 column chemistry. Minimal protein carryover on this column chemistry helps to improve the accuracy of the quantitative results. Through the combination of ACQUITY UPLC and the BEH300 C_4 column chemistry, fast and reliable quantitative results are obtained at any point in the lifecycle of a protein biotherapeutic.

References

1. Protein Separation Technology ACQUITY BEH300 C_4, 1.7 µm, Waters Care & Use Manual, 715001870en.

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Reversed-Phase Analysis of Proteins Using ACQUITY UPLC H-Class Bio System and Auto•Blend Plus Technology

GOAL

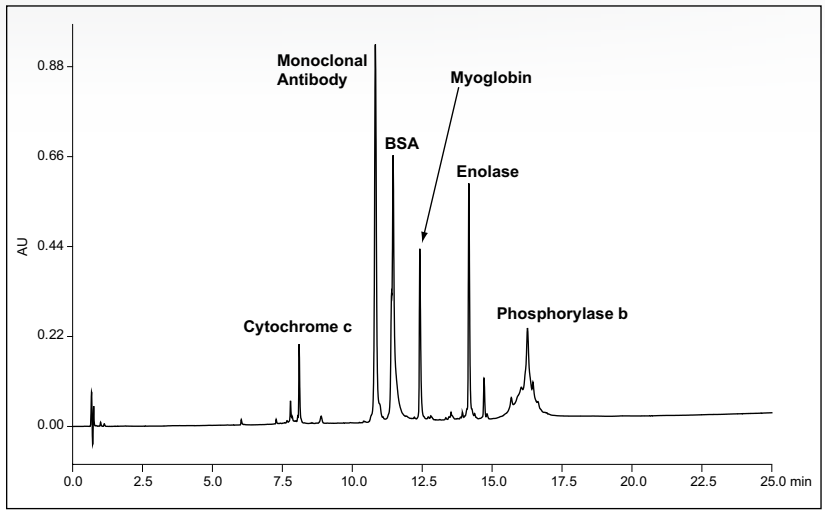
To demonstrate rugged, high-resolution separation of proteins using reversed-phase techniques, four-solvent blending, UPLC® Protein Separation Technology columns, and the ACQUITY UPLC® H-Class Bio System.

BACKGROUND

The characterization and analysis of protein samples must be sensitive to chemical differences in a molecule that, while but a small fraction of the large molecule, can have large biological effects. The general approach to this analytical challenge is to use an array of separation techniques, each sensitive to a specific physical or chemical property. The ACQUITY UPLC H-Class Bio System was designed to be suitable for use with all the common protein analysis techniques, including ion exchange, size exclusion, HILIC, and reversed phase. The experiments described here focus on reversed phase analysis of large, intact proteins.

Reversed phase separations of proteins typically use wide pore columns with short chain bonded phases. The ACQUITY UPLC BEH 300 C_4, 1.7 µm Column was selected for these experiments. The samples are eluted with a gradient of increasing organic solvent in the presence of a polar acid modifier. There are several choices in identity and concentration for both the solvent and the modifier. We can manage these choices by using the four-solvent blending capability of the

A robust separation of intact proteins is readily achieved with this biocompatible UPLC system that streamlines use of desired mobile phase combinations.



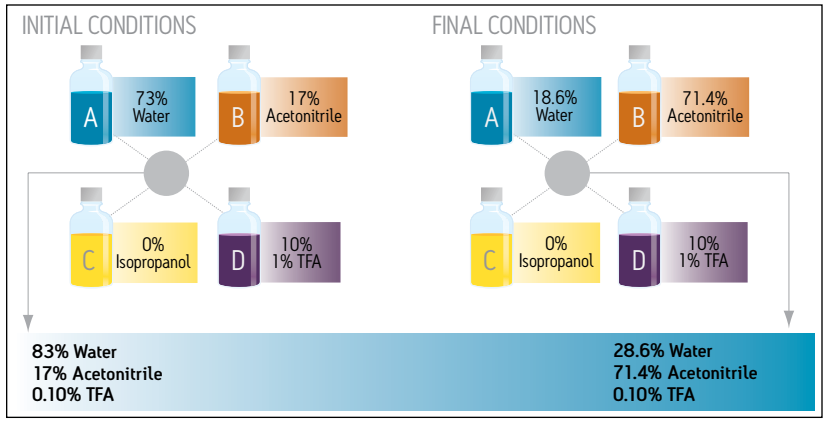
The Auto•Blend Technology method blends the required mobile phase from reservoirs of pure solvents and concentrated modifier stocks. The reversed phase chromatogram generated from this convenient method shows a wide range of proteins as a test of robustness.

ACQUITY UPLC H-Class Bio System's quaternary solvent manager to prepare the different desired mobile phase combinations on-demand from bottles of pure solvents and concentrated modifiers. This Auto•Blend Plus™ Technology is only useful if it reliably produces the programmed mobile phase gradient accurately and precisely. The experiments described here focus on that system performance.

THE SOLUTION

A robust method for protein analysis must include an instrument, a column chemistry, and the set of operating conditions. For developing the separation discussed here, UPLC Technology was chosen as the underlying principle. The use of sub-2-µm particle packing materials on instruments that minimize dispersion ensures the best resolution, sensitivity, and sample throughput. The ACQUITY UPLC family of instruments provides the required performance.

The ACQUITY UPLC H-Class Bio System was chosen for these experiments due to its biocompatible materials of construction, and because its four-solvent blending combines with Auto•Blend Plus Technology for easy methods development and execution. The UPLC Protein Separation Technology BEH300 C_4, 1.7 µm column was selected for the separation because its combination of large pores, short chain bonded phase, and inert base particle gives the best resolution and recovery. Auto•Blend Plus Technology was used to find the best combination of modifier concentration and organic solvent. The convenience of method development with Auto•Blend Plus requires a high degree of accuracy and precision in solvent blends over a long series of runs. The selected four-solvent method and the resultant chromatogram are shown in the accompanying figure.



Injection	Cytochrome *c*	Chimeric Antibody	BSA	Myoglobin	Enolase	Phosporylase b
1	8.169	10.898	11.532	12.497	14.252	16.353
2	8.16	10.882	11.523	12.482	14.237	16.359
3	8.18	10.909	11.548	12.505	14.259	16.356
4	8.176	10.897	11.546	12.497	14.251	16.358
5	8.179	10.898	11.536	12.501	14.244	16.354
6	8.179	10.909	11.548	12.504	14.257	16.358
mean	**8.174**	**10.899**	**11.539**	**12.498**	**14.250**	**16.356**
std dev	**0.008**	**0.010**	**0.010**	**0.008**	**0.008**	**0.002**
%RSD	**0.096**	**0.091**	**0.089**	**0.067**	**0.058**	**0.015**

The accompanying table shows the reproducibility of retention times for this protein mixture. The proteins represent a wide range of properties and sizes, providing a good test of separation consistency. The reproducibility of the separation is suitable for routine use. The application of Auto•Blend Technology for preparation of blended mobile phases reduces labor and the possibility of error while providing reliable results.

SUMMARY

The requirements for a reversed phase analysis of intact proteins are satisfied by the system solution that combines ACQUITY UPLC H-Class Bio instrumentation, UPLC column chemistry, and software. The robustness of the system includes simplified mobile phase preparation, and is demonstrated with reproducibility of the separation.

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Reversed-Phase for Peptide Mapping

Due to the resolving power, RP-LC has long been the preferred analytical technique to characterize and quantify various products.

When used for peptide separations (whether they are from protein digests or resulting from synthetic peptide processes), this technique is useful in the confirmation of amino acid sequences, identification of post-translational modification (PTM), and analysis of impurities.

Any difference in the structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amount of the peptide with and without a particular modification is used to measure the fraction of the protein in a particular sample that carries that modification.

To meet these application requirements, long, shallow gradients are often required. Low abundance peptides, representing these trace modifications, may co-elute with major components. Therefore, these analyses benefit from the use of the most highly resolving chromatographic techniques.

Solution Highlights

The use of **ACQUITY UPLC H-Class Bio System** for peptide mapping optimizes resolution by reducing peak dispersion. While this approach leads to better resolution, sensitivity, and speed, it is still necessary to exploit the chemical and chromatographic operational variables that can be used to optimize retention and selectivity. These variables include the nature of the organic solvent, pH, modifier, and their concentrations along with temperature, flow rate, and gradient steepness. Exploring this separation space requires preparation of many solvent mixtures. **Auto•Blend Plus Technology** makes it easier to manipulate these parameters that can further enhance resolution.

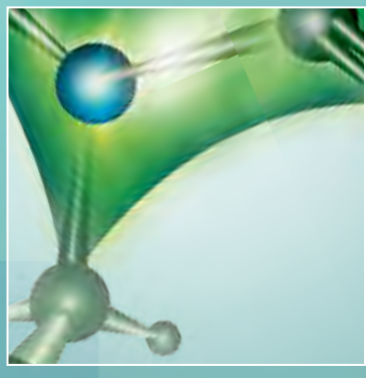
Waters Ethylene Bridged Hybrid (BEH Technology) or the Charged Surface Hybrid (CSH) Peptide Separation Technology columns result in better resolution of complex protein digests and higher throughput. By virtue of having a novel charged surface, the stationary phase in ACQUITY UPLC Peptide CSH C_{18}, 130Å Columns is able to produce extraordinarily high peak capacities even when used with LC-MS friendly mobile phases like formic acid.

Meanwhile, the ACQUITY UPLC Peptide BEH C_{18}, 130Å and 300Å Columns are also available and have proven highly effective for separations of a wide range peptides: large and small, acidic and basic, hydrophilic and hydrophobic. **The MassPREP Digestion Standards and MassPREP Peptide Mixtures** were specifically developed and are QC tested (with available Certificates of Analyses Documentation) for use in RP column evaluation and for verifying instrumentation performance.

The ACQUITY QDa Detector brings the confidence of mass detection to routine peptide analyses.

For meaningful peptide mapping, both quantitative and qualitative reproducibility are required. The ACQUITY QDa Detector is Waters' solution to meet the demands of today's fast-paced biopharmaceutical laboratories by enabling complementary optical and mass analysis techniques. Using on-line MS detection, it is now possible to have a single screening workflow that can improve the productivity of data analysis.

- Monitor peptides from protein digests.
- QC test synthetic peptides.
- Quantify peptide variants or related species.
- Monitor formulation components.
- Add mass data to routine analyses in pilot, manufacturing, and QC.



Effect of Pore Size in UPLC Peptide Mapping

Beth L. Gillece-Castro, Thomas E. Wheat, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, USA

INTRODUCTION

The enhanced chromatographic resolution associated with UltraPerformance LC® (UPLC®) has been demonstrated for peptide mapping (Mazzeo, et al., Biopharm.). The technique improves resolution by a factor of three or more.

In addition, the surface chemistry of the ACQUITY UPLC® BEH Technology™ particles has proven especially advantageous for peptide mapping; good retention and peak shape are observed with either TFA or formic acid as a modifier. The former modifier is preferred for best sensitivity with UV detection, while the latter improves signal-to-noise in electrospray MS experiments. Glycopeptides, which exhibit microheterogeneity, have also been shown to have enhanced resolution and peak shape. These benefits have been demonstrated for tryptic digests of several proteins.

The utility of UPLC peptide mapping for characterizing protein structure can be further extended by expanding the range of column chemistries available. Many peptide chromatographers prefer to use large pore packing materials for peptide separations. The Peptide Separation Technology columns for UPLC include both 130Å and 300Å pore size materials.

The separation of two complex peptide digests on these two pore sizes are compared here. One is a tryptic digest of phosphorylase b, a sample with a large number of smaller peptides; and the other is a LysC digest of phosphorylase b, to give a smaller number of larger peptides. Coupling UPLC chromatography to an oa-TOF mass spectrometer allows high sensitivity identification of peptides and glycopeptides by exact mass measurement.

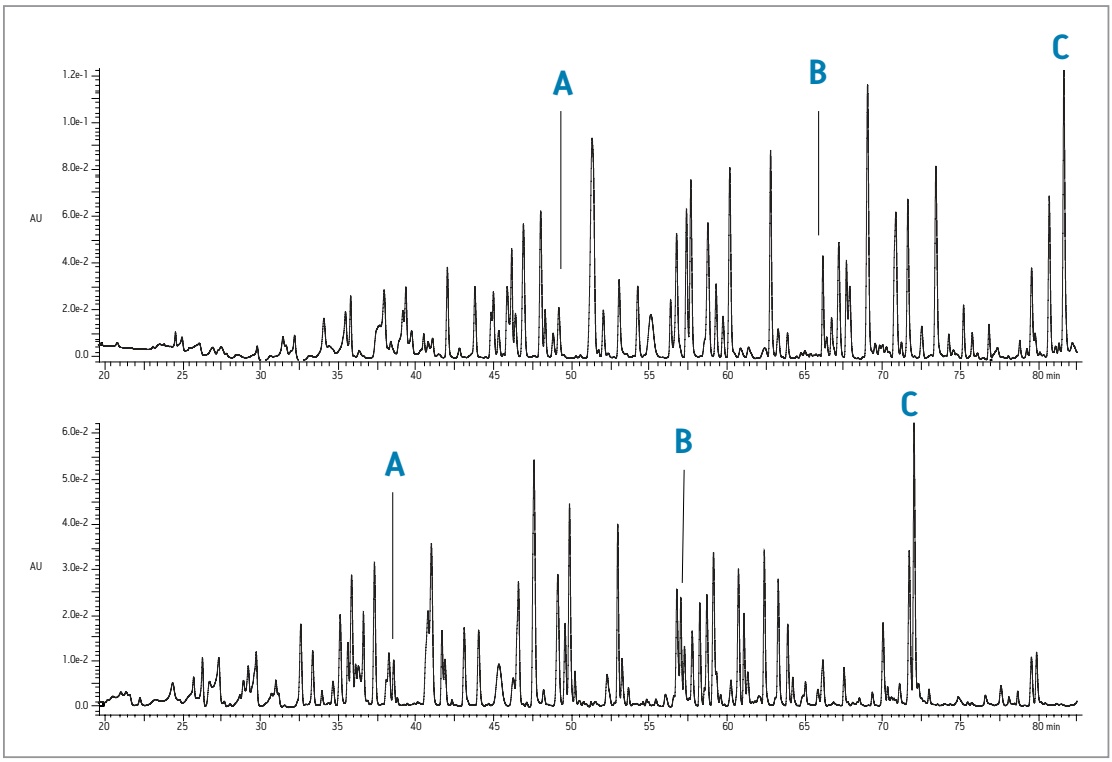


Figure 1. Comparison of the separation of tryptic digest of phosphorylase b on BEH 130, 1.7 µm (top) and on BEH 300, 1.7 µm (bottom) Peptide Separation Technology Columns.

RESULTS AND DISCUSSION

Phosphorylase b was chosen to test chromatography due to the large number of peptides that can be derived from this 97 kDa protein. 110 peptides are expected from a complete tryptic digestion, whereas an IgG protein typically forms 50 to 60 tryptic peptides.

Separations of the phosphorylase b tryptic digest are shown in Figure 1. The same sample was analyzed on both the 130Å and the 300Å columns. Three peptides were chosen to monitor specific changes in the chromatogram. The observed peptide maps both have a large number of reasonably well-resolved, sharp, symmetrical peaks. Retention is lower on the larger pore size material. The change in retention for the specific tracked peaks is equivalent to elution at 4 to 5% lower concentration of acetonitrile. Even with the lower retention on the 300Å column, the four-residue peptide A is easily analyzed.

The selectivity between the two columns is similar, but not identical. For example, a similar pattern of peaks is found between 42 and 46 minutes in the 130Å separation and between 52 and 56 minutes on the 300Å column. Expressed as total resolving power, the calculated peak capacity for the 130Å column is about 1026 and for the 300Å, is 1064. About 230 peaks were recognized on the smaller pore material and 240 on the larger pore.

Digestion of this protein with LysC gives a smaller number of peptides including some larger species. The LysC digests were separated on both columns as shown in Figure 2. Again, peptide retention is generally lower on the larger pore size, and the selectivity is similar, but not identical. The columns, however, still share the same utility described for the separation of the tryptic digests.

The 300Å packing would often be preferred for peptide mapping because the larger molecules are thought to diffuse more freely with larger pores. The chromatographic changes in these experiments do not obviously correlate with the molecular weight of the peptides. For example, the four-residue peptide GRIF is observed as a well-retained and resolved, symmetrical peak in all four maps. A much larger peptide, the LysC peptide representing the 36 residues with a molecular weight of 4477 Da, also elutes as a symmetrical peak from both columns. We are actively investigating the physical and chemical basis of the differences in chromatography.

Both Peptide Separation Technology columns give useful separations of peptides over a wide range of sizes and chemical properties. The differences in selectivity will prove advantageous in the process of developing an optimized peptide map for a given protein.

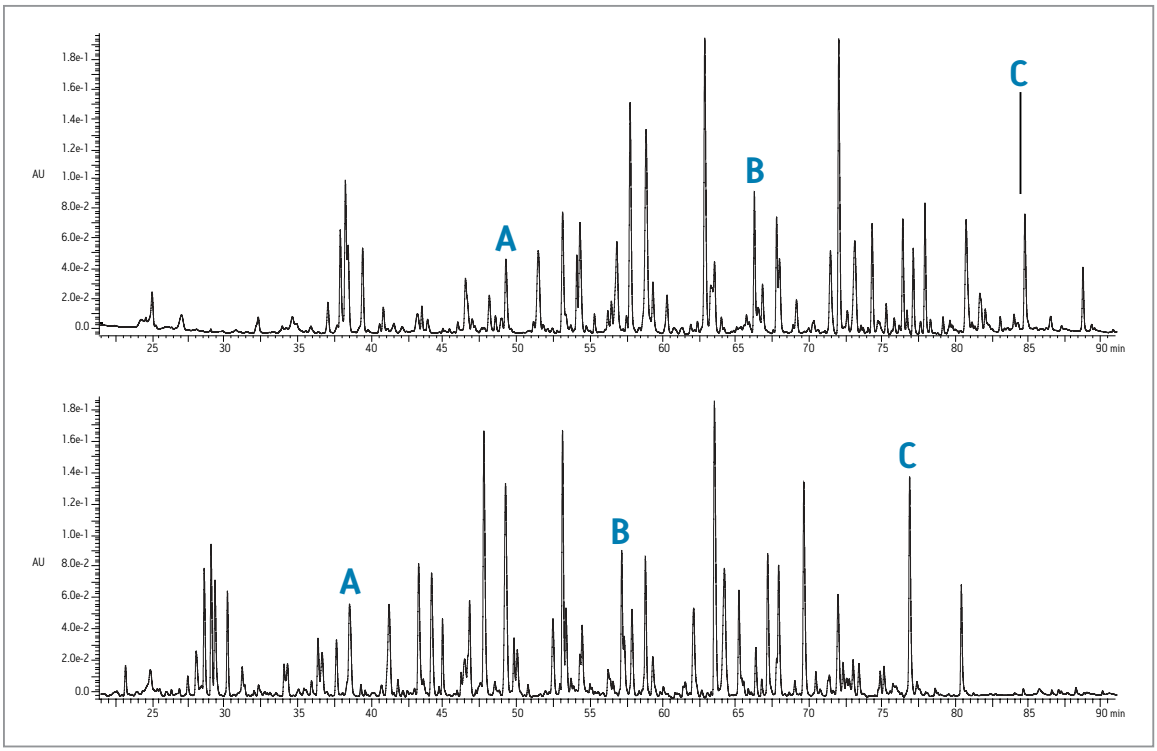


Figure 2. Comparison of the separation of LysC digest of phosphorylase b on BEH 130, 1.7 µm (top) and on BEH 300, 1.7 µm (bottom) Peptide Separation Technology columns.

MATERIALS AND METHODS

System configuration

- Standard ACQUITY UPLC System modules, including the Binary Solvent Manager and Sample Manager, were used for the separation.
- A high-sensitivity mixer for peptide analysis (P/N 205000403) was added to the system. Separations were monitored with a TUV detector at 214 nm and with an LCT Premier™ Q-Tof Mass Spectrometer.

Preparation of digests

Phosphorylase b (rabbit) was dissolved in aqueous ammonium bicarbonate (pH 8) to a concentration of 1 mg/mL, and *Rapi*Gest™ was added to a concentration of 0.1%. LysC or Trypsin was added to separate aliquots of phosphorylase b solution at an enzyme-to-substrate ratio of 1:50 (w/w), and the samples were incubated overnight at 37 °C. The digestions were terminated by addition of trifluoroacetic acid to a concentration of 0.1%, and stored at <20 °C.

SEPARATION METHOD

The same chromatography method was used for both digests on both columns; that is, for all four experiments.

Injection:	Volume: 10 µL
Columns:	Waters Peptide Separation Technology ACQUITY UPLC BEH 130, 1.7 µm 2.1 x 100 mm ACQUITY UPLC BEH 300, 1.7 µm 2.1 x 100 mm
Temperature:	40 °C
Flow rate:	100 µL/min
Solvent A:	0.1% TFA in water
Solvent B:	0.08% TFA in acetonitrile

Gradient Table

Time	%A	%B	Curve
Init.	100	0	6
2	100	0	6
118	50	50	6
120	25	75	6
122.1	100	0	6
122	25	75	6
150	100	0	6

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Efficient Development of Peptide Maps Using the Multi-Solvent Blending Capability of the ACQUITY UPLC H-Class System

GOAL

The ACQUITY UPLC® H-Class System is used for the systematic optimization of peptide mapping conditions in a fully automated protocol using four-solvent blending.

BACKGROUND

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTM), and analyze impurities. Any difference in structure of a protein is reflected in a change in retention time for the peptide containing the modification.

Complete structural characterization of a protein requires resolution of 100 or more peptides that span a wide range of size and molecular properties. The experiments to develop the required separation conditions include changes in the mobile phase modifier and organic solvent composition. Each condition tested requires the preparation of a pair of mixed solvents involving two or three exact measurements.

With the four-solvent blending of the ACQUITY UPLC H-Class System, these solvent mixtures are generated by the instrument from bottles of pure solvent. In this way the labor of preparing many solvent mixtures is reduced, and the possibility of error is minimized. Intermediate compositions can also be easily tested.

The ACQUITY UPLC H-Class System simplifies the development of reliable peptide maps that can be used to characterize the structure of a protein.

The ACQUITY UPLC H-Class System and its four-solvent blending capability enables the user to focus on the quality of the analysis so that reliable results can be obtained efficiently.

THE SOLUTION

MassPREP™ Enolase Digestion Standards were separated on a Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18} Column with various mobile phase combinations. The ACQUITY UPLC H-Class System was configured with water as Solvent A, acetonitrile as Solvent B, isopropanol as Solvent C, and 1% TFA in water as Solvent D. To assess the effect of acid concentration, three identical gradients of increasing acetonitrile were run with 2.5%, 5%, and 10% D, respectively, corresponding to 0.025%, 0.05%, and 0.1% TFA.

The chromatograms in Figure 1 show significant changes in selectivity as a function of acid concentration in the mobile phase. The experiment can be extended by repeating the same experiments but using a gradient of increasing isopropanol. This is conveniently performed by running the gradient from Solvent A (water) to Solvent C (isopropanol) with the same constant percentage of D as in the previous experiments. The change from acetonitrile to isopropanol reduces retention while also giving substantial changes in selectivity.

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This set of experiments evaluates six different mobile phase formulations to identify the optimum peptide map. The changes in selectivity can be used to obtain the best resolution of the critical peptides in the mixture. To perform these experiments with a binary system or with a switching valve, the scientist would need to prepare 12 bottles of mobile phase with at least 24 volume measurements. With the ACQUITY UPLC H-Class System, only one mixture was used, with two measured volumes, in conjunction with three bottles of pure solvents (Figure 2). The ACQUITY UPLC H-Class System makes it possible to reduce the amount of work, time, and potential for error in reaching the optimum separation conditions.

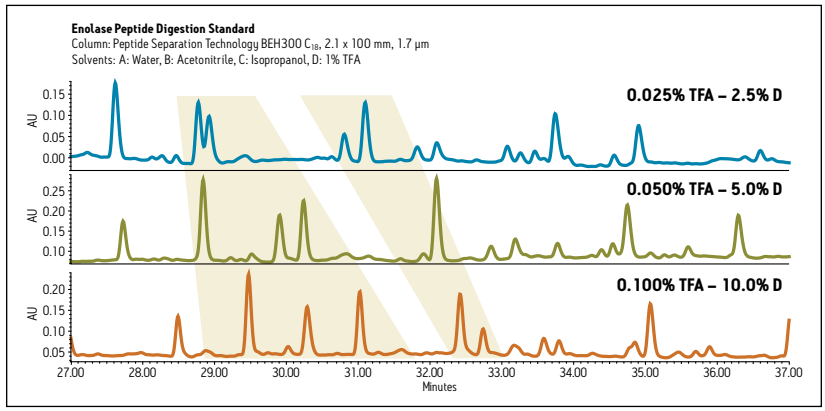

Figure 1. Solvent composition is easily adjusted using the Auto•Blend™ functions of the ACQUITY UPLC H-Class System. In this example, the optimum concentration of TFA for a peptide map is identified simply by varying the percentage of flow taken from the D line. There is no need to make extra bottles of solvent, and intermediate values can be tested with minimal effort.

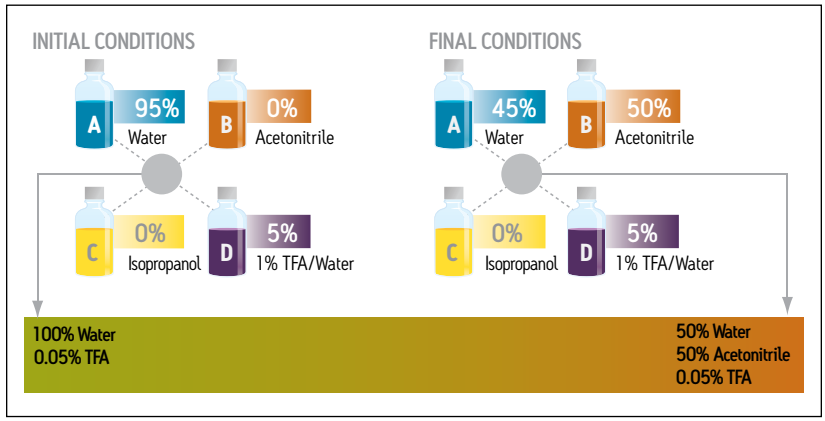

Figure 2. Solvent mixtures are generated from bottles of pure solvent by the ACQUITY UPLC H-Class System's Auto•Blend functionality.

SUMMARY

Biochemists can use the ACQUITY UPLC H-Class System to efficiently develop peptide maps for protein characterization. The system's easy-to-use four-solvent blending capability enables the user to focus on the quality of the analysis so that complete, reliable separations can be developed efficiently. The ACQUITY UPLC H-Class System combines UPLC separation principles with flexible instrument operation to provide the best possible results for bioseparations.

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Future-proofing the Biopharmaceutical QC Laboratory: Integrating Auto•Blend Technology to Improve Routine Peptide Mapping

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Auto•Blend™ Technology for acidic modifier control
- Transfer peptide map applications from HPLC to UPLC®
- Future-proof laboratory for UPLC methods

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

XBridge® C_{18} 130 Å 3.5 µm, 4.6 x 100 mm Column

Empower® 3 Chromatography Data Software

KEY WORDS

Auto•Blend Technology, peptide mapping, automated mobile phase management

INTRODUCTION

As an initial step towards transferring peptide mapping methods from HPLC to UPLC, we previously presented an approach using the ACQUITY UPLC H-Class Bio System for legacy HPLC-based peptide mapping.[1] Our method transfer discussion continues here, focusing on improving the consistency of peptide mapping separations during routine analyses.

Peptide mapping methods generally include an acidic modifier to improve peak shape. However, accurate and reproducible management of the modifier content within mobile phase solvents can be variable, consequently affecting the peptide map quality and reproducibility.

This application note demonstrates the ability of Auto•Blend Technology to control the trifluoroacetic acid (TFA) component of the mobile phase during routine peptide mapping analyses, thereby producing chromatograms of equivalent performance to HPLC-acquired chromatograms with conventionally modified mobile phases.

TFA is commonly used as a modifier in peptide mapping methods with optical detection because it provides peak shape and chromatographic resolution benefits. Concentrations of TFA are typically low in most applications, accounting for 0.02% to 0.20% of the final mobile phase. Subtle changes in the modifier concentration can have profound effects on peptide retention time, resolution, and elution order, causing concern over chromatographic reproducibility and the occurrence of out-of-specification results. Such issues ultimately affect productivity due to time-consuming resolution of QC issues as opposed to moving product to the marketplace.

The reason for this classical approach using TFA in peptide mapping chromatography has been to modify the two mobile phases, normally water and acetonitrile, with a pre-determined amount of TFA. Here, we demonstrate the benefits of allocating TFA to an independent solvent line using Auto•Blend to manage its contribution to the solvent composition throughout gradient delivery. The result is not only consistency in chromatographic performance but a significant benefit in terms of chromatographic reproducibility with minimal solvent preparatory requirements. Auto•Blend Technology in peptide mapping enables QC labs to spend less time in the prep labs, instead focusing on driving productivity.

EXPERIMENTAL

Sample preparation

Two peptide preparations were used in this study: Ribonuclease B (Sigma Aldrich, USA), and infliximab, both prepared as follows. Five hundred µg of protein was reduced with dithiothreitol, alkylated with iodoacetamide, and isolated using NAP-5 columns (GE Healthcare, PA, USA). Sequence-grade trypsin (Promega, CA, USA) was added to each protein to a final composition 1:20 enzyme/substrate with samples subsequently digested overnight at 37 °C. Following digestion, trypsin was deactivated by incubation at 70 °C for 15 minutes, and 60 µL of digested protein material was reconstituted in 40 µL of 5% MeCN/0.1% TFA, generating a final peptide concentration of 0.6 µg/µL.

UPLC conditions

System:	ACQUITY UPLC H-Class Bio with Tunable UV Detector with 10-mm titanium flow cell
Extension loop:	100 µL (p/n 430002625)
Mixer:	250 µL (p/n 205000737)
Column:	XBridge BEH C_{18} 130 Å 3.5 µm, 4.6 x 100 mm
Column temp.:	40 °C
Injection volume:	95 µL
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	1% (v/v) TFA in water

Detection wavelength: 214 nm

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D	Curve
–	0.500	85	5	10	0	6
5.00	0.500	85	5	10	0	6
45.00	0.500	40	50	10	0	6
47.50	0.500	0	90	10	0	6
52.50	0.500	0	90	10	0	6
52.60	0.500	85	5	10	0	6
60.00	0.500	85	5	10	0	6

RESULTS AND DISCUSSION

Evaluation of TFA effect on peptide retention time

To illustrate the impact of changing TFA concentration on peptide retention time, a series of trypsinized Ribonuclease B peptide separations were performed with a TFA concentration ranging from 0.08% to 0.10% in 0.01% increments. The resulting chromatograms (Figure 1) show significant differences in peak retention times with changes in TFA concentration of 0.01%. Changing TFA concentrations is observed to affect not only the peak retention time but also selectivity, where various peaks are observed to change elution order based on TFA concentration (Figure 1). Based on such sensitivity to TFA concentration, accurate and reproducible preparation of solvents containing TFA must be considered critical for improving consistency in peptide mapping.

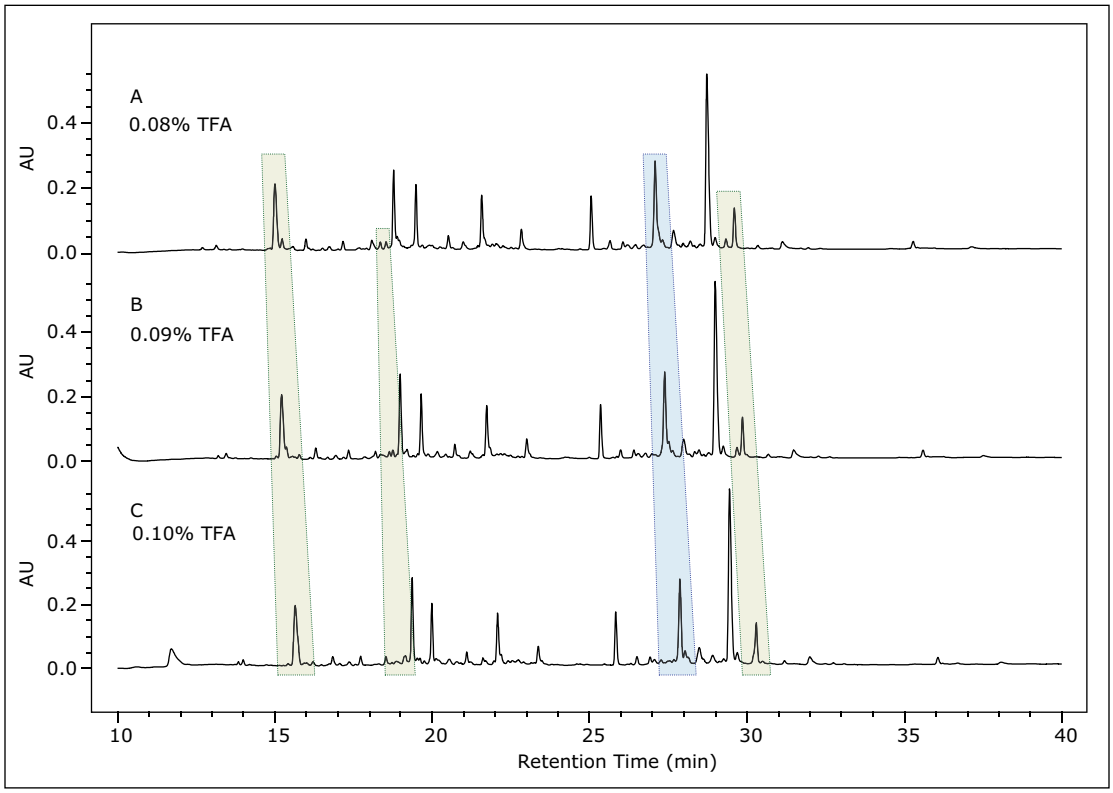

Figure 1. Effect of TFA concentration on peptide separation. Trypsinized Ribonuclease B peptides were separated with varying amounts of TFA ranging from 0.08% to 0.10%. Changes of as little as 0.01% TFA resulted in changes in peak retention times. Peaks within green enclosures indicate a loss of resolution while peaks contained within blue enclosures illustrate an increase in resolution. (A) 0.08% TFA. (B) 0.09% TFA. (C) 0.10% TFA. Adjustments to TFA concentration were obtained using Auto•Blend Technology.

Using Auto•Blend Technology to automate accurate delivery of TFA

One approach for eliminating TFA concentration variability is to remove the additive as a component of each mobile phase, instead providing a stock concentration of TFA as its own solvent line. This results in three solvents contributing to a peptide map gradient, all of which can be accurately managed using Auto•Blend. To demonstrate the use of Auto•Blend in peptide mapping using this solvent arrangement, 1% TFA in water was prepared and configured on solvent line C beside MilliQ dH_2O and acetonitrile as solvents A and B, respectively. Evaluation of Auto•Blend Technology for controlling TFA in peptide mapping was performed using trypsinized infliximab as a model protein therapeutic.

Previous peptide mapping of trypsinized infliximab monitored a total of 56 peaks.[1] For comparative purposes between the HPLC and ACQUITY UPLC H-Class Bio instruments, and instrument methods (with or without Auto•Blend), the same 56 peaks were monitored. Trypsinized infliximab was separated using either a standard configuration of solvents modified with 0.1% TFA or in an Auto•Blend configuration with three solvents (pure acetonitrile, pure water, and 1% formic acid in water) used for gradient formation. Each of these configurations was compared to the legacy method generated on an HPLC instrument (Figure 2a).

In the conventional mobile phase delivery using the ACQUITY UPLC H-Class Bio System, comparable chromatography can be observed to that obtained on the HPLC instrument (Figure 2b). Using the Auto•Blend configuration with 1% TFA as a separate solvent line, no difference in selectivity and nearly identical retention times are observed (Figure 2c). Importantly, no difference was observed between the chromatogram obtained using Auto•Blend Technology and the chromatogram obtained on the HPLC instrument; a result that supports the use of ACQUITY UPLC H-Class Bio and Auto•Blend for running legacy HPLC peptide mapping methods.

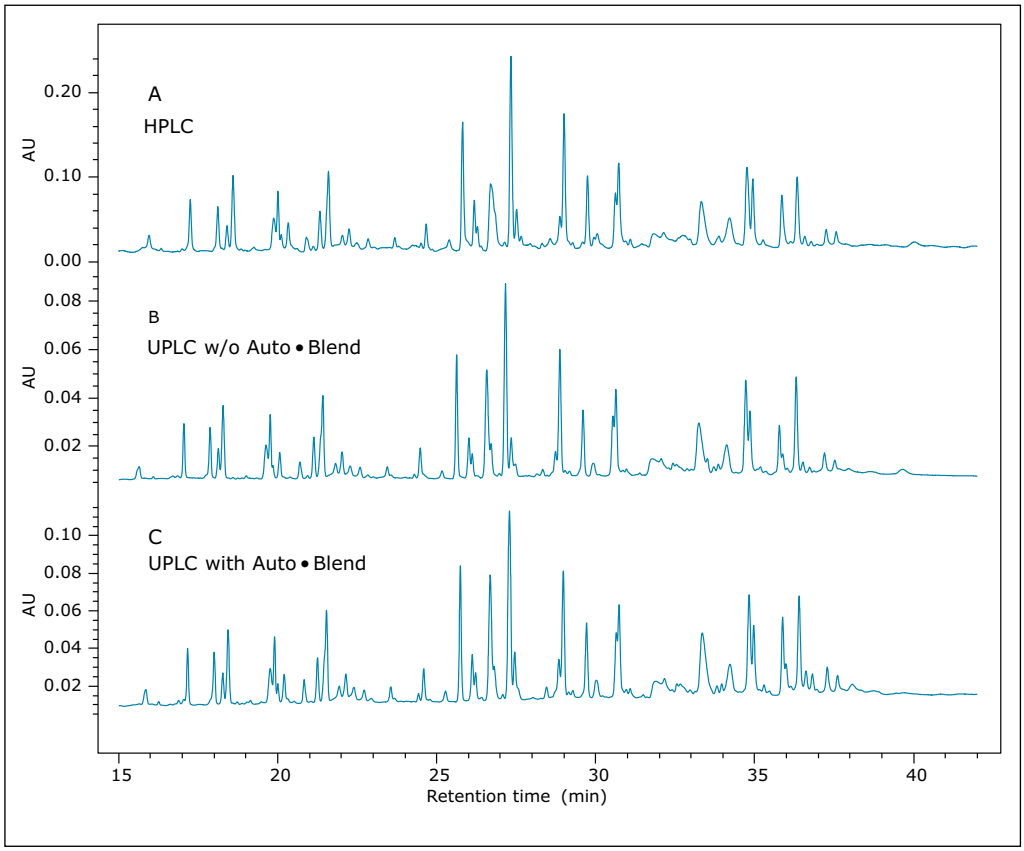

Figure 2. Incorporation of Auto•Blend Technology for peptide mapping produces chromatograms comparable to conventional solvent. The challenge for Auto•Blend control of multiple solvent lines is to replicate chromatographic performance of a legacy HPLC peptide mapping method. A trypsinized infliximab peptide map was produced on an HPLC using a legacy HPLC method with the acidic modifier as a component of two solvents (A), establishing the benchmark for separation on the ACQUITY UPLC H-Class Bio System. The same approach for solvent use was set up on the ACQUITY UPLC H-Class Bio System to establish comparability to the HPLC chromatogram (B). TFA was then removed as a component and provided its own solvent line, where the resulting chromatography was found to be significantly comparable to both the HPLC method and the HPLC method run on the ACQUITY UPLC H-Class Bio System.

Adoption of Auto•Blend Technology for peptide mapping methods allows more consistent mobile phase composition and delivery, which ultimately benefits chromatogram reproducibility over wider time spans and reduces analyst bench time preparing stock solvents adjusted with acidic modifiers.

Auto•Blend control of TFA results in reproducible peptide mapping

For QC labs performing peptide mapping on a routine basis, reproducibility and reliability are key factors that ultimately drive productivity. To determine the reproducibility, and hence consistency, of the ACQUITY UPLC H-Class Bio System with Auto•Blend for peptide mapping applications, five injections of trypsinized infliximab were run on the ACQUITY UPLC H-Class Bio System. Results of the evaluation demonstrated comparable chromatograms with standard deviations no greater than 0.011 (Figure 3a and Table 1). This finding was confirmed by measuring the relative peak area across each chromatogram, where low standard deviation was found with the relative peak area of each monitored peak (Figure 3b).

Measurement of reproducibility within a peptide map is best reported in terms of percent relative standard deviation (%RSD) of peak retention times. The performance of the ACQUITY UPLC H-Class Bio System in terms of retention time reproducibility was also evaluated through determination of relative standard deviation (RSD) with calculated retention times indicating a maximum %RSD value of 0.089% (Table 1). This represents a value significantly lower than that required by existing regulatory guidelines. Despite the inclusion of an additional solvent line, Auto•Blend Technology demonstrated a capacity to generate highly consistent and reproducible chromatograms in both retention time (Figure 3a) and peak area values (Figure 3b).

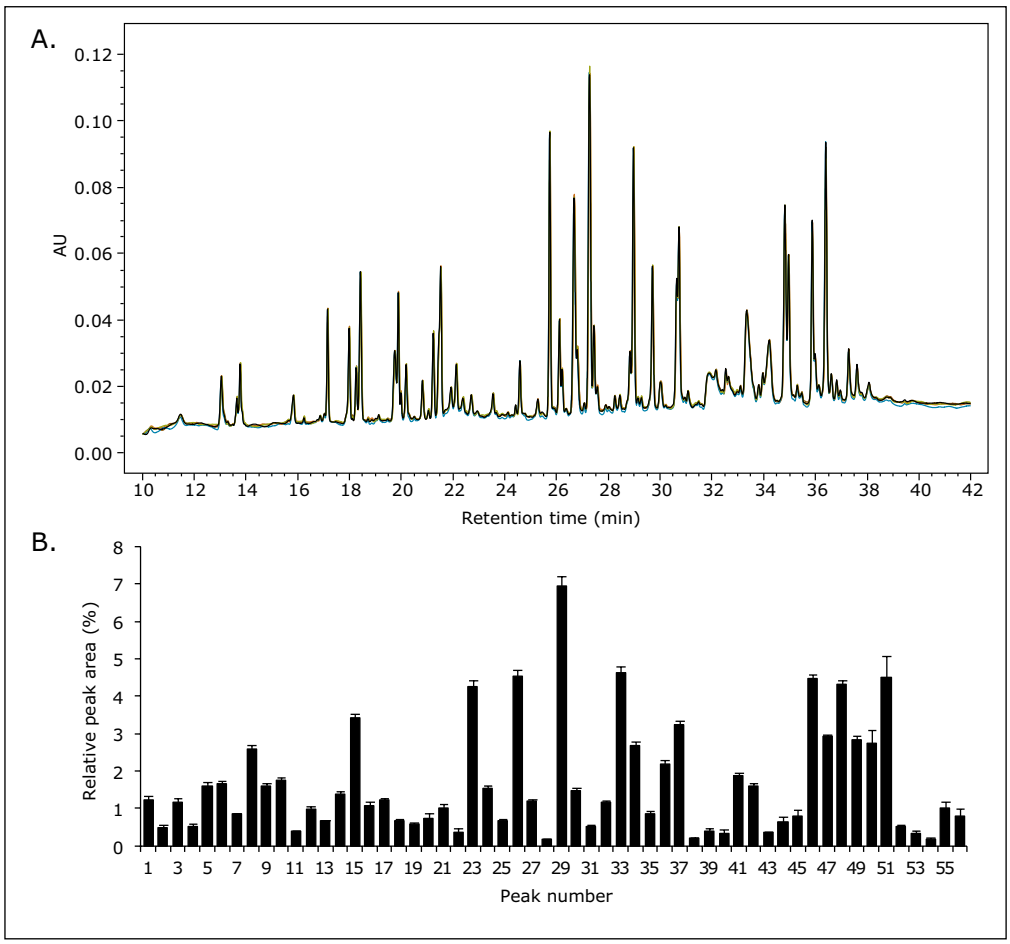

Figure 3. Auto•Blend Technology provides accurate and consistent reproducibility for peptide mapping on the ACQUITY UPLC H-Class Bio System. To ascertain the reproducibility of the ACQUITY UPLC H-Class Bio and Auto•Blend Technology, a total of five injections were performed with retention times tabulated for a total of 56 common peptide peaks identified in the infliximab peptide map. The resulting five chromatograms were overlayed to illustrate the reproducibility of a separation using Auto•Blend (A). Reproducibility of relative peak area was also assessed, with average relative peak areas and associated standard deviations provided as a column chart (B).

Peak	Retention Time (min)					$\bar{x}$	s	% RSD
	1	2	3	4	5			
1	13.034	13.041	13.055	13.063	13.053	13.049	0.012	0.089
2	13.639	13.639	13.647	13.660	13.649	13.647	0.009	0.064
3	13.770	13.769	13.778	13.791	13.780	13.778	0.009	0.065
4	15.826	15.828	15.838	15.847	15.843	15.836	0.009	0.058
5	17.151	17.150	17.161	17.163	17.157	17.156	0.006	0.034
6	17.982	17.982	17.994	18.000	17.990	17.990	0.008	0.043
7	18.254	18.254	18.265	18.271	18.262	18.261	0.007	0.040
8	18.416	18.417	18.428	18.434	18.425	18.424	0.008	0.041
9	19.878	19.881	19.892	19.895	19.890	19.887	0.007	0.037
10	19.980	19.983	19.994	19.995	19.991	19.989	0.007	0.034
11	20.180	20.183	20.194	20.198	20.192	20.189	0.008	0.038
12	20.815	20.813	20.825	20.828	20.822	20.821	0.006	0.031
13	21.049	21.048	21.060	21.065	21.060	21.056	0.008	0.036
14	21.233	21.232	21.244	21.249	21.244	21.240	0.008	0.035
15	21.518	21.518	21.530	21.534	21.527	21.525	0.007	0.033
16	21.916	21.915	21.928	21.937	21.925	21.924	0.009	0.041
17	22.125	22.124	22.135	22.145	22.132	22.132	0.009	0.039
18	22.382	22.377	22.392	22.404	22.386	22.388	0.010	0.046
19	22.705	22.700	22.713	22.721	22.710	22.710	0.008	0.035
20	23.547	23.541	23.555	23.561	23.555	23.552	0.008	0.033
21	24.582	24.579	24.590	24.595	24.592	24.588	0.007	0.028
22	25.270	25.272	25.284	25.285	25.284	25.279	0.007	0.029
23	25.730	25.734	25.745	25.741	25.744	25.739	0.007	0.025
24	26.106	26.108	26.121	26.117	26.122	26.115	0.007	0.028
25	26.211	26.213	26.226	26.222	26.228	26.220	0.008	0.029
26	26.668	26.673	26.689	26.684	26.690	26.681	0.010	0.037
27	26.792	26.800	26.816	26.810	26.817	26.807	0.011	0.040
28	27.063	27.070	27.082	27.076	27.082	27.075	0.008	0.030
29	27.266	27.272	27.287	27.280	27.285	27.278	0.009	0.032
30	27.436	27.440	27.454	27.449	27.455	27.447	0.008	0.031
31	27.569	27.575	27.588	27.585	27.590	27.581	0.009	0.033
32	28.831	28.833	28.849	28.843	28.848	28.841	0.008	0.029
33	28.969	28.971	28.988	28.981	28.986	28.979	0.009	0.030
34	29.701	29.704	29.719	29.711	29.717	29.710	0.008	0.026
35	30.022	30.018	30.029	30.030	30.030	30.026	0.005	0.018
36	30.636	30.640	30.653	30.645	30.653	30.645	0.008	0.025
37	30.726	30.729	30.743	30.735	30.742	30.735	0.008	0.025
38	30.950	30.953	30.967	30.961	30.966	30.959	0.008	0.025
39	31.075	31.078	31.092	31.083	31.090	31.084	0.007	0.024
40	31.484	31.488	31.503	31.493	31.502	31.494	0.008	0.027
41	31.899	31.892	31.914	31.903	31.911	31.904	0.009	0.028
42	32.156	32.152	32.172	32.161	32.169	32.162	0.008	0.026
43	32.410	32.407	32.422	32.404	32.410	32.411	0.007	0.021
44	32.537	32.534	32.550	32.537	32.549	32.541	0.008	0.023
45	33.102	33.102	33.117	33.105	33.115	33.108	0.007	0.022
46	33.342	33.344	33.357	33.350	33.356	33.350	0.007	0.020
47	34.213	34.216	34.231	34.221	34.226	34.221	0.007	0.021
48	34.819	34.821	34.834	34.826	34.829	34.826	0.006	0.017
49	34.962	34.965	34.977	34.969	34.972	34.969	0.006	0.017
50	35.876	35.881	35.893	35.884	35.888	35.884	0.007	0.018
51	36.396	36.401	36.410	36.405	36.406	36.404	0.005	0.015
52	36.614	36.618	36.626	36.622	36.624	36.621	0.005	0.013
53	36.818	36.824	36.830	36.828	36.830	36.826	0.005	0.014
54	36.959	36.966	36.974	36.967	36.971	36.967	0.006	0.015
55	37.281	37.289	37.297	37.292	37.295	37.291	0.006	0.017
56	37.603	37.609	37.619	37.612	37.616	37.612	0.006	0.017

Table 1. Auto•Blend control of TFA delivery generates highly reproducible retention times. Five individual injections of trypsinized infliximab were separated on the ACQUITY UPLC H-Class Bio System using Auto•Blend Technology, where retention times of 56 peaks were monitored. Relative standard deviation (%RSD) was calculated across all chromatograms with a maximum value of 0.089 determined.

CONCLUSIONS

Reliability, robustness, and reproducibility are cornerstones of QC laboratories. For routine analysis of complex peptide maps, LC instrumentation needs to generate consistent chromatography over extended periods of time to adhere to specifications outlined in SOP documents. Modification of mobile phases with acidic modifiers introduces the potential for loss of reproducibility due to sensitivity of peptide maps to subtle changes in modifier concentration. Auto•Blend Technology using the ACQUITY UPLC H-Class Bio System circumvents this issue by simplifying solvent preparation and automating the formulation of the peptide map mobile phase throughout gradient delivery.

As a result, Auto•Blend improves the reproducibility of complex peptide separations, thereby reducing time dedicated to reviewing instrument-related separation issues.

Reference

1. Cosgrave EFJ, McCarthy SM. Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System for HPLC Peptide Mapping. Waters Application Note 720004614en. 2013 June.

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Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio System for HPLC Peptide Mapping

Eoin F.J. Cosgrave and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Transfer peptide map applications from HPLC to UPLC®
- Future-proof laboratory for UPLC methods

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

XBridge® BEH C_{18} 130Å 4.6 x 100 mm, 3.5 µm Column (p/n 186003033)

MassPREP™ peptide mixture standard (p/n 186002337)

Empower® 3 Software

KEY WORDS

Biopharmaceutical analysis, peptide mapping, QC laboratory

INTRODUCTION

This application note represents the first in a series focusing on transitioning HPLC-based biopharmaceutical separations to UPLC-based methods. The procedure for transferring a peptide mapping method from an HPLC quaternary system to the ACQUITY UPLC H-Class Bio System is described here. The goal of this first step is to perform the identical HPLC assay on the UPLC system operating as an HPLC. This approach enables the QC laboratory to employ UPLC technology for legacy HPLC methods while transitioning to UPLC methods at a later time. Improvements in assay reproducibility and, ultimately, incorporating UPLC-based technology will be discussed later in the series.

Peptide mapping represents a typical assay for QC labs dealing with routine analyses of large molecule therapeutics. Peptide separation is a considerable challenge in a QC environment due to the numerous parameters that can influence retention time and selectivity. Consequently, adoption of new separation technology such as UPLC can be a challenge in the QC environment due to the qualification requirements for new instruments together with the need to establish and validate new methods for UPLC instrumentation. Regulatory agencies and pharmacopoeias are becoming increasingly aware of the advantages UPLC offers for product characterization. Correspondingly, regulatory guidelines and pharmacopoeial monographs are beginning to outline new analytical requirements for future drug applications. Companies that manufacture such drugs are, therefore, under pressure to begin the adoption process of new analytical technology.

As a first step in transitioning from HPLC to UPLC Technology for routine biopharmaceutical analysis in the QC laboratory, a simplified process for running legacy HPLC peptide mapping methods on the ACQUITY UPLC H-Class Bio System is demonstrated. This allows the laboratory to implement UPLC Technology without the burden of developing, validating, and qualifying new UPLC separation methods. HPLC-based peptide mapping performed on the ACQUITY UPLC H-Class Bio System generates comparable chromatograms compared to equivalent peptide maps acquired on HPLC instrumentation. With UPLC Technology established in the lab, efforts can be directed to transferring legacy HPLC methods to UPLC without disruption to the HPLC-based analytical workflows. Users can then deploy the ACQUITY UPLC H-Class Bio System for UPLC-based separations at a suitable time in the future, having previously qualified the instrumentation.

EXPERIMENTAL

UPLC conditions

System:	ACQUITY UPLC H-Class Bio
Detector:	ACQUITY UPLC Tunable UV (TUV)
Extension loop:	100 µL
Bio mixer volume:	250 µL
Column:	XBridge BEH C_{18} 130 Å 4.6 x 100 mm, 3.5 µm
Column temp.:	40 °C
Flow rate:	0.500 mL/min
Injection volume:	95 µL
Mobile phase A:	H_2O with 0.1% (v/v) TFA
Mobile phase B:	Acetonitrile with 0.1% (v/v) TFA
Detection wavelength:	214 nm

Gradient:

Time (min)	Flow rate (ml/min)	%A	%B	%C	%D	Curve
–	0.500	95	5	0	0	–
5.00	0.500	95	5	0	0	6
45.00	0.500	50	50	0	0	6
47.50	0.500	5	95	0	0	6
52.50	0.500	5	95	0	0	6
52.60	0.500	95	5	0	0	6
60.00	0.500	95	5	0	0	6

Sample description

Three peptide preparations were used in this study: Waters® MassPREP peptide mixture standard, ribonuclease B (Sigma Aldrich, USA), and infliximab with the latter two undigested protein samples prepared as follows. Five hundred µg of ribonuclease B or infliximab was reduced with dithiothreitol, alkylated with iodoacetamide, and isolated using NAP-5 columns (GE Healthcare, USA). Sequence-grade trypsin (Promega, USA) was added to each protein to a final composition 1:20 enzyme/substrate, and samples were digested at 37 °C overnight. Following digestion, trypsin was deactivated by incubation at 70 °C for 15 min whereupon 60 µL of digested protein material was reconstituted in 40 µL of 5% MeCN/0.1% TFA, generating a final peptide concentration of 0.6 µg/µL.

RESULTS AND DISCUSSION

Adding a gradient start offset to an instrument method

As a first step in transferring the method from HPLC to UPLC, the ability of the ACQUITY UPLC H-Class Bio System to generate a comparable chromatogram using identical conditions outlined in a legacy HPLC peptide mapping method was evaluated. This study included the use of a MassPREP peptide mixture standard containing peptides of varying hydrophobicity eluting regularly across a delivered gradient. The MassPREP peptide mixture was analyzed using both the ACQUITY UPLC H-Class Bio System and an HPLC instrument, each fitted with an XBridge C_{18} 130Å, 3.5 µm Column. As shown in Figure 1A, separation on the ACQUITY UPLC H-Class Bio System resulted in early elution positions for all peaks in the peptide mixture standard. This result indicated that the reduced dwell volume of UPLC compared to the HPLC was causing a change in peak retention times.

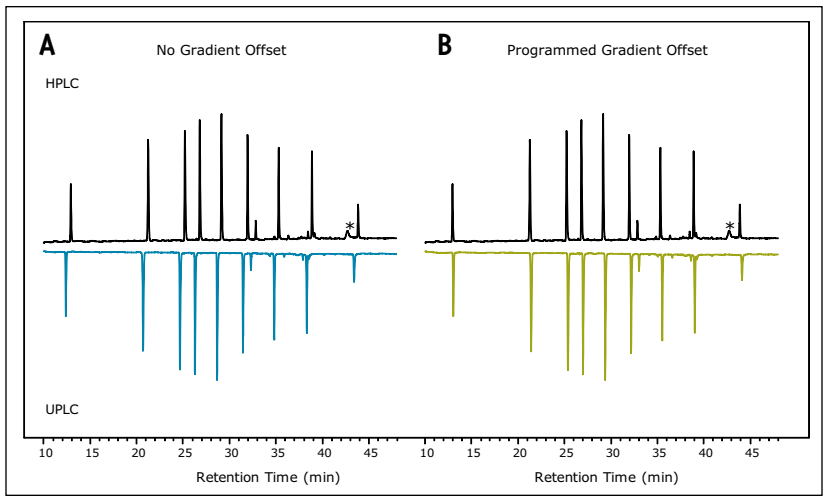

Figure 1. A gradient start offset aligns chromatograms generated on instruments with different dwell volumes. A MassPREP peptide mixture standard was separated using either HPLC or UPLC. (A) Comparison of the peptide mapping method transferred directly across without any modifications to the ACQUITY UPLC H-Class Bio instrument method. (B) The resulting chromatogram after the addition of a 360 µL gradient start offset volume on the ACQUITY UPLC H-Class Bio instrument method. The asterisked peak (*) refers to a system peak observed on the HPLC instrument.

A simple adjustment was made to the ACQUITY UPLC H-Class Bio System method to account for this difference, which worked to simulate a larger dwell volume. To implement this adjustment, the dwell volume was calculated for each instrument where a difference of 360 µL was determined to exist between the instruments. This value was then included as the sole change on the ACQUITY UPLC H-Class Bio instrument method as a gradient start offset volume, as shown in Figure 2.

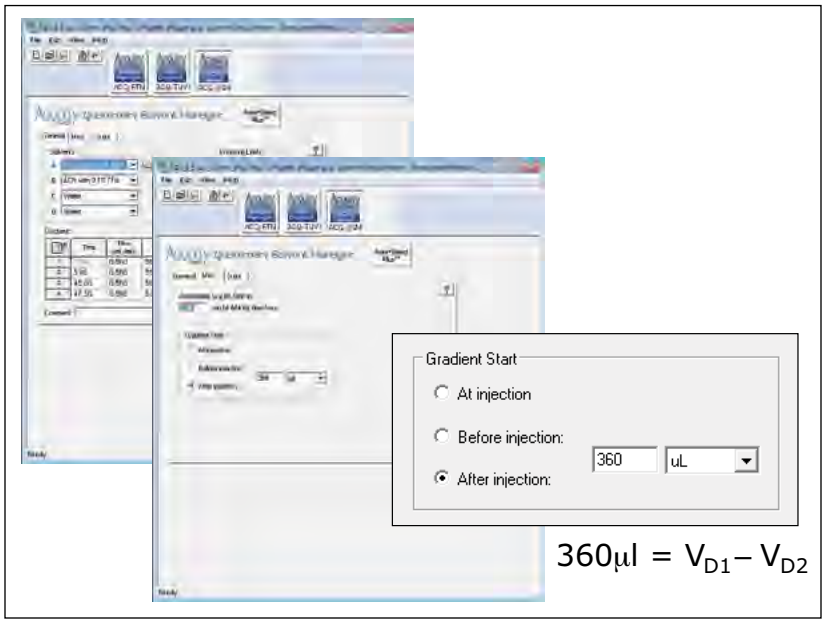

Figure 2. Adjusting existing HPLC peptide mapping instrument method to include a gradient start offset. To access this option within the instrument method, select the QSM tab followed by the "Misc" tab. The resulting window will provide a field to include a gradient start option for the selected peptide mapping method. Choose the "after injection" option, and enter the volumetric difference in dwell volumes for each instrument previously calculated.

The addition of a gradient start offset following sample injection acts to increase the length of the isocratic hold step, providing a means for simulating an instrument with a larger dwell volume. This offset does not affect void markers that may elute within the isocratic hold as the injection step occurs in a typical manner, ensuring all unretained molecules will appear at a similar position in the chromatogram. This allows for continuity in relative retention times should the reference peak(s) of interest be calculated against a void marker. As shown in Figure 1B, separation of the MassPREP peptide mixture standard following implementation of the volume correction yields nearly identical chromatographic performance to that of the original HPLC system.

Impact of a gradient start offset on trypsinized ribonuclease B

While the gradient start offset feature was able to align chromatograms generated on two separate instruments, the MassPREP peptide mixture standard contained a limited number of peaks. To challenge the applicability of this approach, a more complex sample in the form of trypsinized ribonuclease B was selected for analysis. With the gradient start offset enabled, the ribonuclease B peptide map produced a highly homologous chromatogram on the ACQUITY UPLC H-Class Bio System compared to the legacy HPLC, as shown in Figure 3. Slight differences were observed in retention times of similar peaks; however, these differences were limited to no greater than a two-second window, as shown in Table 1. Relative retention times were also calculated for all of the 33 monitored peaks on each instrument to determine the extent of comparability between the HPLC and UPLC instruments, as shown in Table 1. Peptide maps from each instrument generated highly similar relative retention time values.

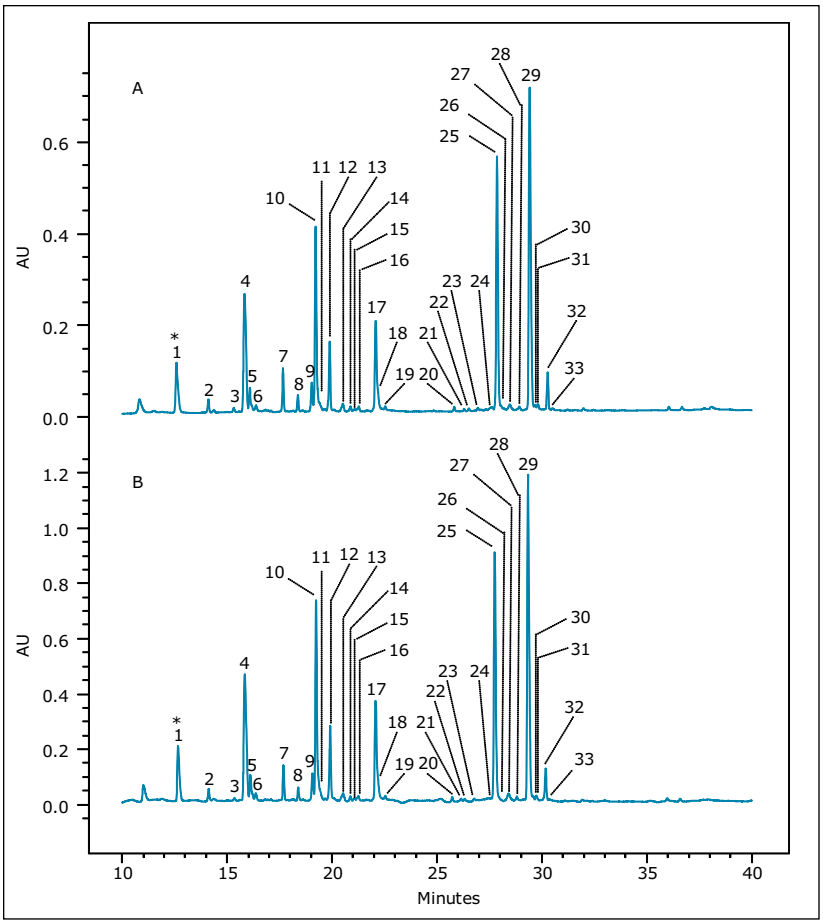

Figure 3. Trypsinized ribonuclease B peptide maps acquired on an HPLC and the ACQUITY UPLC H-Class Bio System are aligned using the gradient start offset feature. A peptide mixture generated from trypsinization of ribonuclease B was used to evaluate the robustness of the gradient start offset feature. Using the same 360 µL offset, the peptide map produced on the HPLC (A) can be generated on the ACQUITY UPLC H-Class Bio System (B) with nearly an identical separation. Peaks denoted by an asterisk (*) refer to the peak used for calculating relative retention times.

Peak	Legacy HPLC		ACQUITY UPLC H-Class Bio		D
	RT (min)	RRT	RT (min)	RRT	
1	12.680	1.000	12.604	1.000	0.000
2	14.140	1.115	14.127	1.121	0.006
3	15.372	1.212	15.332	1.216	0.004
4	15.856	1.250	15.845	1.257	0.007
5	16.128	1.272	16.099	1.277	0.005
6	16.381	1.292	16.396	1.301	0.009
7	17.699	1.396	17.678	1.403	0.007
8	18.403	1.451	18.383	1.459	0.007
9	19.072	1.504	19.042	1.511	0.007
10	19.250	1.518	19.228	1.526	0.007
11	19.680	1.552	19.647	1.559	0.007
12	19.923	1.571	19.900	1.579	0.008
13	20.540	1.620	20.529	1.629	0.009
14	20.886	1.647	20.889	1.657	0.010
15	21.091	1.663	21.109	1.675	0.011
16	21.272	1.678	21.294	1.689	0.012
17	22.083	1.742	22.086	1.752	0.011
18	22.402	1.767	22.408	1.778	0.011
19	22.546	1.778	22.543	1.789	0.010
20	25.822	2.036	25.832	2.050	0.013
21	26.150	2.062	26.301	2.087	0.024
22	26.332	2.077	26.526	2.105	0.028
23	26.780	2.112	26.956	2.139	0.027
24	27.580	2.175	27.590	2.189	0.014
25	27.755	2.189	27.863	2.211	0.022
26	28.030	2.211	28.171	2.235	0.025
27	28.413	2.241	28.474	2.259	0.018
28	28.810	2.272	28.928	2.295	0.023
29	29.338	2.314	29.414	2.334	0.020
30	29.578	2.333	29.674	2.354	0.022
31	29.734	2.345	29.811	2.365	0.020
32	30.181	2.380	30.276	2.402	0.022
33	30.365	2.395	30.512	2.421	0.026

Table 1. Trypsinized ribonuclease B separated on HPLC and the ACQUITY UPLC H-Class Bio System generates nearly identical chromatographic data when the gradient start offset feature is employed. Retention times (RT) and relative retention times (RRT) were recorded for the 33 monitored peaks observed in the ribonuclease peptide map. The difference in relative retention time (D) between the HPLC and the UPLC separations was calculated and recorded.

Gradient start offset approach aligns a trypsinized monoclonal antibody peptide map

To further challenge the applicability of the gradient start offset approach, infliximab, a therapeutic monoclonal antibody, was selected for analysis as it represents a typical protein used in routine peptide mapping analyses in QC labs. A total of 56 infliximab peptide peaks were selected for monitoring between the UPLC and HPLC systems, despite approximately 90 peaks identified within each chromatographic space. Peak selection was based on a number of factors including signal intensity, elution position, and relative resolution. This approach was chosen to simplify the comparative analysis. When separated using the ACQUITY UPLC H-Class Bio System, the infliximab peptide map illustrated significant chromatographic alignment compared to its equivalent acquired on the HPLC instrument, shown in Figure 4. Assessing the relative retention times, virtually no differences were observed with values of less than 0.005 reported, as shown in Table 2. These data provide convincing evidence that employment of a gradient start offset is a suitable strategy for transferring legacy HPLC peptide mapping methods to the ACQUITY UPLC H-Class Bio System.

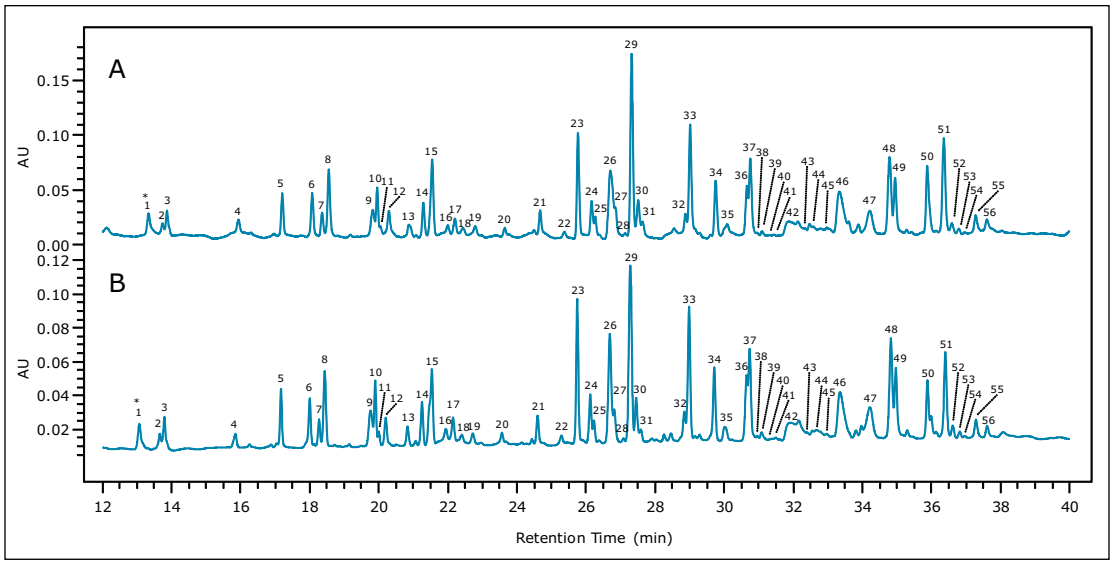

Figure 4. The gradient start offset feature aligns chromatograms for complex trypsinized infliximab. Infliximab was used to evaluate the performance of the gradient start offset option for dwell volume adjustment. The chromatogram generated on the HPLC system (A) demonstrates extensive similarity to the chromatogram generated on the UPLC system (B). Peaks denoted by an asterisk (*) in each chromatogram refer to the peak selected for calculating relative retention times.

Peak	Legacy HPLC		ACQUITY UPLC H-Class Bio		D
	RT (min)	RRT	RT (min)	RRT	
1	13.060	1.000	13.034	1.000	0.000
2	13.655	1.046	13.639	1.046	0.001
3	13.785	1.056	13.770	1.056	0.001
4	15.843	1.213	15.826	1.214	0.001
5	17.165	1.314	17.151	1.316	0.002
6	17.997	1.378	17.982	1.380	0.002
7	18.267	1.399	18.254	1.400	0.002
8	18.430	1.411	18.416	1.413	0.002
9	19.757	1.513	19.741	1.515	0.002
10	19.894	1.523	19.878	1.525	0.002
11	19.995	1.531	19.980	1.533	0.002
12	20.197	1.546	20.180	1.548	0.002
13	20.826	1.595	20.815	1.597	0.002
14	21.247	1.627	21.233	1.629	0.002
15	21.533	1.649	21.518	1.651	0.002
16	21.938	1.680	21.916	1.681	0.002
17	22.147	1.696	22.125	1.697	0.002
18	22.403	1.715	22.382	1.717	0.002
19	22.721	1.740	22.705	1.742	0.002
20	23.563	1.804	23.547	1.807	0.002
21	24.596	1.883	24.582	1.886	0.003
22	25.288	1.936	25.270	1.939	0.002
23	25.745	1.971	25.730	1.974	0.003
24	26.121	2.000	26.106	2.003	0.003
25	26.227	2.008	26.211	2.011	0.003
26	26.686	2.043	26.668	2.046	0.003
27	26.815	2.053	26.792	2.056	0.002
28	27.081	2.074	27.063	2.076	0.003
29	27.281	2.089	27.266	2.092	0.003
30	27.452	2.102	27.436	2.105	0.003
31	27.587	2.112	27.569	2.115	0.003
32	28.844	2.209	28.831	2.212	0.003
33	28.982	2.219	28.969	2.223	0.003
34	29.713	2.275	29.701	2.279	0.004
35	30.015	2.298	30.022	2.303	0.005
36	30.647	2.347	30.636	2.350	0.004
37	30.737	2.354	30.726	2.357	0.004
38	30.963	2.371	30.950	2.375	0.004
39	31.085	2.380	31.075	2.384	0.004
40	31.493	2.411	31.484	2.416	0.004
41	31.934	2.445	31.899	2.447	0.002
42	32.164	2.463	32.156	2.467	0.004
43	32.393	2.480	32.410	2.487	0.006
44	32.553	2.493	32.537	2.496	0.004
45	32.972	2.525	33.102	2.540	0.015
46	33.354	2.554	33.342	2.558	0.004
47	34.222	2.620	34.213	2.625	0.005
48	34.828	2.667	34.819	2.671	0.005
49	34.970	2.678	34.962	2.682	0.005
50	35.887	2.748	35.876	2.752	0.005
51	36.404	2.787	36.396	2.792	0.005
52	36.619	2.804	36.614	2.809	0.005
53	36.824	2.820	36.818	2.825	0.005
54	36.965	2.830	36.959	2.836	0.005
55	37.292	2.855	37.281	2.860	0.005
56	37.616	2.880	37.603	2.885	0.005

Table 2. A gradient start offset produces significantly similar chromatographic data for complex monoclonal antibody peptide maps. Retention times (RT) and relative retention times (RRT) were recorded for the 56 monitored peaks observed in the infliximab peptide map. The difference in relative retention time (D) between the HPLC and UPLC separations was calculated and recorded.

CONCLUSION

Migration from HPLC to UPLC for large molecule-based separations represents a key challenge for the QC laboratory. While the advantages of UPLC are apparent, many labs are reluctant to make the migration due to the time required to qualify and validate both the instrument and new UPLC-based separations. Here, we have illustrated the application of the ACQUITY UPLC H-Class Bio System for HPLC-based peptide mapping. By making a simple adjustment to the legacy HPLC method in the form of a gradient start offset, nearly identical chromatograms were obtained on the ACQUITY UPLC H-Class Bio System compared to the HPLC chromatogram. It is important to note that the approach outlined here should be used on a case-by-case basis as various proteins will contain peptides that respond differentially to changes in separation conditions. By using the gradient start offset to account for differences in dwell volumes between instruments, QC labs can adopt UPLC instrumentation without the need to move directly to UPLC separations, choosing instead to run legacy HPLC methods while efforts are made to transfer methods to UPLC. This enables QC labs to align instrument technology with other divisions within their organization, future-proof their own laboratories, and migrate to UPLC-based technology in a step-wise manner with minimum disruption to analytical workflows.

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High Mass Loading of Peptides with Hybrid Particle C_{18} Columns and Acetic Acid Mobile Phases

Matthew A. Lauber, Stephan M. Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Two hybrid particle column chemistries (BEH130 C_{18} and CSH130 C_{18}) with unique selectivities.
- BEH130 C_{18} and CSH130 C_{18} can produce narrower target peptide peaks and greater resolution when using mobile phases modified with optimal concentrations of HOAc versus those modified with 0.1% TFA. This can be exploited to obtain peptides with a pharmaceutically acceptable counter ion in fewer steps.
- BEH130 C_{18} and CSH130 C_{18} are QC tested with a tryptic digest of cytochrome *c*.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

XSelect® CSH130 C_{18}, 5 µm

XBridge® BEH130 C_{18}, 5 µm

MassPREP™ Peptide Mixture

KEY WORDS

Reversed phase (RP), peptides, acetic acid (HOAc), charged surface hybrid (CSH), CSH130 C_{18}, BEH130 C_{18}, preparative chromatography, peptide separation technology (PST)

INTRODUCTION

Peptides have proven to be very useful as both therapeutic agents and markers in research. It is common practice to purify peptides for these purposes by preparative reversed-phase (RP) chromatography. In most instances, it is imperative that the purification process yield peptides of high purity. Contaminants can render results from biological assays ambiguous, and be a serious concern when present in active pharmaceutical ingredients. As a result, there is a need for high chromatographic resolution to minimize the co-elution of impurities that are often closely related chemically to the target peptide. There is also a need for column chemistries with excellent loadability to ensure that throughput and productivity are optimized. Typically, peptides are separated using mobile phases containing strong ion pairing agents, such as trifluoroacetic acid (TFA). However, when mobile phases containing TFA are employed, additional preparation steps are required. It is necessary to remove or exchange trifluoroacetate (TFA salt), because of its inherent toxicity.[1] Less toxic counter ions, notably acetate, are preferred. In fact, most peptide pharmaceuticals are either acetate salts or acetate-containing liquid formulations.[2-3] It would seem advantageous to avoid TFA mobile phases when possible, instead using acetic acid (HOAc) mobile phases. It has been previously shown that a peptide in a TFA solution, such as a crude synthetic peptide,[4-5] is more than partially converted to an acetate form after using HOAc mobile phases and isocratic RP chromatography.[6] Meanwhile, gradient separations with short wash steps involving a high concentration of an acetate buffer can be employed for more complete conversion of salt forms.[7] In the end, a streamlined purification process involving HOAc mobile phases would facilitate obtaining the desired peptide and counter ion with fewer steps.

This study investigates the use of both BEH C_{18} and CSH™ C_{18} columns for preparative peptide separations. BEH C_{18} is an organo-silica C_{18} stationary phase, based on ethylene bridged hybrid (BEH) technology, and is noted for its robustness and pH stability. Charged Surface Hybrid (CSH) C_{18} is an evolution of BEH C_{18} as it is modified to contain a low level positive surface charge under acidic conditions. The following data demonstrates that each of these stationary phases is amenable to high mass load peptide separations with both TFA- and HOAc-modified mobile phases. The following results also show that both BEH C_{18} and CSH C_{18} can yield narrower target peaks at high mass loads with optimized HOAc mobile phases than those containing 0.1% TFA.

EXPERIMENTAL

Sample description

MassPREP Peptide Mixture (p/n 186002337, shown in Table 1) was reconstituted with either 0.1% TFA or 0.1% HOAc (depending on the mobile phase employed) to a total peptide concentration of either 0.6 or 2.0 mg/mL (depending on the sample load). A low purity (<70%) preparation of the synthetic peptide, DFVGYGVKDFVGVGVK, was reconstituted in 0.1% TFA/0.1% HOAc to a concentration of either 1 or 4 mg/mL.

Method conditions (unless otherwise noted)

LC conditions

System:	ACQUITY UPLC H-Class Bio System with a 20-cm Column Heater
Detection:	ACQUITY UPLC TUV Detector with 500-nL Analytical Flow Cell Xevo® G2 Q-Tof™ Mass Spectrometer
Wavelength:	214 and 250 nm
Scan rate:	2 Hz (filter time constant, 1 s)
Columns:	XBridge BEH130 C_{18} 4.6 x 100 mm, 5 µm, Porous, 130Å (p/n 186003579) XSelect CSH130 C_{18} 4.6 x 100 mm, 5 µm, Porous, 130Å (p/n 186007077)
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	50 to 1000 µL, sample loads noted below
Flow rate:	1 mL/min (split post-UV detector to *ca.* 20 µL/min for infusion into the MS source)
Mobile phases:	See gradient tables
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)

Gradients: MassPREP Peptide Mixture

A: 0.1% (v/v) TFA in water

B: 0.1% (v/v) TFA in 90:10 acetonitrile (ACN)/water

or

A: 0.1% (v/v) HOAc in water

B: 0.1% (v/v) HOAc in 90:10 ACN/water

Time (min)	% A	% B
0	99.5	0.5
1	99.5	0.5
61	40.0	60.0
62	10.0	90.0
65	10.0	90.0
66	99.5	0.5
85	99.5	0.5

Focused Gradients for DFVGYGVKDFVGVGVK

A: 0.1% (v/v) TFA in water

B: 0.1% (v/v) TFA in 90:10 ACN/water

Time (min)	% A	% B
0.0	90	10
3.0	90	10
4.0	80	20
24.2	60	40
29.2	10	90
32.2	10	90
33.2	90	10
52.0	90	10

A: 0.1% (v/v) HOAc in water

B: 0.1% (v/v) HOAc in 90:10 ACN/water

or

A: 99:1 (v/v) water/HOAc – 1% HOAc

B: 90:9:1 (v/v) ACN/water/HOAc – 1% HOAc

Time (min)	% A	% B
0.0	90	10
3.0	90	10
3.3	87	13
23.5	67	33
29.2	10	90
32.2	10	90
33.2	90	10
52.0	90	10

MS conditions

Mass spectrometer:	Xevo G2 Q-Tof
Ionization mode:	ESI+
Analyzer mode:	Resolution
Capillary voltage:	3.00 kV
Cone voltage:	25 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	800 L/h
Calibration:	NaI, 1 µg/µL from 50 to 2000 *m/z*
Acquisition:	50 to 1990 *m/z*, 2 Hz scan rate

Data management

MassLynx Software v4.1

RESULTS AND DISCUSSION

Loading studies of a nine-component peptide mixture

The application of CSH130 C_{18} and BEH130 C_{18} to analytical peptide separations, such as peptide mapping, has been extensively discussed in previous work.[8-9] Briefly, CSH130 C_{18}, with its novel positive surface charge, has been found to provide improved peak shapes and loadability compared to other peptide reversed-phase column chemistries. Significant increases in peak capacity, up to 90%, have been observed for analytical applications, particularly when mobile phases with little to no ion pairing are used. The positive surface charge of CSH130 C_{18} also provides unique selectivity and less retentivity compared to BEH130 C_{18}, which makes the two sorbents excellent companion column chemistries for peptide separations.

To investigate the performance of CSH130 C_{18} and BEH130 C_{18} in preparative separations, loading studies were performed with a number of different peptides and mobile phases that are typically used in manufacturing, namely those containing either TFA or HOAc. Analytical (4.6 mm I.D.) columns packed with 5 µm particles were employed for these method development experiments.

The MassPREP peptide mixture, containing nine different peptides shown in Table 1, was the first sample used to interrogate these columns. Figure 1 displays a set of chromatograms obtained for this mixture at semi-preparative loads using BEH130 C_{18} and CSH130 C_{18} as well as two different mobile phases, one containing 0.1% TFA and the other 0.1% HOAc. With 0.1% TFA, the average 4σ peak width for the BEH column was 0.8 minute. With 0.1% HOAc, this average peak width increased nearly two-fold to 1.5 minutes. HOAc is a much weaker acid than TFA, producing a less acidic mobile phase with significantly less ionic strength and ion pairing ability. Accordingly, peak shape for most C_{18} columns would be expected to be much worse when using HOAc instead of TFA. This assumption holds true for semi-preparative sample loads with BEH130 C_{18}, as shown in Figure 1. However, peak shapes were remarkably well maintained with the CSH130 C_{18} column when TFA was exchanged for HOAc. The average 4σ peak widths observed using the CSH130 column with 0.1% TFA and 0.1% HOAc mobile phases were 0.5 and 0.6 minutes, respectively. These peak width data are summarized in Figure 2, where individual peak widths for the peptides in the mixture are plotted according to column type and mobile phase condition. In addition to the data for the semi-preparative loads, data obtained for analytical sample loads are shown. This figure highlights that BEH130 C_{18} and CSH130 C_{18} produce similar peptide peak shapes under some conditions, including analytical sample loads with 0.1% TFA. However, under other conditions, such as semi-preparative loads with 0.1% HOAc, CSH130 C_{18} yields much narrower peaks. As has been demonstrated before,[8-9] CSH130 C_{18} tends to deliver markedly better peak shapes for peptides in acidic mobile phases with little to no ion pairing. These data also demonstrate that this becomes even more evident for sample loads 20 times greater than those routinely used for analytical separations.

	Peptide	Sequence
1	RASG-1	RGDSPASSKP
2	Angiotensin 1-7	DRVYIHP
3	Bradykinin	RPPGFSPFR
4	Angiotensin II	DRVYIHPF
5	Angiotensin I	DRVYIHPFHL
6	Renin Substrate	DRVYIHPFHLLVYS
7	Enolase T35	WLTGPQLADLYHSLMK
8	Enolase T37	YPIVSIEDPFAEDDWEAWSHFFK
9	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ

Table 1. MassPREP peptide mixture.

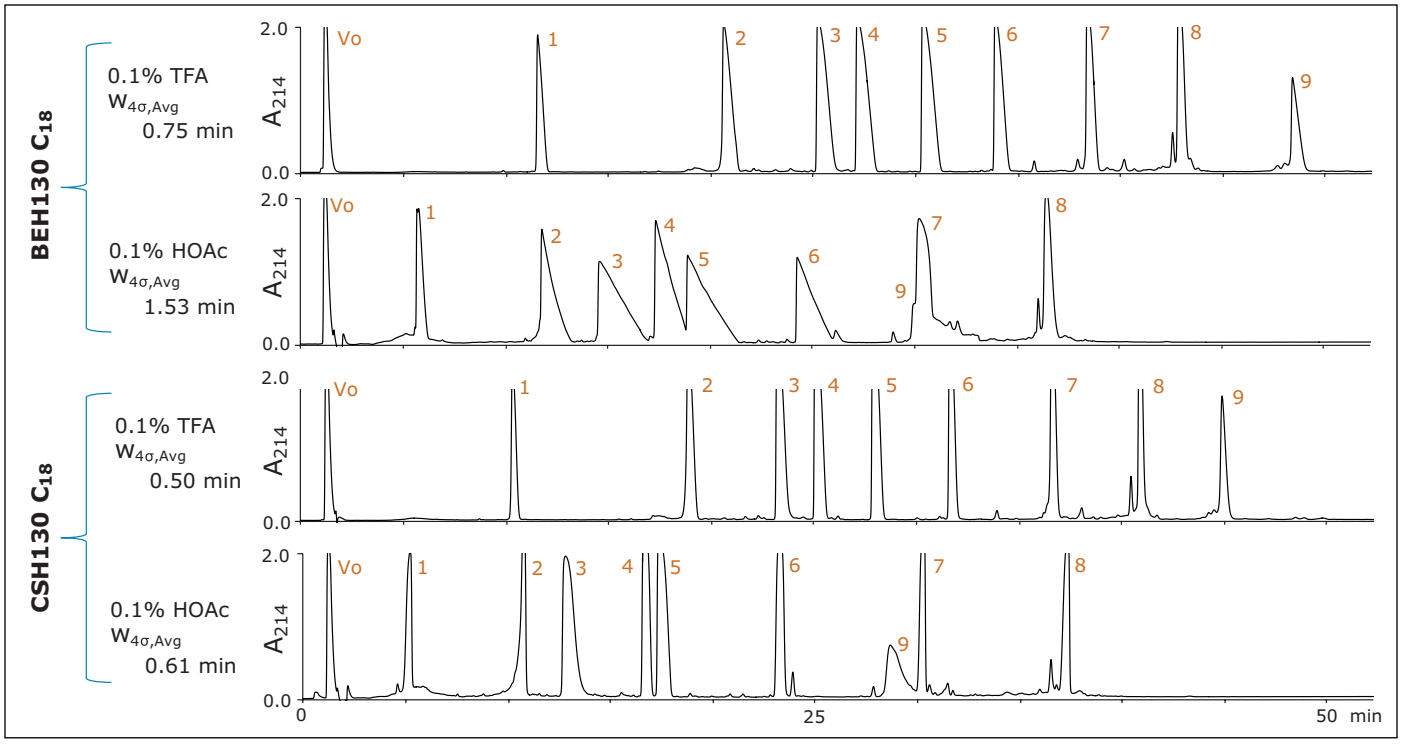

Figure 1. Analysis of the MassPREP peptide mixture at semi-preparative sample loads (500 µg of mixture) with BEH130 and CSH130 C_{18} 4.6 x 100 mm, 5 µm columns.

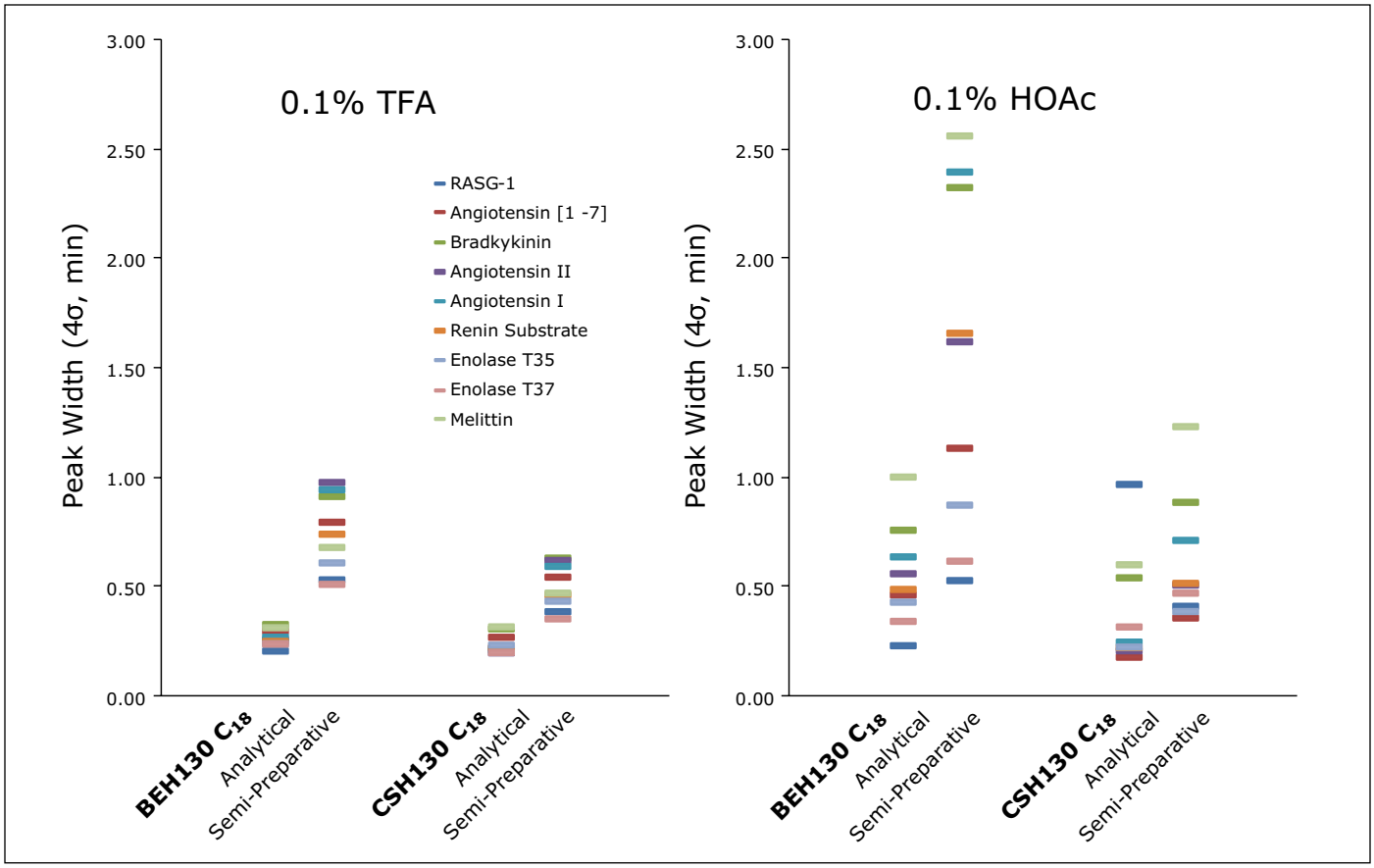

Figure 2. Peak widths of the individual species in the MassPREP peptide mixture observed at analytical (30 µg of mixture) and semi-preparative (500 µg of mixture) sample loads.

Low purity synthetic peptide

Preparative separations must often be performed at sample loads up to and sometimes more than 1000 times greater than those used for analytical separations. Sample loads below and within this regime were investigated using a synthetic, low purity peptide of the sequence DFVGYGVKDFVGVGVK, a neutral peptide (pI = 6) with a molecular weight of 1.7 kDa. Separations were performed on both the BEH and CSH columns using focused gradients to reduce run times, and low sensitivity wavelength (250 nm) detection to assess full peak shapes.

Semi-preparative and preparative sample loads with 0.1% HOAc modified mobile phases were analyzed first, as shown in Figure 3. A semi-preparative load (50 µg) on the BEH column produced the target peptide as a peak with the pronounced tailing that is consistent with commonly observed Langmuirian isotherms. Conversely, at a preparative load (1 mg), the target peptide eluted as a slightly fronting peak, typified by an anti-Langmuirian isotherm. Anti-Langmuirian isotherms are known to occur when peptides are present in zwitterionic form.[10] Consequently, the 0.1% HOAc modified mobile phase was not acidic enough to fully protonate the carboxyl groups of this synthetic peptide. The peptide was likely present in both cationic and zwitterionic forms. The relative amount of the zwitterion would be expected to increase with sample load, particularly when the concentration of the target peptide exceeds the protonation/buffering capacity of the mobile phase. This explains the dramatic change in peak shape with increasing load on the BEH column.

Interestingly, 0.1% HOAc, as seen in Figure 3, appears to be ideal for obtaining a narrow target peptide peak with BEH130 C_{18} at the preparative sample load. Under these conditions, the BEH column yielded a narrower target peptide peak than the CSH column. In fact, CSH130 C_{18} yielded fronting peaks at both sample loads with 0.1% HOAc. This is not surprising given that its positive surface charge minimizes the extent of tailing that occurs for peptides.[8-9] Thus, the fronting peak shape is more readily apparent. Because there is no peak tailing, the width of the target peak at the preparative sample load is actually greater on the CSH column than the BEH column.

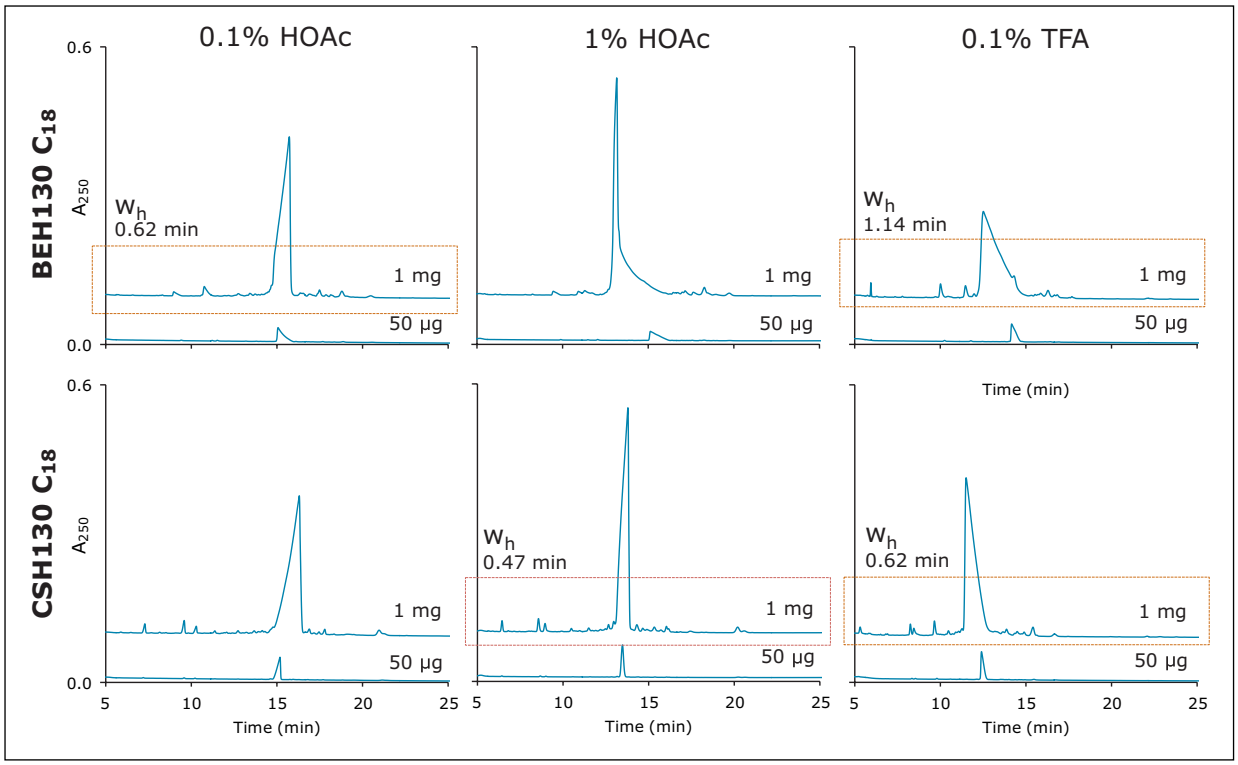

Figure 3. Chromatograms of low purity, synthetic peptide DFVGYGVKDFVGVGVK (pI 6, 1.7 kDa) obtained at semi-preparative (50 µg) and preparative (1 mg) sample loads. The outlined chromatograms are shown in Figure 4.

Based on this, CSH130 C_{18} and BEH130 C_{18} would be expected to produce optimal peak shapes under different mobile phase conditions. To this end, separations were also performed with a mobile phase modified with 10 times more acid (1% HOAc). Intermediate concentrations were not evaluated, although they may be of value for purification development. This change in the mobile phase composition significantly improved the peak shape for the CSH column, but actually worsened peak shape for the BEH column, as shown in Figure 3. With their optimal HOAc mobile phases, both the BEH (0.1% HOAc) and CSH (1% HOAc) columns produced narrow target peptide peaks (half height widths of 0.5 and 0.6 minutes, respectively). To benchmark these results, separations were also performed with 0.1% TFA as an ion-pairing agent, as seen in the right panel of Figure 3. With TFA, target peptide peak widths were 0.6 minute on CSH130 C_{18} and 1.1 minutes on BEH130 C_{18}. Regardless of column chemistry, the optimized HOAc mobile phases yielded significantly narrower peptide target peaks than those containing TFA. These results demonstrate that acetic acid mobile phases have utility for peptide preparative chromatography.

Narrower target peptide peaks often coincide with greater resolution of impurities and, in turn, provide the opportunity to collect higher purity fractions. The effect of column chemistry and mobile phase additive on the preparative loads of DFVGYGVKDFVGVGVK is shown again in Figure 4, where the baselines of the chromatograms and the capability of each separation to resolve a number of MS-identified impurities from the target peak are the focus. As previously noted, narrower target peaks were observed with the HOAc mobile phases. Figure 4 also demonstrates that co-elution of the monitored impurities was likewise minimized through the use of the HOAc mobile phases. In addition, it is apparent that through the use of the different mobile phase additives and the two different column chemistries, chromatographic selectivity between the target peptide and the impurities was dramatically altered. For the parameters screened in this loading study, the CSH column with a 1% HOAc mobile phase appeared to provide both the narrowest target peptide peak and the least co-elution with monitored impurities. Nonetheless, a comparable separation could be achieved with the BEH column and a 0.1% HOAc mobile phase. The availability of column chemistries with different selectivity and optimal additive concentrations can be of benefit when developing challenging preparative separations.

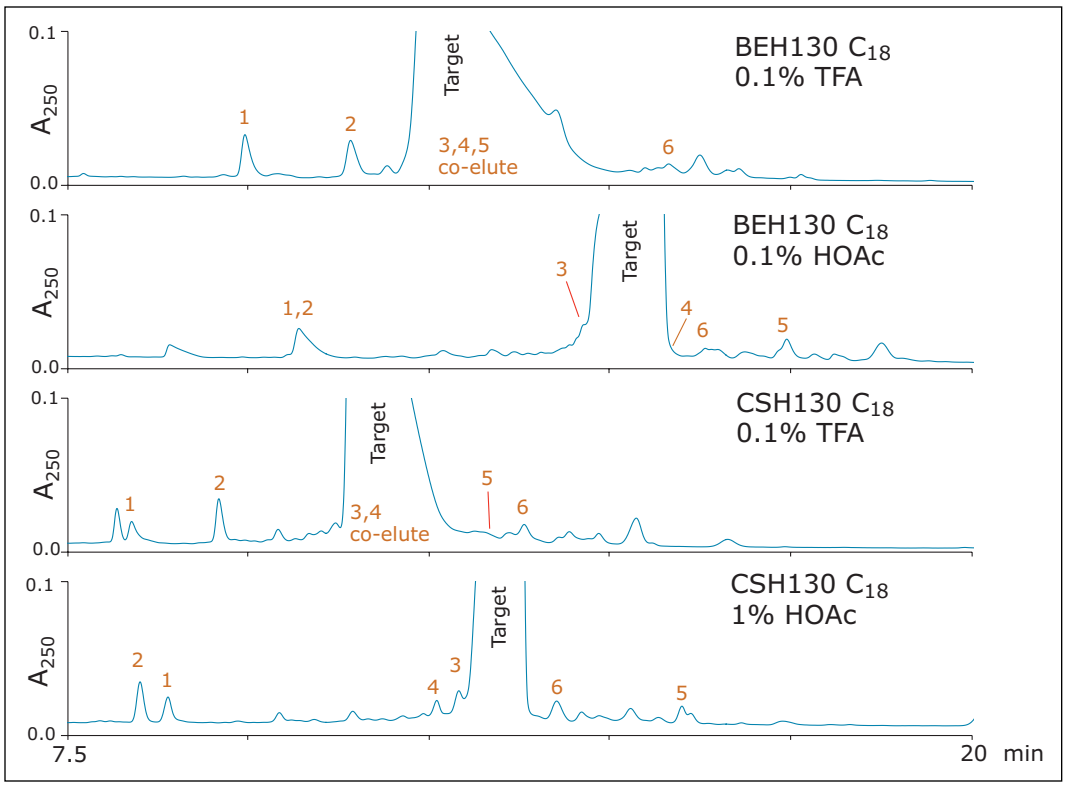

Figure 4. Chromatograms for a preparative sample load (1 mg) of DFVGYGVKDFVGVGVK obtained with BEH130 C_{18} and CSH130 C_{18} using either optimal HOAc mobile phases or mobile phases modified with 0.1% TFA. Retention times of the target peptide and the noted impurities were assigned by ESI-MS. The following mass shifts were observed for the assigned impurities: (1, -147.1 Da), (2, -263.1 Da), (3, -470.2 Da), (4, +548.4 Da), (5, -227.2 Da), and (6, +988.6 Da).

Thus far, results for only a moderate preparative sample load (1 mg) of the synthetic peptide, DFVGYGVKDFVGVGVK, has been discussed. Chromatograms acquired for 4 mg loads of this peptide are shown in Figure 5. This sample load would correspond to 0.5 g of material and, thus, very high productivity per injection on a larger 50-mm I.D. column. From these data, it is clear that both the CSH and BEH columns are amenable to high sample loads. It is notable, nevertheless, how strikingly consistent the peak profiles are for the CSH column from semi-preparative (50 µg) to preparative (4 mg) loads. Such predictability may prove useful when needing to develop an isolation method without consuming large amounts of sample.

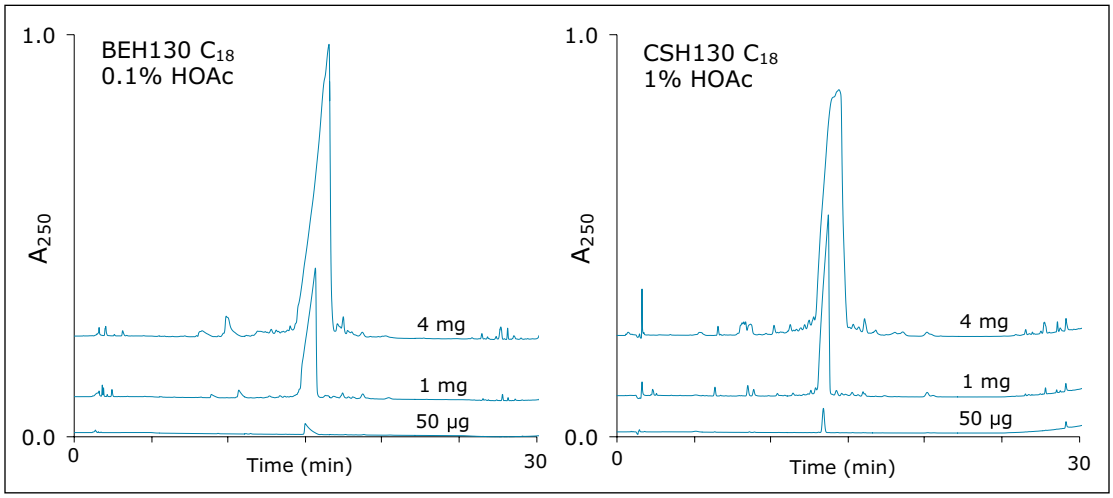

Figure 5. High mass loading (up to 4 mg) on BEH130 C_{18} and CSH130 C_{18} with optimized acetic acid mobile phases.

CONCLUSIONS

Based on loading studies with analytical bore columns, the use of 5-µm BEH130 C_{18} and 5 µm CSH130 C_{18} shows significant promise for facilitating preparative peptide separations with either TFA or HOAc containing mobile phases. Both BEH130 C_{18} and CSH130 C_{18} column chemistries have useful attributes. Under acidic conditions, CSH130 C_{18} tends to exhibit improved loadability and generally narrower target peaks compared to BEH130 C_{18}. Lower volume fractions can, therefore, be obtained with CSH130 C_{18}, which could be exploited to aid subsequent purification and solvent removal steps. BEH130 C_{18}, on the other hand, is perfectly suited to neutral/basic pH preparative chromatography, due to its longer term and higher temperature stability at such conditions. Finally, CSH130 C_{18} and BEH130 C_{18} also exhibit unique selectivity, making them useful companions for resolving challenging impurity/target peptide profiles.

Perhaps more interesting than these aforementioned attributes is the fact that each stationary phase optimizes with different concentrations of mobile phase additive and yields best peak shapes for preparative loads of an example synthetic peptide with optimized HOAc mobile phases, rather than those containing 0.1% TFA. This suggests an opportunity to leverage these hybrid particle C_{18} columns to streamline purification processes, since using HOAc mobile phases would mean that peptides with a pharmaceutically acceptable counter ion could be obtained in fewer steps.

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Increasing Specificity and Sensitivity in Routine Peptide Analyses Using Mass Detection with the ACQUITY QDa Detector

Robert E. Birdsall and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Simple add-on to existing Empower® Software based GMP compliant workflows
- Supplement optical data with mass data for routine peptide assays for biotherapeutics
- Increased assay specificity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable UV (TUV) Detector

ACQUITY UPLC Peptide CSH™ Column

Empower 3 Software

KEY WORDS

Peptides, specificity, mass detection

INTRODUCTION

Peptide analyses are frequently used during protein-based biotherapeutics development, to assess critical quality attributes (CQAs) of candidate molecules.[1,2] Throughout the development process, many optical based assays, often developed from mass spectrometric based characterization, are used to assess product identity, purity, and monitor CQAs.

While optical detection provides a level of assurance, often there is a need to add an orthogonal detection technique, such as mass detection, to improve specificity, expand detection limits, and increase confidence about peak homogeneity.[3]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector (Figure 1) provides a simple and cost-effective solution for the acquisition of optical and MS-based data, for increased specificity in routine peptide monitoring assays associated with monoclonal antibodies (mAbs), while strengthening confidence in the biotherapeutic production environment.

Figure 1. The ACQUITY QDa Detector. The compact footprint of the ACQUITY QDa Detector allows for convenient integration into laboratories, for improving productivity and strengthening process control and quality assurance in the biotherapeutic production environment.

EXPERIMENTAL

An ACQUITY UPLC Peptide CSH Column was conditioned as outlined by the column care and use manual. Chemical reagents were purchased from Sigma Aldrich and used as received. Sequence grade modified trypsin from Promega was used to prepare a digest of trastuzumab (reduced and alkylated) at a concentration of 0.5 mg/mL, as outlined by the manufacturers' protocol.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	215 nm
Vials:	Total recovery vial, 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	ACQUITY UPLC Peptide CSH C_{18}, 130Å, 1.7 µm, 2.1 mm x 100 mm (p/n 186006937)
Column temp.:	65 °C
Sample temp.:	10 °C
Injection volume:	8 µL
Mobile phase A:	H_2O, 0.1% FA
Mobile phase B:	Acetonitrile, 0.1% FA
Mobile phase C:	H_2O
Mobile phase D:	H_2O

Gradient table:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	99	1	0	0
3.00	0.200	99	1	0	0
120.00	0.200	67	33	0	0
127.00	0.200	20	80	0	0
130.00	0.200	20	80	0	0
131.00	0.200	99	1	0	0
140.00	0.200	99	1	0	0

ACQUITY TUV Detector settings

Sample rate:	20 Hz
Detector λ:	215 nm
Filter TC:	normal

ACQUITY QDa Detector settings

Sample rate:	2 points/sec
Mass range:	350–1250 Da
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Data management

Empower 3 SR2 Chromatography Data Software (CDS) with mass analysis

RESULTS AND DISCUSSION

Optical based assays are often used in the biotherapeutic production environment to assess CQAs that impact product efficacy and safety. For biotherapeutics, such as mAbs, monitoring peptides that contain complementary determining region (CDR) sequences are critical in assuring product identity, and to ensure safety standards.[4,5] Optical based techniques that are easily deployed and universally accessible across sites are often used in the development of peptide map profile assays.

As shown in Figure 2A, a chromatographic region containing critical peptides such as the CDR peptide (L3) and its associated deamidated form (L3D) are eluting with non-related neighboring peptides.[5] These factors, combined with baseline noise associated with the use of formic acid, raise the possibility of peak heterogeneity, which can impact the accurate assessment of CQAs. Optimization of such peptide map profiles may require an iterative process where parameters such as gradient, column, temperature, solvent, and ion-pairing agents are systematically changed and evaluated to produce robust separations and ensure peak homogeneity.

With the addition of the ACQUITY QDa Detector into existing workflows, accurate assessment of CQAs can be made with minimal impact on productivity, or the need to modify chromatographic methods.

Using the Empower CDS Software, optical data (Figure 2A) was analyzed to generate an extracted ion chromatogram (XIC) profile (Figure 2B) using mass spectral information acquired with the ACQUITY QDa Detector. From the XIC profile, multiple species were confirmed to be coeluting with the critical pair of interest, demonstrating the ACQUITY QDa Detector's ability to enable rapid assessment of peak homogeneity through the addition of mass detection.

Difficult separations that contain partial or perfectly coeluting peptide species can require extensive optimization and often result in marginal improvements in separation efficiency and subsequent quantification. The ACQUITY QDa Detector features the ability to perform Single-Ion-Recording (SIR) for maximum specificity and sensitivity in routine assays such as peptide mapping profiles.

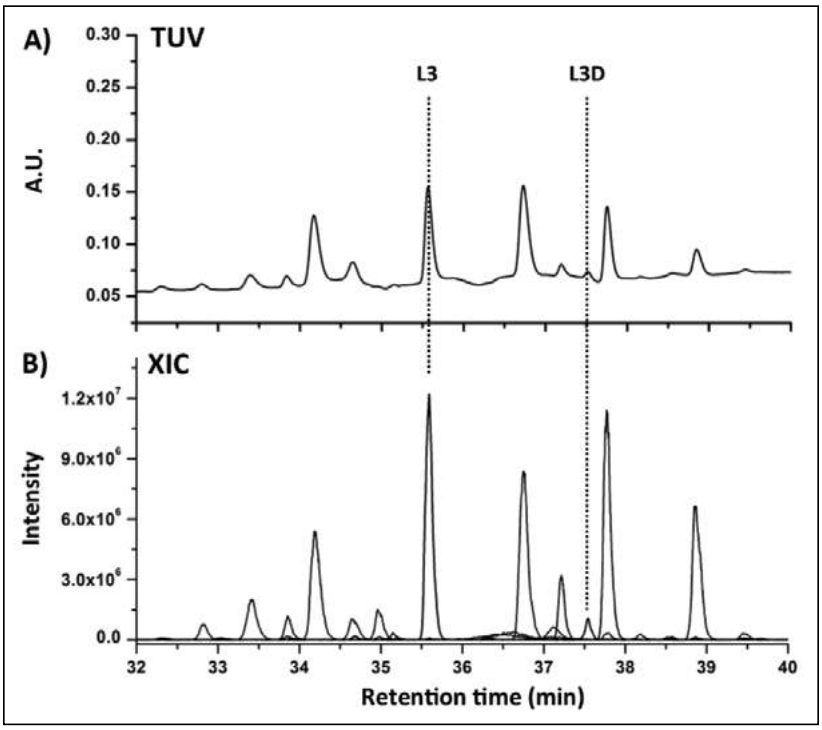


Figure 2. Peptide mapping with the ACQUITY QDa Detector. Using an ACQUITY UPLC Peptide CSH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 100 mm. A peptide map of trastuzumab was acquired using A) optical and B) mass spectral detectors simultaneously. Unique masses identified from the mass spectra collected for each peak were used to generate an XIC, confirming multiple species were co-eluting in the chromatographic region containing the CDR peptide L3 and its deamidated form L3D.

For peptides with characterized elution times, such as the L3 peptide species containing a critical CDR sequence, timed SIR events can be entered under the advanced option through the instrument method setup screen within the instrument method. As shown in Figure 3A, two timed SIR events were programmed to acquire the +2 and +3 charge state for the non-deamidated (L3), and main deamidated (L3D) form of the L3 peptide species.

Through the use of SIRs, only ions exhibiting the mass of interest are recorded as shown in Figure 3B, resulting in two chromatograms that exhibits high signal to noise ratio (SNR), and are free of co-eluting species (Figure 2) that could otherwise impact the accuracy of quantification, when using optical based techniques.

Determining the relative amount of each peptide form across two data channels is addressed through the use of an inter-channel calculation processing feature of Empower CDS Software, as shown in the workflow of Figure 4.

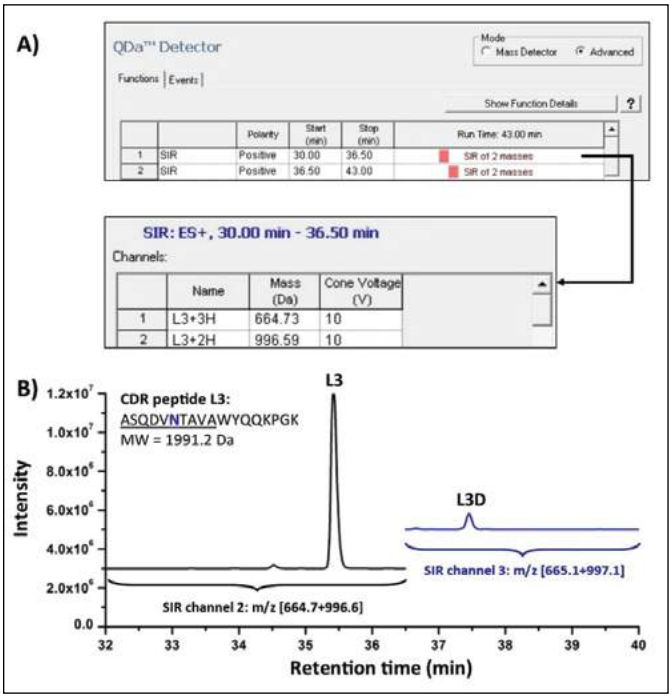


Figure 3. SIR with the ACQUITY QDa Detector. A) Timed SIRs were programmed to acquire non-deamidated (m/z 664.73, m/z 996.59) and deamidated (m/z 665.05, m/z 997.08) masses from 30.00 – 36.50 min and 36.50–43.00 min, respectively for the L3 peptide containing a CDR sequence (underlined). B) Resulting ion chromatograms for each species were summed using a derived channel and plotted with a y-axis offset of 3 x 10^6 (non-deamidated form) and 5 x 10^6 (deamidated form) to contrast the two unique data channels.

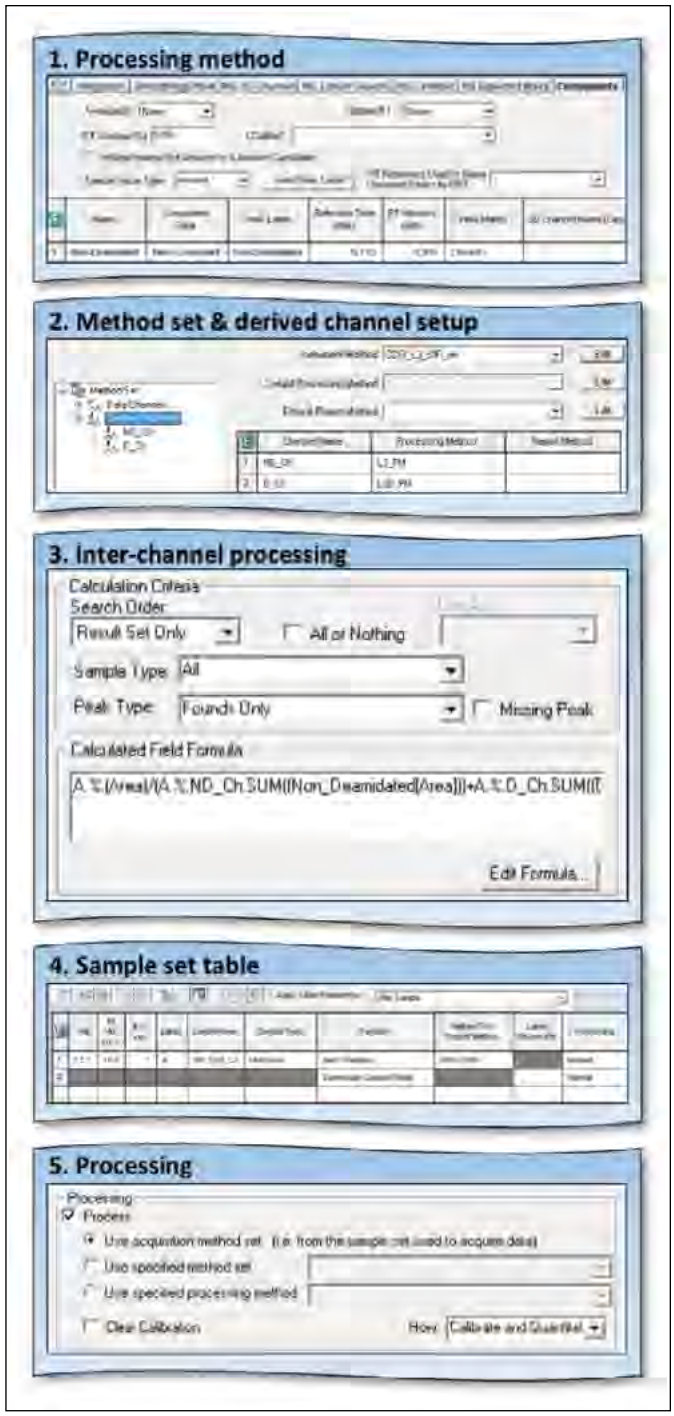


Figure 4. Inter-channel calculation workflow. A generic workflow for determining relative abundance of peaks between two data channels using the inter-channel calculations feature of Empower Software is shown for CDR sequence containing peptide L3.

As an example, the CDR peptide species L3 is used to illustrate the process of inter-channel quantification. As shown in step 1 of Figure 4, a unique processing method for each species form should be created, and include an integration window spanning the peak of interest with the individual component listed in the component table, along with its expected retention time for identification.

In this example the non-deamidated component of L3 is listed in the component table. Once processing methods are defined for each peptide species form, a method is set-up where derived channels are created (Figure 4, step 2), corresponding to the SIR channels from the instrument method. In this example the derived channels ND_Ch and D_Ch correspond to the non-deamidated and deamidated SIR channels, respectively, used for data acquisition in Figure 3A. Upon creation, the individual processing methods defined in step 1 are assigned to the derived channels.

An inter-channel calculation is then set-up using custom fields using the standardized syntax: Label.Injection.Channel.Summary Function(Field)

For this study, relative percent was calculated using the algebraic expression

$$\frac{(\text{Area})}{\text{Area1} + \text{Area2}} \times 100$$

using the following formulas

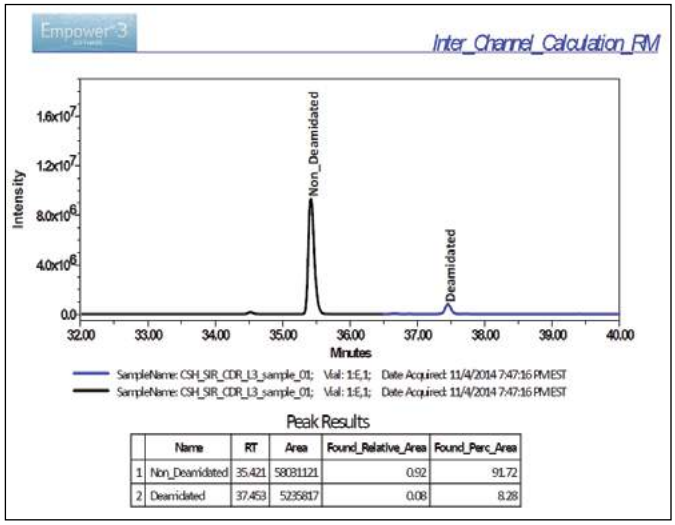
Area: A.%.(Area)

Area1 (non-deamidated area): (A.%.ND_Ch.SUM((Non_Deamidated[Area]))

Area2 (deamidated area): A.%.D_Ch.SUM((Deamidated[Area]))

which were combined in the Calculated Field Formula window as shown in Figure 4, step 3.

Upon entering the inter-channel calculation, a sample set can be created to process the data (Figure 4, step 4). In this example, a sample set was created using the injection named SIR_CDR_L3 and labeled as (A) with the method CDR_L3_MS selected. The summarize custom fields function is selected as shown in Figure 4, step 4, to facilitate the inter-channel quantification. Processing the sample set using the acquisition method set as shown in Figure 4, step 5 will generate a result set containing the two derived channels. Using the preview/publisher option with both derived channels selected allows for the ability to design custom reports for the review of inter-channel processed data, as shown in Figure 5.



	Name	RT	Area	Found_Relative_Area	Found_Perc_Area
1	Non_Deamidated	35.421	5808112	0.92	91.72
2	Deamidated	37.453	523581	0.08	8.28

Figure 5. Reporting results using inter-channel calculations. Integration, identification, and relative quantification of the non-deamidated (black trace) and deamidated (blue trace) L3 peptide species using SIRs was performed using the inter-channel calculation feature of Empower CDS Software. A report template was created for the review of processed data.

Data acquired using timed SIR channels can be overlayed for easy review, while individual metrics for identified peaks are displayed along with the inter-channel calculations and shown in the last two columns of the associated data tables, as shown in Figure 5.

For this study, the CDR containing peptide L3 was calculated to contain 8.28% deamidation. From this data, it can be seen that the ACQUITY QDa Detector, in combination with Empower 3 CDS Software, affords improved productivity in development of routine peptide monitoring assays in the biotherapeutic environment.

CONCLUSION

Developing efficient methods for the accurate assessment of CQAs that impact product efficacy and safety are highly desirable in the biopharmaceutical production environment. This study has demonstrated orthogonal detection techniques, such as mass detection, can be readily adapted to existing workflows for increased specificity and sensitivity in routine monitoring assays.

The ACQUITY QDa Detector in conjunction with Empower 3 CDS Software affords a means for cost-effective mass detection, with increased productivity, and confidence in the development and analysis of routine monitoring assays.

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Peptide Mapping and Small Protein Separations with Charged Surface Hybrid (CSH) C_{18} and TFA-Free Mobile Phases

Matthew A. Lauber, Stephan M. Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Greater peak capacity and unique selectivity compared to other C_{18} columns
- Compatibility with formic acid and ESI-MS
- High resolution separations of species up to approximately 10 kDa
- CSH130 C_{18} is QC tested with a tryptic digest of cytochrome c

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ACQUITY UPLC® H-Class Bio System

Xevo® G2 Q-Tof™ Mass Spectrometer

ACQUITY UPLC CSH130 C_{18}, 1.7 µm Column

MassPREP™ Enolase Digestion Standard

LCGC Certified Clear Glass Qsert Vial

KEY WORDS

Reversed phase, peptides, UPLC,® trifluoroacetic acid, TFA, formic acid, FA, ion pairing, charged surface hybrid, CSH, C_{18}, CSH130 C_{18}, LC-MS, peptide mapping, small proteins

INTRODUCTION

Peptide mapping of a biopharmaceutical, when employed for quality control, has traditionally involved detection by UV absorbance. However, to characterize the species in a peptide map, LC separations often must be coupled with ESI-MS. Mobile phases containing trifluoroacetic acid (TFA) have been almost exclusively used for peptide mapping, likely because the performance of many C_{18} columns is highly dependent on strong ion pairing agents to improve peak shape. For LC-MS applications, it is desirable to avoid strong ion pairing agents such as TFA due to ion suppression, which can be more than an order of magnitude in MS intensity.[1-3] Weaker acid modifiers with reduced ion pairing properties, such as formic acid (FA), are preferred for LC-MS as they permit more sensitive detection.[4-5]

In a separate application note,[6] the performance of columns packed with a novel C_{18} stationary phase containing a low level positive charge was compared to existing state-of-the-art peptide analysis columns. Using a nine-peptide mixture, it was demonstrated that the charged surface hybrid (CSH™) C_{18} stationary phase offers greater peak capacity and, unlike most column chemistries, minimal dependence on strong ion pairing agents to obtain optimal peak capacity. This attribute suggests it is ideal for LC applications that require characterization using mass spectrometry.

In this study, the use of CSH130 C_{18} with FA mobile phases is further investigated with the analysis of more demanding separations. LC-MS of an enolase tryptic digest is compared among a CSH130 C_{18}, BEH130 C_{18}, and a superficially porous C_{18} column. In addition, the applicability of these columns for separations of polypeptides up to 12 kDa is evaluated.

EXPERIMENTAL

LC conditions

System: Waters ACQUITY UPLC H-Class Bio System with a 20 cm Column Heater

Detection: ACQUITY UPLC TUV Detector with 500 nL Analytical Flow Cell

Xevo G2 Q-Tof Mass Spectrometer

(Only MS detection employed for peptide mapping. Both UV and MS detection used for analyses of the large peptides/small proteins.)

Wavelength: 214 nm

Scan rate: 10 Hz

Columns: C_{18}, 2.1 x 150 mm, 1.7 µm, superficially porous (1.25 µm core, 0.22 µm shell) 100Å (competitor product)

ACQUITY UPLC BEH130 C_{18} 2.1 x 150 mm, 1.7 µm, porous, 130Å (p/n 186003556)

ACQUITY UPLC BEH300 C_{18} 2.1 x 150 mm, 1.7 µm, porous, 300Å (p/n 186003687)

ACQUITY UPLC CSH130 C_{18} 2.1 x 150 mm, 1.7 µm, porous, 130Å (p/n 186006938)

Column temp.: 40 °C

Sample temp.: 10 °C

Injection volume: 10 µL for enolase digest

1 µL for large peptide/small protein mixture

Flow rate: 0.3 mL/min

Mobile phases: A: 0.1% FA (v/v) in water
B: 0.1% FA (v/v) in acetonitrile
C: 0.1% TFA (v/v) in water
D: 0.1% TFA (v/v) in acetonitrile

Vials: LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)

Gradient for 0.1% FA:

Time (min)	%A	%B	%C	%D
0	98.0	2.0	0.0	0.0
1	98.0	2.0	0.0	0.0
61	50.0	50.0	0.0	0.0

Gradient for 0.1% TFA (only used for comparison in Figure 1):

Time (min)	%A	%B	%C	%D
0	0.0	0.0	98.0	2.0
1	0.0	0.0	98.0	2.0
61	0.0	0.0	50.0	50.0

MS conditions

Mass spectrometer: Xevo G2 Q-Tof

Ionization mode: ESI+

Analyzer mode: Resolution

Scan rate: 10 Hz

Capillary voltage: 3.00 kV

Cone voltage: 25 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas flow: 0.0 L/h

Desolvation gas flow: 800 L/h

Calibration: NaI 2 µg/µL from 50 to 2000 *m/z*

Acquisition: 50 to 1990 *m/z*, 10 Hz scan rate

Data management: MassLynx® Software

Sample description

Waters® MassPREP Enolase Digestion Standard (p/n 186002325) was reconstituted with 0.1% FA in water to a total peptide concentration of *ca.* 0.05 mM. Large peptides and small proteins obtained from Sigma were reconstituted with 0.1% FA in water, and combined into a mixture containing 1 mg/mL of each component.

Calculations:

The following tryptic peptides from enolase were used to evaluate separation performance: T6, T10, T14, T23, T27, T35, T37, T38, T40, T42, T45, and T51. Their peak widths at half-height (w_h) were measured from extracted ion chromatograms (XICs), averaged, then used to calculate peak capacity according to the following equation:

$$P_{c,4\sigma} = 1 + \left[\left(\frac{2.35}{4}\right)\left(\frac{t_{gradient}}{w_{h,avg}}\right)\right]$$

RESULTS AND DISCUSSION

Peptide mapping

Reversed phase peptide separations are routinely employed in analyses of proteolyzed proteins, as in peptide mapping experiments. The utility of a C_{18} column for peptide separations is best evaluated through the analysis of a digestion standard, such as a tryptic digest of enolase. Using such a sample, the performance of an ACQUITY UPLC CSH130 C_{18}, 1.7 µm Column was assessed for LC-MS-based peptide mapping. Figure 1 shows total ion chromatograms of enolase tryptic peptides obtained with mobile phases containing either FA (blue trace) or TFA (orange trace). The deleterious effect of TFA on MS sensitivity is readily apparent. The use of TFA rather than FA as the modifier in this LC/MS analysis resulted in an order of magnitude drop in MS sensitivity.

Previous work with a nine-peptide mixture demonstrated that the performance of CSH130 C_{18} for peptide separations exhibits little to no dependence on strong ion pairing agents, such as TFA. Peptide peak shapes were found to be excellent with either FA- or TFA-containing mobile phases.[6] The most profound consequence of this is that CSH130 C_{18} is capable of producing high peak capacity peptide mapping separations even under MS-friendly conditions. To illustrate this point, we measured the peak capacity for the enolase peptide map that was obtained with the FA mobile phase (see experimental for calculation). The 12 peptides labeled in Figure 1, with their wide ranging retention times, were selected to calculate the peak capacity of the separation.

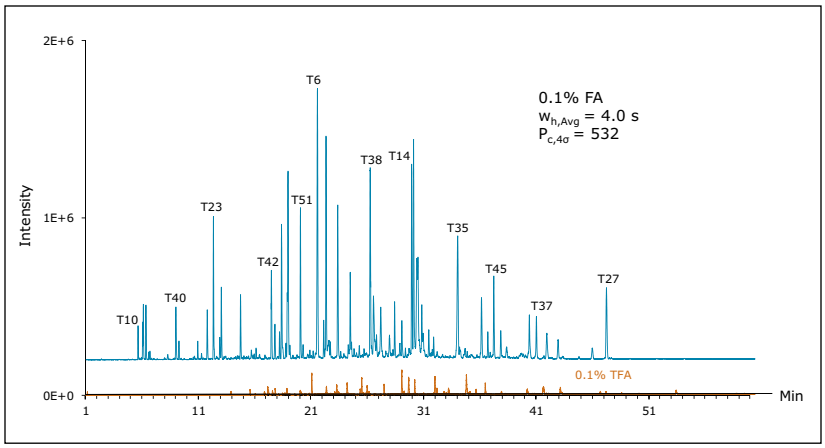

Figure 1. LC/MS of an enolase tryptic digest using a CSH130 C_{18}, 1.7 µm Column. Total ion chromatograms obtained with mobile phases containing either 0.1% FA or 0.1% TFA are shown in blue and orange, respectively. Both chromatograms are displayed on the same scale. Peptides used in the calculation of peak capacity are labeled.

Based on these measurements, the CSH130 C_{18}, 1.7 µm Column produced a peak capacity of 532, which is remarkably high for an LC-MS platform amenable to routine work. To provide perspective, the enolase digest was likewise analyzed by LC-MS using two 1.7 µm C_{18} columns that do not have a low level positive charge applied to the particle surface, as shown in Figure 2. The fully porous BEH130 C_{18}, 1.7 µm Column produced a peak capacity of 399, and the superficially porous C_{18}, 1.7 µm column produced a similar peak capacity of 405. The novel CSH130 C_{18} stationary phase, thus, yielded a significant performance advantage for this application with 30% greater peak capacity.

The retentivity and selectivity of peptides also varied between the three columns shown in Figure 2. An early time segment of the enolase peptide maps capturing this observation is shown in Figure 3. Peaks corresponding to six different peptides are labeled. The most immediate observations resulting from this comparison include: 1) CSH130 C_{18} provides better peak shape; and 2) CSH130 C_{18} is slightly less retentive than the other stationary phases. Elution of the labeled peptides from the CSH130 C_{18} Column occurred approximately 5 min earlier compared to the BEH130 C_{18} Column and approximately 2 min earlier compared to the superficially porous C_{18} column. In terms of elution strength, these are differences estimated at 4% and 2% acetonitrile, respectively. A more detailed analysis of these chromatograms shows the unique selectivity of the CSH130 C_{18} Column. Elution order of the peptides changes quite dramatically when changing from the BEH130 C_{18} to CSH130 C_{18} Column. Peptides with the largest selectivity differences in this chromatographic window appear to be peptides T10 and T19. Most tryptic peptides, such as T3, T5, T12, and T40, contain only two basic moieties, one N-terminus and one C-terminal lysine or arginine residue. Peptides T10 and T19, in contrast, also contain basic histidine residues, causing them to have an additional positive charge. This is most likely the reason for their relatively larger shift in retention time. The retention of peptides on CSH130 C_{18}, therefore, seems to be influenced by their charge (or possibly their charge density), which in turn has an effect on selectivity. This suggests it is advantageous to screen both BEH130 C_{18} and CSH130 C_{18} Columns when developing challenging peptide maps, particularly when aiming to separate critical pairs of peptides.

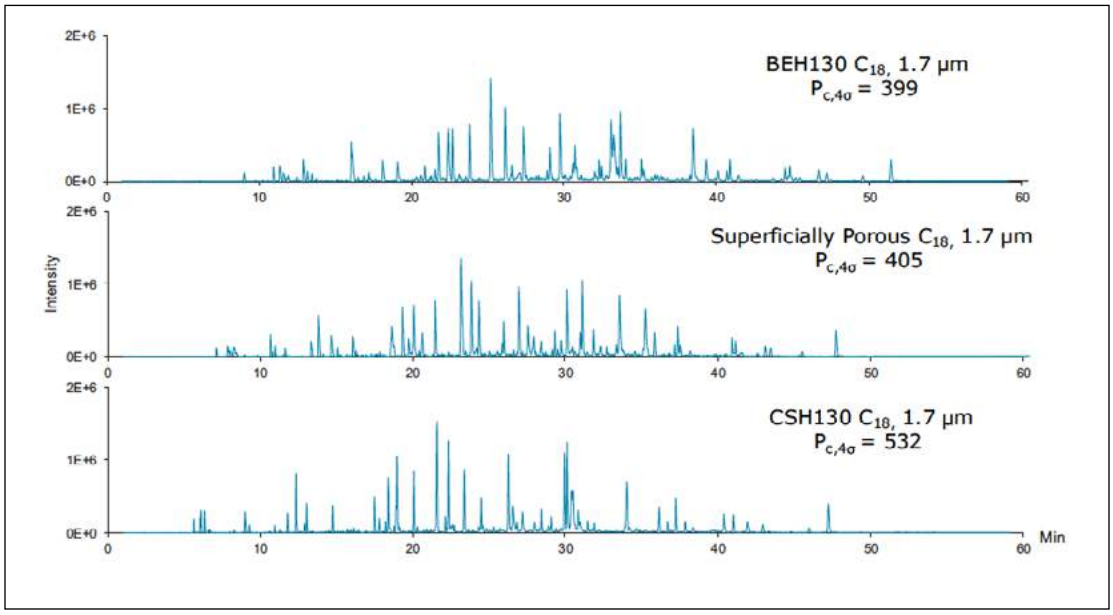

Figure 2. Total ion chromatograms of an enolase tryptic digest obtained with 0.1% FA mobile phases and three different columns.

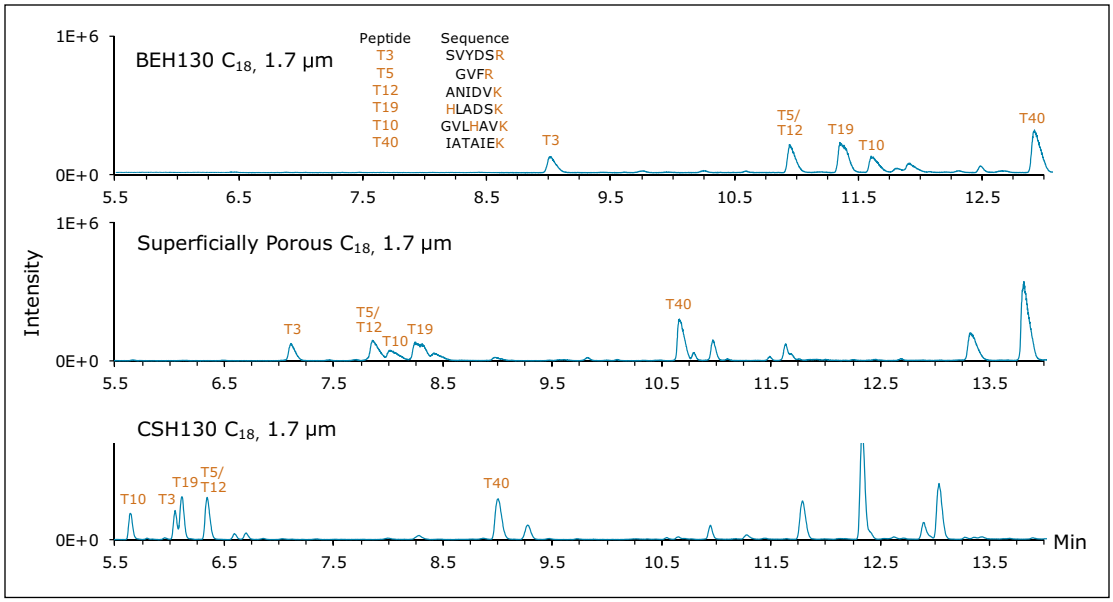

Figure 3. Expanded view of an early retention window from the enolase peptide maps shown in Figure 2 highlighting differences in retentivity and selectivity. Six different peptides were tracked across chromatograms obtained with three different columns. The sequences of these peptides are provided in the table on the top panel.

Analysis of large peptides and small proteins

There are many variables encountered when choosing an optimal column chemistry for a given peptide separation. The aforementioned work has shown a pronounced effect for surface charge. Another variable is pore size. Based on these results, CSH130 C_{18} is very effective at separating tryptic peptides. It was of interest, nevertheless, to evaluate the use of CSH130 C_{18}, even with its 130Å pores, for separations of larger peptides and small proteins.

Six polypeptides ranging in mass from 1 to 12 kDa were separated on four columns containing stationary phases with pores varying from 100 to 300Å in diameter, as shown in Figure 4. By comparing these chromatograms, it is clear that the CSH130 C_{18} Column produced the best peak shapes for most of the peptide species, including insulin (5.8 kDa). As a result, CSH130 C_{18} has already become a stationary phase of choice for the bioanalysis of therapeutic insulin analogs.[7]

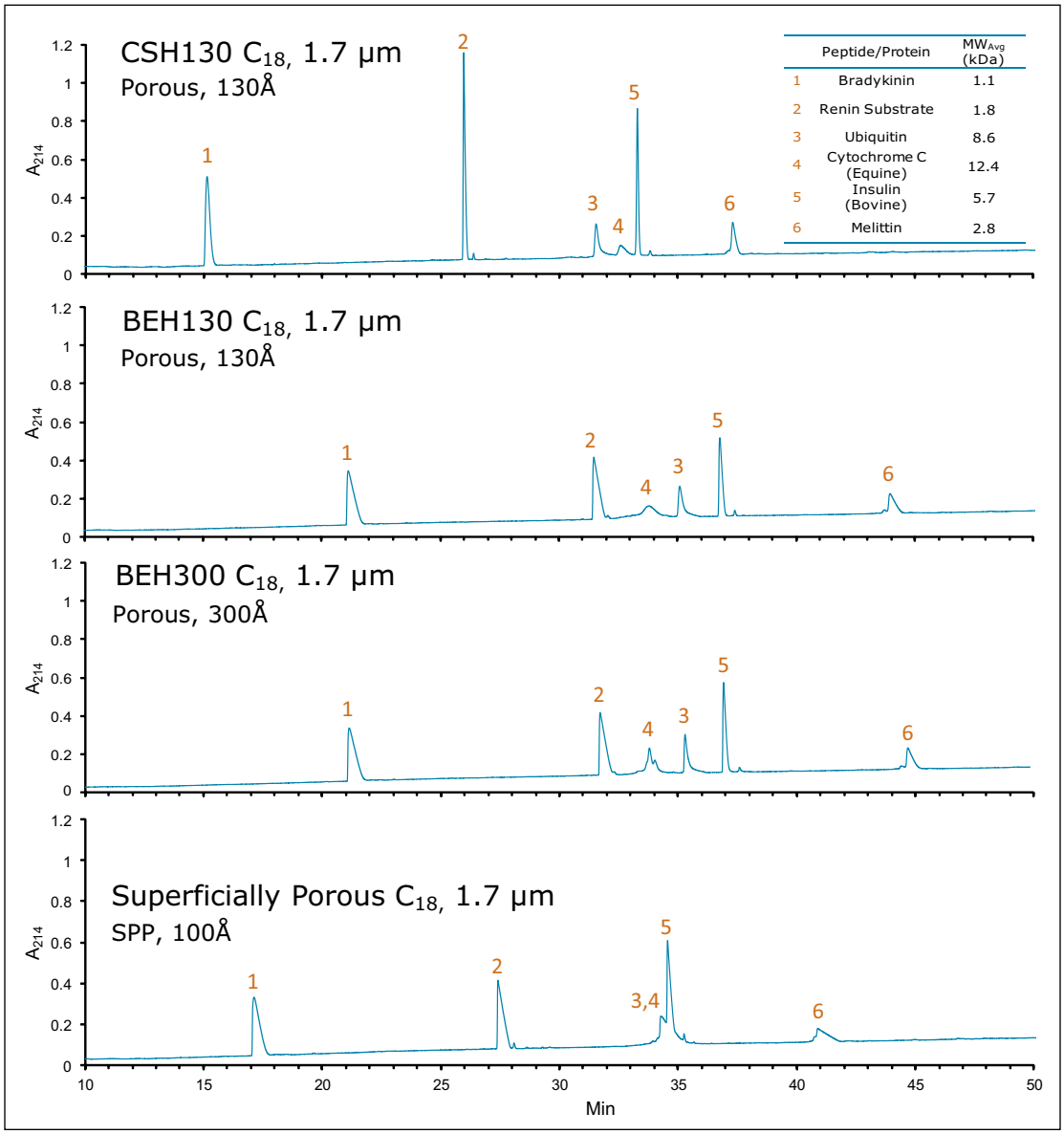

Figure 4. Chromatograms of large peptides/small proteins obtained with 0.1% FA mobile phases and four different columns. Peaks were identified by ESI-MS.

Analysis of the largest polypeptides, ubiquitin (8.6 kDa) and cytochrome *c* (12.4 kDa), better defined the effect of using 300Å versus 130Å pore size sorbents. Ubiquitin was found to exhibit only slightly better peak shape on the BEH300 C_{18} (300Å) Column versus both the CSH130 C_{18} (130Å) and BEH130 C_{18} (130Å) Columns. In contrast, the largest polypeptide, cytochrome c, was separated with markedly better peak shape using BEH300 C_{18}. The BEH300 C_{18} Column was actually capable of resolving cytochrome *c* into multiple peaks, indicating protein heterogeneity. Most peptide separations, such as those derived from proteolytic digests, will contain few, if any, species this large. For this reason, the use of a 130Å pore size particle, like CSH130 C_{18}, may impact the separation of a protein digest more positively than the use of a larger pore size particle, since it will offer more surface area and likely greater retention of small, hydrophilic peptides. A larger pore size particle, like 300Å pore size C_{18}, may be preferred when primarily analyzing large peptides, for example, those weighing more than 6 kDa. Such an analysis might involve the study of disulfide-linked peptides from a Lys-C digest of an IgG when it may not be crucial to retain or separate efficiently smaller non-linked peptides. It is also worth noting that different pore sizes can sometimes be used to alter the selectivity in a peptide map.[8]

The 100Å pore size superficially porous column was capable of separating the smallest peptides with peak widths and shapes comparable to the BEH C_{18} Columns. However, peak shapes for the largest peptides (3 to 12 kDa) were noticeably worse. In addition, this column did not resolve the three largest polypeptides. These data suggest that the superficially porous C_{18} column is limited to the analysis of smaller peptides, whereas the CSH130 and BEH130/300 C_{18} can separate a wider range of peptides and small proteins.

CONCLUSIONS

Peptide mapping with FA instead of TFA facilitates more sensitive detection using ESI-MS. As a result, CSH130 C_{18} is ideal for LC-MS-based peptide mapping because its performance is excellent with either acid modifier. For an LC-MS analysis of tryptic peptides from enolase with FA mobile phases, a CSH130 C_{18}, 1.7 µm Column provided 30% greater peak capacity than fully porous or superficially porous conventional C_{18} columns with equivalent particle size. Moreover, the analysis of enolase tryptic peptides demonstrated that CSH130 C_{18} provides unique selectivity in separating peptides. Consequently, CSH130 C_{18} should be screened along with conventional C_{18} when developing a peptide map. In addition to offering greater peak capacity, this may give the desired selectivity for critical pairs of peptides. Finally, through analysis of both large peptides and small proteins, it was established that a CSH130 C_{18} Column, even with its 130Å pore size sorbent, is well suited to separating polypeptides up to at least 10 kDa.

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Reliability of Peptide Mapping using the ACQUITY UPLC H-Class System

GOAL

To test the analytical reproducibility of the ACQUITY UPLC® H-Class System in applications that require long shallow gradients to resolve complex mixtures, such as peptide maps.

The ACQUITY UPLC H-Class System provides reproducible and accurate control of peptide mapping separations over extended series of runs.

BACKGROUND

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTM), and analyze potential impurities. Any difference in the structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amount of the peptide with and without a particular modification is used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification.

To meet these application requirements, long, shallow gradients are required. In the past, such separation conditions have been regarded as challenging for single-pump gradient systems. The ACQUITY UPLC H-Class System was tested with a typical peptide mapping protocol.

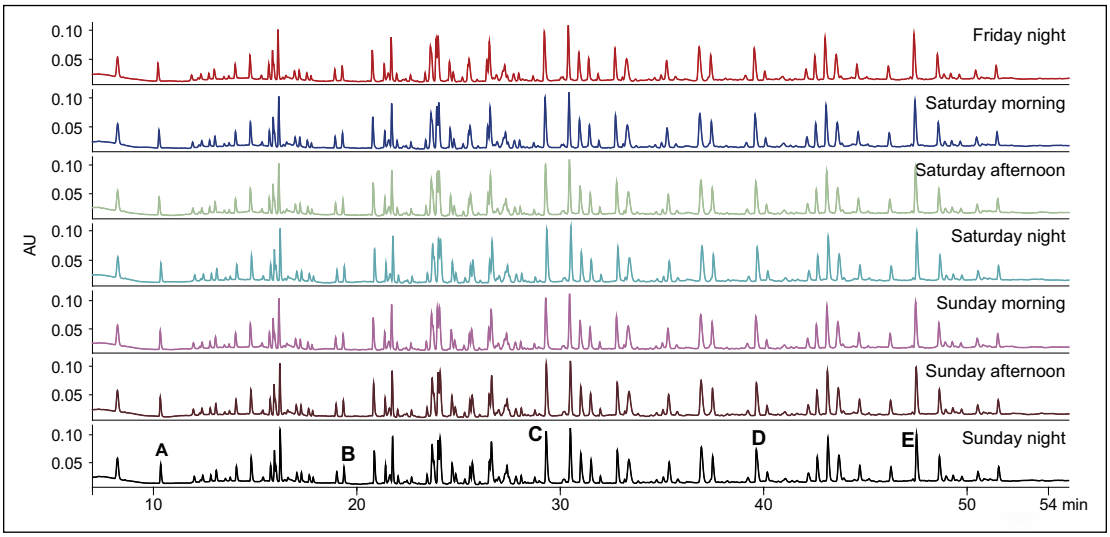

Figure 1. The peptide mapping sample list started on Friday and ran automatically through the weekend. The data was ready to review upon return to work on Monday. Every third separation is shown, demonstrating both excellent reproducibility, resolution, and retention. The five labeled peaks, A to E, were chosen as representative for quantitative analysis, as summarized in Table 1.

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THE SOLUTION

The ACQUITY UPLC H-Class System consisted of the Quaternary Solvent Manager (QSM), Flow-Through-Needle Sample Manager (SM-FTN), Column Heater, and Photodiode Array (PDA) Detector. The optional 250 µL mixer was installed. The MassPREP™ BSA Digestion Standard was separated on a Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18} Column. A shallow gradient of 1.0% per column volume, about 0.6% per minute, was selected as typical of peptide mapping gradients.

The protocol takes advantage of the Auto•Blend™ capabilities of the instrument. Reservoirs of pure solvents and stocks of concentrated modifiers are used in place of binary, pre-formatted solvent. In this example, a gradient is formed between pure water and pure acetonitrile in lines A and B, while a fraction of the flow is drawn from reservoir D that contains 1% TFA in water. During the gradient, the percentage from line D decreases from 5% to 4%, corresponding to 0.05% to 0.04% TFA, to minimize baseline drift.

The peptide maps are shown in Figure 1 with both retention time and peak area statistics summarized in Table 1. Relative retention and resolution remain constant over this long series of runs. The retention times are sufficiently reproducible as to ensure that peaks will always be correctly identified. Peak area ratios were calculated comparing peaks B, C, D, and E to peak A. The consistency of these area ratios (Table 1) meets the requirements for estimating proportion of modified protein in a set of samples.

	Retention Reproducibility	Relative Quantitation
Peak	Time ± Std. Dev.	Ratio ± Std. Dev.
A	10.311 ± 0.093	1
B	19.331 ± 0.064	0.727 ± 0.008
C	29.284 ± 0.064	3.621 ± 0.014
D	39.614 ± 0.073	2.801 ± 0.009
E	47.470 ± 0.086	3.504 ± 0.020

Table 1. A summary of peak retention times and area ratios. Representative peaks, evenly spaced throughout the separation at 10-minute intervals, were selected for quantitative comparison.

SUMMARY

For meaningful peptide mapping, both quantitative and qualitative reproducibility are required. The ACQUITY UPLC H-Class System provides precise control of peptide mapping separations over large sample sets, so that the analyst can be confident that any deviation in retention time indicates a change in sample composition rather than instrument variability. The observed separations meet this objective while taking advantage of the multi-solvent blending capability. The system has been designed to ensure that both qualitative and quantitative results meet the requirements of modern analytical biochemistry.

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Increasing Peak Capacity in Reversed-Phase Peptide Separations with Charged Surface Hybrid (CSH) C_{18} Columns

Matthew A. Lauber, Stephan M. Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Higher peak capacity RP peptide separations
- Compatibility with formic acid mobile phases
- Availability in both UPLC® and HPLC particle sizes
- CSH130 C_{18} is QC tested with a tryptic digest of cytochrome c

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

Xevo® G2 Q-Tof Mass Spectrometer

ACQUITY UPLC CSH130 C_{18}, 1.7 µm Column

XSelect® CSH130 C_{18} *XP*, 2.5 µm Column

MassPREP™ Peptide Mixture

LCGC Certified Clear Glass Qsert Vial

KEY WORDS

Reversed-phase, peptides, trifluoroacetic acid (TFA), formic acid (FA), ion pairing, charged surface hybrid (CSH), peak capacity

INTRODUCTION

Peptide separations are of paramount importance, whether studying proteins by bottom-up proteomics[1] or thoroughly characterizing biopharmaceuticals using peptide mapping.[2] The mixtures encountered in these analyses are inherently complex. Reversed-phase (RP) chromatography has become the separation mode of choice, as it offers relatively high resolving power and easily interfaces with mass spectrometry (MS).

The performance metric most relevant to gradient RP chromatography is peak capacity, or the maximum number of peaks that can fit into the gradient time.[3] Interestingly, chemical modifications of a stationary phase, in addition to the use of smaller particle sizes, can significantly impact peak capacity. Charged surface hybrid (CSH™) C_{18} column chemistry, for example, is an evolution of the ethylene bridged hybrid (BEH) C_{18} stationary phase[4] as its surface is modified to contain a low-level positive charge in addition to the C_{18} bonded phase.[5] This modification has been shown to improve peak shapes, loading behavior, and peak capacities for small, ionized molecules.[5-10]

In this study, we evaluate CSH130 C_{18} for peptide separations, expecting similar improvements in chromatographic performance since most peptides under commonly employed acidic conditions contain a positive charge. This work with peptides demonstrates that this novel stationary phase exhibits greater peak capacity, unique selectivity, and less dependence on MS signal suppressing, strong ion pairing agents when compared to the state-of-the-art in peptide analysis.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class Bio System with a 20 or 30 cm Column Heater (20 cm Column Heater for 150 mm columns and 30 cm Column Heater for 250 mm columns)
Detection:	ACQUITY UPLC TUV Detector with 500 nL Analytical Flow Cell; Xevo G2 QTof Mass Spectrometer
Wavelength:	214 nm
Scan rate:	10 Hz
Columns:	ACQUITY UPLC BEH130 C_{18}, 2.1 x 150 mm, 1.7 µm, Porous, 130Å (p/n 186003556) ACQUITY UPLC CSH130 C_{18}, 2.1 x 150 mm, 1.7 µm, Porous, 130Å (p/n 186006938) XSelect CSH130 C_{18} ***XP***, 2.1 x 150 mm, 2.5 µm porous, 130Å (p/n 186006943) C_{18}, 2.1 x 250 mm, 5 µm, porous, 300Å (competitor product) C_{18}, 2.1 x 150 mm, 1.7 µm, superficially porous (1.25 µm core, 0.22 µm shell) 100Å (competitor product)
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.3 mL/min
Mobile phases:	A: 0.1% FA (v/v) in water B: 0.1% FA (v/v) in acetonitrile (ACN) C: 0.1% trifluoroacetic acid (TFA) (v/v) in water D: 0.1% TFA (v/v) in ACN
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)
Gradient:	2% ACN for 1 min, then to 50% ACN for 60 min (Acid composition was controlled by proportioning mobile phases A/B and C/D.)

MS conditions

Mass spectrometer:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution
Capillary voltage:	3.00 kV
Cone voltage:	25 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	800 L/h
Calibration:	NaI 2 µg/µL from 50 to 2000 *m/z*
Acquisition:	50 to 1990 *m/z*, 10 Hz scan rate
Data management:	MassLynx® Software

Sample description

MassPREP Peptide Mixture (p/n 186002337), as shown in Table 1, was reconstituted with 0.1% FA in water to a total peptide concentration of *ca.* 0.6 mg/mL.

	Peptide	Sequence
1	RASG-1	RGDSPASSKP
2	Angiotensin 1-7	DRVYIHP
3	Bradykinin	RPPGFSPFR
4	Angiotensin II	DRVYIHPF
5	Angiotensin I	DRVYIHPFHL
6	Renin Substrate	DRVYIHPFHLLVYS
7	Enolase T35	WLTGPQLADLYHSLMK
8	Enolase T37	YPIVSIEDPFAEDDWEAWSHFFK
9	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ

Table 1. MassPREP peptide mixture.

Calculations:

Peak capacities for each separation were calculated by measuring peak widths for peptides 2 through 7. Peptides 8 and 9 co-eluted in several of the separations, precluding them from the calculations. Similarly, peptide 1 (RASG-1) was excluded from the calculation because it eluted from the CSH130 C_{18} Column near the void volume using 0.1% FA in the mobile phase. Peak widths were measured from the UV chromatograms at peak half-height (w_h), averaged, then subsequently converted to 4σ peak capacities according to the following equation:

$$P_{c,4\sigma} = 1 + \left[\left(\frac{2.35}{4}\right)\left(\frac{t_{gradient}}{w_{h,avg}}\right)\right]$$

RESULTS AND DISCUSSION

Peptide separations

The MassPREP Peptide Mixture contains nine different peptides, varying in amino acid composition, mass, length, and polarity. Peptide sequences are shown in Table 1. Since this mixture is composed of such a diverse set of peptides, it is useful for evaluating mass spectrometric as well as chromatographic performance. To this end, the peptide mixture was employed to benchmark the separation performance of CSH130 C_{18} against other commonly used stationary phases for peptide analysis. Included in this study were phases with pore sizes large enough in diameter (100 to 300Å) to efficiently separate the peptides in the mixture, which are all less than 3 kDa in mass. In a separate application note, the use of CSH130 C_{18}, with its 130Å pores for separating even larger polypeptides is discussed.[11] For the sake of relevance, mass loads for the individual peptides in the mixture were made approximate to conditions common for peptide mapping of antibodies, wherein 20 to 50 µg of Lys-C or tryptic digests are typically analyzed on 2.0 or 2.1 mm I.D. columns.[2,12-13] Similarly, separations were completed with mobile phases containing a strong ion pairing agent, TFA, a weaker ion pairing agent, FA, or a combination thereof. Performance under these various conditions was of interest, since RP peptide separations are routinely coupled with mass spectrometry. In such applications, formic acid is often preferred over TFA as a mobile phase additive because it permits more sensitive detection.[14-15]

Figure 1 shows three chromatograms of the MassPREP Peptide Mixture obtained using a 5 µm silica C_{18} column under different mobile phase conditions. Separations with TFA provided peaks that were generally symmetrical. Most peaks did not exhibit excessive broadening or tailing. However, peak shape was found to be extremely poor with the FA containing mobile phases. In addition, the largest peptide in the mixture, melittin, was not detected, either because it eluted as too broad a peak or simply failed to elute. Results such as these are typical for HPLC-based peptide separations.

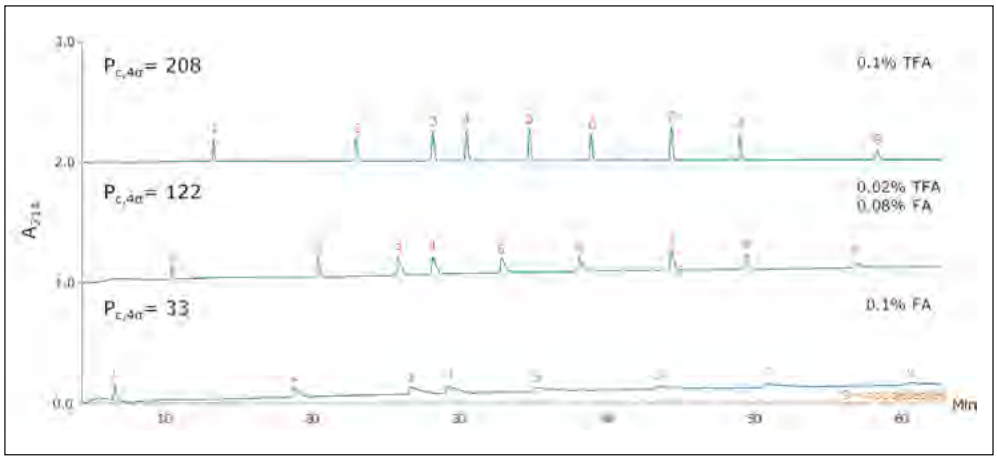

Figure 1. Analysis of MassPREP Peptide Mixture on a porous silica C_{18} 5 µm column using three different mobile phase conditions. Peptides were identified by MS and are labeled according to Table 1.

The use of sub-2-µm particles provides performance benefits to peptide separations. Chromatograms obtained for two different columns both packed with 1.7 µm particles are displayed in Figures 2 and 3. The chromatograms in Figure 2, in particular, were obtained using a superficially porous C_{18} column. With TFA in the mobile phase, the peaks were symmetrical and generally narrower than peaks obtained with the 5 µm, porous C_{18} column, shown in Figure 1. With little or no TFA in the mobile phase, peaks became broader and exhibited significant tailing. The fully porous BEH130 C_{18} Column produced comparable separations to the superficially porous C_{18} column, shown in Figure 3, in all conditions tested.

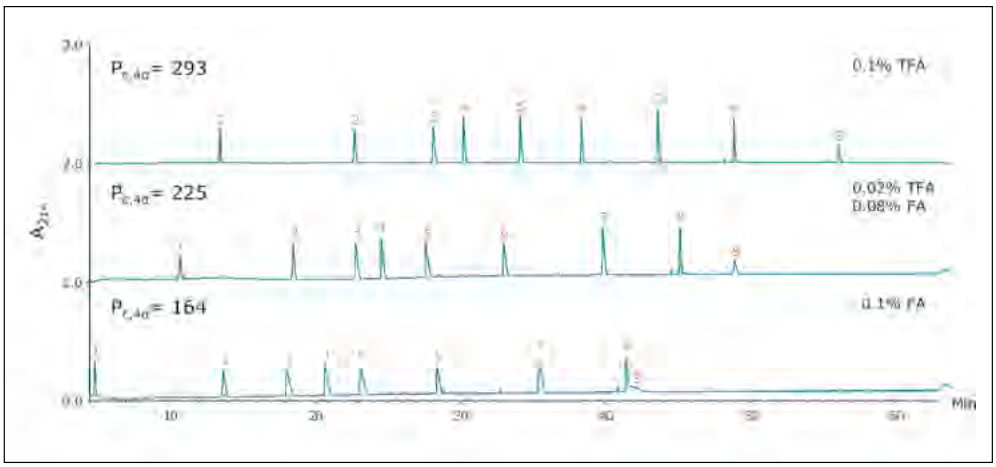

Figure 2. Analysis of MassPREP Peptide Mixture on a superficially porous C_{18}, 1.7 µm column using three different mobile-phase conditions.

The separations shown in Figures 1 through 3 served to benchmark the performance of a CSH130 C_{18}, 1.7 µm Column for peptide analyses. Chromatograms corresponding to separations with this column are shown in Figure 4. Comparison of Figures 1 through 4 clearly indicates that, unlike the other columns, CSH130 C_{18} provides the best peak shape with both TFA and FA mobile phases. Additionally, it is evident that CSH130 C_{18} exhibits unique selectivity, particularly when TFA is excluded from the mobile phase. For instance, with only 0.1% FA in the mobile phase, peptides 8 and 9 were not well resolved on any column used in this study other than the CSH130 C_{18}, when the two peptides eluted as symmetrical peaks and were separated by more than 3 min. Retention differences were also observed among these columns. Most notably, the CSH130 C_{18} Column was found to be slightly less retentive than each of the other columns included in this study. This is consistent with CSH130 C_{18} being the only sorbent with a purposely incorporated low level positive surface charge. Coulombic repulsion between the positively charged peptides and the CSH130 C_{18} surface is likely to be the cause of reduced retention. The insert in Figure 4 displays the earliest portion of the 0.1% FA gradient on CSH130 C_{18}. Under those conditions, the most hydrophilic peptide RASG-1 (peptide 1) eluted near the void marker (Vo). On average, peptides eluted from the CSH130 C_{18} Column with 2% to 4% less ACN when compared to the BEH130 C_{18} Column. Differences in retention were least pronounced with 0.1% TFA and most pronounced with 0.1% FA. When analyzing very polar peptides, initial gradient conditions for the CSH130 C_{18} may require adjustment accordingly.

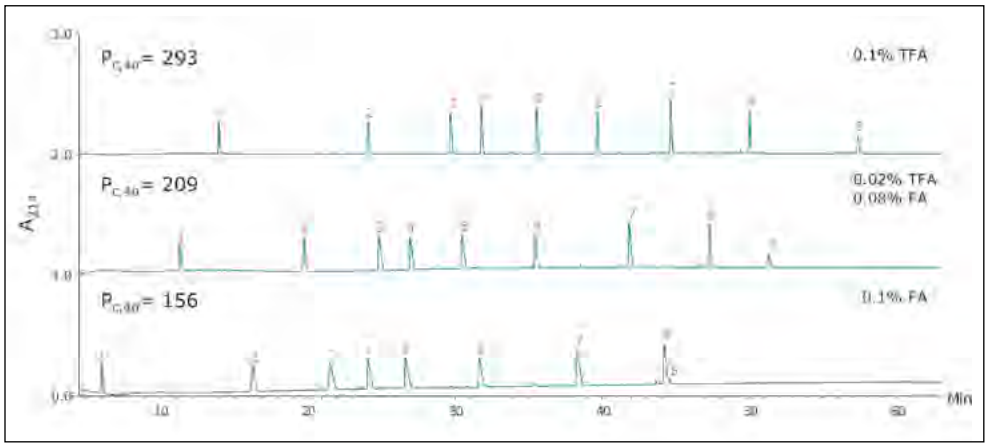

Figure 3. Analysis of MassPREP Peptide Mixture on a Waters ACQUITY UPLC BEH130 C_{18}, 1.7 µm Column using three different mobile-phase conditions.

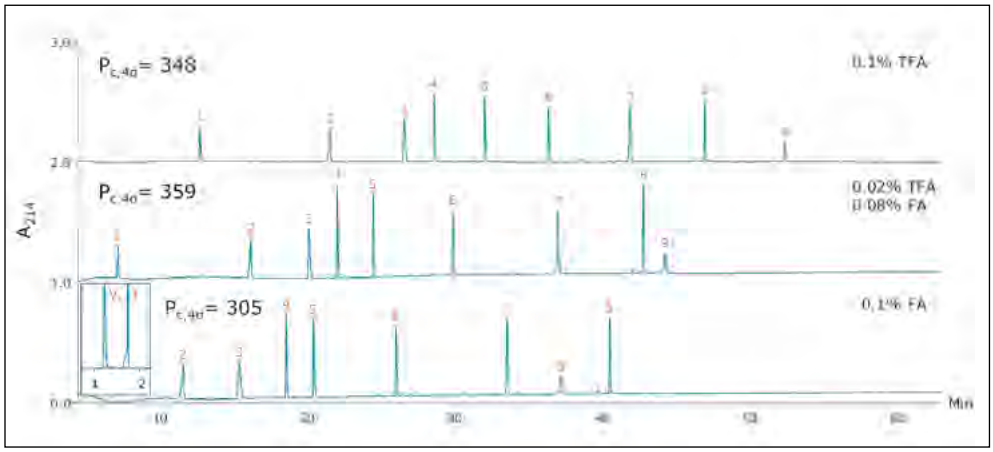

Figure 4. Analysis of MassPREP Peptide Mixture on a Waters ACQUITY UPLC CSH130 C_{18}, 1.7 µm Column using three different mobile-phase conditions.

Peak capacity and MS signal

A qualitative comparison of these separations indicates that the CSH130 C_{18} Column offers a performance advantage over the other columns. However, a quantitative comparison is even more compelling. Peak capacities observed for each separation were calculated as described in the experimental section. Figure 5A shows the relationship between acid modifier composition and the peak capacities obtained with each column. The left side of Figure 5A shows the peak capacity values for separations achieved with only FA and no TFA ion pairing agent. Moving right along the x-axis presents peak capacity values corresponding to decreasing concentrations of FA and increasing concentrations of TFA.

The performance advantage of the CSH130 C_{18} Column is easily visualized. With 0.1% TFA, the CSH130 C_{18} Column has 20% higher peak capacity than the other 1.7 µm C_{18} columns. However, when the mobile phase contains 0.1% formic acid, the CSH130 C_{18} Column provides 90% greater peak capacity compared to the other 1.7 µm columns. Thus, not only does the CSH130 C_{18} Column exhibit greater peak capacity than the other columns, its performance is far less dependent on TFA. A profound consequence of this is that CSH130 C_{18} is highly compatible with mass spectrometry. The effect of TFA on the MS detection of the peptides in these analyses is shown in Figure 5B. It is well known that TFA causes significant ion suppression during electrospray ionization.[16-17] Here, the use of TFA instead of FA caused a 12-fold decrease in MS signal. CSH130 C_{18} does not require much, if any, TFA for optimal peak capacity, making it ideal for LC-MS applications when high sensitivity is desired.

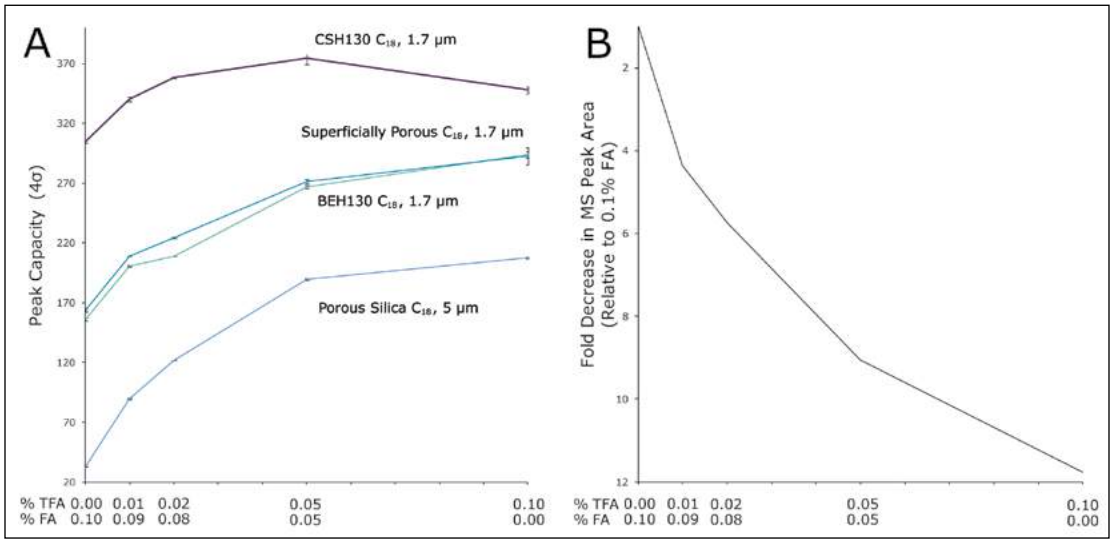

Figure 5. Effect of TFA on peak capacity and MS signal. (A) Peak capacity as a function of acid modifier. Values were derived from two replicates. (B) Decrease in MS peak area as a function of acid modifier.

Particle size and UPLC/HPLC scalability

Many peptide separations are still performed on traditional HPLC instruments. Due to their prohibitively high back pressures, columns packed with sub-2-µm particles are generally not well suited for use with HPLC systems. Conversely, columns packed with 2.5 µm particles can be used on any LC instrument due to their lower back pressure. To determine if the high peak capacity peptide separations obtained using a CSH130 C_{18}, 1.7 µm Column could be successfully transferred to a CSH130 C_{18} ***XP***, 2.5 µm Column, an HPLC compatible method for a 2.5 µm ***XP*** Column was programmed from the method for a 1.7 µm column. This was done by decreasing flow rate and increasing the gradient time by a factor of 1.5, as suggested by the ACQUITY UPLC Column Calculator.[18] Figure 6 shows that separations with the 1.7 µm column were successfully transferred to the 2.5 µm ***XP*** Column. The consistency of the selectivity factors underscores this observation, as shown in Table 2. Separations with the 2.5 µm ***XP*** Particle Column resulted in considerably lower back pressures compared to the 1.7 µm particle column (~3000 versus ~8000 psi), thereby demonstrating that CSH Technology can be readily employed for high peak capacity peptide separations using either UPLC or HPLC instrumentation.

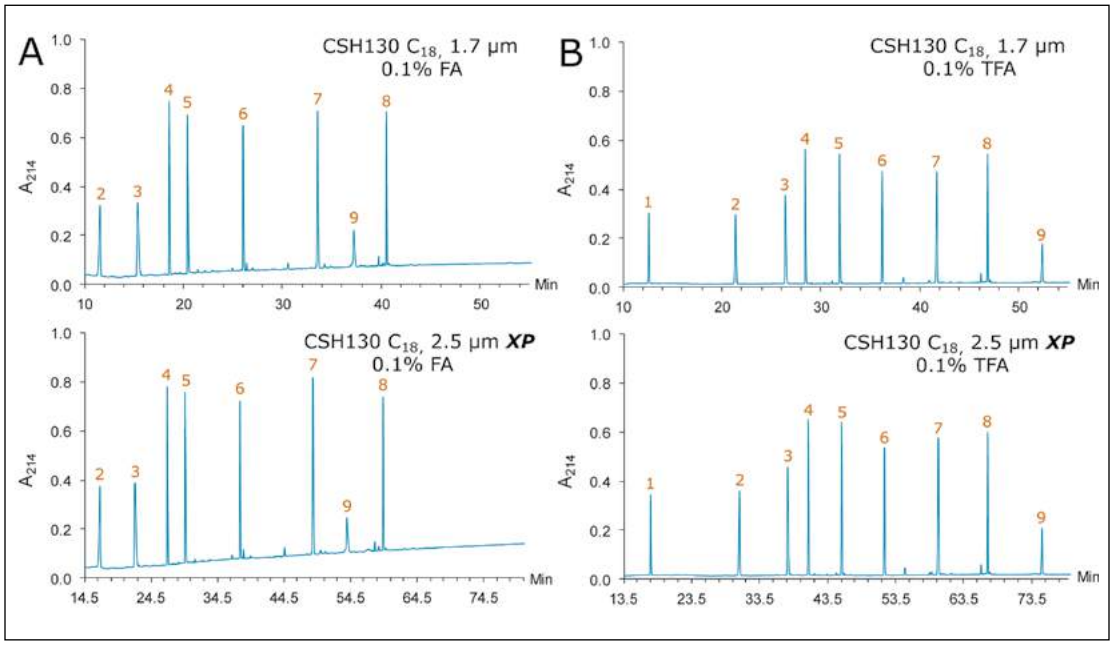

*Figure 6. UPLC/HPLC (1.7/2.5 µm particle size) scalability of CSH130 C_{18} Column. Chromatograms obtained with (A) 0.1% FA, and (B) 0.1% TFA mobile phases. The method for the Waters XSelect CSH130 C_{18}, 2.5 µm **XP** Column was programmed from the method for the Waters ACQUITY CSH130 C_{18}, 1.7 µm Column using the ACQUITY UPLC Column Calculator.*

	0.1% FA		0.1% TFA	
	CSH130 C_{18} 1.7 µm	CSH130 C_{18} 2.5 µm *XP*	CSH130 C_{18} 1.7 µm	CSH130 C_{18} 2.5 µm *XP*
$\alpha_{2,3}$	1.36	1.35	$\alpha_{1,2}$ 1.76	1.83
$\alpha_{3,4}$	1.22	1.24	$\alpha_{2,3}$ 1.25	1.25
$\alpha_{4,5}$	1.11	1.11	$\alpha_{3,4}$ 1.08	1.08
$\alpha_{5,6}$	1.29	1.30	$\alpha_{4,5}$ 1.13	1.13
$\alpha_{6,7}$	1.30	1.30	$\alpha_{5,6}$ 1.14	1.14
$\alpha_{7,9}$	1.11	1.11	$\alpha_{6,7}$ 1.16	1.16
$\alpha_{9,8}$	1.09	1.10	$\alpha_{7,8}$ 1.12	1.12
			$\alpha_{8,9}$ 1.12	1.12

Table 2. Selectivity factors for the separations shown in Figure 6.

CONCLUSIONS

The CSH130 C_{18} Column, with its novel incorporation of a low level positive charge, has proven to be an enabling technology for peptide separations. Based on analyses of a nine-peptide mixture, it was found that CSH130 C_{18} Columns exhibit greater peak capacity as well as unique selectivity when compared to columns containing C_{18} stationary phases without a purposely incorporated positive surface charge. The performance of CSH130 C_{18} Columns was also observed to be significantly less dependent on strong ion pairing agents, such as TFA, which suppress electrospray ionization. Furthermore, separations achieved with a CSH130 C_{18}, 1.7 µm Column were transferrable to a CSH130 C_{18}, 2.5 µm ***XP*** Column under lower pressure HPLC conditions, albeit at the cost of analysis time. The availability of different particle size CSH130 C_{18} Columns should facilitate the adaptation of UPLC separations for routine use in laboratories restricted to HPLC instrumentation as well as expand the capabilities of UHPLC instrumentation with 9000 to 12,000 psi pressure limits.

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ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm Column, 2.1 x 100 mm

Empower 3 Software

KEY WORDS

Peptides, mass detection

INTRODUCTION

Recently it was shown that the Waters ACQUITY QDa Detector, as a complementary orthogonal detection technique, provides a cost effective means of obtaining mass spectral data for peptides within an existing optically based LC workflow.[1]

This proof-of-principle work, while compelling, was confined to a reference set of seven peptides that ranged in molecular weight from 899 to 2,848 Da.

In contrast, biotherapeutics, such as monoclonal antibodies, when enzymatically treated can produce peptides that span nearly three orders of magnitude in size (150 to 7,000 Da).[2] In addition, ion pairing agents such as trifluoroacetic acid (TFA) are often used for peptide mapping. Given that TFA negatively impacts ionization efficiency, it can be rather challenging to obtain mass spectrometry (MS) based peptide maps.[3,4] As previously established, orthogonal techniques that add value and can be employed with minimal cost and effort are highly desirable in the pharmaceutical industry.[5]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting peptides across a wide molecular weight range (one more typical of biotherapeutic peptide maps) and that this mass detection capability is fully compatible with traditional optically based LC peptide monitoring assays that incorporate TFA or formic acid (FA). To this end, we have employed an existing method for the monitoring of peptide maps of trastuzumab, a therapeutic monoclonal antibody (mAb), using the ACQUITY QDa Detector.

Figure 1. An ACQUITY UPLC H-Class System, with the ACQUITY QDa Detector highlighted. The compact footprint of the detector allows for convenient integration into laboratories for improving productivity and strengthening process control and quality assurance in the biotherapeutic production environment.

EXPERIMENTAL

The ACQUITY UPLC CSH 130Å, C_{18} (2.1 x 100 mm, 1.7 µm) and ACQUITY UPLC BEH 300Å, C_{18} (2.1 x 100 mm, 1.7 µm) Columns were conditioned as outlined by the column care and use manual. Chemical reagents were purchased from Sigma Aldrich and used as received. Sequence grade modified trypsin from Promega was used to prepare a digest of trastuzumab (reduced and alkylated) at a concentration of 0.5 mg/mL as outlined by the manufacturers' protocol.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV ACQUITY QDa
Absorption wavelength:	215 nm
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Columns:	ACQUITY UPLC Peptide CSH 130Å, C_{18}, 1.7 µm, 2.1 x 100 mm (p/n 186006937) ACQUITY UPLC BEH 300Å, C_{18}, 1.7 µm, 2.1 x 100 mm (p/n186003686)
Column temp.:	65 °C
Sample temp.:	4 °C
Injection vol.:	8 µL
Mobile phase A:	H_2O, 0.1% TFA
Mobile phase B:	Acetonitrile, 0.1% TFA
Mobile phase C:	H_2O, 0.1 % FA
Mobile phase D:	Acetonitrile, 0.1% FA

Gradient table (BEH column)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	97	3	0	0
3.00	0.200	97	3	0	0
120.00	0.200	65	35	0	0
127.00	0.200	20	80	0	0
130.00	0.200	20	80	0	0
131.00	0.200	97	3	0	0
140.00	0.200	97	3	0	0

Gradient table (CSH column)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	0	0	99	1
3.00	0.200	0	0	99	1
120.00	0.200	0	0	67	33
127.00	0.200	0	0	20	80
130.00	0.200	0	0	20	80
131.00	0.200	0	0	99	1
140.00	0.200	0	0	99	1

MS detector settings

Sample rate:	2 points/sec
Mass range:	350 – 1250 Da.
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Informatics for data collection and processing

Empower 3 Software, SR2

RESULTS AND DISCUSSION

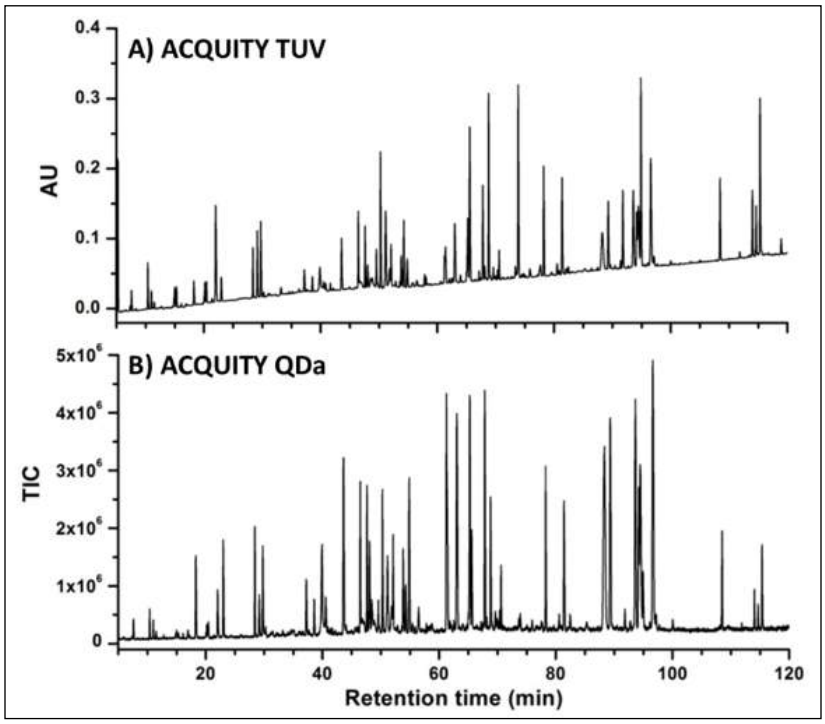

Figure 2. Peptide mapping with the ACQUITY QDa Detector using TFA and an ACQUITY UPLC BEH 300Å, C_{18}, 1.7 µm, 2.1 x 100 mm Column. Mobile phases prepared with 0.1% v/v TFA were used to acquire an A) optical and B) MS based peptide map of trastuzumab simultaneously. A high degree of correlation was observed between both detectors establishing the compatibility of the ACQUITY QDa with legacy methods that incorporate TFA.

From our initial proof-of-principle work we established that the ACQUITY QDa Detector is well-suited for confirming the identity and purity of peptides across a wide molecular weight range.[1] For that work, the TFA concentration in the mobile phases was kept relatively low, at 0.02%, in order to minimize ion supression.[3,4] However, higher concentrations of TFA are often incorporated in optical based (UV) peptide analyses for improving the performance of conventional C_{18} columns.[6]

To demonstrate that the ACQUITY QDa Detector is compatible with such legacy methods, a peptide map of trastuzumab was acquired using conventional concentrations of TFA. For this experiment, a 0.5 mg/mL solution of trypsin digested, reduced, and alkylated trastuzumab was analyzed using mobile phases prepared with 0.1% TFA v/v. As shown in Figure 2A, a 120 minute gradient (see experimental) was used to generate an optical based peptide map using an ACQUITY UPLC BEH 300Å, C_{18}, 1.7-µm Column. The corresponding mass detector response from the ACQUITY QDa, which was in-line post optical detector, is shown in Figure 2B. A high degree of correlation was observed for the peptides detected using the orthogonal detection configuration. From this data, it is evident the ACQUITY QDa Detector is capable of providing mass spectral data using legacy methods that incorporate ion pairing agents such as TFA.

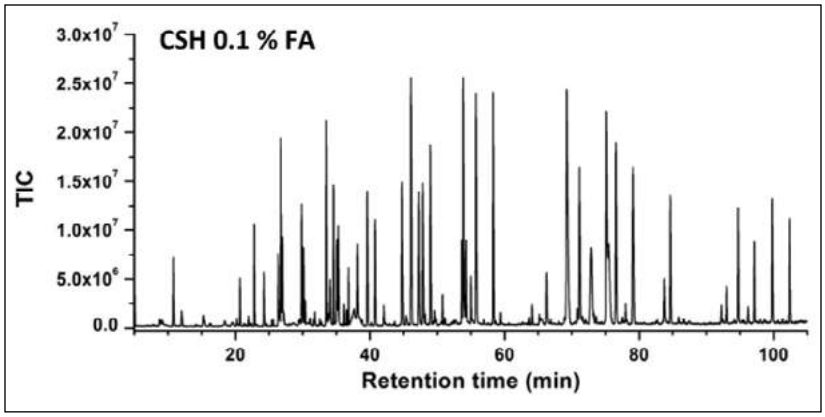

Figure 3. Peptide mapping with an ACQUITY UPLC Peptide CSH C_{18} Column. Mobile phases prepared with 0.1% v/v FA were used to acquire an MS based peptide map of trastuzumab using the ACQUITY QDa Detector. The peptide profile was observed with improved detector response when compared to the one shown in Figure 2B using TFA.

As new columns are introduced to the market with improved performance with FA versus TFA,[7] the question arises if legacy methods can be transferred to new columns more efficiently with the addition of a mass detector.

To test this, an ACQUITY UPLC Peptide CSH C_{18}, 130Å, 1.7 µm Column was used to generate a peptide map using the same sample as shown in Figure 2. For this experiment, mobile phases were prepared with FA at a concentration of 0.1% v/v, and a 120-minute gradient was implemented to generate a peptide map.

As shown in Figure 3 and demonstrated in previous work,[8] the unique surface chemistry of charged surface hybrid (CSH) columns facilitates the use of FA, resulting in increased detector response. Given that both columns offer the ability to monitor peptides with the ACQUITY QDa Detector, further interrogation of the data was performed to evaluate the use of different ion-pairing agents.

Charge State

Peptide	Average Mass	$[M+1H]^{+1}$	$[M+2H]^{+2}$	$[M+3H]^{+3}$	$[M+4H]^{+4}$	$[M+5H]^{+5}$	$[M+6H]^{+6}$	$[M+7H]^{+7}$	$[M+8H]^{+8}$	$[M+9H]^{+9}$	$[M+10H]^{+10}$
T39	574.3	575.3	288.2	192.4	144.6	115.9	96.7	83.0	72.8	64.8	58.4
T7	681.3	682.3	341.7	228.1	171.3	137.3	114.6	98.3	86.2	76.7	69.1
T5	830.0	831.0	416.0	277.7	208.5	167.0	139.3	119.6	104.7	93.2	84.0
T21	835.0	836.0	418.5	279.3	209.7	168.0					84.5
T30	838.0	839.0	420.0	280.3	210.5	168.6					84.8
T9	969.1	970.1	485.5	324.0	243.3	194.8					97.9
T6	1084.2	1085.2	543.1	362.4	272.1	217.8					109.4
T3	1089.2	1090.2	545.6	364.1	273.3	218.8					109.9
T36*	1161.4	1162.4	581.7	388.1	291.3	233.3					117.1
T2*	1167.4	1168.4	584.7	390.1	292.8	234.5					117.7
T8-9	1182.3	1183.3	592.2	395.1	296.6	237.5					119.2
T13	1186.4	1187.4	594.2	396.5	297.6	238.3	198.7	170.5	149.3	132.8	119.6
T10	1310.5	1311.5	656.3	437.8	328.6	263.1	219.4	188.2	164.8	146.6	132.1
T4-5	1311.5	1312.5	656.8	438.2	328.9	263.3	219.6	188.4	164.9	146.7	132.2
T14*	1321.5	1322.5	661.8	441.5	331.4	265.3	221.3	189.8	166.2	147.8	133.2
T11*	1334.4	1335.4	668.2	445.8	334.6	267.9	223.4	191.6	167.8	149.3	134.4
T23	1677.8	1678.8	839.9	560.3	420.5	336.6	280.6	240.7	210.7	187.4	168.8
T33-34	1724.9	1725.9	863.5	576.0	432.2	346.0	288.5	247.4	216.6	192.7	173.5
T26	1808.1	1809.1	905.1	603.7	453.0	362.6	302.4	259.3	227.0	201.9	181.8
T38	1874.1	1875.1	938.0	625.7	469.5	375.8	313.3	268.7	235.3	209.2	188.4
T1	1882.1	1883.1	942.1	628.4	471.5	377.4	314.7	269.9	236.3	210.1	189.2
T22*	2139.4	2140.4	1070.7	714.1	535.8	428.9	357.6	306.6	268.4	238.7	214.9
T26-27	2228.6	2229.6	1115.3	743.9	558.1	446.7	372.4	319.4	279.6	248.6	223.9
T2-3*	2238.6	2239.6	1120.3	747.2	560.6	448.7	374.1	320.8	280.8	249.7	224.9
T37	2544.7	2545.7	1273.3	849.2	637.2	509.9	425.1	364.5	319.1	283.7	255.5
T12	2785.0	2786.0	1393.5	929.3	697.3	558.0	465.2	398.9	349.1	310.4	279.5
T41*	2802.1	2803.1	1402.1	935.0	701.5	561.4	468.0	401.3	351.3	312.3	281.2
T24-25*	3117.1	3118.1	1487.0	1040.4	780.3	624.4	520.5	446.3	390.6	347.3	312.7
T19-20*	3335.9	3336.9	1669.0	1113.0	835.0	668.2	557.0	477.6	418.0	371.7	334.6
T15*	6716.5	6717.5	3359.2	2239.8	1680.1	1344.3	1120.4	960.5	840.6	747.3	672.6
T15-16*	7058.9	7059.9	3530.4	2354.0	1765.7	1412.8	1177.5	1009.4	883.4	785.3	706.9
T15-17*	7187.0	7188.0	3594.5	2396.7	1797.8	1438.4	1198.8	1027.7	899.4	799.6	719.7

Legend:
Trifluoroacetic acid
Formic acid
= not observed

Table 1. Peptide map charge state table. A charge state table constructed from a simulated digest of the heavy chain of trastuzumab was used to identify the observed charge states of peptides detected with the ACQUITY QDa when using TFA or FA as an ion pairing agent. For comparison sake, only peptide fragment assignments that were observed in both the TFA and FA experiments were included in the table.

The volatility and acidic nature of the ion-pairing agent used in the mobile phase can impact the ionization and charge state distribution observed in multiply charged species such as peptides.[3,4] To investigate the effect of TFA versus FA on the charge state distribution of the peptides resulting from the enzymatically treated trastuzumab sample, a charge state table comprised of the heavy chain peptides was constructed from a simulated tryptic digest of trastuzumab (Table 1).

For both the TFA and FA peptide map experiments, the ACQUITY QDa Detector was set to a scan from 350 *m/z* to 1250 *m/z* (maximum) and is highlighted by the thicker line traces inside Table 1. Green and blue highlights were used to indicate if the peptide charge state was observed with TFA or FA, respectively.

Both colors indicate the charge state was observed in both peptide map experiments whereas a grey box represents a charge state within the scan range, but not observed.

From Table 1, it is clear that with only one exception (peptide T12), all peptides were observed to have multiple charge states with either TFA or FA as an ion-pairing agent. In addition, the peptides observed represent 93% of the heavy chain of trastuzumab. Similarly, 92% of the light chain peptide fragments were observed with multiple charge states for the trastuzumab digest when using TFA or FA. Peptides not observed were either not retained or were in a charge state below the 350 *m/z* experimental setting.

From these data, it can be seen that the ACQUITY QDa Detector is compatible with both TFA and FA based methods, affording significant flexibility in method development of monitoring assays.

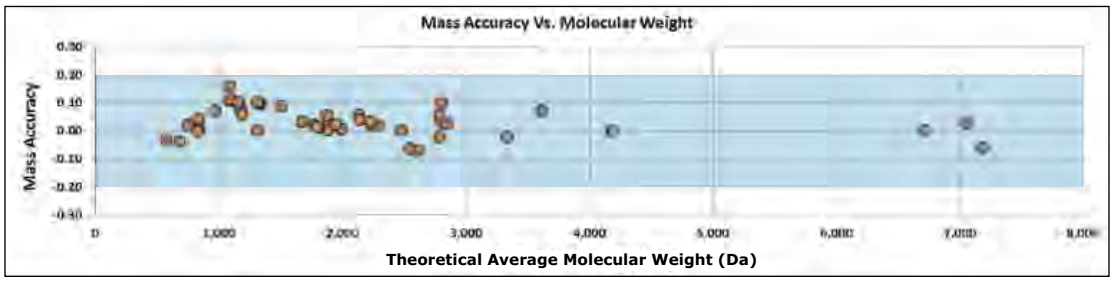

Figure 4. Peptide mass accuracy. A plot of the m/z difference between the theoretical and observed m/z was plotted against the average molecular weight for the peptides detected with the ACQUITY QDa. The mass accuracy of the peptides detected were within the instrument specification of ± 0.2 Dalton.

A natural question that arises from these results is: What is the accuracy with which we are detecting the charge states of the observed peptides?

An assessment of mass accuracy of the ACQUITY QDa was thus performed using the base peak ion (BPI) for the observed peptides. For this assessment, the difference between the observed and theoretical charge state *m/z* value was calculated for the BPI using the average molecular weight of each peptide.

A plot of the mass difference versus the theoretical average molecular weight was constructed as shown in Figure 4. It can be seen from Figure 4 that the BPI derived masses for the observed peptides are within the instrument specification of ± 0.2 Dalton (blue highlight), with a large portion of the peptides falling within ± 0.10 Dalton, demonstrating the ACQUITY QDa Detector is capable of providing accurate mass information for peptides over a broad molecular weight range in assays routinely employed during the analysis of biotherapeutics.

CONCLUSIONS

From previous work, it was shown that the ACQUITY QDa Detector provides a complementary detection technique to optical detection and can thereby improve the productivity of a single workflow. Cost effective techniques that add value and can be implemented into existing workflows with minimal effort are highly desirable in the pharmaceutical industry.

A natural extension of this work was to evaluate the performance of the ACQUITY QDa Detector using a representative biotherapeutic drug. From this work it has been demonstrated that the ACQUITY QDa Detector is compatible with conventional LC mobile phases and is able to detect and accurately report mass information for peptides over a wide molecular weight range typical of biotherapeutic peptide maps.

Collectively, these results establish the ACQUITY QDa Detector as an ideal addition for the biopharmaceutical analyst's lab that will afford increases in productivity and the confidence of data analysis for routine peptide analysis assays.

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Complementing Routine Peptide Monitoring Using the ACQUITY QDa Mass Detector

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APPLICATION BENEFITS

- Increased productivity through the use of on-line orthogonal detection techniques
- Efficient data acquisition and analysis within a single compliant software package

WATERS SOLUTIONS

ACQUITY QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY UPLC Peptide CSH Column

Empower® 3 Software

KEY WORDS

Peptide, UV detection, mass detection, screening, monitoring, biopharmaceutical QC

INTRODUCTION

For established biotherapeutic drugs in the pharmaceutical industry, screening protocols are often employed at different stages during the manufacturing process to ensure regulatory guidelines are met with regards to product efficacy, identity, and purity. Improved productivity and lower development costs are often associated with the implementation of targeted analytical protocols to assess critical quality attributes (CQA). Orthogonal techniques that add value and can be employed with minimal cost and effort without compromising productivity are highly desirable.

The ACQUITY® QDa Detector is Waters' solution to meet the demands of today's fast-paced pharmaceutical work environment by enabling complementary optical and mass spec analysis techniques to be run on-line in a single screening workflow for improved productivity in data analysis.

Figure 1. The ACQUITY QDa Detector. The compact footprint of the ACQUITY QDa allows for easy integration into existing instrument stacks. Its plug-and-play design allows for the implementation of complementary orthogonal detection techniques with minimal effort into a single integrated system and workflow.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class
Vials:	Total Recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	ACQUITY UPLC Peptide CSH 130Å, C_{18}, 1.7 µm, 2.1 x 100 mm (p/n 186006937)
Column temp.:	60 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
UV detector:	ACQUITY UPLC TUV
Wavelength:	215 nm
MS detector:	ACQUITY QDa
MS sample rate:	2 points/sec
Mass range:	200 to 1250 Da
Cone voltage:	7 V
Capillary voltage:	0.5 kV
Probe temp.:	600 °C
Mobile phase A:	H_2O, 0.1 % TFA
Mobile phase B:	Acetonitrile, 0.1 % TFA
Mobile phase C:	H_2O, 0.1 % FA
Mobile phase D:	Acetonitrile, 0.1 % FA

Gradient table

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.300	19	1	76	4
2.00	0.300	19	1	76	4
22.00	0.300	11	9	44	36
25.00	0.300	3	17	12	68
28.00	0.300	3	17	12	68
28.01	0.300	19	1	76	4
30.00	0.300	19	1	76	4

Informatics for data collection & processing

Empower 3 Chromatography Data Software, SR2 with Mass Analysis

The ACQUITY QDa Detector offers the ability to combine straightforward mass spectral data with optical data in a single workflow with minimal effort and cost. The ACQUITY QDa is pre-optimized to work without the need for sample-specific user adjustments that are typical of traditional mass spectrometers, making it simple and easy to use. With a purposeful design that allows for integration into existing UPLC® instrumentation (Figure 1), the ACQUITY QDa Detector is well-suited for improving productivity and strengthening quality assurance in the biotherapeutic production environment.

The objective of this application is to demonstrate the use of the ACQUITY QDa Detector as a complementary orthogonal detection technique that enables the integration of optical (UV) and basic mass spectral data within a single workflow using a known peptide mixture.

EXPERIMENTAL

Sample preparation

The ACQUITY UPLC Peptide CSH Column (130Å, C_{18}, 1.7 µm, 2.1 x 100 mm) 1.7 µm) was conditioned prior to use per the care and use manual. Chemical reagents were purchased from Sigma Aldrich and used as received. A peptide mix containing angiotensin (frag. 1-7), bradykinin, angiotensin II, angiotensin I, renin substrate, enolase T37, and melittin was prepared at a concentration of 0.3 mg/mL in initial mobile phase conditions.

RESULTS AND DISCUSSION

Confirmation of Peak Identity Using On-line Complementary Orthogonal Detection Techniques

Proteolytic digests are often analyzed with optical detectors as a monitoring technique for protein composition of biotherapeutic drugs. The volatility and acidic nature of the mobile phases used in conventional peptide analyses makes the technique amenable to mass spectrometry. Lower *m/z* values are often observed for peptides because they are often multiply charged during the ionization process, particularly when low TFA or TFA-free mobile phases are employed. With a detection range between 30–1250 *m/z*, the ACQUITY QDa Detector is well-suited for providing complementary mass data in peptide analyses for multiply charged species.

To demonstrate this, a peptide mixture containing seven peptides was prepared at a concentration of 0.3 mg/mL. With an ACQUITY QDa on-line as a mass detector post optical detection, 3 µg of the peptide mixture was injected onto an ACQUITY UPLC Peptide CSH 130 Å C_{18} Column. A 20-minute reversed-phase gradient was used to elute the peptides (see Experimental).

As shown in Figure 2A, the ACQUITY QDa Detector provides sufficient sensitivity to clearly detect the individual peptides. Extracted MS spectra (Figure 2A inset) of the detected peaks can be examined in Purity, Combined, or Apex mode (shown) using Empower 3 Software. The complementary mass data are seamlessly integrated into the optical data as shown in Figure 2B, where the mass from the most intense base peak ion is displayed next to the retention time of the identified peak.

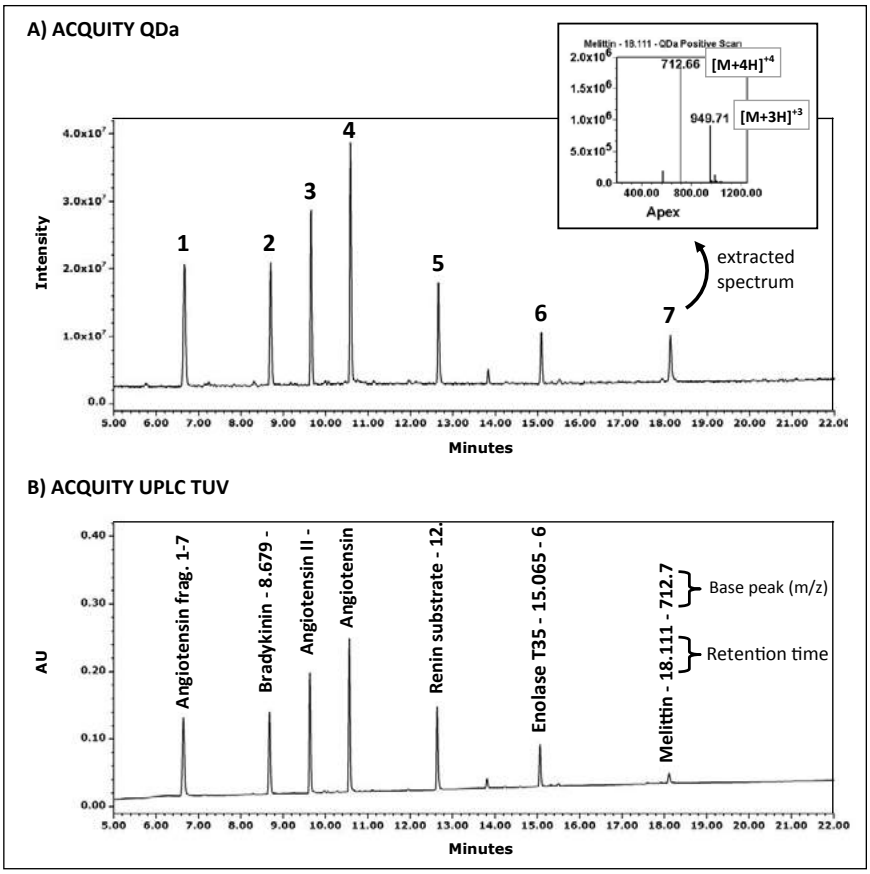

Figure 2. On-line Complementary Detection Techniques. A 3-µg injection of a peptide mixture containing seven peptides was separated using a 20-minute reversed-phase gradient. Detection of the separation was performed on-line with an A) ACQUITY QDa as well as an B) ACQUITY UPLC TUV.

	Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	Int Type	Base Peak (m/z)
1	Angiotensin frag. 1-7	6.646	404491	17.88	116116	BV	450.5
2	Bradykinin	8.679	348049	15.39	122312	VV	531.0
3	Angiotensin II	9.632	434250	19.20	179209	BV	524.0
4	Angiotensin I	10.564	542397	23.98	227720	VV	433.2
5	Renin substrate	12.639	309110	13.66	123245	VV	587.4
6	Enolase T35	15.065	163047	7.21	62493	BV	625.4
7	Melittin	18.111	60868	2.69	15275	VB	712.7

Table 1. Component management. Through the use of the integrated component manger, Empower automatically labels identified peaks and reports corresponding metrics in tabular format.

Peak	Peptide	Average mass (Da)	Charge state	Calculated (*m/z*)	Base peak (*m/z*)
1	Angiotensin (frag. 1-7)	899.02	$[M+2H]^{+2}$	450.5	450.5
2	Bradykinin	1060.22	$[M+2H]^{+2}$	531.1	531.1
3	Angiotensin II	1046.19	$[M+2H]^{+2}$	524.1	524.1
4	Angiotensin I	1296.49	$[M+3H]^{+3}$	433.2	433.2
5	Renin substrate	1759.04	$[M+3H]^{+3}$	587.4	587.4
6	Enolase T35	1873.21	$[M+3H]^{+3}$	625.4	625.4
7	Melittin	2847.49	$[M+4H]^{+4}$	712.9	712.7

Table 2. Charge state analysis. Analysis of the base peaks identified in Empower indicate the ACQUITY QDa Detector is capable of detecting multiply charged peptides ranging in mass from 900 – 2800 Da within detector response specifications.

Utilizing Empower's integrated informatics tools such as the Component Manager, optical peaks are automatically identified and labeled with their corresponding peptide name within the chromatogram (Figure 2B) as well as in tabular format (Table 1) for efficient data review.

The base peak ion information reported by Empower 3 Software was compared to the calculated *m/z* for increasing charge states of the peptides using the average mass. As shown in Table 2, the ACQUITY QDa Detector readily detected peptides ranging in mass from 900 – 2800 Da. The base peaks detected corresponded to the peptides at charge states ranging from $[M+2H]^{+2H}$ – $[M+4H]^{+4H}$. Accuracy of the detector response was tested over three injections and found to be within the instrument specification of ± 0.2 Da.

The performance of the ACQUITY QDa Detector as part of the ACQUITY UPLC H-Class System, combined with Empower 3 Software's ability to seamlessly integrate mass spectral data with optical data, makes the ACQUITY QDa an ideal solution as an on-line complementary orthogonal detection technique for improving productivity in screening protocols for well characterized biopharmaceutical drugs.

CONCLUSIONS

The high costs associated with meeting regulatory guidelines for biotherapeutic drug safety has renewed interest in efficient methods that reduce costs and increase productivity in the manufacturing process. The ACQUITY QDa Detector, used with an ACQUITY UPLC H-Class System, offers the ability to employ mass spec analysis as an orthogonal detection technique in a single workflow, greatly improving monitoring capabilities in the production environment with minimal cost and effort.

With a plug-and-play design, and integrated compliant data acquisition and analysis through Empower 3 Software, the ACQUITY QDa Detector is a powerful tool that can increase productivity and confidence in data analysis within the biotherapeutic production environment.

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Reversed-Phase for Amino Acid Analysis (AAA)

Amino acids are the building blocks of proteins. For that reason, the amino acid composition of hydrolyzed protein and peptides can be analyzed for identifying a material and for measuring its concentration. Amino acids are also intermediates in a myriad of metabolic pathways and are therefore measured as elements of physiological and nutritional studies. This has proven particularly important in monitoring the cell culture and fermentation processes that are used for the production of biopharmaceuticals.

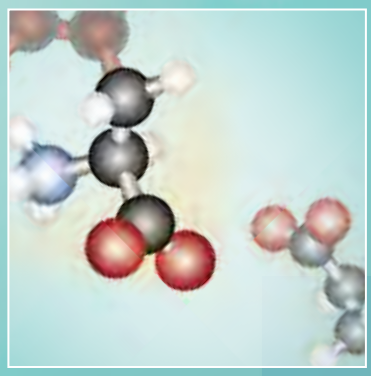
Amino Acid Analysis (AAA) can be challenging because amino acids have diverse, yet also sometimes very similar, chemical properties. They also lack common chemical features that can be used for convenient detection.

Solution highlights

The Waters UPLC Amino Acid Application Solution provides you with control over the major factors that cause variability in an amino acid analysis. From optimized instrumentation and quality controlled consumables to standardized software projects, you can rely on methods that are both rugged and reliable.

The UPLC Amino Acid Analysis Application Solution consists of:

- The AccQ•Tag Ultra derivatization and separation consumables chemistries including quality-control tested columns, reagents, and eluents.
- The AccQ•Tag Ultra C_{18} Column (1.7 µm particles), specifically certified for use with the AccQ•Tag method, delivers the excellent column efficiency and resolution that is required for AAA.
- ACQUITY UPLC (binary), ACQUITY UPLC H-Class (quaternary), or ACQUITY UPLC H-Class Bio (quaternary) System with a Tunable UV Detector, with enhanced chromatographic resolution and maximum-sensitivity detection. The system's superior resolution of all amino acids provides unambiguous peak identification and integration, which assures accurate results.
- Empower Software pre-configured projects, methods, and report formats.
- Connections INSIGHT® ISDP instrument diagnostics to ensure continuous, consistent, and reliable operation in your lab.
- Installation, Operation, and Performance Qualification (IQ/OQ/PQ) protocols that ensure every element of the system is performing, for reproducible results day-to-day, instrument-to-instrument, and lab-to-lab.



Amino Acid Analysis of Pure Protein Hydrolysate with Waters UPLC Amino Acid Analysis Solution

Hillary B. Hewitson, Thomas E. Wheat, and Diane M. Diehl
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INTRODUCTION

Amino acid analysis is used in the protein structure laboratory to provide two kinds of information. First, the total quantity of amino acids is a direct measure of the amount of protein in a sample. Second, the measurement of the proportions of amino acids provides information to confirm the identity of the protein and to detect modifications. Both applications require robust, accurate, and sensitive measurements that both identify and quantitate the amino acids. There is increasing need for these labs to provide these correct results faster and more economically.

The Waters UPLC® Amino Acid Analysis Application is a turnkey solution to address these needs. This total system solution includes a well established and understood sample derivatization kit, eluents, chromatographic column, and a separation system based on the ACQUITY UPLC® using UV detection under Empower® Software control.

In this experiment, this system solution is used to measure the composition and concentration of a known protein. The accuracy of the determination is compared to the known correct results.

Figure 1. Waters UPLC Amino Acid Analysis Solution

EXPERIMENTAL

Sample

Acid-hydrolyzed bovine serum albumin (BSA) samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in sealed ampoules. Samples were stored at -80 °C until analysis.

Sample derivatization

The sample was diluted 1:10 with 0.1 M HCl prior to derivatization. The standard derivatization protocol was modified to include neutralization of excess acid with 0.1 M NaOH. Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide (P/N 71500129702).

- 10 µL of samples diluted 1:10 with 0.1 M HCl
- 10 µL 0.1 N NaOH
- 60 µL AccQ•Tag™ Ultra Borate Buffer
- 20 µL AccQ•Tag Ultra Reagent

LC conditions

LC system:	ACQUITY UPLC System with TUV detection at 260
Column:	AccQ•Tag Ultra 2.1 x 100 mm, 1.7 µm, (P/N: 186003837)
Column temp.:	55 °C
Flow rate:	700 µL/min
Mobile phase A:	1:20 Dilution of AccQ •Tag Ultra Eluent A concentrate (P/N: 186003838)
Mobile phase B:	AccQ•Tag Ultra Eluent B (P/N: 186003839)
Gradient:	AccQ•Tag Ultra Hydrolysate Method
Injection vol.:	1 µL

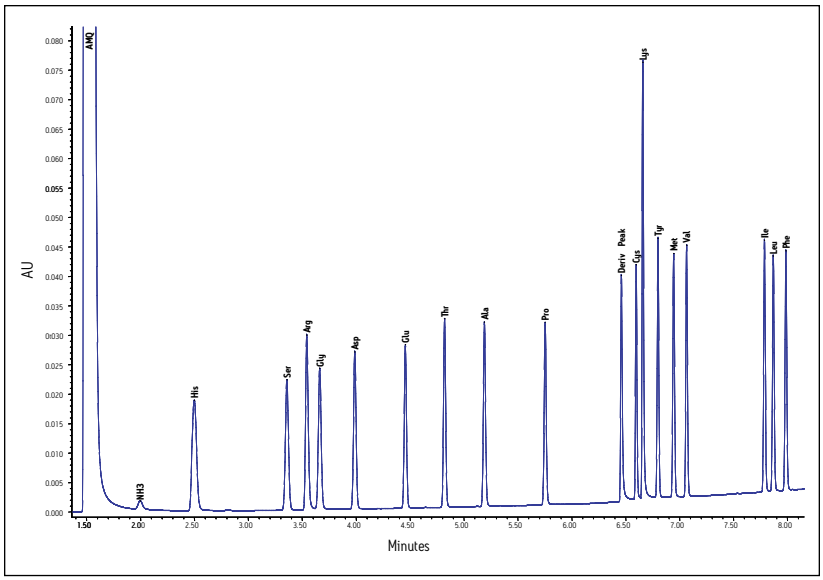

Figure 2. Analysis of Amino Acid Hydrolysate Standard, 10 pmoles each on column.

RESULTS

Figure 2 shows the chromatogram of a standard that contains amino acids typically found in protein hydrolysate samples. Each amino acid is 10 pmoles on column. For quantitative analyses, a three point calibration at 0.5, 10, and 25 pmoles on column was applied.

The analysis of a typical BSA hydrolysate is shown in Figure 3. The estimated starting concentration is consistent with this chromatogram representing a total of 9 ng of protein on column. This analysis was repeated a total of 75 times, over five separate days, with two columns and a total of five mobile phase preparations. The 75 injections represent five independent sample dilutions, each dilution derivatized five separate times. Each derivatized sample was injected in triplicate.

The amino acid composition, expressed as residues/mole of protein, was compared to the value expected from the known sequence. Table 1 shows the mean and standard deviation for each amino acid over all 75 analytical injections. Tryptophan and cysteine/cystine are excluded from the calculation because they are destroyed by acid hydrolysis. The measured molar composition agrees well with the expected values from the sequence. The reliability of the UPLC Amino Acid Analysis Application is confirmed by the reproducibility of results over the large number of determinations that intentionally includes the variability that could arise from multiple columns, eluents, and derivatizations.

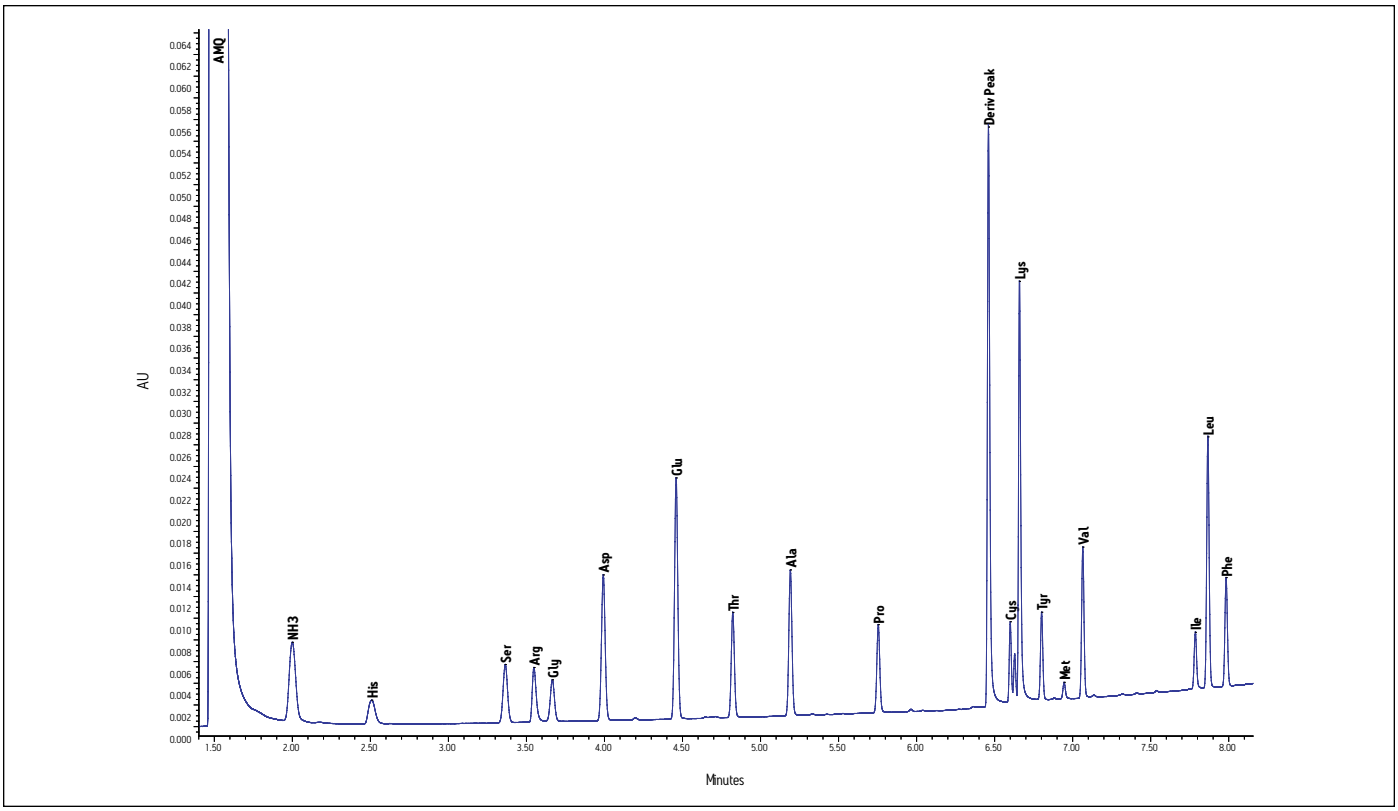

Figure 3. Analysis of BSA Hydrolysate Sample, approximately 9 ng on column.

Amino acid	Expected residues	*Observed residues
His	17	15.36 ± 0.19
Ser	28	26.00 ± 0.08
Arg	23	22.37 ± 0.08
Gly	16	17.68 ± 0.20
Asp	54	55.47 ± 0.21
Glu	79	80.68 ± 0.20
Thr	33	31.92 ± 0.06
Ala	47	47.51 ± 0.15
Pro	28	28.35 ± 0.14
Lys	59	57.78 ± 0.38
Tyr	20	20.19 ± 0.08
Met	4	4.16 ± 0.15
Val	36	35.67 ± 0.16
lle	14	13.15 ±0.15
Leu	61	63.13 ± 0.19
Phe	27	26.57 ± 0.13

Table 1. Comparison of Observed with Expected Composition Derived from Known Sequence of BSA.
*Average of 75 data points (25 derivatizations, each injected in triplicate; mean value ± standard deviation)

Sample I.D.	Total µg AA/ mL hydrolysate
BAS 1-1	699.64
BAS 1-2	694.09
BSA 1-3	697.88
BSA 1-4	698.21
BSA 1-5	695.43
BSA 2-1	716.66
BSA 2-2	717.87
BSA 2-3	715.65
BSA 2-4	709.75
BSA 2-5	707.93
BSA 3-1	714.93
BSA 3-2	711.50
BSA 3-3	708.46
BSA 3-4	708.14
BSA 3-5	708.10
BSA 4-1	674.43
BSA 4-2	678.43
BSA 4-3	683.07
BSA 4-4	678.62
BSA 4-5	678.94
BSA 5-1	591.40
BSA 5-2	604.30
BSA 5-3	571.78
BSA 5-4	571.57
BSA 5-5	599.18
Mean	677.43
Standard Deviation	47.98
% RSD	7.08

Table 2. Reproducibility of method for amount of BSA protein in sample.

The analytical data was used to calculate absolute amount of protein in the sample. The amount of each amino acid were expressed as the residue molecular weight. The sum of weights of the amino acids is equal to the weight of the protein. Table 2 summarizes the result of the 75 determinations with the mean for the triplicate injections shown for each derivatization. The measured amount corresponds to 0.7 mg/mL in the starting material. The estimated amount used to prepare the hydrolysate has not been independently verified. It should be noted in addition that this measurement of protein amount does not include the contribution of cysteine/cystine and tryptophan, as they are mostly destroyed by the hydrolysis of the protein.

The reproducibility of determination of the 75 analyses gives a RSD of 7%. Detailed examination shows that much of this variance is due to the difference of experiment 5 from the other four analyses. Since all of the replicates in experiment 5 are lower than the others, this variance is consistent with a difference in pipetting in the initial sample preparation. The addition of an internal standard to the sample to be hydrolyzed will improve the reliability of the final analytical result. Norvaline is the preferred internal standard for this purpose

CONCLUSION

Protein structure and biopharmaceutical laboratories rely on accurate quantitation of amino acids to confirm the identity and amount of protein in their samples. The analyses shown here demonstrate that Waters UPLC Amino Acid Analysis Application can provide assured results for these laboratories.

The molar ratios of amino acids are reproducible over multiple derivatizations and replicate injections. The measured composition agrees with that expected from the sequence of BSA.

Determination of the absolute amount of protein in the samples is determined by summing the residue weights of amino acids. The reproducibility of this measurement is on the order of ±7%. The largest contribution to this variance is the initial sample dilution. Incorporation of an internal standard in hydrolysis will improve the precision of the determination. Norvaline is recommended as an internal standard for the method.

The Waters UPLC Amino Acid Analysis Application provides a complete turnkey analytical method for analyzing protein hydrolysate samples. The ACQUITY UPLC System gives very high resolution for certain peak identification and ease of integration. With standard UV detection, all the derivatized amino acids have similar extinction coefficients to facilitate quantitative analysis. Sensitivity levels corresponding to nanograms of protein can be achieved routinely. The ruggedness of this turnkey system solution ensures rapid and unequivocal identification of proteins, with no interference or ambiguity. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. The pre-defined methods and reports simplify reporting of results. These analytical benefits are obtained with a short analysis time for the high throughput required for the routine determination of protein composition and concentration.

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Enhancement of the UPLC Amino Acid Analysis Solution with Flexible Detector Options

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TECHNOLOGY BENEFITS

The use of UV and fluorescence detection is demonstrated for the identification and quantitation of amino acids in pure protein and complex animal feed hydrolysate samples, using the UPLC® Amino Acid Analysis Solution.

- Three detection options now available with the UPLC Amino Acid Analysis Solution – TUV, PDA, FLR
- All detectors give the same quantitative and qualitative results
- Similar sensitivity is achieved for full amino acid profile with TUV and FLR detectors
- Detector choice is based on other application needs

WATERS SOLUTIONS

Total Solution: Accurate results with a system solution including hardware, derivatization chemistry, separation chemistry, software, and support

ACQUITY® with TUV, PDA, or FLR detectors

AccQ•Tag™ Ultra Derivatization Kit

AccQ•Tag Ultra Eluents and 1.7 µm Columns

Waters Empower® Software

KEY WORDS

INTRODUCTION

The measurement of amino acids is important in many applications. Protein structure laboratories use it to confirm the identification and modification of proteins and peptides. Also, the sum of the amounts of amino acids gives the total concentration of the samples. Biopharmaceutical manufacturing facilities can optimize drug yield through careful monitoring and adjustments to the nutrient levels in cell cultures used in its production. In the animal feed industry, amino acid levels are measured as part of determining nutritional content. In each of these applications, it is essential to be able to quickly and accurately identify and quantitate amino acid levels. Incorrect results could result in poor batch yields, delay of product to market, or loss of product.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

Waters provides a complete turnkey solution to meet the needs for each of these applications. The Waters UPLC Amino Acid Analysis Solution was initially offered in 2006 as a total system solution that was available to users with a tunable UV (TUV) detector. Through the use of the application-specific quality tested columns, eluents, and derivatization chemistry, users can count on accurate results. Inclusion of pre-defined Empower Software methods provides users with powerful data generation and handling capabilities and allows rapid analysis and reporting of sample results. Recently, photodiode array and fluorescence detection have been added as options in the defined system, providing the users with equipment flexibility to satisfy the requirements of their laboratories, while maintaining the same quality results regardless of which detection option is chosen.

In this experiment, hydrolyzed samples of pure protein and of animal feed were analyzed using the Waters UPLC Amino Acid Analysis Solution with a TUV detector, photodiode array detector (PDA), and with fluorescence detection (FLR). Absolute amounts of amino acids as well as molar ratios were compared between TUV and FLR detection options for reproducibility, consistency, and accuracy as compared to expected values.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm
Column temp.:	55 °C
Sample temp.:	20 °C
Flow rate:	700 µL/min
Mobile phase A:	1:20 Dilution of AccQ•Tag Ultra Eluent A Concentrate (prepared fresh daily)
Mobile phase B:	AccQ•Tag Ultra Eluent B
Needle washes:	Weak – 95:5 Water: Acetonitrile Strong – 5:95 Water: Acetonitrile
Gradient:	AccQ•Tag Ultra Hydrolysate Method (provided in the UPLC Amino Acid Analysis Solution)
Total run time:	9.5 min
Injection volume:	1 µL, Partial Loop with Needle Overfill (2 µL loop installed)
Detection:	UV (TUV), 260 nm UV (PDA), 260 nm, using 2D mode Fluorescence (FLR), λEx 266 nm, λEm 473 nm

Samples

Acid-hydrolyzed bovine serum albumin (BSA) and soybean meal samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in ampoules. Samples were stored at -80 °C until analysis.

Sample dilution and derivatization

The supplied samples were diluted with 0.1 M HCl prior to derivatization, as necessary, to assure accurate pipetting and complete derivatization. The samples were derivatized in batches, and were stable for up to one week at room temperature when tightly capped. Conditions, including suggested neutralization, for pre-column derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide. The following sequential modified derivatization conditions were used for these samples.

1. 60 µL AccQ•Tag Ultra Borate Buffer
2. 10 µL diluted sample
3. 10 µL 0.1 N NaOH
4. 20 µL reconstituted AccQ•Tag Ultra Reagent

Acquisition and processing methods

The Waters UPLC Amino Acid Analysis Solution is provided with a CD that contains all the Empower methods necessary for acquisition and processing of the samples, as well as reporting of results. Details of the methods can be found in the Waters UPLC Amino Acid Analysis System Guide.

RESULTS AND DISCUSSION

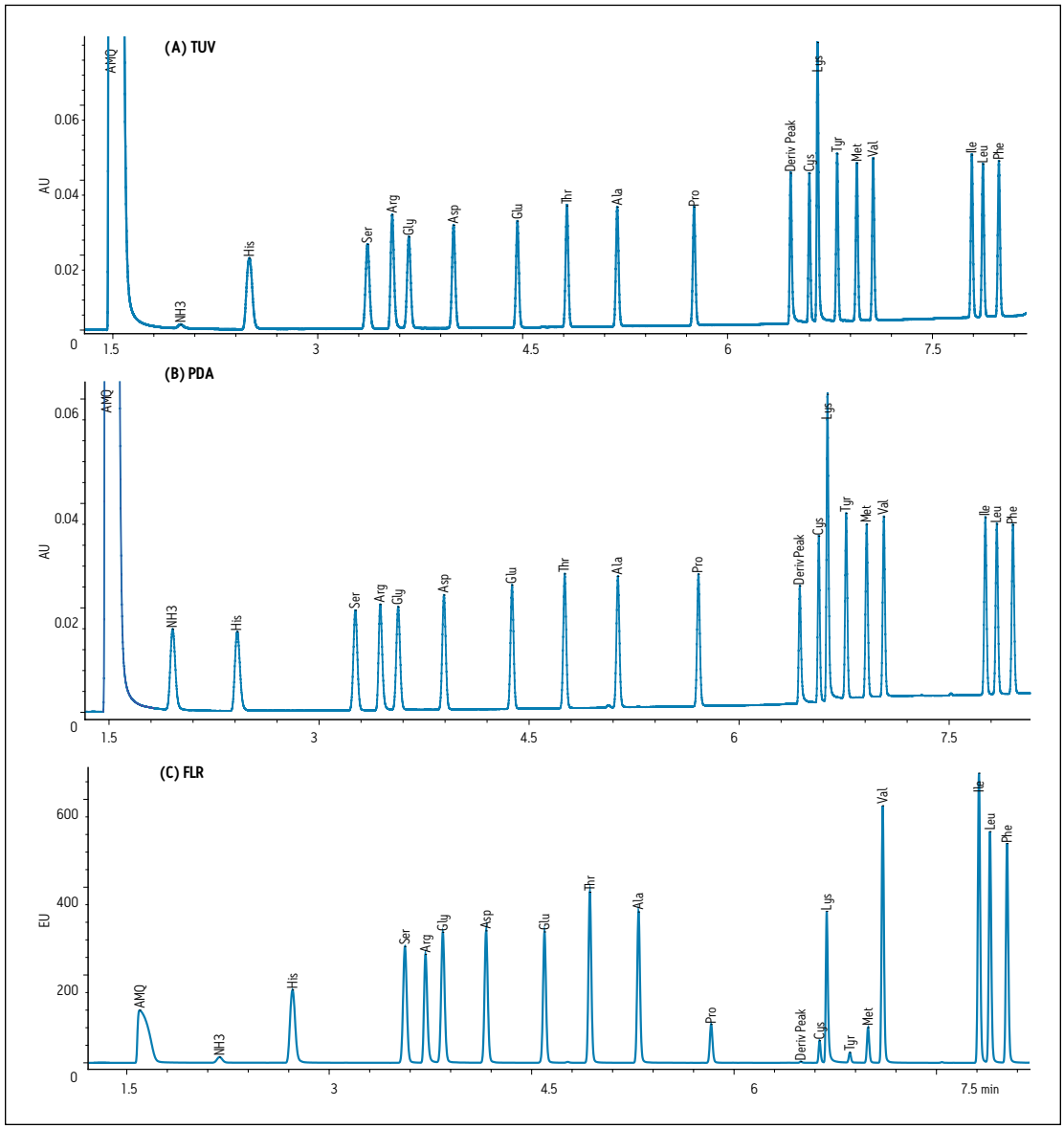

Figure 2. Analysis of Hydrolysate Standard, 10 pmoles of each amino acid on column (except Cystine at 5 pmoles), with (A) TUV detector, (B) PDA detector, and (C) FLR detector.

The operating conditions were optimized for each of the three detectors to give the highest signal-to-noise ratio. The results were compared, and a representative chromatogram of an amino acid hydrolysate standard is shown for each detector in Figure 2.

It has been widely accepted that a TUV is more sensitive than a PDA, and that a FLR is much more sensitive than UV detection. In addition, it is also believed in general that a FLR detector will give more selectivity, while a PDA can give UV spectral information to confirm peak identity and purity. The data was analyzed with these assumptions in mind, to see if they were true in this application solution.

The response for 10 pmoles on column is almost identical for the TUV and PDA detectors, while the FLR gives quite a different response. The TUV has lower noise than the PDA detector by approximately a factor of two, so the sensitivity as signal-to-noise is higher for the TUV by about the same factor.

With the FLR detector, we observe that the derivatives of the different amino acids have different fluorescence yields, and thus different sized peaks. The excitation and emission spectra are identical for all the amino acids. The differences do not seem to be related to spectral shifts. Tyrosine is the smallest peak in the fluorescence chromatogram, and, therefore, dictates the limit of quantitation. The usable range for both the TUV and FLR detectors in the application is 50 fmoles to 50 pmoles on column.

Peak identity and purity are often assessed based on spectral properties using a PDA detector. Figure 3 shows the UV spectra for five examples of AccQ•Tag derivatized amino acids, including acids, bases, neutrals, and doubly-derivatized molecules. The chemical distinctions between amino acids do not yield any useful spectral differences that could be used for peak identification. Therefore, the major value of using a PDA detector in the UPLC Amino Acid Analysis Solution is in the instrument flexibility created for other applications that require its use.

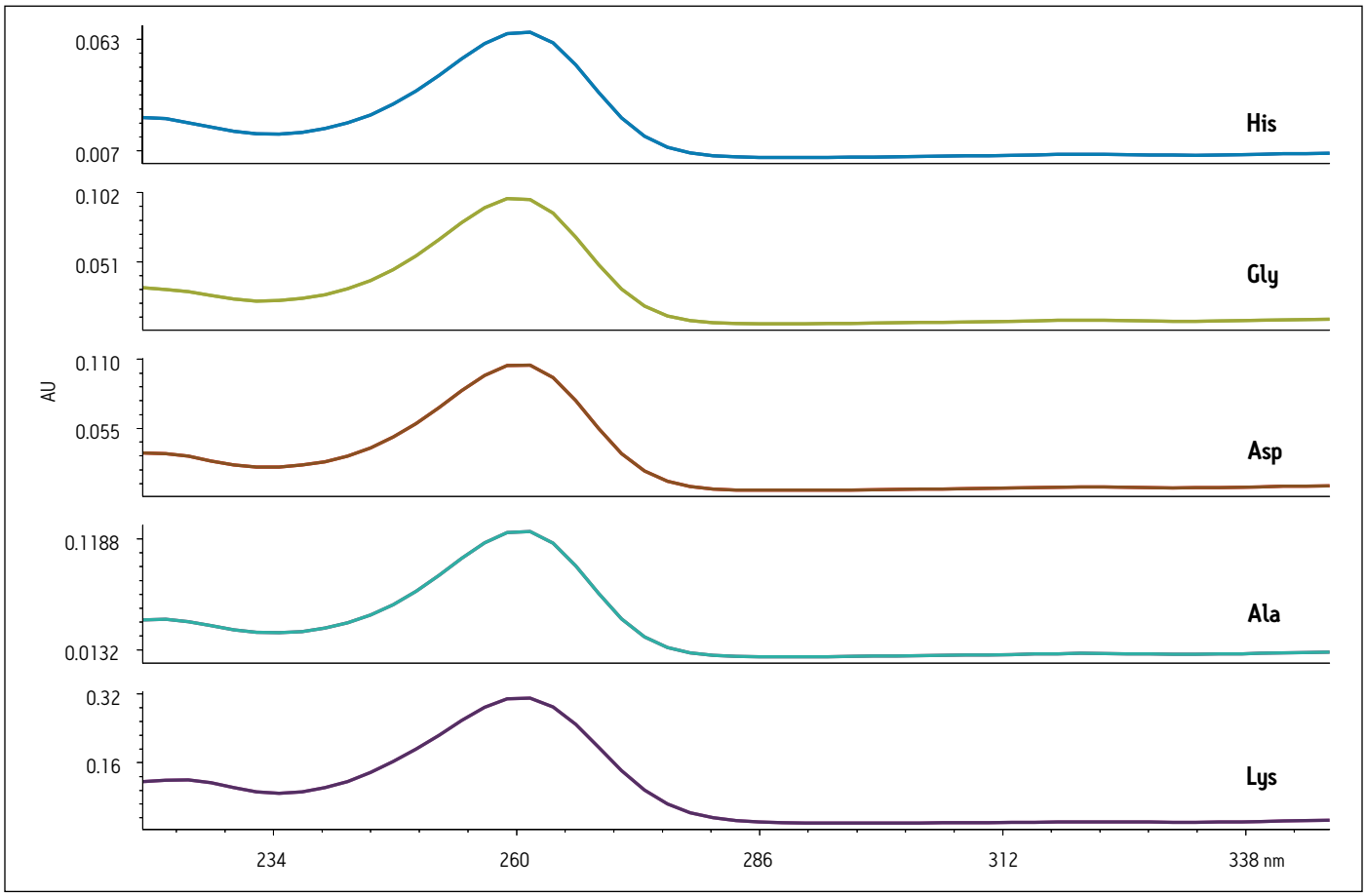

Figure 3. UV Spectra for various AccQ•Tag derivatized amino acids.

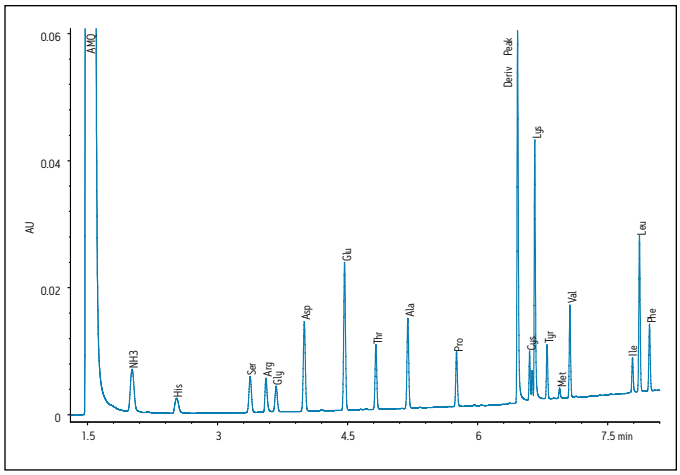

Figure 4. Analysis of BSA hydrolysate sample with UV detection, approximately 9 ng on column.

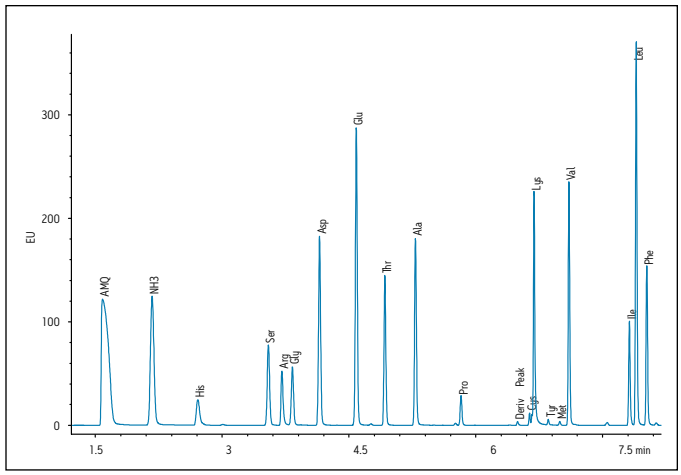

Figure 5. Analysis of BSA hydrolysate sample with fluorescence detection, approximately 9 ng on column.

Figures 4 and 5 show chromatograms with the same load of BSA hydrolysate on the column. Again, there is a difference in response for the amino acid peaks between the UV and fluorescence detectors. However, since the sample analysis is calibrated against a standard analyzed under the same conditions, no differences in the final result should be expected.

The accuracy of the results for both detectors is demonstrated by the quantitative results seen in Table 1. For all sample types, the 75 data points represent five days of analysis, each with independent sample dilutions, fresh mobile phase preparation, and each diluted sample derivatized five separate times, and injected in triplicate. The amino acid composition is expressed as residues per mole of BSA. Tryptophan and cysteine/cystine are excluded from the calculations because they are destroyed by the acid hydrolysis. The measured results for each detector match each other very well in addition to agreeing with the expected composition values.

		*Observed Residues	
Amino Acid	**Expected Residues**	**TUV**	**FLR**
His	17	15.36 ± 0.19	15.73 ± 0.16
Ser	28	26.00 ± 0.08	25.90 ± 0.41
Arg	23	22.37 ± 0.08	22.39 ± 0.20
Gly	16	22.37 ± 0.08	16.65 ± 0.42
Asp	54	55.47 ± 0.21	55.18 ± 0.32
Glu	79	80.68 ± 0.20	80.27 ± 0.44
Thr	33	31.92 ± 0.06	32.01 ± 0.07
Ala	47	47.51 ± 0.15	47.40 ± 0.16
Pro	28	28.35 ± 0.14	28.92 ± 0.13
Lys	59	57.78 ± 0.38	57.83 ± 0.99
Tyr	20	20.19 ± 0.08	20.67 ± 0.34
Met	4	4.16 ± 0.15	4.04 ± 0.05
Val	36	35.67 ± 0.13	35.38 ± 0.13
Ile	14	13.15 ± 0.16	13.44 ± 0.16
Leu	61	63.13 ± 0.28	63.18 ± 0.28
Phe	27	26.57 ± 0.13	27.00 ± 0.33

Table 1. Comparison of observed with expected composition derived from known sequence of BSA for both UV and fluorescence detection.

**Average of 75 data points (25 derivatizations, each injected in triplicate).*

The analysis of complex animal feed hydrolysate samples with both UV and fluorescence detection is shown in Figures 6 and 7. As with the analysis of the BSA hydrolysate, the difference in response for amino acids between the detectors does not mean that one detector is more suitable for quantitation than the other. This fact is further supported by the comparison of measured absolute amounts of the same samples with both detectors. Table 2 shows the mean weight % values for both TUV and FLR for the 75 data points. The ratio of amount of each amino acid to amount of feed hydrolysate was expressed using the residue molecular weights of the amino acids. Since each analysis was calibrated relative to a standard with the same detector, the quantitative results are the same.

The reliability of the method is demonstrated with the reproducibility of the results over a large number of determinations that intentionally includes the variability that would be possible in routine analysis. These variations include multiple columns, eluents, and derivatizations. The largest contribution to variability in the method is due to the pipetting steps in the sample preparation. The addition of an internal standard to the sample to be hydrolyzed will correct for pipetting variability. Norvaline is the preferred internal standard for this purpose.

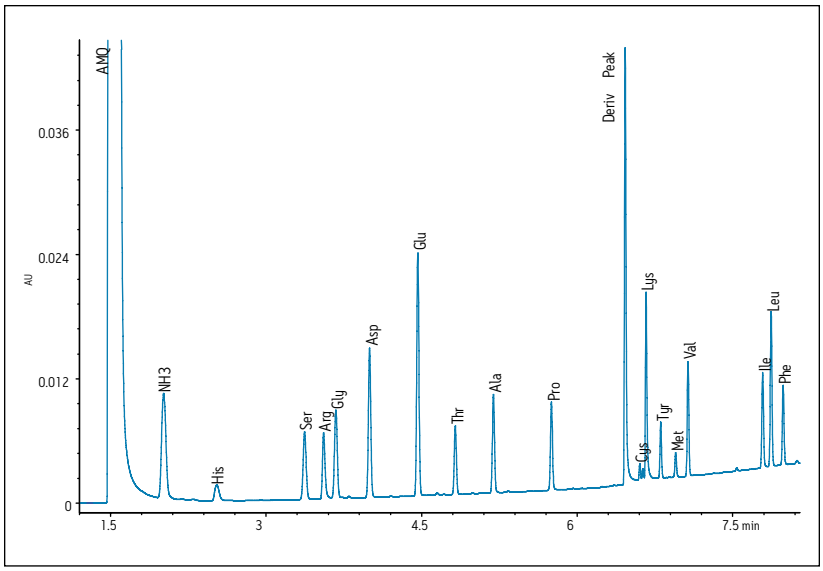

Figure 5. Analysis of BSA hydrolysate sample with fluorescence detection, approximately 9 ng on column.

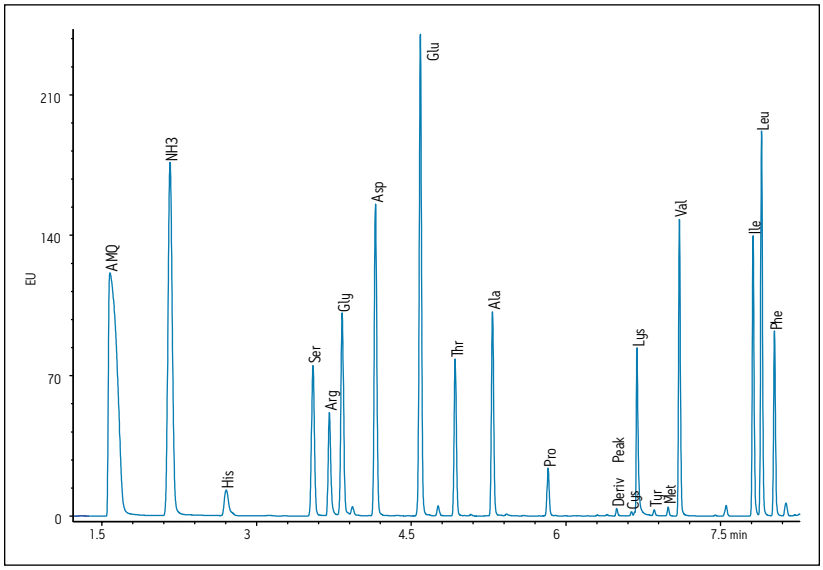

Figure 7. Analysis of soybean meal hydrolysate sample with fluorescence detection, approximately 6 ng on column.

	*Combined Mean	
Amino Acid	TUV	FLR
His	1.87 ± 0.06	1.98 ± 0.13
Ser	3.65 ± 0.07	3.58 ± 0.21
Arg	5.82 ± 0.13	5.74 ± 0.35
Gly	2.98 ± 0.07	2.87 ± 0.16
Asp	9.06 ± 0.17	8.92 ± 0.54
Glu	14.49 ± 0.28	14.36 ± 0.87
Thr	2.92 ± 0.06	2.90 ± 0.17
Ala	3.17 ± 0.08	3.15 ± 0.18
Pro	3.86 ± 0.08	3.87 ± 0.23
Lys	4.76 ± 0.11	4.80 ± 0.32
Tyr	2.90 ± 0.07	3.06 ± 0.21
Met	1.08 ± 0.03	1.08 ± 0.06
Val	3.66 ± 0.07	1.08 ± 0.06
Ile	3.45 ± 0.07	3.48 ± 0.20
Leu	6.12 ± 0.13	6.10 ± 0.35
Phe	3.92 ± 0.09	3.94 ± 0.22

Table 2. Weight/Weight % Comparison of TUV and FLR results for soybean meal hydrolysate; approximately 6 ng hydrolysate injected on column.

**Average of 75 data points (25 derivatizations, each injected in triplicate) ± Standard Deviation.*

CONCLUSION

The Waters UPLC Amino Acid Analysis is extended to three detector choices: TUV, PDA, and FLR. All three detectors give the same qualitative and quantitative result.

Historically, fluorescence detection has often been desired in amino acid analysis to provide enhanced sensitivity and to give specificity in the analysis of complex samples. The low variable fluorescence yield for the amino acids means that sensitivity is limited to the least responsive amino acid, specifically tyrosine. The analyses of pure protein and complex animal feed hydrolysates in this experiment shows that fluorescence and UV detectors both give accurate and consistent results with the Waters UPLC Amino Acid Analysis Solution.

It is generally true that cleanliness limits the usable sensitivity in any amino acid analysis method. Both the UV and fluorescence detectors give good analytical results well below the typical background limits. The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for the analysis of hydrolysate samples that allows the selection of a detector that not only meets the needs of the application, but also that of other assays in the laboratory as well. Regardless of the detector option chosen for the application, the ruggedness of the total system solution ensures highly reliable and rapid identification and quantitation of amino acids, with no interference or ambiguity.

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Monitoring Cell Culture Media with the Waters Amino Acid Analysis Solution

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INTRODUCTION

Cell culture techniques are routinely used to produce proteins intended for use as biopharmaceuticals. The culture conditions must be optimized to ensure that the protein is produced without structural modification and in the highest possible yield. These preferred conditions will often be different for each clone investigated, so a large number of optimization experiments may be required. This assessment of growth conditions must also consider the changes in the media that occur as a consequence of cell growth, that is, the consumption of nutrients and the release of waste products. The monitoring and optimization are complex because of the large number of physical and chemical parameters that have an effect. The experiments described here are focused on one particular class of components, the free amino acids.

Amino acids are important as the constituents of proteins, but they also serve as intermediates in many metabolic pathways. They are provided as individual amino acids in the growth media to satisfy both types of nutritional requirements. The concentration of amino acids in the media changes both from consumption of some amino acids and release of others by the growing cells. Monitoring these dynamic conditions is part of the optimization process, and the observed changes in concentration can be used to schedule a "feeding" of the culture or replacement of the medium. The Waters UPLC® Amino Acid Analysis Solution (Figure 1) provides a suitable way to monitor these changing nutrient levels.

The Waters UPLC Amino Acid Analysis Solution is a turnkey offering that encompasses instrumentation, derivatization chemistry, separation chemistry, software, and support. The solution includes defined conditions suitable for the assay of the amino acids commonly found in mammalian cell culture media. We show here the use of this defined method in monitoring a growing culture.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

EXPERIMENTAL

Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Solution System Guide.

Samples of serum-free cell culture medium were obtained at daily time intervals from a bioreactor that was actively producing a biopharmaceutical protein. The medium was diluted 1:4 with 0.1 M HCl. A 10 µL aliquot of the dilution, with no additional sample preparation, was derivatized using the standard AccQ•Tag™ Ultra protocol.

LC conditions

LC System:	WATERS ACQUITY UPLC® System with TUV detection at 260nm
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm PN: 186003837
Column temp:	60 °C
Flow rate:	700 µL/min
Mobile phase A:	1:10 Dilution of AccQ•Tag Ultra A concentrate
Mobile phase B:	AccQ•Tag Ultra B PN: 186003838
Gradient:	AccQ•Tag Ultra Cell Culture Method PN: 186003839
Injection volume:	1 µL

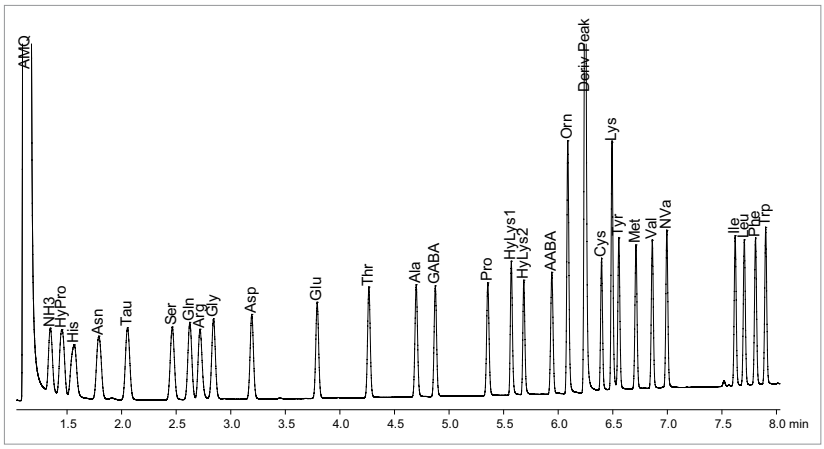

Figure 2. Analysis of standards of amino acids commonly found in cell culture media.

RESULTS

An analysis of amino acid standards representing the compounds commonly found in cell culture media is shown in Figure 2. This separation is obtained using the mobile phases and separation conditions that are part of the standard UPLC Amino Acid Analysis Solution. No adjustment of mobile phase pH or changes in composition are required. The resolution and reproducibility are sufficient for unambiguous peak identification and for reliable quantitation.

This method was applied to samples taken from an active bioreactor at daily intervals. These results are overlaid in Figure 3, and a second overlay in Figure 4 magnifies the region of the chromatogram which includes the amino acids that change most significantly during this growth experiment. The chromatographic characteristics observed with the standards are preserved with the authentic samples. The significant amino acids are readily identified and are sufficiently well-resolved for quantitation. There are a few small unidentified peaks that do not interfere with the amino acids.

The comparison of the 1, 3, and 6 day samples clearly shows the decline in concentration for some amino acids, notably glutamine, and the increase in others, such as alanine. These changes can be expressed quantitatively as plotted in Figure 5. All the amino acids can be quantitated, but only a few are shown as examples, including glutamine which increases in concentration with feeding.

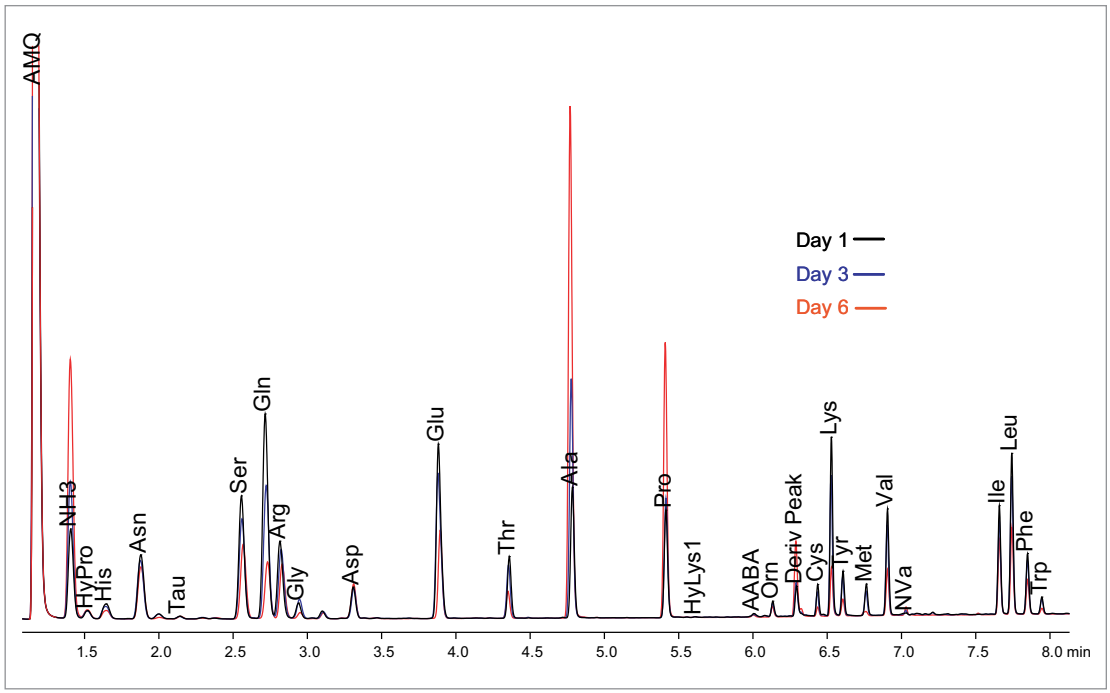

Figure 3. Analysis of amino acids in cell culture media after 1, 3, and 6 days of culture.

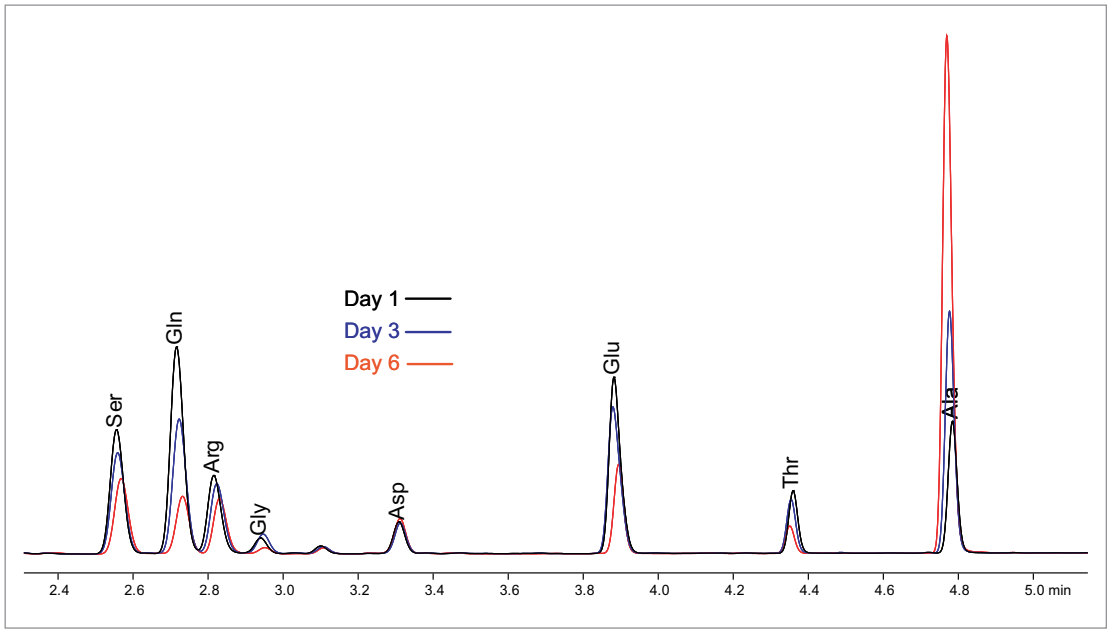

Figure 4. Analysis of critical amino acids in cell culture media after 1, 3, and 6 days of culture.

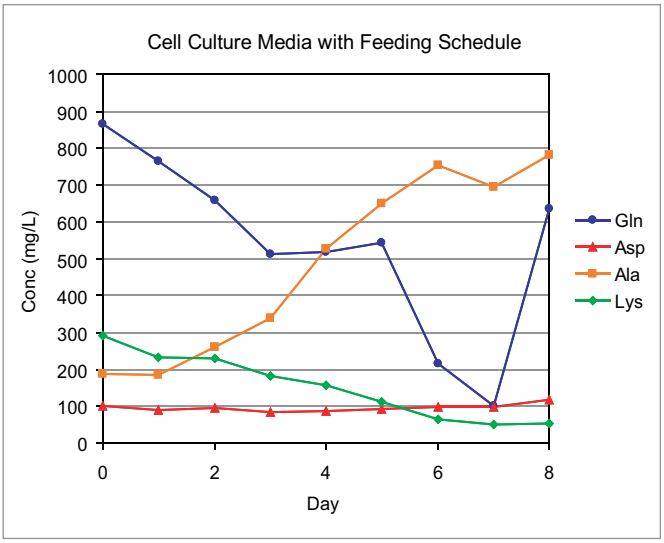

Figure 5. Quantitative trends in amino acid concentration during cell culture.

CONCLUSION

The Waters UPLC Amino Acid Analysis Solution has been used for the analysis of mammalian cell culture media. The standard method provides the chromatographic resolution required for peak identification. No sample preparation beyond simple dilution is required.

The analysis proves rugged and reproducible over a series of samples. No interferences are observed. The quantitative analysis is suitable for monitoring changes in concentration over time and for recognizing the proper time for a scheduled feeding. These analytical results are obtained with a short run time compatible with the high throughput requirements for optimizing growth conditions.

The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for monitoring amino acids in mammalian cell culture media. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. These analytical benefits are obtained with a short analysis time for the high throughput required for the optimization of cell culture conditions.

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UPLC Amino Acid Analysis Solution

Thomas E. Wheat, Eric S. Grumbach, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, USA

INTRODUCTION

Analysis of amino acids is required in several different areas of research and is also a fundamental tool in various product analysis activities. These applications impose different requirements on the analytical method because the amino acids play different roles.

Amino acids are the basic constituents of proteins. For that reason, qualitative and quantitative analysis of the amino acid composition of hydrolyzed samples of pure proteins or peptides is used to identify the material and to directly measure its concentration.

Amino acids are also intermediates in a myriad of metabolic pathways, often not directly involving proteins. The amino acids are, therefore, measured as elements of physiological and nutritional studies. This has proven particularly important in monitoring the growth of cells in cultures, as used in the production of biopharmaceuticals.

Similar considerations lead to the analysis of foods and feeds to ensure that nutritional requirements are met. These diverse sample applications will all benefit from improved amino acid methods.

A comprehensive system-based solution for the analysis of amino acids has been recently developed. This solution provides better resolution and sensitivity, all achieved in a shorter analysis time than with previous methodologies. Its enhanced separation ensures that the analysis yields accurate and precise qualitative and quantitative results and that the method is exceptionally rugged.

This application solution is based on the well-understood and widely-used Waters® AccQ•Tag™ pre-column derivatization chemistry The derivatives are separated using the Waters ACQUITY UltraPerformance LC® (UPLC®) System for optimum resolution and sensitivity. System control, data acquisition, processing, and flexible reporting are provided within Empower® Software. The integrated total application solution ensures successful analyses.

UPLC Amino Acid Analysis Solution.

METHODS AND DISCUSSION

Ultimately, a new amino acid method must provide the right answer. Increased ruggedness, preferably with reduced labor and run times, are also desired characteristics of a successful laboratory system. These needs are met by combining AccQ•Tag Ultra amino acid analysis chemistries with the proven separation technology of the ACQUITY UPLC System; together they comprise the turnkey application solution called the Waters UPLC Amino Acid Analysis Solution.

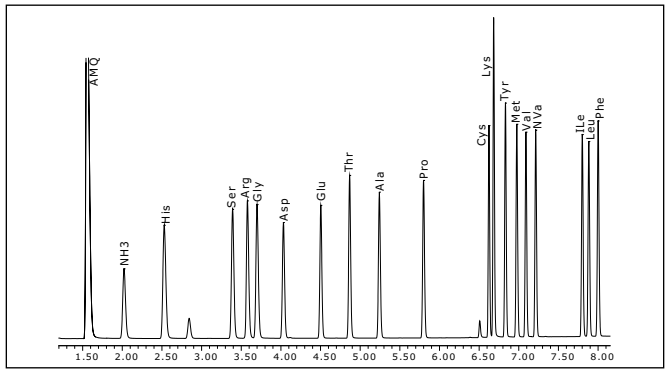

Figure 1. Separation of 50 pmoles of the amino acid hydrolysate standard with the UPLC Amino Acid Analysis Solution.

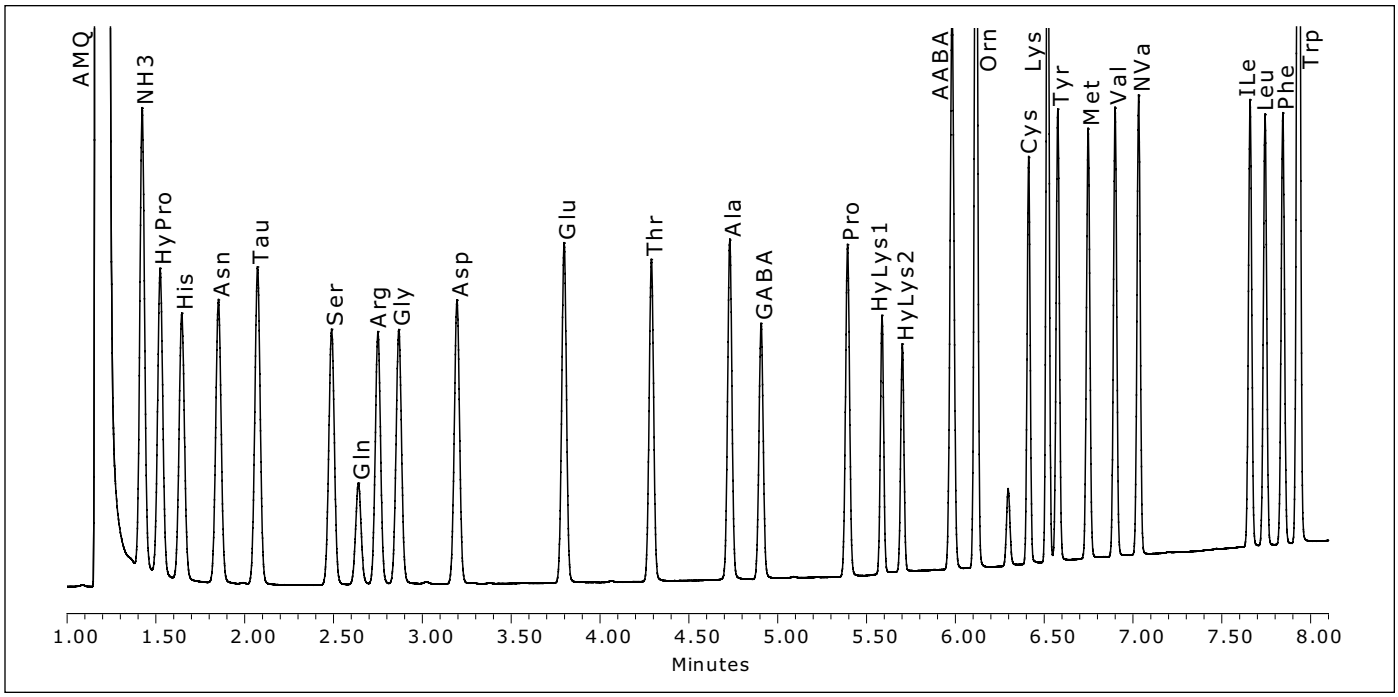

Figure 2. Separation of 10 pmoles of the amino acids commonly found in cell culture media. The UPLC Amino Acid Analysis Solution includes this modified separation method.

Analysis of a hydrolysate standard is shown in Figure 1. The amino acids are derivatized using AccQ•Fluor™ Ultra Reagent (Part Number: 186003836) (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Both primary and secondary amino acids react in a simple batch-wise derivatization, and samples are stable for several days. No special sample preparation is required, and the reaction occurs in a largely aqueous solution so it is very tolerant of buffer salts and other sample components. The excess reagent naturally hydrolyzes, and the by-product is chromatographically resolved from the derivatives. No special handling or extraction is required.

The derivatives are separated on an AccQ•Tag Ultra Column, 2.1 x 100 mm (Part Number: 186003837), a bridged ethyl hybrid (BEH) C18 1.7 µm particle specifically tested for separation of the amino acids. Packaged eluents are quality control (QC) tested with amino acid separations. They are provided as concentrates requiring only dilution with water before use. The instrument is the ACQUITY UPLC System with UV detection at 260 nm.

The resolution of the amino acids is 1.6 or greater to ensure accurate quantitation. Retention time reproducibility is on the order of hundredths of minutes, much less than a peak width, to ensure unambiguous identification of the amino acids. The detection is linear, over more than three orders of magnitude, to permit quantitative analysis of samples with disparate ratios of amino acids with an ample margin for samples of different concentration. The sensitivity of the method gives adequate signal-to-noise to quantitate at the level of 50 femtomoles on-column.

The method can be successfully used for a range of applications. The standard method shown in Figure 1 can also resolve the derivatives of cysteine commonly used in protein structure analysis. The products of performic acid oxidation that are part of assessing the nutritional quality of foods and feeds are also well-separated.

For monitoring the composition of media during the growth of cells in culture, additional amino acids must be resolved. This requires a different dilution of the AccQ•Tag Ultra Eluent A Concentrate and a higher separation temperature. The chromatogram used for monitoring cell culture media is shown in Figure 2.

CONCLUSION

These results describe the new Waters UPLC Amino Acid Analysis Solution, an Assured Performance Solution (APS), that come complete with application-focused chemistries, innovative UltraPerformance LC and MS technologies, methodology, documentation, and support to deliver the answers you need about amino acids, every time. Successful results are assured through the use of pre-tested derivatization and separation chemistry and the high resolution provided with the ACQUITY UPLC System.

This integrated analytical approach will give accurate and precise qualitative and quantitative results for a wide range of applications including protein and peptide hydrolysates, monitoring cell culture media, and measuring the nutritional value of food and feeds.

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Separation of Low Levels of Isoleucine from Leucine Using the ACQUITY UPLC H-Class Amino Acid System

Richard C. Daw
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Resolution of leucine and isoleucine at levels as low as 0.05% Ile/Leu using the UPLC® Amino Acid Analysis Solution.

WATERS SOLUTIONS

UPLC Amino Acid Analysis Solution

ACQUITY UPLC® H-Class System

AccQ•Tag™ Ultra Chemistry

Empower® 3 Software

KEY WORDS

Amino acid analysis, leucine, isoleucine, European Pharmacopoeia, USP

INTRODUCTION

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid and is produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of 1.5 to levels as low as 0.05%. This application note is intended to demonstrate that the Waters ACQUITY UPLC H-Class Amino Acid System can be used to suitably resolve isoleucine from leucine at these low levels.

The Waters ACQUITY UPLC H-Class Amino Acid System combines UPLC separation technology with AccQ•Tag Ultra derivatization chemistry, providing improved resolution and sensitivity, leading to improved sample characterization, all achieved within a shorter analysis time than conventional methodologies.

The amino acids are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) under largely aqueous conditions. The derivatives are then separated utilizing the ACQUITY UPLC H-Class System, enabling analysts to achieve accurate, precise, and robust amino acid analysis utilizing a reversed-phase separation, and quantification with either UV or fluorescence detection.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV at 260 nm
Column:	AccQ•Tag Ultra $C_{18,}$ 2.1 x 100 mm, 1.7 µm
Sample temp.:	20 °C
Column temp.:	43 °C
Injection vol.:	0.8 µL
Flow rate:	0.7 mL/min
Mobile phase A:	AccQ•Tag Eluent A
Mobile phase B:	10/90 Water/AccQ•Tag Eluent B
Mobile phase C:	Water
Mobile phase D:	AccQ•Tag Eluent B

Gradient:

Time	%A	%B	%C	%D	Curve
0.00	10.0	0.0	90.0	0.0	N/A
0.29	9.9	0.0	90.1	0.0	11
5.49	9.0	80.0	11.0	0.0	7
7.10	8.0	15.6	57.9	18.5	6
7.30	8.0	15.6	57.9	18.5	6
7.69	7.8	0.0	70.9	21.3	6
7.99	4.0	0.0	36.3	59.7	6
8.59	4.0	0.0	36.3	59.7	6
8.68	10.0	0.0	90.0	0.0	6
10.20	10.0	0.0	90.0	0.0	6

Data Management

Empower 3 Software, SR2

Standards, reagents, separation column, and turnkey methodologies within Empower Software projects, are sold as a system solution.

Sample preparation

To a leucine solution, different amounts of isoleucine were spiked to prepare isoleucine/leucine mixtures at 0.0, 0.05, 0.1, and 0.2%. A calibration standard and samples were prepared by transferring 70 µL Borate buffer and 10 µL of the standard/sample to a Waters total recovery vial, vortexing to mix. The derivatization reagent was dissolved in 1 mL of acetonitrile and then 20 µL of the solution was transferred to each vial. Each vial was capped, vortexed, and then heated to 55 °C for 10 minutes prior to analysis.

RESULTS AND DISCUSSION

A calibration standard of 17 amino acids was prepared. The standard consisted of a single point calibration curve with each standard at a concentration of 50 pmoles/µL (except cystine at 25 pmoles/µL). As can be seen from the figure below, isoleucine and leucine were resolved at around 7.8 minutes.

The 0.0% Ile/Leu (unspiked) was analyzed and the leucine sample was found to be free of interferences at the retention time of isoleucine. The spiked Ile/Leu samples at 0% (Black line) 0.05% (Blue line), 0.1% (Brown line), and 0.2% (Green line) were analyzed and the peaks were found to have a USP resolution of 2.0 for the 0.05%, 0.1%, and 0.2% levels.

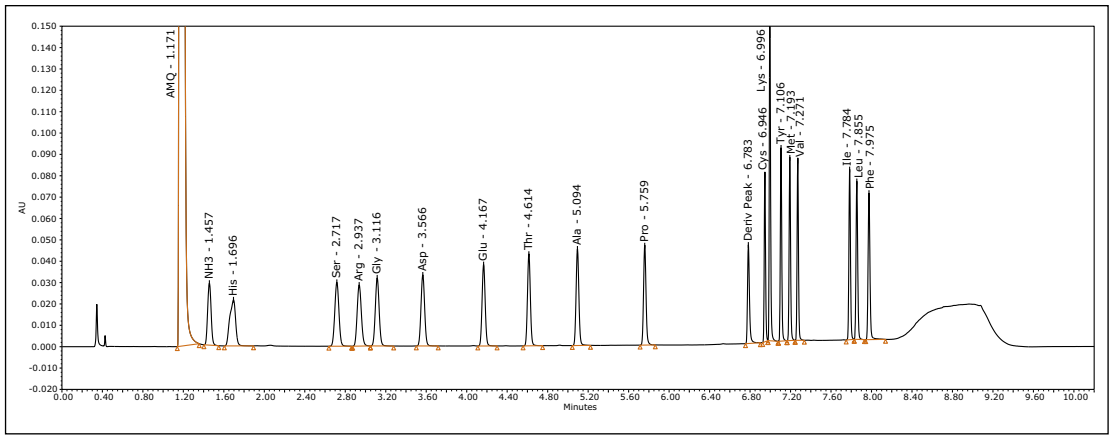

Figure 1. Standard chromatogram.

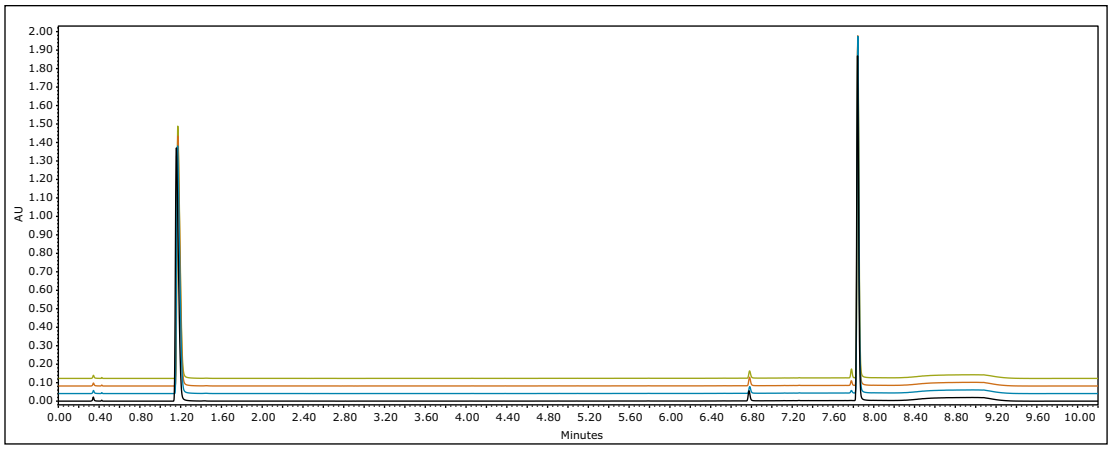

Figure 2. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay.

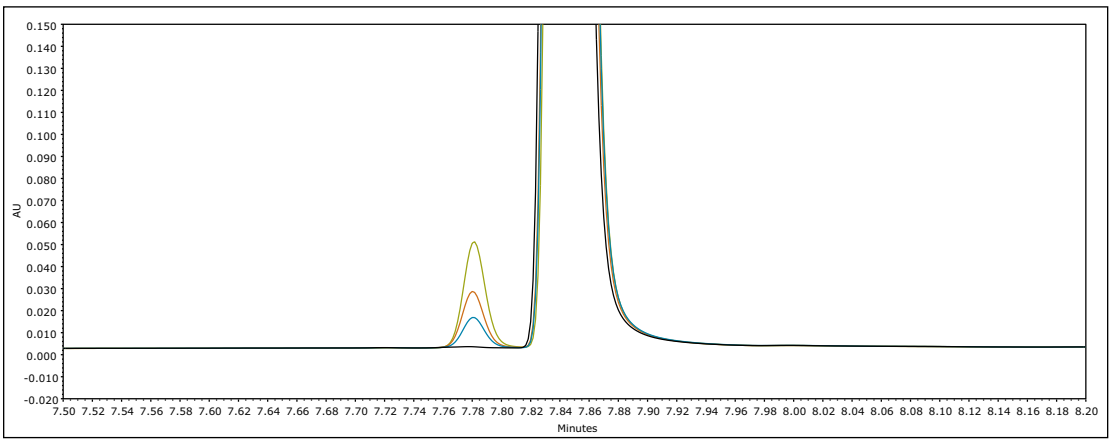

Figure 3. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay (zoomed).

The Ile area was found to be linear when compared to the %Ile/Leu, with an R^2 of 0.9999.

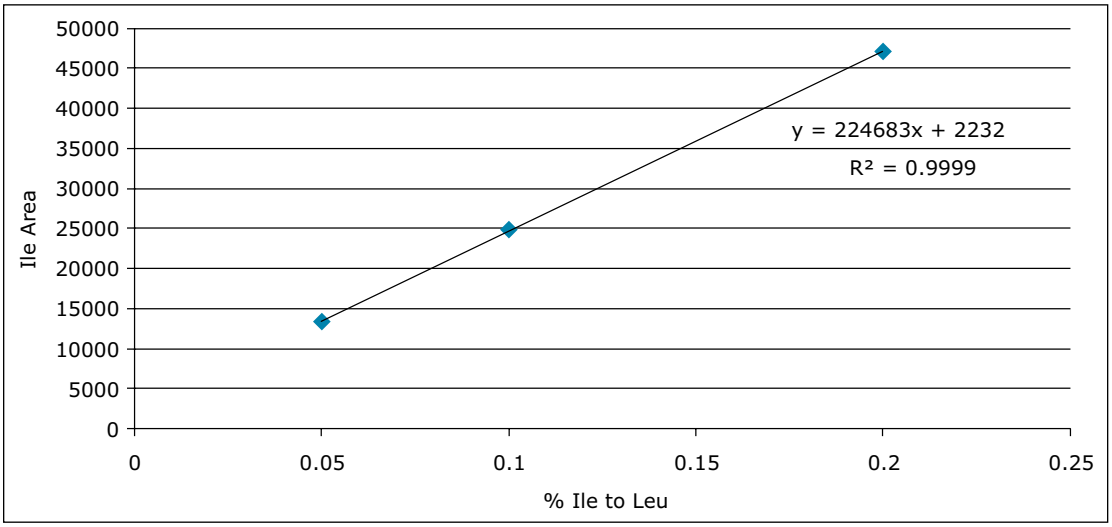

Figure 4. Linearity of Ile area vs. %Ile/Leu.

CONCLUSIONS

The Waters ACQUITY UPLC H-Class System provided chromatographic separation of all 17 amino acids in a commercially available amino acid mix within a very short run time. Baseline resolution of isoleucine and leucine was confirmed at levels as low as 0.05% Ile/Leu, meeting the regulatory requirements for these components.

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HILIC for Released Glycans and Glycoproteins

Glycosylation is a post-translational modification of proteins that occurs in all eukaryotic cells. The sugar chains on glycoproteins can mediate biological activity, play a role in receptor-mediated recognition, increase solubility, regulate half-life and exert a stabilizing influence upon confirmation. Specific glycan structures are, therefore, associated with safety and efficacy attributes of many protein drugs. Correct glycosylation is essential if a glycoprotein is to achieve and maintain its full biological activity.

As a result, the measurement of glycans is important in biopharmaceutical development projects. The relative amounts of the individual glycan structures are monitored during process development to establish the stability of the growth and purification steps of manufacturing. The same measurements are required in the development of formulations and during stability testing.

Solution highlights

There are many analytical techniques available to determine and quantify protein glycosylation. Each analytical technique measures glycosylation based on different physical and chemical characteristics of the glycoprotein. Since no single technique can routinely provide complete characterization, various techniques are used together to obtain the glycosylation information for a glycoprotein.

Waters offers complete, workflow-based approaches for analyzing glycoproteins at all structural levels by bringing together complementary techniques to streamline glycan analysis including:

- Released glycan characterization and monitoring
- Glycoprotein profiling
- Middle up/down–Subunit analysis
- Glycopeptide mapping
- Monosaccharide/Sialic acid composition

Released Glycan Characterization and Monitoring

Released N-glycan analysis is an information-rich technique, but it has involved complicated sample preparation procedures. The value of a *Rapi*Fluor-MS N-glycan sample preparation is that it can be used in place of legacy 2-AB methods without requiring significant adaptations to existing analytical techniques. With just 3 easy steps, and as little as 30 minutes, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit reduces complicated, time-consuming sample preparation. **The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit** comprises of everything you need for released glycan analysis.

You can now use a single label that provides valuable information from characterization to routine monitoring. The unique chemical attributes of *Rapi*Fluor-MS provide increased fluorescence sensitivity along with supreme mass spectral response. The increased ionization efficiency of *Rapi*Fluor-MS allows for quantitative fluorescence assays to be paired with **mass detection with the ACQUITY QDa Detector**.

Glycoprotein Profiling (N-links and O-links), middle up/down–Subunit Analysis, Glycopeptide Mapping

The ACQUITY UPLC Glycoprotein BEH Amide 300Å Column's wide pore stationary phase and optimized amide bonding, provides unprecedented, MS compatible HILIC-based separations for the analysis of intact glycoproteins, glycoprotein fragments, and glycopeptides. This new, patent pending column technology allows access to information about site-specific glycan structures and glycan occupancy.

The Glycoprotein Performance Test Standard can be used for intact and subunit workflows, while the **Intact mAb Mass Check Standard** can be applied to Glycopeptide Mapping Workflows.

Amide-Bonded BEH HILIC Columns for High Resolution, HPLC-Compatible Separations of N-Glycans

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APPLICATION BENEFITS

- Proven stationary phase for labeled glycan separations available in HPLC-compatible particle sizes (2.5 µm ***XP*** and 3.5 µm) and column diameters (2.1 and 4.6 mm I.D.)
- Noteworthy resolution with HPLC, albeit with analysis times longer than UPLC
- Consistent glycan profiles and GU values upon LC method transfer between different particle size columns
- BEH Glycan stationary phase is QC tested via separation of human-like, 2-AB labeled N-glycans to ensure consistent batch-to-batch column performance

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC BEH Glycan Column (1.7 µm)

XBridge® BEH Glycan Column (2.5 µm ***XP*** and 3.5 µm)

Alliance® HPLC System

Dextran Calibration Ladder Standard (2-AB labeled)

Glycan Performance Test Standard (2-AB labeled)

KEY WORDS

N-glycans, BEH Glycan, Glycan Separation Technology, Glycan Performance Test Standard, HILIC, Dextran Calibration Ladder Standard, HPLC, ACQUITY UPLC, Alliance

INTRODUCTION

With little dispute, it is recognized that the glycosylation of biotherapeutics must be thoroughly and routinely characterized, since changes in glycan profiles can affect efficacy and immunogenicity or be indicative of manufacturing process instability.[1] In a common characterization approach, N-linked glycans from glycoproteins are released enzymatically, labeled with a fluorescent tag, subsequently separated by hydrophilic interaction chromatography (HILIC), and detected via fluorescence (FLR).[2-9]

The ACQUITY UPLC BEH Glycan Column, packed with sub-2-µm particles, has proven to be an enabling technology for the HILIC-based separation of these labeled glycans. With an optimized amide-bonded ethylene bridged hybrid (BEH) HILIC stationary phase, this column provides faster and higher resolution separations than alternative HPLC-based columns.[10]

However, many laboratories have not yet fully transitioned to UPLC® Technology. To enable them access to this amide-bonded BEH Technology,™ HPLC-compatible particle size (2.5 µm ***XP*** and 3.5 µm) columns are now available with the proven BEH glycan stationary phase.

In the following work, we demonstrate that BEH Glycan Columns packed with 1.7, 2.5, and 3.5 µm particle sizes afford scalability between glycan separations performed under UPLC and HPLC-compatible conditions. Using standard LC method transfer principles accounting for differences in particle diameter (d_p), we additionally show that noteworthy resolution can be achieved with the larger particle size columns at HPLC-compatible pressures, albeit with an increase in analysis time. Lastly, the following work demonstrates that highly similar glycan profiles and experimental glucose unit (GU) values are obtained, no matter the particle size or LC system employed.

EXPERIMENTAL

Method conditions (unless otherwise noted)

LC conditions

LC systems:	ACQUITY UPLC H-Class Bio System/ Alliance 2695 HPLC
Detection:	ACQUITY UPLC FLR Detector/2475 FLR Detector
Excitation:	330 nm
Emission:	420 nm
Scan rate:	10 Hz
Time const.:	0.2 sec
Gain:	1.00
Column:	ACQUITY UPLC BEH Glycan, 1.7 µm, 130Å, 2.1 x 150 mm (p/n 186004742) XBridge BEH Glycan, 2.5 µm ***XP***, 130Å, 2.1 x 150 mm (p/n 186007265) XBridge BEH Glycan, 3.5 µm, 130Å, 2.1 x 150 mm (p/n 186007504) 100% Silica-based, HILIC Amide Bonded, 3 µm, 80 Å, 2.0 x 150 mm (Competitor product)
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	2.5 µL (Glycan Performance Test Standard + A3 Mixture), 2.0 µL (Glycan Performance Test Standard), 1.0 µL (Dextran Calibration Ladder Standard)
Flow rate:	0.5 mL/min (0.25 mL/min for the highly aqueous regeneration step in the gradient)
Mobile phase A:	100 mM ammonium formate, pH 4.4
Mobile phase B:	Acetonitrile (ACN)
Vials:	LC/GC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)
Gradient*:	Curves styles were linear. For a 1.7 µm, 2.1 x 150 mm column

Time (min)	%A	%B	Flow rate (mL/min)
0	22.0	78.0	0.5
38.5	44.1	55.9	0.5
39.5	80.0	20.0	0.25
44.5	80.0	20.0	0.25
46.5	22.0	78.0	0.5
50	22.0	78.0	0.5

*This gradient should not be used when aiming to assign identifications by means of the NIBRT GlycoBase.[6] For best agreement between experimental and database GU values, the method NIBRT has used for generating GU values should be employed.

For a 2.5 µm, 2.1 x 150 mm ***XP*** column

Time (min)	%A	%B	Flow rate (mL/min)
0	22.0	78.0	0.34
56.62	44.1	55.9	0.34
58.09	80.0	20.0	0.17
65.44	80.0	20.0	0.17
68.38	22.0	78.0	0.34
73.53	22.0	78.0	0.34

For a 3.5 µm, 2.1 mm x 150 mm column

Time (min)	%A	%B	Flow rate (mL/min)
0	22.0	78.0	0.24
79.26	44.1	55.9	0.24
81.32	80.0	20.0	0.12
91.62	80.0	20.0	0.12
95.74	22.0	78.0	0.24
102.94	22.0	78.0	0.24

Data Management: UNIFI® (v1.6)
Waters Empower® Pro (v2)

Sample description

Glycan Performance Test Standard (p/n 186006349) was mixed with 2-AB (2-aminobenzamide) labeled trisialylated A3 glycans (ProZyme®) in water to make a solution of 3 pmol/µL. Aliquots (10 µL) of this mixture were diluted with 15 µL of ACN prior to injection.

For the evaluation of method transfer from a UPLC to an HPLC, Glycan Performance Test Standard (p/n 186006349) was reconstituted in 200 µL of 50:50 mobile phase A/mobile phase B to make a solution of 1.14 pmol/µL.

Dextran Calibration Ladder Standard (p/n 186006841) was reconstituted in 100 µL of Milli-Q® water. This aliquot was then mixed with an equal volume of ACN prior to injection.

CALCULATIONS

Peak capacities for each separation were calculated by measuring peak widths for species 1 through 16. Species 8 and 9 co-eluted in some separations and were therefore not included in the calculations. Peak widths were measured from the FLR chromatograms at peak half-height ($w_{half\text{-}height}$), averaged, and converted to peak capacities according to the following equation. To be rigorous among the comparisons, the gradient time in these calculations was defined as the difference in retention time of the first and last eluting component of the studied glycan mixture. Peak capacities were also calculated, in a similar manner, based on species 1 through 14 for the evaluation of method transfer from UPLC to HPLC instrumentation.

$$P^{*}_{c,half\text{-}height} = 1 + \left[\left(\frac{\Delta RT_{1,16}}{W_{half\text{-}height,avg}}\right)\right]$$

RESULTS AND DISCUSSION

Performance of HILIC glycan columns varying in particle size at the same linear velocity

The performance of columns packed with BEH-based, amide-bonded HILIC stationary phase of varying particle sizes was evaluated for separations of released and fluorescently labeled N-glycans. A test mixture for this purpose was prepared by combining the 2-AB (aminobenzamide) labeled N-glycans of the Glycan Performance Test Standard, which contains 2-AB labeled N-glycans derived from pooled human serum IgG spiked with high mannose glycans (Man5 and Man6), with 2-AB labeled trisialylated A3 glycans. This is a mixture with complexity extending beyond that commonly found on human or human-like IgGs, making it challenging to fully resolve and thus an excellent probe to measure column performance.

In order to evaluate the separation capabilities of the noted columns in an unbiased manner, we first evaluated performance on a low dispersion, Waters H-Class Bio UPLC System capable of operating under both UPLC and HPLC pressures. A typical chromatogram obtained for this sample with an ACQUITY UPLC BEH Glycan 1.7 µm Column and a method optimized for high resolving power is shown at the top of Figure 1. In this example UPLC separation, column pressures of up to 8700 psi were observed during the column wash step. This method could, as a result, not be implemented on an HPLC or most UHPLC instruments, given that such chromatographs are often limited to operating pressures of approximately 5,000–6,000 and 8,000–9,000 psi, respectively.

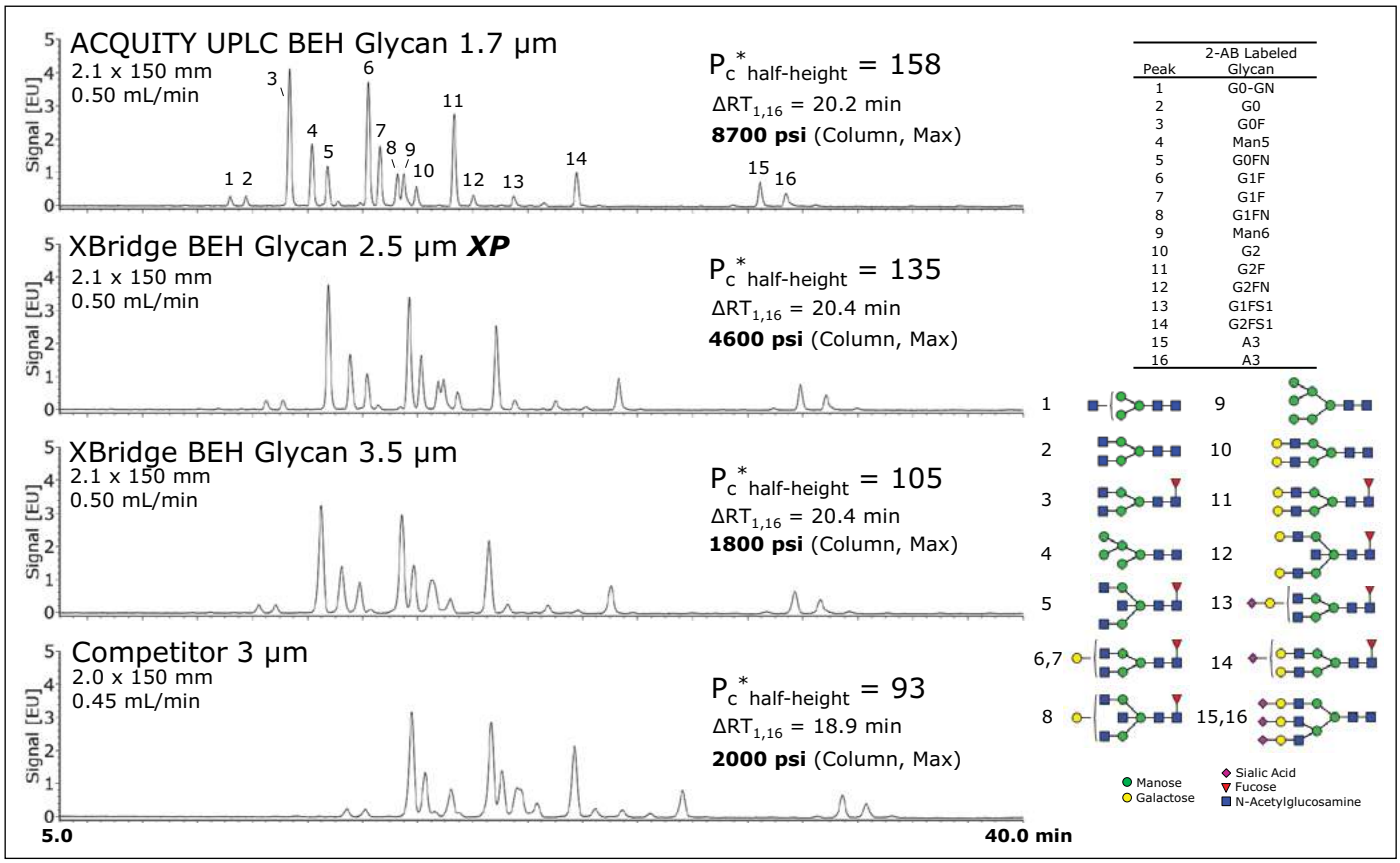

Figure 1. HILIC-FLR analysis of 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans with columns packed with BEH-based, amide-bonded 1.7, 2.5, and 3.5 µm versus competitor's 100% silica, amide-bonded 3 µm particles. Separations were performed with an ACQUITY UPLC H-Class Bio using the same linear velocity with 3 pmol of sample injected in a volume of 2.5 µL. Peak capacities measured for each separation are shown as well as the maximum column pressures observed during column wash step.

Column pressure is inversely proportional to particle diameter.[11] HPLC-compatible methods can thereby be obtained through the use of larger particle columns, for instance those packed with 2.5 and 3.5 µm particles. Separations achieved with such columns are also shown in Figure 1. Both columns exhibited significantly lower pressures, namely 4600 and 1800 psi, making them compatible with modern-HPLC systems. At the same linear velocity, the 1.7 µm particle column yielded superior peak capacity, as a result of the enhanced kinetic efficiencies that coincide with sub-2-µm particle diameters. Peak capacities for the different BEH Glycan Columns ranged from 158 to 105, decreasing with increasing particle diameter, as would be expected. It is, however, noteworthy that all three of these BEH Glycan Columns afforded peak capacities superior to those obtained with a commonly-used, 100% silica-based, HPLC amide 3 µm particle column (competitor). Further, the 3.5 µm BEH Glycan column provides comparable, if not better, performance at a lower pressure compared to the competitor column.

In all, the peak capacities and separations demonstrated in Figure 1 underscore the advantage of being able to use and fully exploit the performance of columns packed with sub-2-µm particles. Higher peak capacities can be achieved with faster analysis times. Kinetic efficiency and amenability to faster linear velocities accompany decreases in particle diameter. Because of this, it is widely understood that in order to achieve similar peak capacities with columns packed with larger particle size diameters, it is necessary to decrease the linear velocity of the mobile phase, thus increasing the analysis time.

Scalability and HPLC-compatible separations

The highly resolving method developed for the UPLC column (1.7 µm) was scaled to methods suitable for the HPLC columns (2.5 µm ***XP*** and 3.5 µm) using the ACQUITY UPLC Column Calculator.[12] Linear velocities were decreased and gradient times increased by the calculated percentage difference between the differing particle size diameters (column dimensions were identical). For example, in going from the 1.7 µm to the 2.5 µm ***XP*** Column, flow rate was decreased 1.5 fold, while the gradient was increased by the same factor, such that the change in acetonitrile per column volume remained constant. Figure 2 presents chromatograms obtained with these transferred methods. For the HPLC particle size separations, there were noticeable, up to 20%, increases in calculated peak capacities with the lower, properly scaled flow rates compared to data reported in Figure 1. Moreover, there were significant decreases in column pressure, further confirming the compatibility of the 2.5 µm ***XP*** and 3.5 µm Columns with HPLC instrumentation.

It is worth noting that the 2.5 µm ***XP*** Column, upon method transfer, was able to approach the peak capacity of the UPLC method (Pc 155 vs 158). As with the UPLC method, the HPLC-compatible method with the 2.5 µm ***XP*** Column yielded significant resolution (Rs>1) between species 8 (G1FN) and 9 (Man6), which can often pose a challenge for most HILIC separations given the highly similar hydrophilicities of the two glycans. So, the 2.5 µm ***XP*** Column, upon appropriate method scaling, is capable of noteworthy resolving power, albeit at the cost of increased analysis time when compared to true UPLC with a sub-2-µm column. As can be seen in comparing Figures 1 and 2, the 3.5 µm column also exhibited increases in peak capacity upon method transfer. The peak capacity of this column was lower than that of the 1.7 and 2.5 µm ***XP*** Columns (as expected), but was significantly higher than the peak capacity observed for the competitor's, silica-based, amide column containing slightly smaller, 3 µm particles (Figure 2).

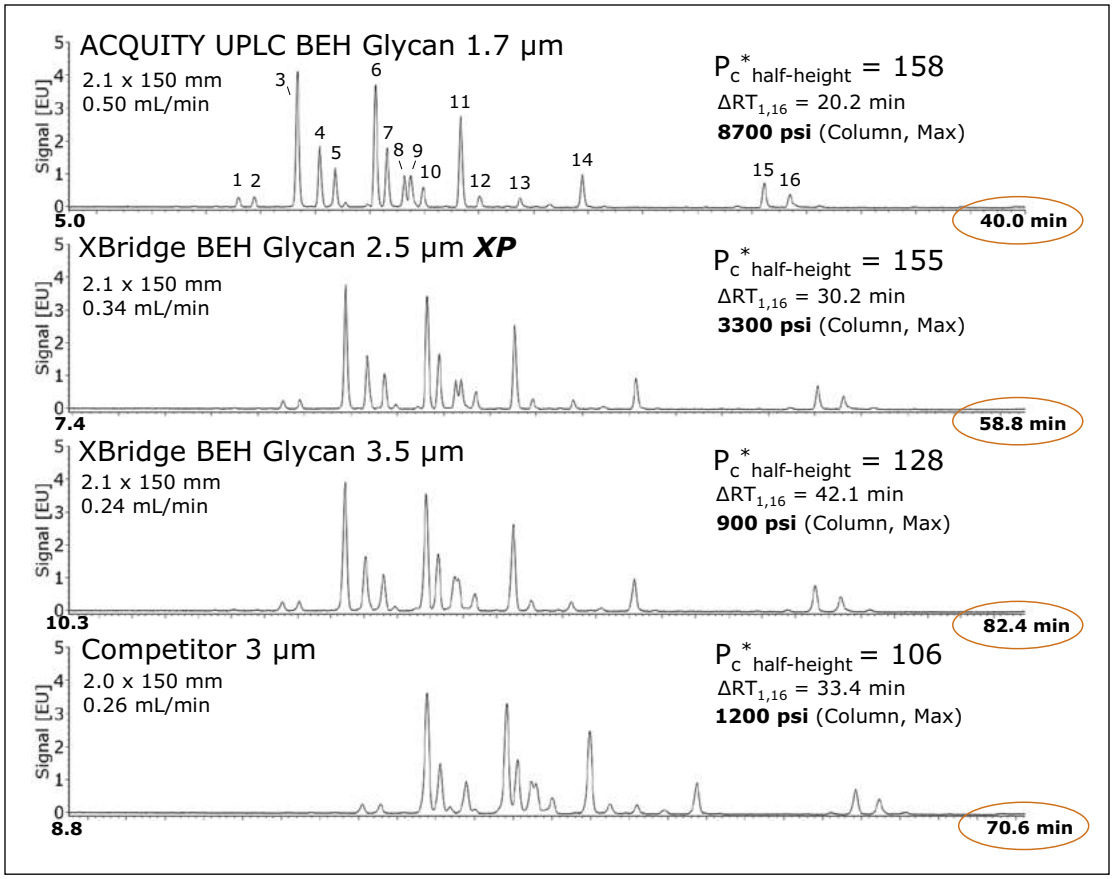

Figure 2. HILIC-FLR analysis of 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans using an ACQUITY UPLC H-Class Bio System and Columns packed with 1.7, 2.5, 3, and 3.5 µm particles. Linear velocities and gradient times were scaled according to the change in d_p according to the ACQUITY UPLC Column Calculator.

Successful method transfer among different particle sizes relies not only on obtaining similar resolving power but also maintaining comparable chromatographic selectivity. This is of utmost significance, as altered elution orders and changing chromatographic profiles would make it infeasible to develop any correspondence between data. For this reason, the separations with the different BEH Glycan Columns, shown in Figure 2, were additionally assessed in terms of comparability of chromatographic selectivity. Selectivity factors for three different sets of peaks spread across the profile of the glycan test mixture were determined and found to be comparable for the various methods and particle sizes (Table 1).

	1.7 µm, 0.50 mL/min	2.5 µm *XP* 0.34 mL/min	3.5 µm 0.24 mL/min
α3,6	1.22	1.20	1.20
α6,11	1.20	1.18	1.18
α11,15	1.59	1.54	1.53

Table 1. Selectivity factors for the separations shown in Figure 2 that were obtained using appropriately scaled flow rates and gradient durations. Note: Peak IDs are shown in Figure 1.

Glycan profiles and GU values

Consistency in the data derived from separations with BEH Glycan Columns packed with different particle sizes is of importance in biopharmaceutical laboratories. Most critically, these columns and their associated methods should deliver consistent determinations of a glycan profile. Figure 3 presents relative abundances determined from the separations shown in Figure 2. Very similar abundances for the separated glycan species were determined, regardless of which column had been used. The largest discrepancy in the glycan abundance values corresponded to labeled glycan species 8 and 9, where there were approximately 20% deviations in calculated results generated with columns containing the 3.5 µm versus 1.7 and 2.5 µm particles. In absolute terms, these were differences of 4.8% versus ~4.1% (peak 8) and 3.6% versus ~4.5% (peak 9). Reviewing the separations in Figure 2 gives a clear explanation for this discrepancy; resolution of species 8 and 9 was significantly poorer on the 3.5 µm column, which in turn impaired the accuracy of determining the abundances of the two species. In contrast, all other relative abundance determinations were in agreement to within 10%, indicating that this newly expanded suite of BEH Glycan Columns is indeed capable of yielding highly consistent analyses of a 2-AB labeled glycans.

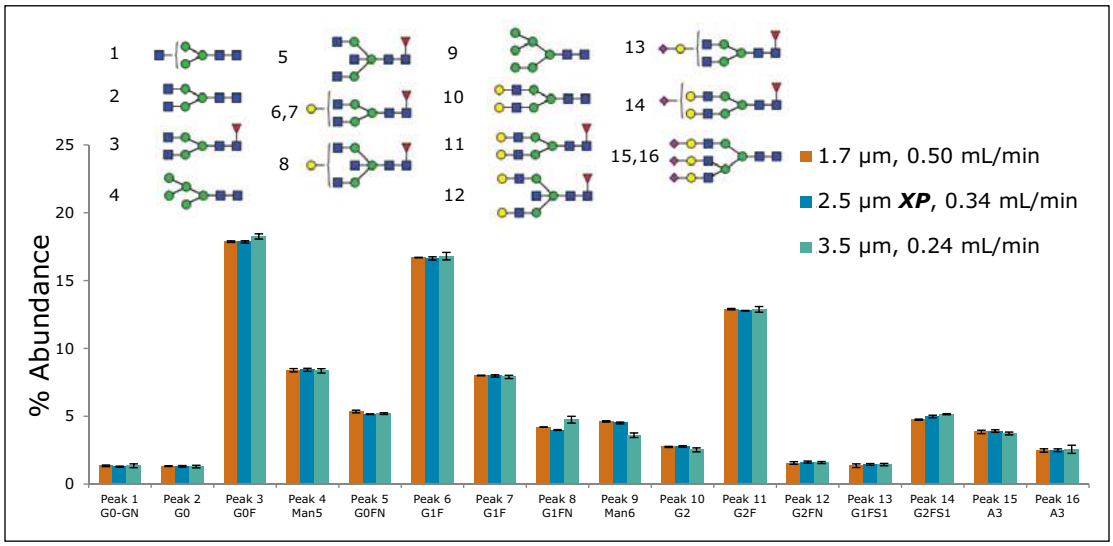

*Figure 3. Relative abundances of 2-AB labeled glycans in the test mixture as determined using the 1.7 µm column and the 2.5 **XP** and 3.5 µm Columns with methods scaled for d_p. Values shown were determined based on the three replicate analyses. Note that resolution of peaks 8 and 9 was poor with the 3.5 µm column, leading to a slight discrepancy in their abundances.*

Separations with these BEH Glycan Columns may also be used in conjunction with glucose unit (GU) values. The concept of GU values was developed as a means to calibrate HILIC-based glycan separations. In essence, its use minimizes subtle retention time variations of separated glycans between runs by expressing the results in terms of standardized GU values.[13] To assign GU values, a dextran ladder (comprised of glucose multimers of increasing length) is used as an external calibrant. The retention times of the separated glycans are then converted to GU values via use of a software-calculated, GU Ladder calibration table. This process, represented in Figure 4, helps to address variability in retention times due to a method being run on different instruments and in different laboratories. It also has value for use with BEH Glycan separations transferred between different particle size columns. The GU values derived from the various columns and methods were compared (Figure 5) and found to be highly similar. Nevertheless, an average increase in GU values of 0.03 and 0.05 was observed in transition from the 1.7 µm column to the 2.5 µm ***XP*** and 3.5 µm Columns, respectively. Yet, the magnitude of these increases is no greater than the experimental error in database GU values.[13]

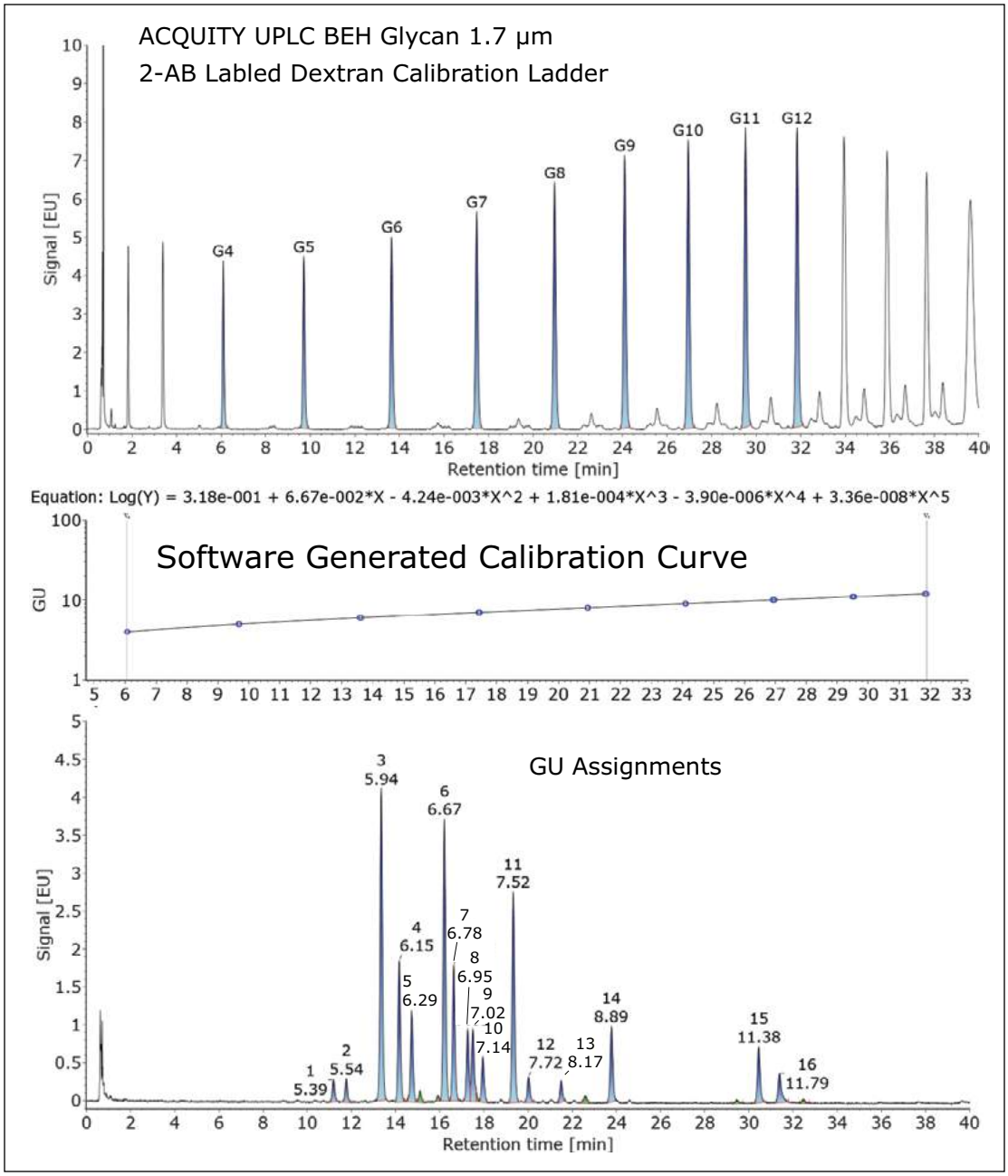

Figure 4. Workflow for the application of the 2-AB Labeled Dextran Calibration Ladder Standard and the assignment of GU values from Figure 2.

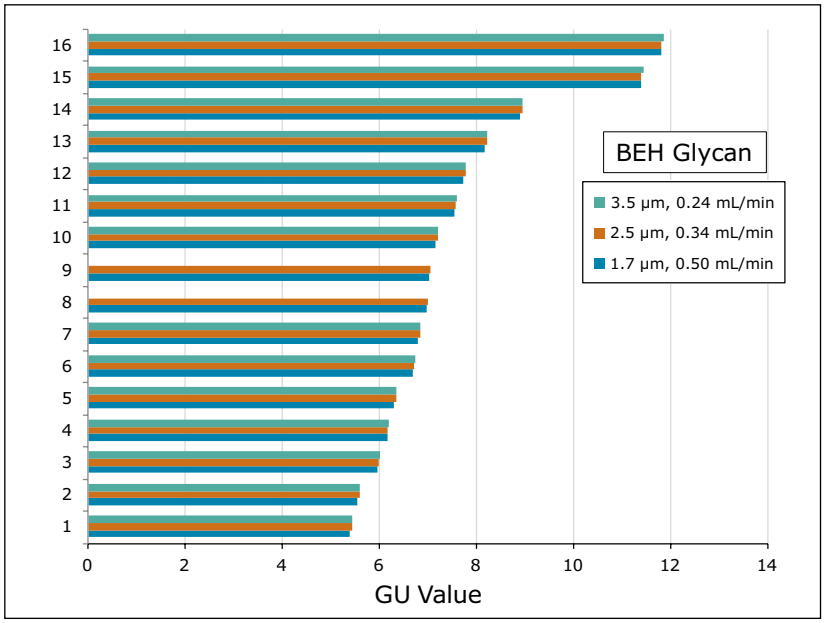

*Figure 5. GU values assigned to components of the 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans when using the UPLC BEH Glycan 1.7 µm Column and the two, HPLC BEH Glycan (2.5 µm **XP** and 3.5 µm) Columns with the scaled methods (all separations performed with an ACQUITY UPLC H-Class Bio). Values were derived from three replicate runs and a separation calibration based on two bracketing analyses of the Dextran Calibration Ladder Standard. Note: to generate GU values that can be searched against the NIBRT GlycoBase, different method conditions should be used (see experimental).*

HPLC Instrumentation

As shown with the preceding results, the BEH Glycan Columns afford excellent scalability of a glycan separation across different UPLC and HPLC-based particle sizes. A more challenging proposition, however, is having the desired scalability and transferability across both different particle size columns and UPLC/HPLC instrumentation. In pursuit of this, we evaluated the transferability of a glycan separation from a UPLC and 1.7 µm column to an HPLC and a 2.5 µm ***XP*** Column. Chromatograms obtained under these conditions for the 2-AB labeled Glycan Performance Test Standard (without sialylated A3 glycans) are displayed in Figure 6. Peak capacities observed across the 14 identified peaks were measured and found to be 110 with the UPLC separation and 78 with the HPLC separation. This corresponds to a 29% decrease in performance for the HPLC-based analysis. This metric of relative performance is shown for this and other separations in Table 2.
What becomes clear from these data is that although the 2.5 µm ***XP*** Column is capable of producing near UPLC resolving power with a properly scaled method and a UPLC (Figure 2), such performance may not be easily attained with HPLC instrumentation due to additional system dispersion (A band spread of 30 µL was measured for the Alliance HPLC, while 6 µL was measured for the ACQUITY® UPLC H-Class Bio). Nonetheless, it is worth noting that the resolving power achieved with the 2.5 µm ***XP*** Column and an Alliance HPLC is greater than that accessible with a competitor's 3 µm amide HILIC column, even when used in combination with a low dispersion UPLC.

$P^*_{c\ half\text{-}height}$ / $\Delta RT_{1,14}$	BEH Glycan			
	1.7 µm	2.5 µm ***XP***	3.5 µm	Amide-bonded Silica 3 µm
ACQUITY UPLCH-Class Bio Generated	110 (0%)	100 (-9%)	82 (-25%)	70 (-36%)
Alliance HPLC Generated	not possible	78 (-29%)	55 (-50%)	

Table 2. Peak capacities (half-height, ΔRT1,14) and percent decreases in peak capacity relative to a UPLC separation with an ACQUITY UPLC BEH Glycan 1.7 µm Column. Larger particle amide columns were used in combination with scaled methods and either UPLC or HPLC instrumentation. Note: see Figure 1 for Peak IDs.

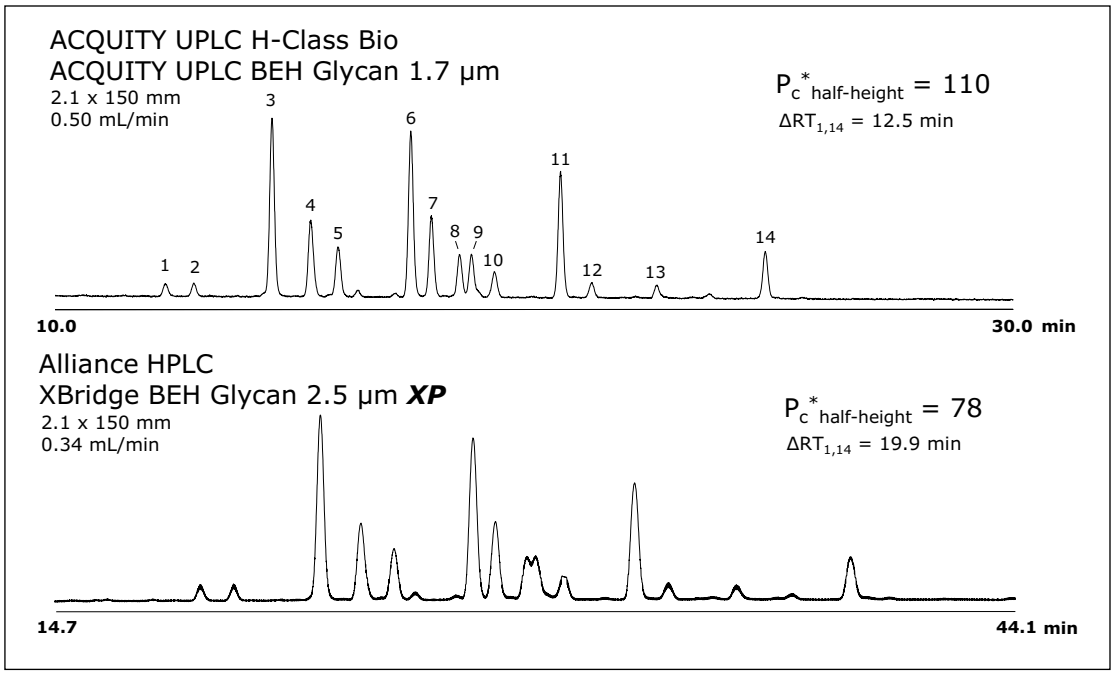

*Figure 6. Comparison of HILIC-FLR chromatograms for 2-AB labeled Glycan Performance Test Standard as obtained with an ACQUITY UPLC H-Class Bio and an ACQUITY UPLC BEH Glycan 1.7 µm Column versus an Alliance HPLC and an XBridge BEH Glycan 2.5 µm **XP** Column. Separations were performed with ca. 2.3 pmol of sample injected in a volume of 2 µL.*

The transfer of the BEH Glycan separations from UPLC to HPLC was also evaluated in terms of GU value assignment. Figure 7 displays GU values assigned using the UPLC method versus the HPLC method. It can be seen that GU values were highly similar no matter the employed method. The largest difference in a GU value assignment was in fact only 0.02, which again, is no greater than the typical experimental error in database GU values.

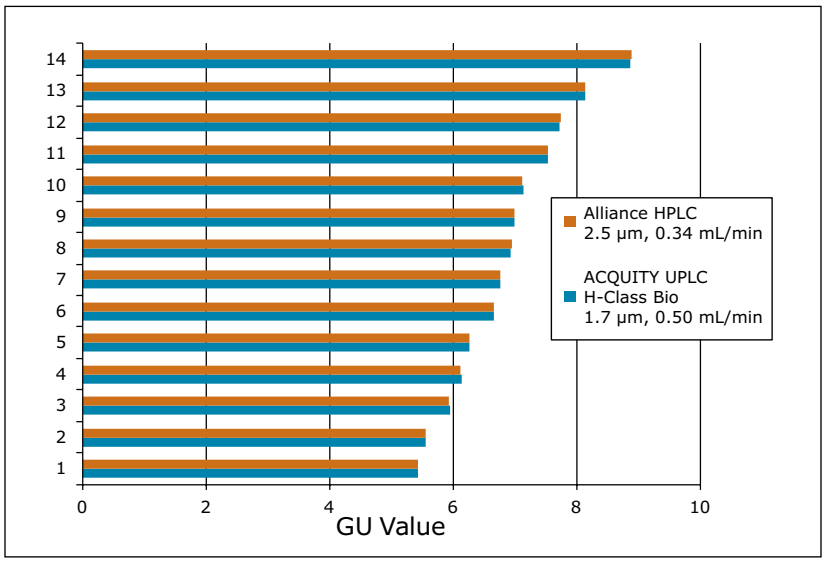

*Figure 7. GU values assigned to components of the 2-AB labeled Glycan Performance Test Standard when using an ACQUITY UPLC H-Class Bio with an ACQUITY UPLC BEH Glycan 1.7 µm Column versus an Alliance HPLC with an XBridge BEH Glycan 2.5 µm **XP** Column and scaled methods.*

CONCLUSIONS

In this application note, we have demonstrated that BEH Glycan Columns packed with 1.7, 2.5, and 3.5 µm particles indeed afford scalability between 2-AB labeled glycan separations performed under UPLC and HPLC-compatible conditions. With method transfer accounting for differences in particle diameter (d_p), we additionally show that noteworthy resolution can be achieved with columns packed with larger particles at HPLC-compatible pressures, albeit with an increase in analysis time compared to UPLC-based separations using BEH Glycan 1.7 µm columns. Lastly, this study demonstrates that highly similar glycan profiles and experimental glucose unit (GU) values are obtained, independent of the particle size or LC system (ACQUITY UPLC or Alliance HPLC) employed for a separation. The availability of these various BEH Glycan Columns should be of interest to laboratories looking for flexibility during method development or a facile means of transferring glycan separation methods between UPLC and HPLC instrumentation.

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Development of a Glycan Database for Waters ACQUITY UPLC Systems

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APPLICATION BENEFITS

ACQUITY UPLC/FLR analysis under the control of an Empower workstation represents a powerful and fully integrated analytical platform for analyzing the oligosaccharides attached to glycoproteins.

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC FLR Detector

Empower® Software

GlycoBase 3.0 Database

KEY WORDS

HILIC-UPLC with florescence detection, 2AB-labeled glycans, glycan database

INTRODUCTION

Glycosylation is the most complex post-translational protein modification. More than half of all proteins are glycosylated, and, to elucidate their function, it is important to define the structures of the glycans that are covalently attached to their surfaces. The manufacturing of glycoprotein life sciences products can be challenging and a number of factors can have a major impact upon their glycosylation, including the cell type in which the protein is expressed, media composition, and processing parameters such as dissolved oxygen, pH, the carbon source, and temperature.

The glycosylation of biotherapeutics is often a critical product attribute for therapeutic efficacy and safety. Fluctuations in the process can put product integrity at risk and therefore it is important to monitor glycosylation accurately throughout the process.

www.nibrt.ie

In collaboration with Waters, the National Institute for Bioprocessing Research and Training (NIBRT), based in Ireland, has developed a glycan database for use in conjunction with UPLC glycan separations.

The analytical platform utilized by NIBRT is described in Figure 1. Briefly: N-glycans are released by PNGase F and fluorescently labeled with 2-aminobenzamide before separating the labeled glycan pool by hydrophilic interaction chromatography (HILIC).

The Waters ACQUITY UPLC® System applies an optimized hydrophilic interaction chromatographic separation (HILIC) using columns containing sub-2-µm particles. The combination of the ideal selectivity of diol-bonded Bridged Ethyl Hybrid (BEH) particles and exceptional peak capacity at higher flow rates results in overall increases in speed, sensitivity, and resolution compared to standard HPLC systems.

EXPERIMENTAL

Sample

The monoclonal antibody Herceptin was immobilized in acrylamide gel blocks and N-linked glycans released using peptide-*N*-glycosidase F. The glycan pool was labelled with 2-aminobenzamide (2AB). A linear gradient of 50 mM ammonium formate buffer, pH 4.4, and acetonitrile was used for glycan separation.

LC conditions

UPLC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH Glycan Column (2.1 x 150 mm)
Column temp.:	40 °C
Sample temp.:	5 ± 5 °C
Flow rate:	0.561 mL/min
Mobile phase A:	50 mM ammonium formate (pH 4.4)
Mobile phase B:	100% acetonitrile

Gradient table

Time (min)	Flow rate	%A	%B	Curve
1. Initial	0.561	30.0	70.0	6
2. 1.47	0.561	30.0	70.0	6
3. 24.81	0.561	47.0	53.0	6
4. 25.50	0.400	70.0	30.0	6
5. 26.25	0.400	70.0	30.0	6
6. 26.55	0.400	30.0	70.0	6
7. 28.55	0.561	30.0	70.0	6
7. 72.00	0.400	30.0	70.0	6

Detection

UPLC detector:	ACQUITY UPLC FLR Detector
Wavelengths:	λ_{ex} =330 nm, λ_{em} = 420 nm
Data rate:	10 pts/sec
PMT gain:	20
Time constant:	Normal

Auto Zero On Injection Start (check)

Data management

Empower Software

GlycoBase 3.0 database

RESULTS AND DISCUSSION

The preliminary assignment of glycan structures is obtained by interrogating the NIBRT database (http://glycobase.nibrt.ie/glycobase) in which peak retention times are expressed in glucose units (GU values) by alignment to a dextran hydrolysate ladder. Chromatographic resolution and reproducibility play a vital role in obtaining accurate and reproducible GU measurements and quantitative data. In the ACQUITY UPLC System, the GU value standard deviation is below ± 0.01 GU from 8 to 10 independent analyses, illustrating the superb peak resolution and run-to-run reproducibility.

Improved peak capacity and resolution of glycan pools

An ACQUITY UPLC System combined with a BEH glycan column provides significant enhancement in terms of peak capacity and resolution in comparison to classical HPLC technologies.

In Figure 2, a 30-minute separation of N-glycans from human serum on a (1.7-µm) BEH glycan column is compared with chromatograms that were generated using a classical HPLC system with amide columns (5-µm and 3-µm particle size).

Figure 3 shows that the ACQUITY UPLC System rapidly resolves glycans that were released from Herceptin in 12 minutes. 24 glycans were identified and quantified using the NIBRT database and exoglycosidase array digestions; the major glycan species are annotated in the figure.

GlycoBase 3.0; an aid to data interpretation

Included in GlycoBase 3.0 (http://glycobase.nibrt.ie/glycobase) is the Waters collection of UPLC GU values of the glycans of a diverse group of nine samples (Herceptin, Human and Mouse serum IgG, recombinant Erythropoietin, Bovine Fetuin, Bovine Ribonuclease B, Yeast Invertase and Human serum glycoproteins).These values were obtained by the systematic analysis of released glycans using Waters HPLC and UPLC technologies and the NIBRT glycan analytical platform. The profiles of the glycan pools and the enzymatic digestions can be obtained from a link (http://glycobase.nibrt.ie/glycobase/documents/enzyme.pdf). In brief, the N-linked glycan pool is separated equally into 5 (0.5 mL) eppendorfs and appropriate exoglycosidase enzymes are sequentially added with 10x 50 mM sodium acetate pH 5.5 buffer to build up the array. These are labeled (i) undigested control; (ii) ABS; (iii) ABS+BFK; (iv) ABS+BFK, BTG; and finally (v) ABS+BFK, BTG, GUH. Enzyme nomenclature and amounts are described below.

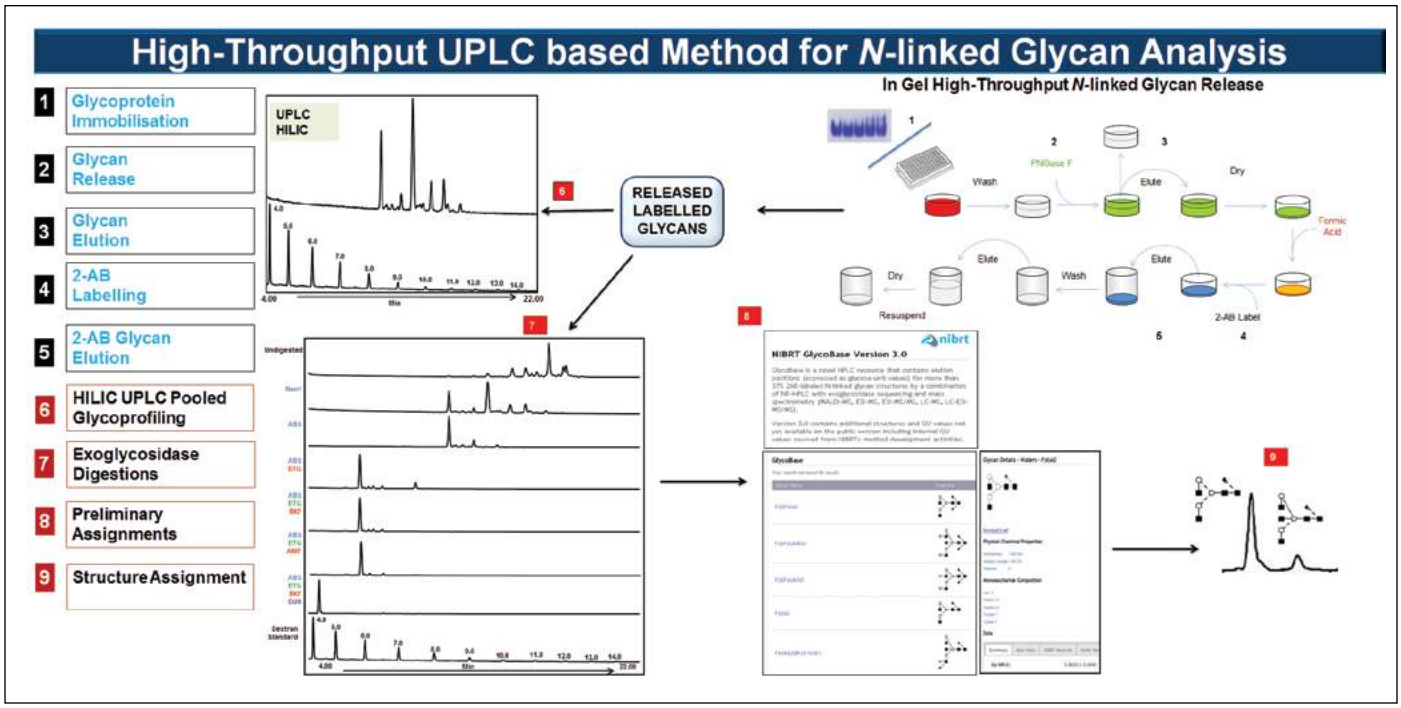
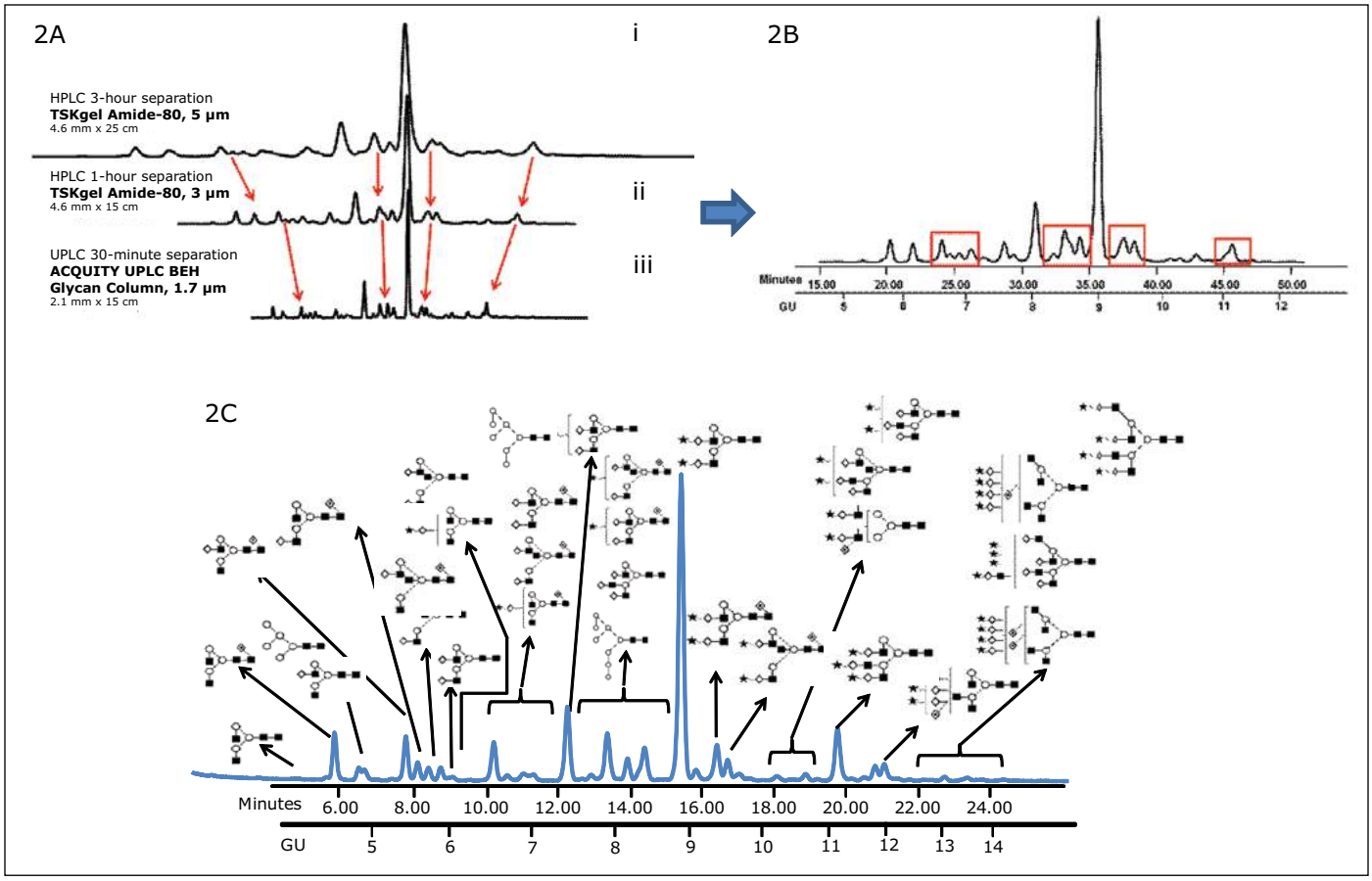


Figure 1. High-throughput UPLC-based method for N-linked glycan analysis.



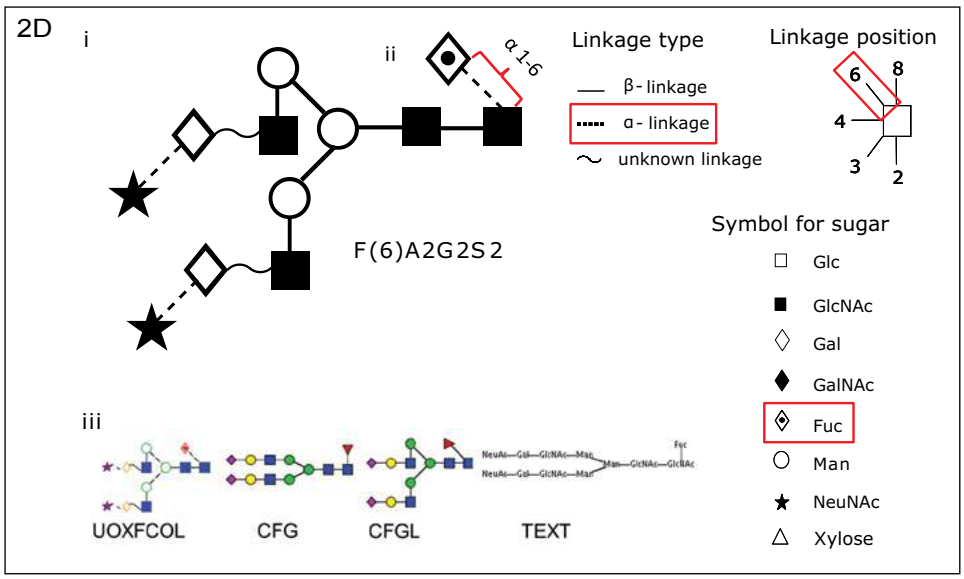


Figure 2A. Comparison of the 3-hour HPLC method and 1-hour HPLC separation of complex human serum glycans on (i) 5-µm and (ii) 3-µm TSK-GEL Amide-80 columns using a 30 minute Waters UPLC method (iii) BEH glycan column 1.7-µm, 2.1 mm x 15 cm.

Figure 2B. Separation of human serum protein glycans with classical HPLC methods, TSK-GEL 3-µm Amide-80 column, 1 hour run time.

Figure 2C. The same human serum glycome analyzed on Waters ACQUITY UPLC. The most abundant of the 136 glycans that were identified are annotated (Figure 2D) There is significant decrease in run times and improved separation of the glycans compared with the classical HPLC methods.

Figure 2D. (i) A cartoon depicting the linkage type, linkage position and symbol for common sugars used with the Oxford notation. (ii) An example using F(6)A2G2S2, where core fucose F(6) α 1-6 linkage is depicted with Oxford notation highlighted in red for linkage type, linkage position and symbol for associated sugar. (iii) Glycobase has a conversion function (developed by Eurocarb DB) that can represent glycans in other common formats as can be see with F(6)A2G2S2.[1,2]

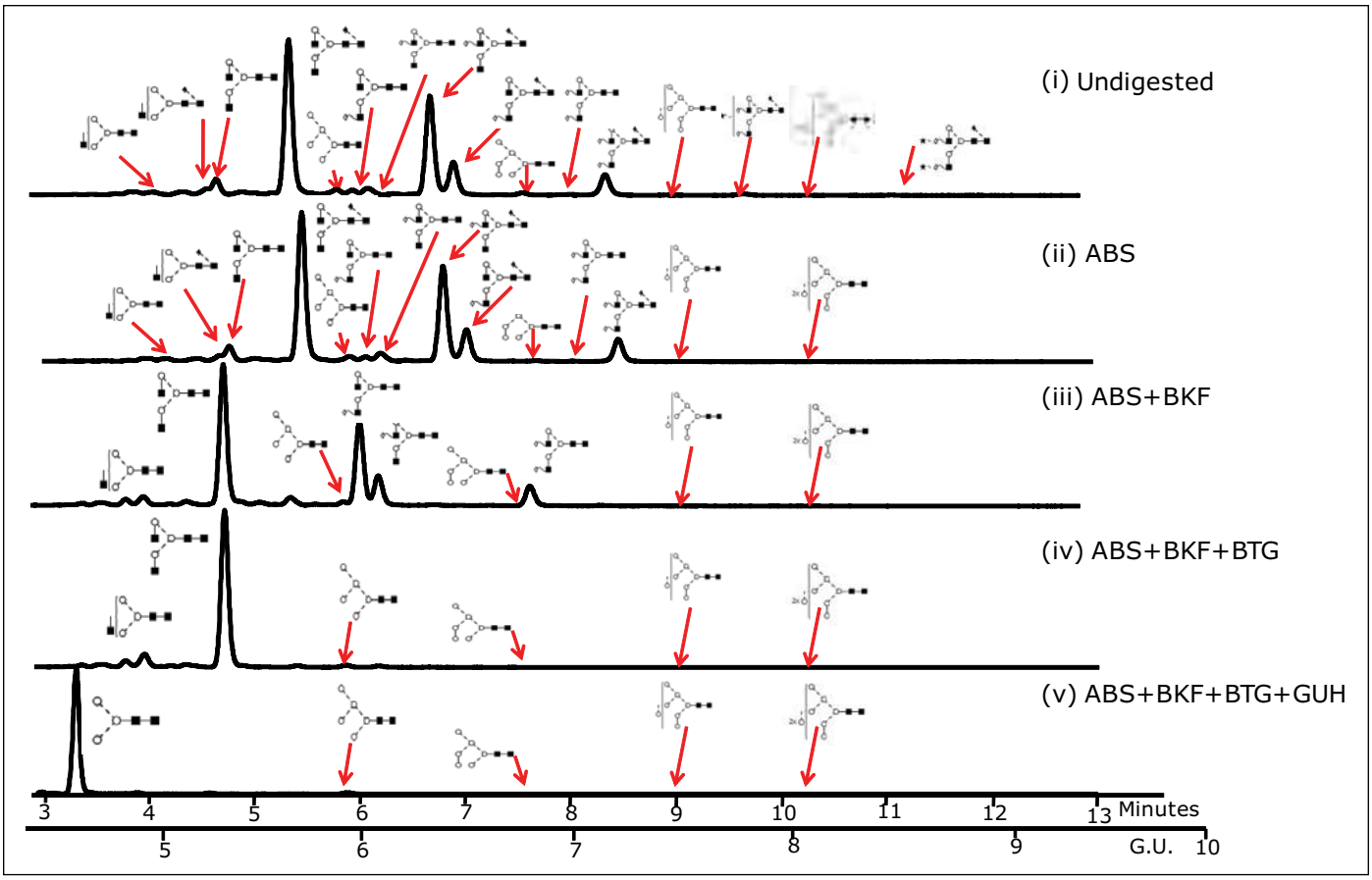


Figure 3. UHPLC analysis of the pool of glycans released from Herceptin (Trastuzumab) monoclonal antibody and the exoglycosidase array digestions that provide monosaccharide sequence and linkage information. 10 ug of Herceptin was used for the initial release by PNGaseF and intact pool represents approximately 2% of the total released N-glycan pool.

(i) Intact N-glycan pool released by PNGase F; (ii) ABS, Arthrobacterureafacienssialidase (1 U/mL) digestion. ABS releases α2-3 /6/8 sialic acids; (iii) ABS + BKF, bovine kidney alpha-fucosidase (BFK) (1 U/mL) array digestion. BKF releases α1-6/2 fucose (iv) ABS +BKF+ BTG, bovine testes beta-galactosidase (BTG) (1 U/mL) array digestion. BTG releases galactose β1-3>1-4 linkages and (v) ABS+BKF+BTG+N-acetylglucosaminidase (GUH) (4 U/mL) array digestion.

CONCLUSIONS

- HPLC technologies are relatively straightforward, robust, and inexpensive. They can separate arm specific isomers and when coupled with exoglycosidase digestions they provide monosaccharide sequence and linkage information.
- Non-selective fluorescent labeling (2AB) provides quantitative data and high sensitivity (glycans from <1 ng protein on a 2D gel can be analyzed)[3, 4]
- Enhancements in peak resolution, peak capacity, and reproducibility of fluorescently labeled (2AB) glycan separations can be achieved using an ACQUITY UPLC System and a BEH Glycan Column operated in HILIC mode.
- The enhanced resolution of UPLC enables the more accurate assignment of GU values compared to conventional HPLC because overlapping peaks are better separated.

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Optimization of GlycoWorks HILIC SPE for the Quantitative and Robust Recovery of N-Linked Glycans from mAb-Type Samples

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- GlycoWorks HILIC µElution Plate provides an efficient mechanism for glycan cleanup
- Quantitative and consistent recoveries for a diverse range of N-glycans with optimized SPE conditions
- Optimized SPE conditions also confer excellent method robustness
- Availability of a 2-AB labeled glycan standard for ensuring the performance of sample preparation and analysis methods

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC GST Amide (BEH Glycan) Column

GlycoWorks™ 96-well HILIC µElution Plate

Glycan Performance Test Standard

KEY WORDS

N-glycans, GlycoWorks, BEH glycan, Glycan Performance Test Standard, HILIC, HILIC SPE

INTRODUCTION

More than half of all proteins are estimated to be glycosylated.[1,2] This post-translational modification, involving the attachment of oligosaccharides, plays a very significant role in many biological processes.[3] Therapeutic antibodies are a salient example of a set of proteins affected by glycosylation, given that their efficacy and immunogenicity can be considerably attenuated by changes in their glycan profile. Glycan profiles of therapeutic antibodies are often, therefore, a critical quality attribute (CQA) that must be assessed during cell line selection and monitored during development and batch releases.

A highly effective analysis platform for evaluating N-glycans from glycoproteins involves the release of glycans by PNGase F, their labeling with fluorescently active 2-aminobenzamide (2-AB), subsequent separation by hydrophilic interaction chromatography (HILIC), and detection by fluorescence (FLR), as shown in Figure 1.[3-10]

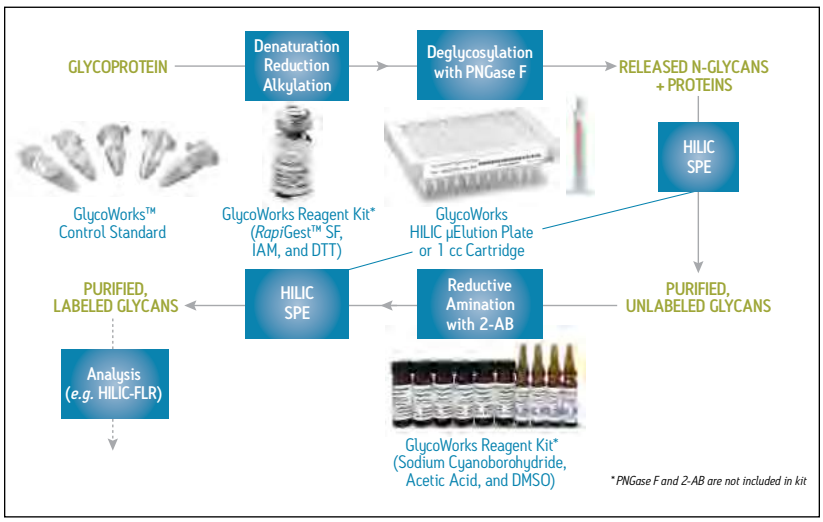


Figure 1. Schematic for preparing 2-AB labeled glycans from a glycoprotein using GlycoWorks. Consumables that are included as part of the GlycoWorks solution are highlighted in blue. Note that PNGase F and 2-AB are not included as part of the GlycoWorks Reagent Kit.

EXPERIMENTAL

Sample description

For the labeled oligosaccharide recovery studies, the Glycan Performance Test Standard (p/n 186006349) was mixed with 2-AB labeled trisialylated A3 (ProZyme) in water to make a solution of 3 pmol/µL. Aliquots (10 µL) of this mixture were diluted with 15 µL of acetonitrile (ACN) to make control samples. Aliquots (10 µL) were also dried under vacuum to prepare lyophilization control samples. In addition, 10-µL aliquots were processed by HILIC SPE according to the protocol found in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual (p/n 715004079). Various eluents were studied and are noted in Figures 3 through 6. Dried glycans were reconstituted in 10 µL of water and 15 µL of ACN prior to injection.

For the unlabeled oligosaccharide recovery studies, unlabeled Man5 and trisialylated A3, obtained from ProZyme, were reconstituted in water, and mixed to equal molarity (6.7 µM). Aliquots (6 µL) of this mixture were diluted with 6 µL of ACN to make control samples. Aliquots (6 µL) were also dried under vacuum to prepare lyophilization controls. In addition, 6 µL aliquots were processed by HILIC SPE, according to the protocol found in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual. Elution was performed with an eluent comprised of 100 mM ammonium acetate (NH_4OAc) in 5% ACN (pH 7). Dried glycans were reconstituted in 6 µL of water and 6 µL of ACN prior to injection.

A schematic for the HILIC SPE steps used in this study is shown below:

WASH
200 µL H_2O

CONDITION
200 µL 85% ACN

LOAD
10 µL sample + 100 µL ACN (2-AB labeled)
6 µL sample + 44 µL 25 mM NH_4HCO_3 (pH 8) + 350 µL ACN (unlabeled)

WASH
3 x [200 µL 85% ACN]

ELUTE
3 x [50 µL 100 mM NH_4OAc, 5% ACN, pH 7]
(unless otherwise noted)

LYOPHILIZE AND RECONSTITUTE

Note: Eluates were transferred to micro-centrifuge tubes then lyophilized. Thereafter, samples were reconstituted and transferred to sample vials for injection.

The procedure for preparing samples for this analysis can be complicated. GlycoWorks products help make the workflow more straightforward by bringing together many of the needed consumables. Moreover, GlycoWorks products provide a solution to the cleanup steps that are needed throughout the process of preparing labeled glycans for analysis. In particular, HILIC solid-phase extraction (SPE)[11,12] has been developed to purify released glycans from proteins and buffer/formulation constituents, which can disrupt derivatization. HILIC SPE can also purify labeled glycans after derivatization from excess reagents, which can potentially interfere with downstream chromatography, reduce the lifetime of a column, and thereby impair method robustness.

This application note evaluates HILIC SPE sample preparation to ensure quantitative recovery of both unlabeled and labeled N-glycans. A test mixture, including a complex array of 2-AB labeled human IgG glycans spiked with both high mannose and trisialylated glycans, was used to interrogate and optimize SPE recoveries as well as study the robustness of optimized elution conditions. In addition, an LC-MS assay was employed to demonstrate the quantitative recovery of unlabeled glycans during HILIC SPE with the optimized conditions.

Method conditions (unless otherwise noted)

LC conditions

System:	ACQUITY UPLC H-Class Bio with a 20-cm Column Heater
Detection:	Waters® ACQUITY UPLC FLR Detector
Excitation:	330 nm
Emission:	420 nm
Scan rate:	10 Hz
Time constant:	0.2 s
Gain:	1.00
Column:	ACQUITY UPLC GST Amide (BEH Glycan), 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	2.5 µL (HILIC-FLR), 10 µL (HILIC-MS)
Flow rate:	0.5 mL/min (0.25 mL/min for the highly aqueous regeneration step in the gradient)
Mobile phase A:	100 mM Ammonium formate, pH 4.4
Mobile phase B:	Acetonitrile (ACN)
Sample collection plate:	1 mL Round Well Collection Plate (p/n 186002481)
Vials:	LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)

Gradients:

HILIC-FLR	Time (min)	% A	% B	Flow rate (mL/min)
	0	22.0	78.0	0.5
	38.5	44.1	55.9	0.5
	39.5	80.0	20.0	0.25
	44.5	80.0	20.0	0.25
	46.5	22.0	78.0	0.5
	50.0	22.0	78.0	0.5

HILIC-MS	Time (min)	% A	% B	Flow rate (mL/min)
	0	27.9	72.1	0.5
	19.25	50.0	50.0	0.5
	20.25	80.0	20.0	0.25
	25.25	80.0	20.0	0.25
	27.25	22.0	78.0	0.5
	31.00	22.0	78.0	0.5

MS conditions

Mass spectrometer:	Xevo® G2 Q-Tof
Ionization mode:	ESI+
Analyzer mode:	Sensitivity
Capillary voltage:	3.20 kV
Cone voltage:	37 V
Source temp.:	100 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	800 L/h
Calibration:	NaI, 1 µg/µL from 50 to 2000 *m/z*
Acquisition:	700 to 3000 *m/z*, 1 Hz scan rate
Lock mass:	0.5 µM [Glu^1]-fibrinopeptide in 50:50 ACN/water, 0.1% formic acid

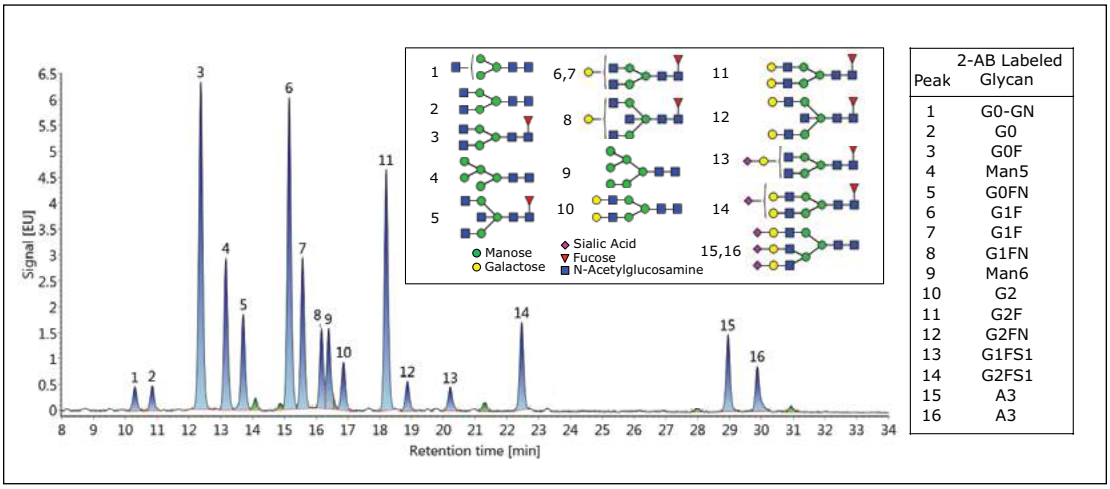
Data management

UNIFI® and MassLynx® software

RESULTS AND DISCUSSION

Optimizing the recovery of glycans from GlycoWorks HILIC SPE

A test mixture, capable of rigorously interrogating the recovery of N-glycans from GlycoWorks HILIC SPE, was prepared by combining the Glycan Performance Test Standard with 2-AB labeled trisialylated A3 glycans. The Glycan Performance Test Standard is comprised of 2-AB labeled N-glycans derived from pooled human serum IgG spiked with high mannose glycans (Man5 and Man6). The addition of the trisialylated A3 glycans further extends the complexity of this mixture, as the A3 glycans are larger, more acidic, and bind more strongly in a HILIC-based separation than glycans commonly found on human or human-like IgG. Figure 2 shows a HILIC-FLR analysis of this modified test mixture using an ACQUITY UPLC GST Amide (BEH Glycan) Column along with UNIFI Software for instrument control and data interpretation.



Peak	2-AB Labeled Glycan
1	G0-GN
2	G0
3	G0F
4	Man5
5	G0FN
6	G1F
7	G1F
8	G1FN
9	Man6
10	G2
11	G2F
12	G2FN
13	G1FS1
14	G2FS1
15	A3
16	A3

Figure 2. HILIC-FLR analysis of 2-AB labeled glycan performance test standard and trisialylated A3 glycans. 3 pmol of sample injected in 2.5 µL onto an ACQUITY UPLC GST Amide (BEH Glycan), 1.7 µm, 2.1 x 150 mm Column. Peaks detected by UNIFI processing are shaded in blue (expected component) or green (discovered component).

Based on this analytical approach, the HILIC SPE of the GlycoWorks solution was evaluated. A silica-based aminopropyl sorbent is contained in the GlycoWorks Kit (p/n 176003090). This sorbent was selected from several tested because it is highly polar and, consequently, useful for HILIC separations. Since this sorbent possesses a weakly basic surface and potential for anion exchange, it was, however, assumed that the relative and total recovery of glycans from a GlycoWorks HILIC SPE device could be particularly sensitive to elution conditions. To evaluate this step, elution from the GlycoWorks HILIC sorbent was studied in detail. 2-AB labeled glycans were loaded onto a 96-well HILIC µElution Plate according to the protocol provided in the GlycoWorks High-throughput Sample Preparation Kit Care and Use Manual.[13] Various eluents were then employed for elution of the labeled glycans, and recoveries for each major species in the test mixture was subsequently determined. These data were compared alongside the recoveries of the glycans from just the lyophilization and reconstitution steps that were performed after the HILIC SPE procedure, in preparation of the samples for HILIC-FLR. A series of eluents comprised of 20% ACN and increasing concentrations of ammonium bicarbonate (NH_4HCO_3, pH 8–9) were first investigated. A volatile salt was chosen, due to requisite lyophilization steps. Interpretation of the recoveries led to the observation that the recovery of the glycans was biased, based on eluent choice, with smaller, neutral species recovered better than larger, acidic species. With an eluent comprised of simply 20% ACN/80% water (H_2O) and no other components, acidic glycans in the test mixture were either poorly recovered or not recovered at all; meanwhile, neutral glycans were obtained with reasonable recovery (≥70%). The addition of NH_4HCO_3, to concentrations of 25 mM or higher minimized this apparent and non-desired ionic retention mechanism. Nevertheless, even with 100 mM NH_4HCO_3, there was a noticeable correlation between recovery and the hydrophilicity, or glucose unit (GU) values, of the glycans (Figure 3A).

Biased recovery, or speciation, can be problematic for a sample preparation procedure. In addition to not providing an accurate representation of the species present in the sample, it can be indicative of a method that is not robust and that the relative abundance profiles obtained may not be reproducibly determined, particularly with respect to the most poorly recovered species. As a result, a study was performed to improve these observed 2-AB labeled glycan recoveries. Given that retention of polar analytes to a polar sorbent is dominated by hydrogen bonding and ionic interactions, eluents with more aqueous content (decreased ACN concentrations) were evaluated (Figure 3B). As predicted, NH_4HCO_3 eluents comprised of lower concentrations of organic solvent yielded both higher and less biased recoveries of the glycan profile. Within the range of this study, an eluent composition of 25 mM NH_4HCO_3/5% ACN was found to produce optimal recoveries.

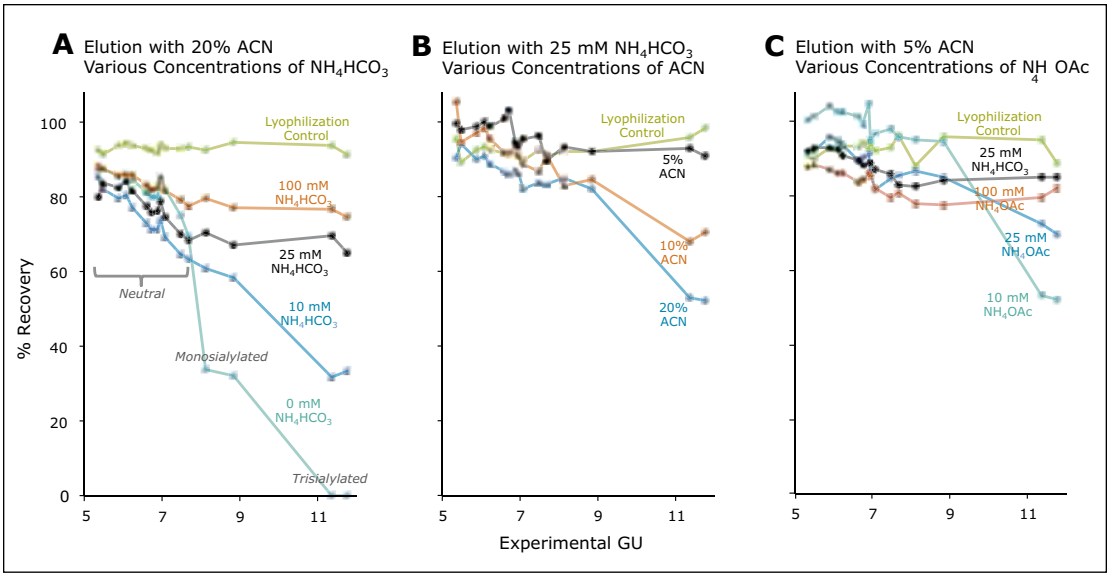


Figure 3. Recovery of 2-AB labeled N-glycans (Figure 2) from GlycoWorks HILIC SPE 96-well µElution Plates (30 pmol of glycans processed). Percent recoveries as a function of experimentally determined glucose units (GU) are shown for various elution conditions. Values are based on the average of three replicate analyses.

Unfortunately, eluents containing NH_4HCO_3 posed a challenge in this application as their basicity (typically pH 8 but increased toward pH 9 upon exposure to air) may result in noticeable dissolution of the silica SPE particles and problematic levels of precipitate in the reconstituted samples. To eliminate this potential issue and establish a more robust procedure, we investigated alternative eluents based on neutral solutions of ammonium acetate (pH 7). The effect of ammonium acetate (NH_4OAc) eluents on the recoveries of the 2-AB labeled glycans is shown in Figure 3. A 100-mM NH_4OAc, 5% ACN eluent was selected as the optimal elution condition, since it provided high as well as relatively unbiased analyte recoveries, similar to those obtained using the 25-mM NH_4HCO_3, 5% ACN eluent.

The set of chromatograms shown in Figures 4A and 4B demonstrates that the test mixture, before and after HILIC SPE treatment, exhibits highly consistent glycan profiles. Relative abundance determinations for control samples as well as a processed sample are shown in Figure 4C. Compared to the control, differences in relative abundances were ≤7% across the entire profile. For example, the relative abundance of G0F (peak 3) was determined to be 17.9% and 18.6%, before and after SPE, respectively. The relative abundance of trisialylated A3 (peak 16) before and after SPE was 2.8% and 2.7%, respectively (Figure 4C). These optimized elution conditions provide quantitative recoveries for both glycans typical of human IgGs and heavily sialylated glycans, as demonstrated with the recovery of the A3 glycan. With these conditions, the GlycoWorks HILIC µElution Plate is well suited for the preparation of N-glycans from a range of glycoproteins, including those with primarily low GU value neutral glycans as well as those decorated with high GU value, heavily sialylated glycans.

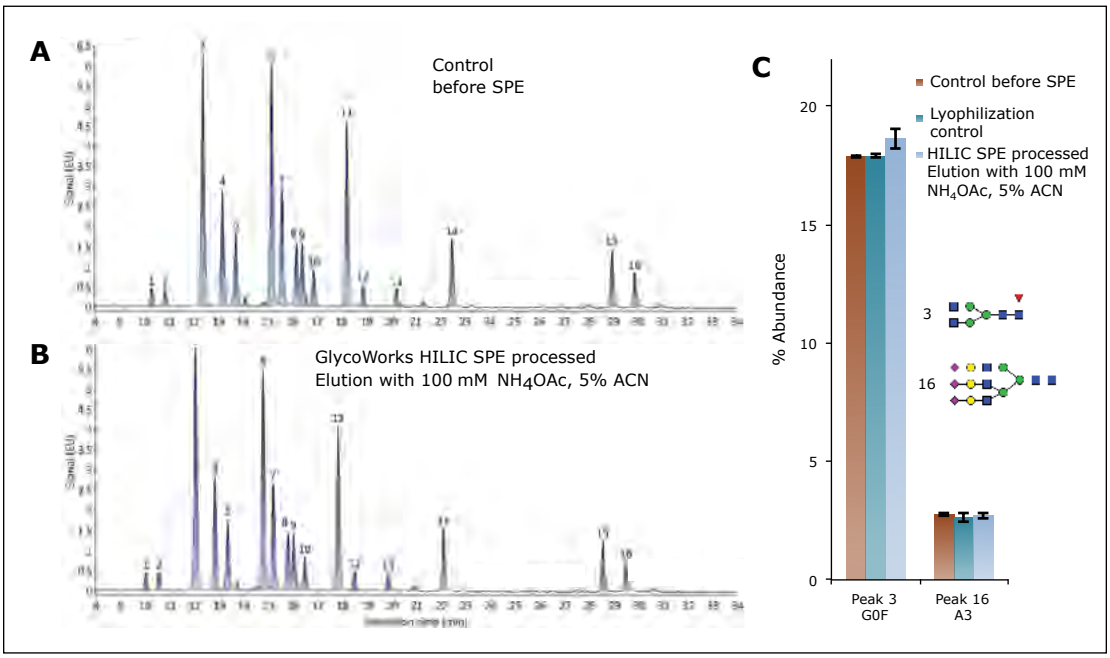


Figure 4. Glycan profile (A) before, and (B) after GlycoWorks HILIC SPE with the optimized elution conditions. Representative chromatograms and relative abundances (C) measured for two 2-AB labeled glycans (low and high GU values) from the test mixture before and after GlycoWorks HILIC SPE are displayed (n=3).

Robustness testing of optimized SPE elution conditions

The GlycoWorks HILIC µElution Plate was optimized to yield desired recoveries and, more importantly, to be robust. Elution conditions were purposely optimized so that even relatively large changes in critical elution parameters, namely organic concentration and ionic strength, would have minimal effect on the obtained glycan profile. To demonstrate this, the HILIC SPE method was subjected to robustness testing. Glycan profiles obtained using SPE eluents with the optimized concentrations of ACN and NH_4OAc concentrations were compared to those obtained with eluents comprised of ACN and NH_4OAc concentrations varied by 10%. The impact of changes in ionic strength and ACN concentration were purposely compounded in these studies. A strong eluent with 110-mM NH_4OAc, 4.5% ACN as well as a comparatively weak eluent with 90-mM NH_4OAc, 5.5% ACN were employed. Figure 5 shows the relative abundances for each of the major constituents in the test mixture obtained using these varied conditions. The glycan profiles obtained were comparable to the conditions tested. The largest percent change observed between relative abundances from the optimal to extreme conditions was only 7%, corresponding to the recovery of trisialylated A3 (peak 16). This result demonstrates that clean-up of 2-AB labeled glycans using the GlycoWorks HILIC µElution Plate with the optimized elution conditions exhibits noteworthy ruggedness, and is, therefore a robust solution for N-glycan preparations even in quality control applications.

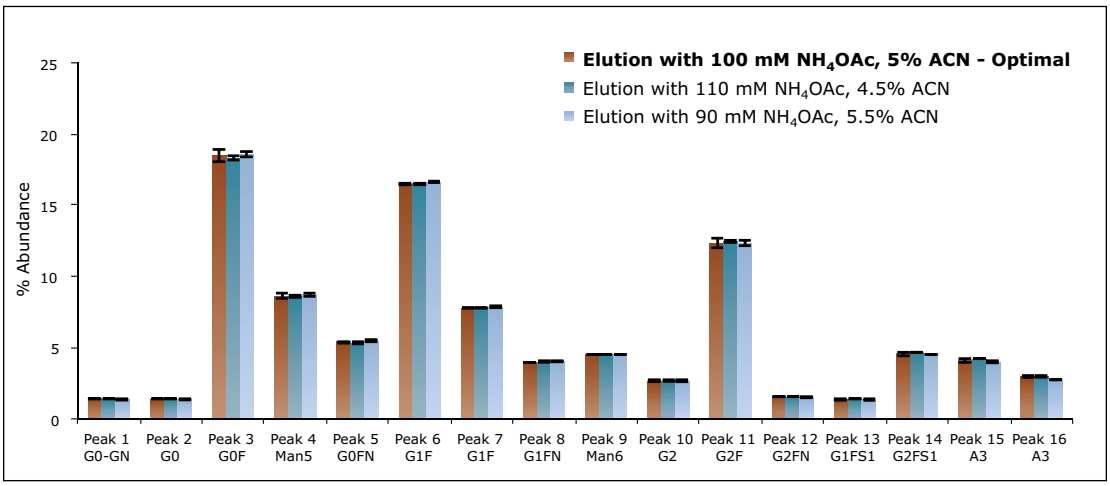


Figure 5. Robustness testing of the SPE elution conditions. Glycan profile for the test mixture obtained after GlycoWorks HILIC SPE with the optimized elution conditions and elution conditions wherein critical parameters were varied by 10% (n=3).

Assaying unlabeled glycans before and after GlycoWorks HILIC SPE by LC-MS

The GlycoWorks HILIC µElution Plate is also suggested for the initial purification of unlabeled glycans cleaved from the target glycoprotein via enzymatic digestion. 2-AB labeled glycans, as previously studied, are slightly less hydrophilic than unlabeled glycans due to the hydrophobicity of the benzamide fluorescent tag. To confirm that recoveries of unlabeled glycans were similar in comparison to the recoveries of 2-AB labeled glycans, an additional study was performed. A HILIC-MS assay was established to determine the relative abundances of two unlabeled glycans representing the extremes of most IgG N-glycan profiles. The mixture tested was comprised of equal amounts of a neutral, low GU value glycan (Man5) and an acidic, high GU value glycan (trisialylated A3). An extracted ion chromatogram (XIC) obtained for this mixture with a Xevo G2 QTof is shown in Figure 6A. Interestingly, two major peaks were observed for both unlabeled Man5 and A3, indicating the presence of different isoforms. Mass spectral windows wide enough to capture both protonated and salt adduct species of the unlabeled glycans were used to construct the chromatogram. XICs obtained in this manner were integrated, and the obtained peak areas were used to calculate relative abundances of the unlabeled glycans before and after HILIC SPE (Figure 6B). As with 2-AB labeled glycans, the profile of the unlabeled glycan mixture before and after SPE was highly comparable, indicating that the optimized GlycoWorks HILIC SPE process also yields minimally biased recoveries of unlabeled glycans.

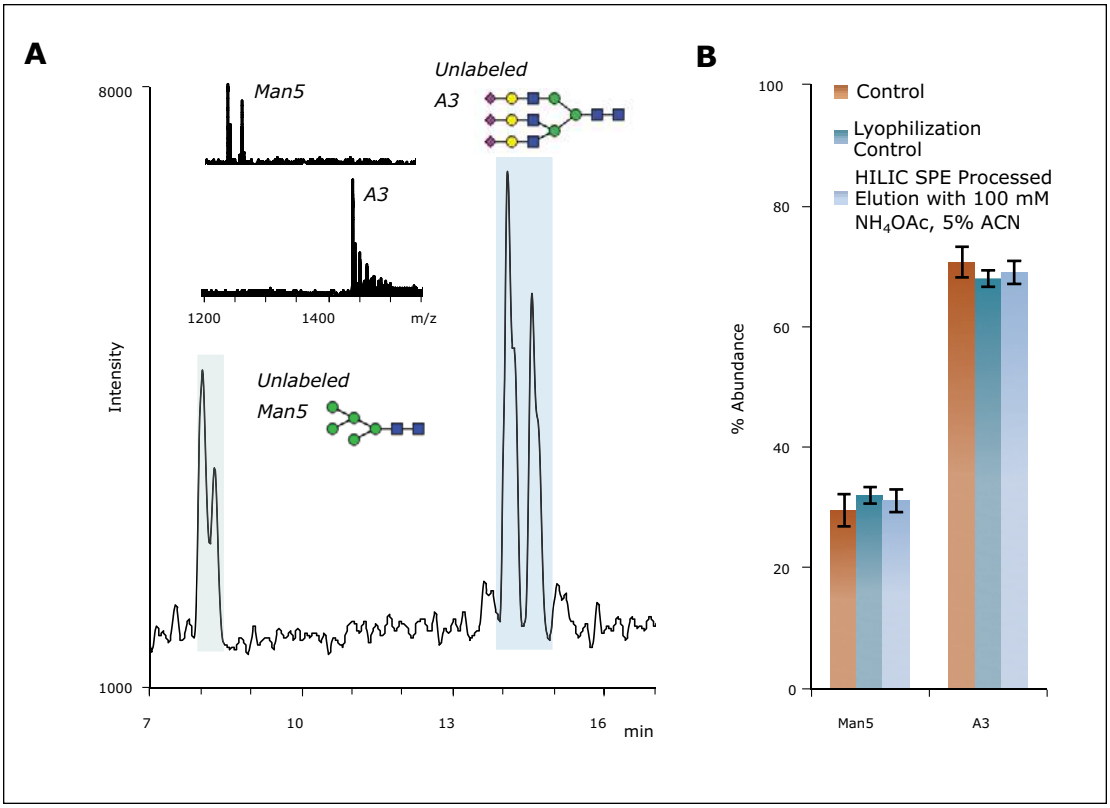


Figure 6. Assessing the effect of GlycoWorks HILIC SPE on the distribution of unlabeled glycans. An extracted ion chromatogram (XIC, 1235-1270+1435-1500 m/z) from a HILIC-ESI-MS analysis of a mixture containing a low GU, neutral glycan (Man5) and a high GU, acidic glycan (trisialylated A3) is shown on the left (A). Relative abundances determined via such an analysis for the mixture before and after GlycoWorks HILIC SPE are shown on the right (B). In this study, 40 pmol of each unlabeled glycan were processed by GlycoWorks HILIC SPE and 10 µL of a 12-µL reconstitution for a total maximum load of 33 pmol of each glycan was loaded onto an ACQUITY UPLC GST Amide (BEH Glycan), 1.7 µm, 2.1 x 150 mm Column for analysis.

CONCLUSIONS

HILIC SPE was rigorously studied and optimized to provide quantitative recoveries of 2-AB labeled and unlabeled N-glycans. A test mixture containing a diverse array of 2-AB labeled N-glycans was employed to interrogate GlycoWorks HILIC µElution Plate performance, and develop optimized elution conditions for a robust and reproducible method. In ruggedness testing of the optimized SPE, only minimal changes in a glycan profile were observed despite significant changes in the critical parameters of the SPE eluent. Moreover, an LC-MS assay showed that unlabeled glycans, like 2-AB labeled glycans, are recovered with minimal bias using the newly optimized elution conditions. These studies highlight the development of the GlycoWorks solution and its value in facilitating the release, labeling, and purification of N-glycans.

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UPLC-FLR Method Development of 2-AB Labeled Glycan Separation in Hydrophilic Interaction Chromatography (HILIC)

Joomi Ahn, Ying Qing Yu, and Martin Gilar
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The ACQUITY UPLC® System used with Glycan Separation Technology Columns delivers the ability to achieve highly-resolved glycan separations in less time. This provides significant benefits to high-throughput glycosylation monitoring and profiling in biopharmaceutical drug development.

WATERS SOLUTIONS

ACQUITY UPLC
ACQUITY UPLC FLR Detector
Glycan Separation Technology Column

KEY WORDS

Recombinant mAb, glycosylation, HILIC, optimizing gradient conditions, flow rate, buffer concentration, mobile phase pH, column temperature

INTRODUCTION

Monoclonal antibody (mAb) drug development has been the most active area in the biopharmaceutical industry in recent years. One of the important aspects in recombinant mAb development is to profile glycosylation patterns. Since the glycans play key functions in biological activities, the glycosylation variances during protein production affect the pharmaceutical properties such as efficacy and elimination rate. One of many analytical approaches for glycan analysis is hydrophilic interaction chromatography (HILIC) with fluorescence detection. It provides high sensitivity, good reproducibility, and the ability to separate complex glycan mixtures.

The Waters ACQUITY UPLC System with fluorescence detection (FLR) combined with a Glycan Separation Technology (GST) Column provides superior resolution compared to HPLC systems. These glycan columns, packed with 1.7-µm amide sorbent, efficiently separate the fluorescent-labeled glycans in HILIC mode. Highly-resolved glycan separations, especially for positional isomers and coeluting minor peaks, now can be more accurately measured in UPLC®/FLR.

In this application note, a general guideline for researchers to optimize HILIC gradient conditions is shown. The focus is to develop a UPLC method with optimal resolution for 2-aminobenzamide (2-AB) labeled N-linked glycans released from human IgG.

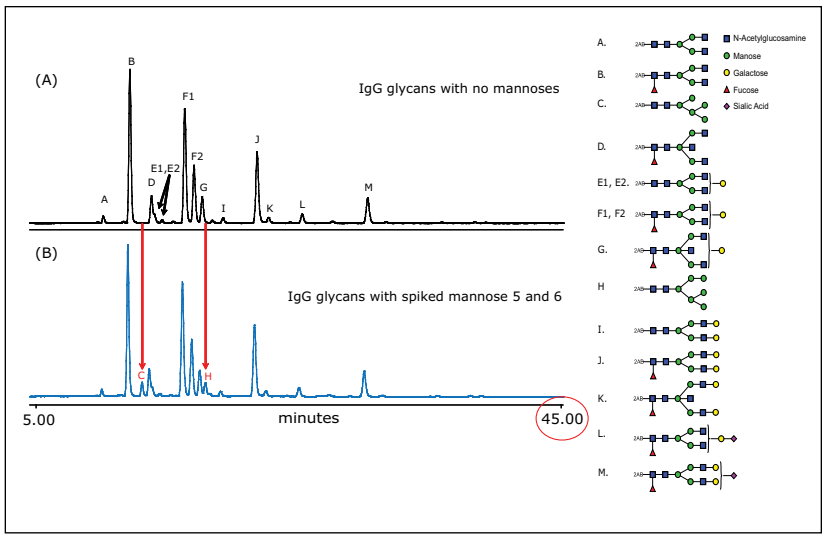


Figure 1. (A) 2-AB labeled glycans released from Human IgG, (B). The same IgG glycans with spiked mannose 5 and mannose 6.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan Column 2.1 x 150 mm, 1.7 µm
Column temp.:	40 °C*
Flow rate:	400 µL/min*
Mobile phase A:	100 mM ammonium formate, pH 4.5*
Mobile phase B:	Acetonitrile
Gradient:	72 to 62% B in 45 min*
Weak wash:	72% acetonitrile
Strong wash:	20% acetonitrile
Injection:	1.0 µL partial loop

(*All other conditions are indicated in figure legends.)

MS conditions

Detector:	ACQUITY UPLC Fluorescence Detector
Excitation:	330 nm
Emission:	420 nm
Data rate:	5 pts/sec
PMT gain:	1.00
Time constant:	Normal

Samples

2-AB labeled N-linked glycans released from human IgG (ProZyme, San Leandro, CA, U.S.) were used. In addition, 2-AB labeled mannose 5 and mannose 6 were spiked into the sample to make a more chromatographically challenging sample.

We demonstrate the capabilities of glycan columns to separate fluorescent-labeled glycans in HILIC mode, including the positional isomers and coeluting minor peaks previously unresolved by HPLC.

RESULTS AND DISCUSSION

2-AB labeled glycan separation was performed using the HILIC method. For method optimization, the focused gradient with shallow gradient slope was used to effectively separate the region where the interested glycans were closely eluting. Other chromatographic settings such as flow rate, buffer concentrations, pH, and column temperature were also varied to maximize the peak resolution in the same region. The optimized HILIC condition used in Figure 1 was established based on the examination of various settings, as illustrated in Figures 2 through 6.

The complex mixture of 2-AB labeled glycans released from IgG was separated with superior resolution, as shown in Figure 1. All glycans were resolved including isomers E1/E2 and F1/F2. The gradient shown in all figures was run in 45 minutes and the entire run was completed in 1 hour using a 2.1 x 150 mm column. When the glycan HILIC method is transferred from a 3.0-µm HPLC to a 1.7-µm UPLC Column, improved resolution in shorter run time can be achieved with a UPLC system.

We demonstrate in Figure 1B that mannose 5 (peak C) and mannose 6 (peak H) can be successfully separated from their neighboring peaks, which often coelute.

Initial acetonitrile concentration in HILIC gradient

The impact of initial gradient strength on the separation is illustrated in Figure 2. The best separation was achieved at gradient condition 72% to 62% B. The resolutions in highlighted regions were changed positively and/or negatively. For example, the boxed region from A to B shows improved resolution; however the second boxed region from B to C shows the decreased resolution.

The gradient slope was set to 10% acetonitrile change in 45 min in all experiments in Figure 2 (0.22% B/min) to eliminate the gradient slope effect as a variable.

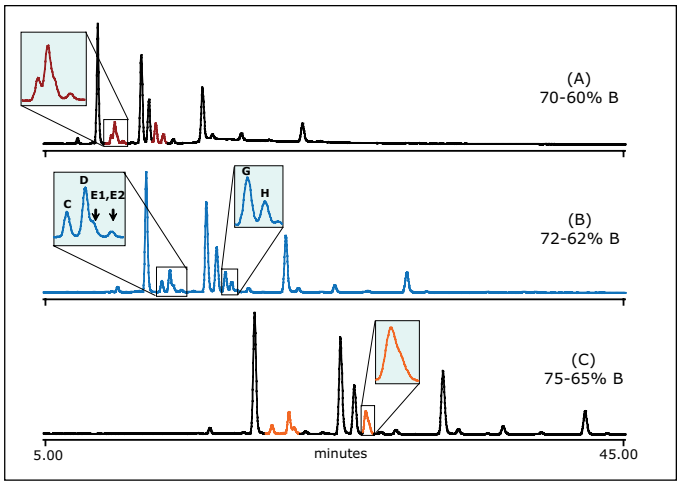


Figure 2. The impact of initial gradient strength on the separation of 2-AB labeled glycans. The flow rate was set at 0.4 mL/min with column temperature at 40 °C. Mobile phase A and B were 100 mM ammonium formate pH 4.5 and 100% acetonitrile respectively (see Figure 1 for peak denotation).

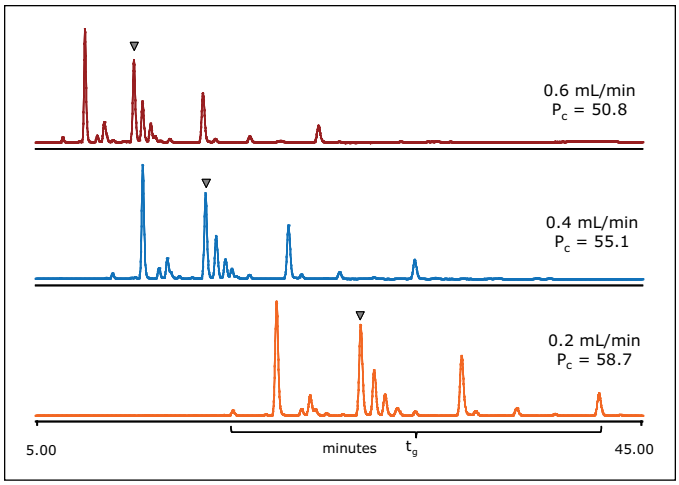
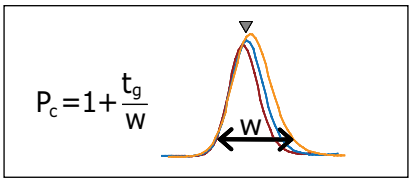


Figure 3. The impact of flow rate. The gradient was run in 72% to 62% B in 45 min at 40 °C. Mobile phase A and B were 100 mM ammonium formate (pH 4.5) and 100% acetonitrile respectively.

Flow rate

The separations in Figure 3 show the effect of the flow rate. Changing the flow rate influences the peak capacity as well as the resolution due to the contribution of the gradient slope change. When the flow rate increases from 0.2 to 0.6 mL/min the peak widths (w) become narrower; however, the effective separation window (tg) is simultaneously reduced (see Figure 3). Therefore, the impact of flow rate on peak capacity (defined as Pc = 1+tg/w) was moderate.



The flow rate 0.4 mL/min was chosen because it showed minor improvements in resolution. These gradient conditions were selected for further separation as described in the next section.

Buffer concentration

The buffer concentration affects the resolution and the selectivity, as shown in Figure 4. When elevating the ammonium formate concentration, the retention time of charged sialylated glycans is affected more significantly than the retention of neutral glycans. With mobile phase containing 250 mM ammonium formate we observed complete resolution of E1 from D, which was very difficult to achieve by varying other chromatographic conditions.

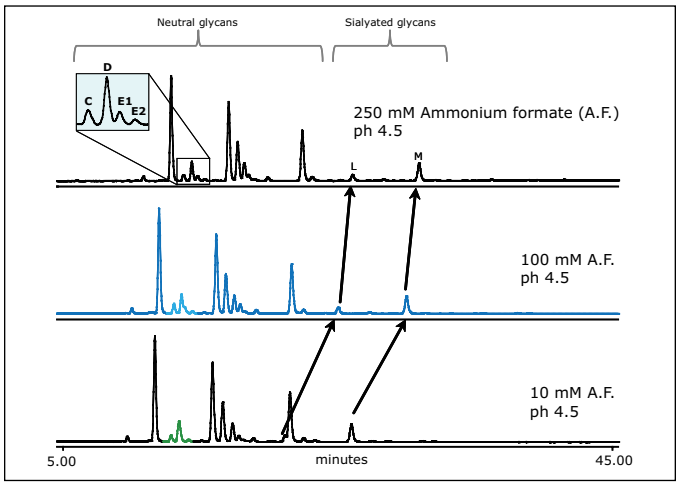


Figure 4. The impact of the buffer concentrations on the sialylated glycans. The column was run in gradient 72% to 62% B for 45 min at 0.4 mL/min. The column temperature was set at 40 °C (see Figure 1 for peak denotation).

Mobile phase pH

As shown in Figure 5, there is no significant change in separation between pH 4.5 and pH 7.5 in mobile phase A (100 mM ammonium formate). The mobile phase pH was adjusted by titrating ammonium with formic acid. However, the low pH in mobile phase will contribute to the retentivity shift of sialylated glycans (figure not shown).

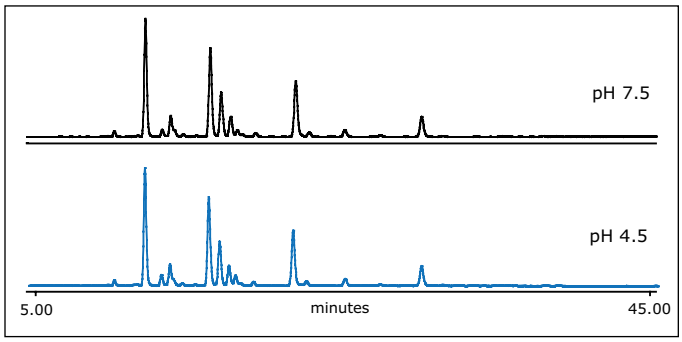


Figure 5. No significant retention change in two different pH conditions at 100 mM ammonium formate. The gradient was run in gradient 72% to 62% B for 45 min at 0.4 mL/min. The column temperature was 40 °C.

Column temperature

The column temperature affects the analyte retentively; all glycans shifted to earlier retention times with increased column temperatures as expected by chromatographic theory. The changes in highlighted regions marked in red and purple were also observed as a result of column temperature. Since the resolution is important for separating complex glycan mixture, the column temperature can be another parameter to be optimized (Figure 6).

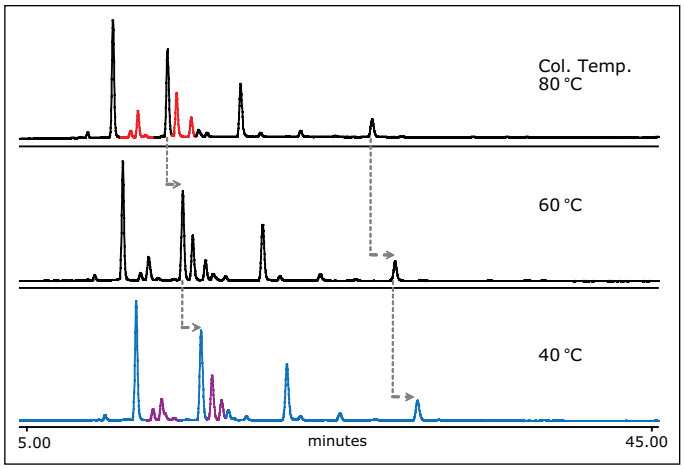


Figure 6. The retention shift due to the column temperature change.

CONCLUSIONS

- High quality of resolution and selectivity of the Glycan Separation Technology Column is demonstrated for 2-AB labeled glycans released from IgG. The impact of chromatographic settings in glycan separations is described as a guideline of HILIC method optimization. This guideline helps save the time and effort of method development to identify and quantify the glycans with confidence.
- Analysis time was significantly reduced with the ACQUITY UPLC System. Typically, a 2- to 3-hour HPLC gradient was needed to resolve 2-AB labeled IgG glycans to the full extent; our optimization method showed that a 1-hour UPLC run time was sufficient to separate the complex glycoforms.
- This system solution provides the capability to achieve highly-resolved glycan separation in less time. This adds value to high-throughput glycosylation monitoring and profiling in biopharmaceutical drug development.

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Profiling Released High Mannose and Complex N-Glycan Structures from Monoclonal Antibodies Using *Rapi*Fluor-MS Labeling and Optimized Hydrophilic Interaction Chromatography

Scott A. McCall, Matthew A. Lauber, Stephan M. Koza, and Erin E. Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Optimized LC method to improve the HILIC profiling of common mAb N-glycan species.
- Improved resolution of high mannose structures, in addition to sialylated species and N-glycan structures containing alpha-linked galactose units.
- The use of *Rapi*Fluor-MS™ High Mannose Standard in system suitability testing and peak identification.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Intact mAb Mass Check Standard

*Rapi*Fluor-MS High Mannose Standard

ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

UNIFI® 1.7 Software

MassLynx® 4.1 Software

KEY WORDS

ACQUITY UPLC H-Class Bio System, ACQUITY UPLC Glycan BEH Amide Column, glycans, glycosylated protein, glycoprotein, Glycosylation, N-Linked glycans, HILIC, *Rapi*Fluor-MS labeling, Intact mAb Mass Check Standard, IgG, monoclonal antibody (mAb), high mannose N-glycans, *Rapi*Fluor-MS High Mannose Standard

INTRODUCTION

Glycan characterization is at the forefront of the biopharmaceutical industry, since most protein therapeutics possess N-glycosylation, if not also O-glycosylation. The most common therapeutic modality, an IgG monoclonal antibody (mAb), is, for instance, typically N-glycosylated at two conserved sites in its Fc domain. Not surprisingly, the nature of the N-glycans on a mAb can impact its circulation half-life and efficacy. As a result, it is particularly important that the N-glycans of a mAb be well characterized and routinely monitored.

A powerful strategy for such an analysis involves releasing N-glycans enzymatically, labeling them with a reagent to improve their detectability, and profiling them using hydrophilic interaction chromatography (HILIC).[1] Recent developments in released N-glycan profiling, made possible by the novel *Rapi*Fluor-MS labeling reagent, have eclipsed conventional N-glycan techniques by simplifying the steps in the procedure, reducing the overall sample preparation times, and enabling unprecedented sensitivities for both fluorescence and mass spectrometric detection.[2] However, regardless of how quickly a sample can be prepared or with what sensitivity it can be detected, it is imperative for N-glycan profiling to exhibit optimal chromatographic resolution. With there being many different sample types, it is often necessary to consider tailoring liquid chromatographic (LC) methods to ensure that robust and optimal chromatographic performance can indeed be achieved.

In this application note, we highlight the development of an LC method that optimizes the chromatographic resolution for the released N-glycans that are commonly found on mAbs, including the high mannose N-glycan structures that are known to negatively affect circulation half-life as well as indicate aberrant cell culture conditions. In this work, N-glycans from a mAb were rapidly released with PNGase F, labeled with *Rapi*Fluor-MS and profiled by HILIC using sensitive fluorescence and mass spectrometric detection. The separation method was optimized to improve the resolution of high mannose glycans, those terminated with N-glycolyl neuraminic acid (Sg) (a member of the broad class of sialic acids), and species containing alpha-linked galactose monosaccharides. Within this work, the utility of this newly developed LC method is demonstrated by means of system suitability testing with high mannose spiked samples.

EXPERIMENTAL

Sample description

The Intact mAb Mass Check Standard (p/n 186006552) was reconstituted in water to a concentration of 2 mg/mL. N-glycans were released from a 15-µg aliquot of this murine mAb and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606) following the instructions provided in its care and use manual (715004793). *Rapi*Fluor-MS-labeled N-glycans were prepared for injection at a concentration of 0.5 pmol/µL (as a mixture in a solvent composed of 90 µL SPE eluate, 100 µL dimethylformamide, and 210 µL acetonitrile).

*Rapi*Fluor-MS High Mannose Standard (p/n 186008317) was reconstituted in water to produce a 5 pmol/µL solution. A series of spiked samples were then prepared by mixing *Rapi*Fluor-MS labeled glycans from Intact mAb Mass Check Standard with the *Rapi*Fluor-MS High Mannose Standard and water. In this way, four spiked samples containing 0.45 pmol/µL of sample-derived N-glycans were prepared along with varying concentrations of the high mannose glycans. Spiking produced samples with mannose-5 (M5) at a relative abundance ranging from approximately 0.2% to 2%.

*Rapi*Fluor-MS Dextran Calibration Ladder (p/n 186007982) was reconstituted with 100 µL of water to produce a 0.5 µg/µL solution. The GU assignments were calculated using a cubic spline fitting method and UNIFI 1.7 Software.

LC conditions for *Rapi*Fluor-MS Released N-Glycans

Chromatographic separations were performed using the following conditions, unless otherwise noted:

Universal N-Glycan Profiling Method

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Injection volume:	10 µL for DMF/ACN-diluted samples or 1 µL for aqueous samples
Column:	ACQUITY UPLC Glycan BEH Amide, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile phase A:	50 mM aqueous ammonium formate, pH 4.4 (LC-MS grade water; from a 100x ammonium formate concentrate (p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

mAb N-Glycan Profiling Method

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	45 °C
Flow rate:	0.5 mL/min
Injection volume:	10 µL (DMF/ACN-diluted samples), 1 µL (aqueous samples)
Column:	ACQUITY UPLC Glycan BEH Amide 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate (p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.00	0.5	20	80	
3.00	0.5	27	73	6
35.0	0.5	37	63	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	20	80	6
47.6	0.5	20	80	6
55.0	0.5	20	80	6

MS conditions for *Rapi*Fluor-MS Released N-Glycans	
MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 0.1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray™:	100 fmol/µL human Glu-fibrinopeptide B prepared in a solution composed of 70:30:0.1% water/acetonitrile/formic acid, sampled every 90 seconds
Data management:	MassLynx 4.1 Software, UNIFI 1.7 Software

RESULTS AND DISCUSSION

A Universal N-Glycan Profiling Method

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit facilitates the robust analysis of many, very diverse N-glycans. Accordingly, we first aimed to establish a separation method for an ACQUITY UPLC Glycan BEH Amide Column that can be universally applied to all types of N-glycans, from small biantennary structures up to highly sialylated, tetraantennary species. It is very useful to run this so-called 'universal N-glycan profiling method' when analyzing new samples. Given that this method is the basis of an upcoming glucose unit (GU) database for *Rapi*Fluor-MS labeled glycans, it will also be the technique recommended for future workflows involving GU based peak assignments.

Being a generic tool, the universal N-glycan profiling method is not optimized for any particular N-glycan sample, including the N-glycans obtained from the Intact mAb Mass Check Standard that is provided as a control sample in each GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Since this standard is a mAb expressed from a murine cell line, it is a relevant surrogate to many mAb therapeutics and will, in fact, produce a highly similar N-glycan profile. The universal N-glycan profiling method produces chromatograms where the mAb glycans elute in only the first half of the analytical gradient, as shown in Figures 1A and 1B. Using online, mass spectrometric detection, at least 14 different N-glycans can be readily identified (Table 1). In addition to having been eluted in a narrower retention window, several of these glycans are only partially resolved and consequently are difficult to monitor, and reliably quantitate, on an LC system that is not fully optimized for low extra-column dispersion. Such peaks, or those that are unresolved, require the use of MS detection and an analysis of extracted ion chromatograms (Figures 1C and 1D) to be deciphered.
Notable critical pairs exhibiting at least partial co-elution include M5/A2G1 and FA2G2Sg1/FA2G2Ga2. Given the significance of monitoring M5 and immunogenic glycans, like those containing the noted N-glycolyl neuraminic acid (Sg) and alpha-linked galactose monosaccharides, these separations were optimized for increased resolution between species containing these types of sugars.

	Glycan	*Rapi*Fluor-MS labeled glycan composition	Mi (Da)	2+	3+
1	A2	$C_{67}H_{105}O_{37}N_9$	1627.66	814.84	543.56
2	FA2	$C_{73}H_{115}O_{41}N_9$	1773.72	887.87	592.25
3	M5	$C_{63}H_{99}O37N_7$	1545.61	773.81	516.10
4	FA1G1	$C_{71}H_{112}O_{41}N8$	1732.69	867.35	578.57
	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
5	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
6	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
7	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
8	FA2G2	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
9	FA2G1Ga1	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
10	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
11	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
12	FA2G2Sg1	$C_{96}H_{152}O_{60}N_{10}$	2404.92	1203.46	802.65
13	FA2G2Ga2	$C_{97}H_{155}O_{61}N_9$	2421.93	1211.97	808.32
14	FA2G2Ga1Sg1	$C_{102}H_{162}O_{65}N_{10}$	2566.97	1284.49	856.66

Table 1. RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard, a murine monoclonal antibody.

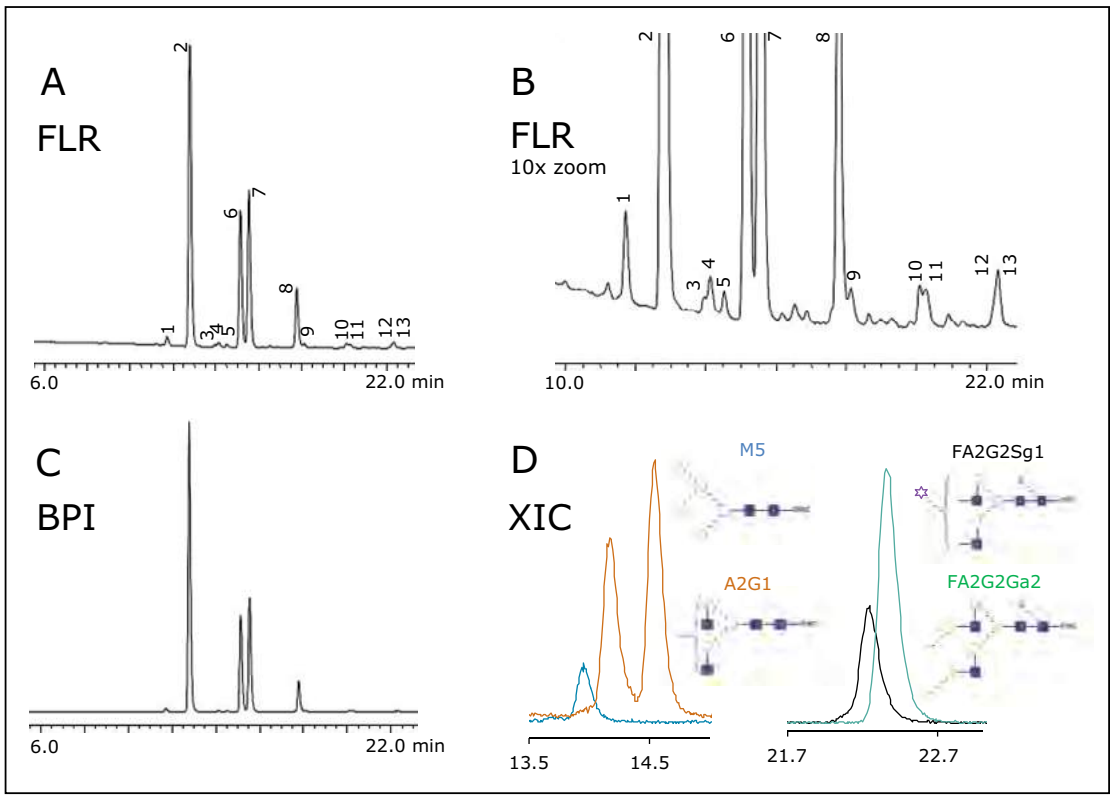


*Figure 1. (A) Fluorescence (FLR) chromatogram of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard obtained using the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) Fluorescence chromatogram scaled with a 10x zoom to show low abundance N-glycans (C) Base peak intensity (BPI) chromatogram of Rapi*Fluor-*MS labeled N-glycans obtained with the universal N-glycan profiling method (D) Extracted ion chromatograms (XICs) of critical pairs to highlight issues with partial co-elution. N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment.*

Developing a Higher Resolution mAb N-Glycan Analysis Method

The significant co-elution of the members of these critical glycan pairs necessitated the development of an LC method specifically tailored for N-glycans released from mAbs. Modifying a HILIC separation by reducing the slope of the gradient can produce more resolution between labeled N-linked glycans with similar partition coefficients. As a result, the first change made to the method was to reduce the gradient slope while retaining the overall run time. A new gradient running from 26%–37% mobile phase A (versus 25% to 46%) indeed showed improvement in peak resolution. In another step, the retention time of the glycans was reduced by increasing the flow rate of the separation from 0.4 to 0.5 mL/min. This flow rate adjustment shifted the mAb N-glycan profile to be well within the gradient window, while at the same time, the maximum pressure of the analysis was maintained well below the pressure limit of the system. Increasing the flow rate of the separation also yielded an improvement in the resolution of the critical pairs (Figures 2A and 2B), but caused an average of a 90% increase in peak widths. This peak broadening was attributed to poor band formation when the injected sample approached the head of the column. To improve band formation, the eluent strength at the onset of the separation was reduced. The gradient was changed to have two segments, an initial ramp from 20% to 27% mobile phase A over 3.2 minutes followed by 27% to 37% mobile phase A over 31.8 minutes. Indeed, these changes facilitated better band formation at the head of the column and obtaining correspondingly sharper glycan peaks (Figure 2C). Despite improving the method in multiple ways, the two noted critical pairs of *Rapi*Fluor-MS labeled N-glycans remained only partially resolved. One final adjustment to the running conditions proved highly effective in improving the resolution of these critical pairs. Lowering the column temperature from 60 °C to 45 °C increased their separation to the point that near baseline resolution could be achieved (Figure 2D). With these final conditions, half-height resolution of the M5/A2G1+FA1G1 and the FA2G2Sg1/FA2G2Ga2 peaks were found to be 1.61 and 1.13, respectively. The extent of separation has been improved for all major species throughout the mAb profile, except for the alpha-linked galactose isomers of the FA2G2Ga1 glycan eluting at approximately 25 minutes.

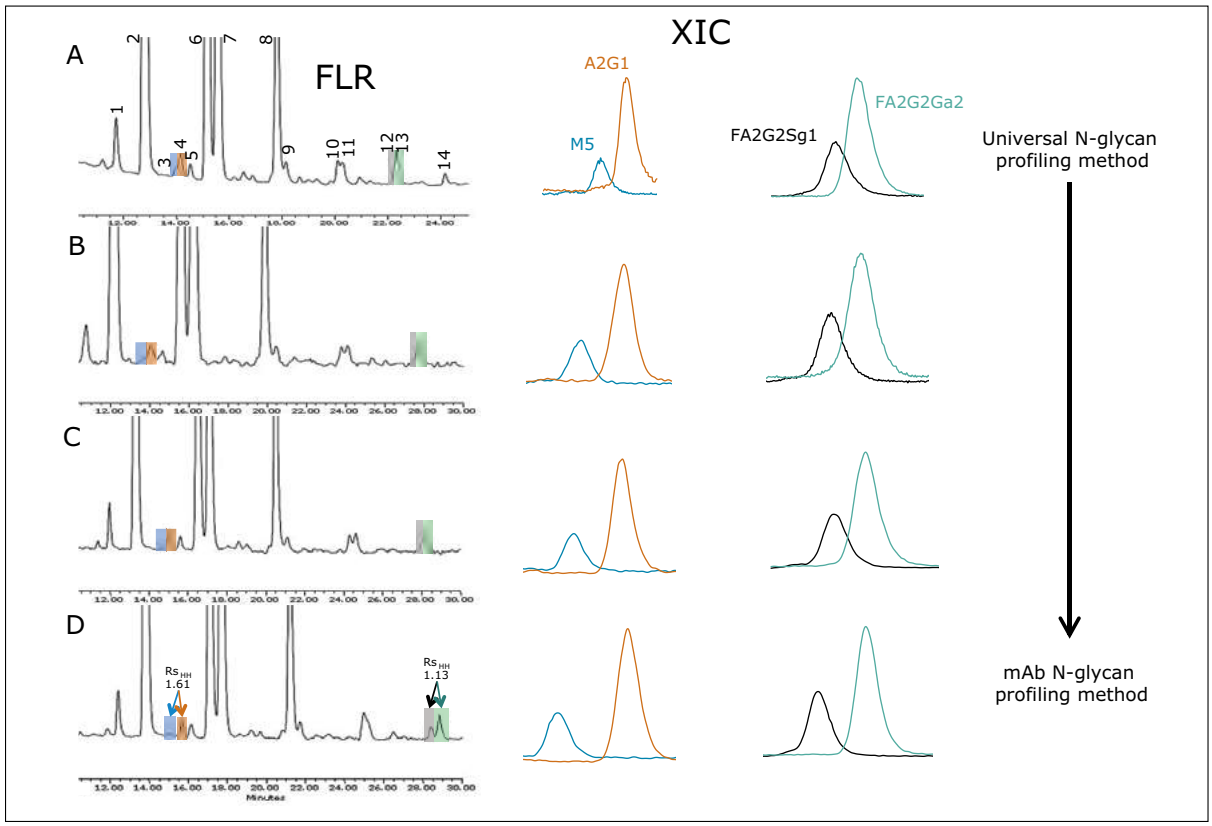
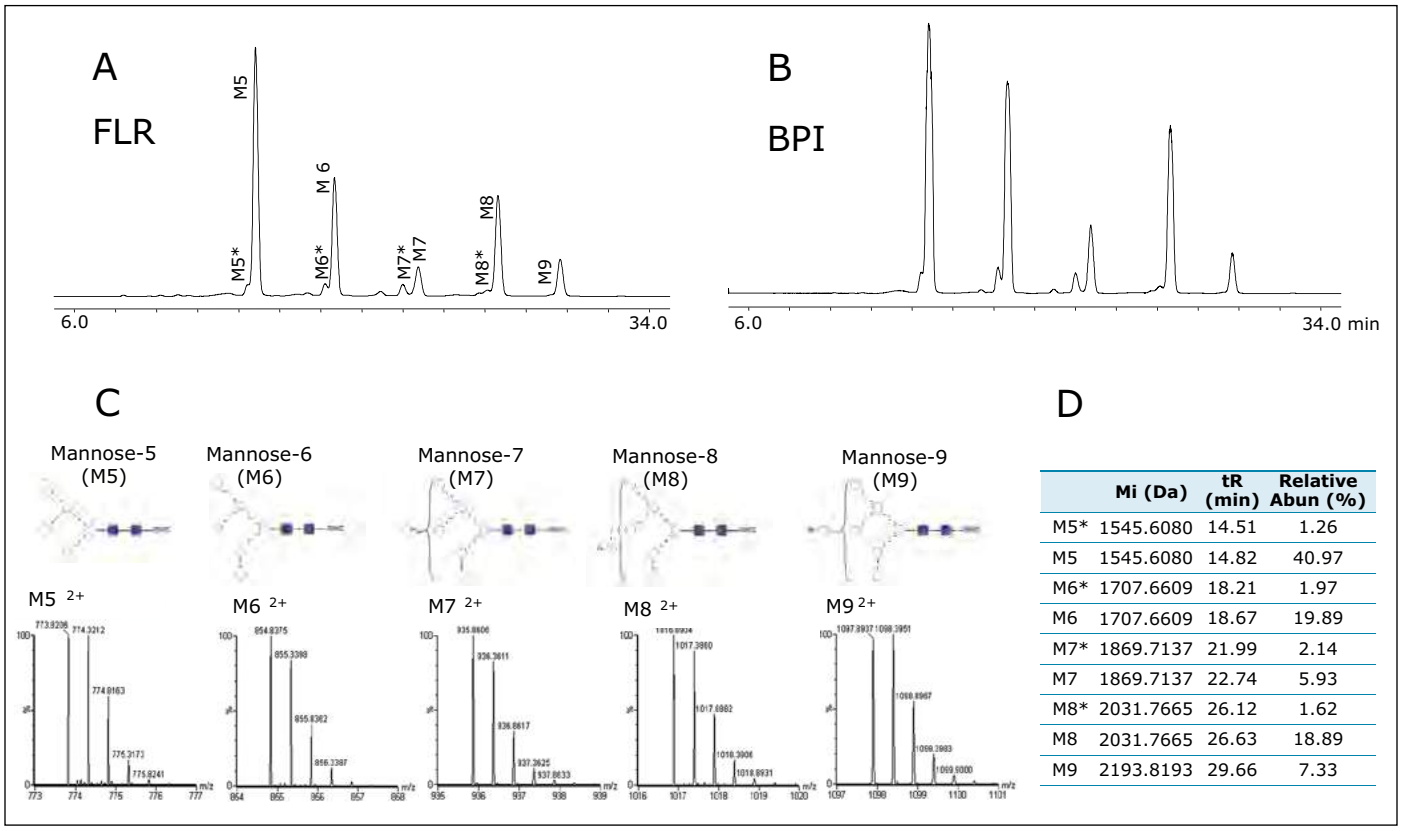


Figure 2. (A) Fluorescence (FLR) chromatogram and extracted ion chromatograms (XICs) obtained with the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a 35 minute gradient from 26% to 37% H_2O (C) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a two-step gradient of 20% to 27% H_2O in 3.2 min followed by 27% to 37% H_2O in 31.8 min. (D) FLR chromatogram and XICs obtained as listed in (C) except with a column temperature of 45 °C (the mAb N-glycan profiling method). N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. RsHH denotes peak resolution measured at half-height (HH).

Using the *Rapi*Fluor-MS High Mannose Standard to Demonstrate System Suitability

The resolution gains afforded by the new mAb N-glycan profiling method allows for better monitoring of high mannose structures. To this end, we have employed a new proficiency standard, the *Rapi*Fluor-MS High Mannose Standard, to demonstrate its ability to precisely monitor high mannose structures. This new standard contains *Rapi*Fluor-MS-labeled M5, M6, M7, M8, and M9. When separated on its own, it is evident that the *Rapi*Fluor-MS High Mannose Standard is a relatively simple mixture (Figure 3).



	Mi (Da)	tR (min)	Relative Abun (%)
M5*	1545.6080	14.51	1.26
M5	1545.6080	14.82	40.97
M6*	1707.6609	18.21	1.97
M6	1707.6609	18.67	19.89
M7*	1869.7137	21.99	2.14
M7	1869.7137	22.74	5.93
M8*	2031.7665	26.12	1.62
M8	2031.7665	26.63	18.89
M9	2193.8193	29.66	7.33

Figure 3. (A) Fluorescence (FLR) and base peak intensity (BPI) chromatograms (B) of the Waters RapiFluor-MS High Mannose Standard as obtained using a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column and the mAb N-glycan profiling method. Asterisks denote isomers of M5, M6, and M7. (C) ESI mass spectra of each major component. (D) Representative chromatographic data. Approximately 5 pmoles of high mannose N-glycans were analyzed in these experiments. An asterisk (*) denotes a linkage isomer.

As a result, it can potentially be used to support the identification of high mannose species when MS detection may not be available. Moreover, this high mannose glycan mixture is well suited for use in spiking studies, which can be performed to establish system suitability. In this work, *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were spiked with varying concentrations of the *Rapi*Fluor-MS High Mannose Standard (as outlined in the experimental section). Four *Rapi*Fluor-MS labeled glycan samples were prepared with M5 relative abundances ranging from 0.2% to 2.0% and analyzed as illustrated in Figure 4A. In these samples, M5, M6, and M8 are readily detected, while M7 and M9 are not due to their lower relative abundances in the spiking standard. The high resolution of the method allows for better integration of the high mannose glycan species. This can be clearly demonstrated by plotting the fluorescence peak areas of M5, M6, and M8 as functions of the spiking level. The linearity of these data ($R^2 \geq 0.974$) underscores the suitability of this technique for monitoring high mannose structures (Figure 4B). These spiking experiments also demonstrate that the Intact mAb Mass Check Standard is effectively free of high mannose species and that the M5 previously monitored during the development of the separation is near the limit of quantitation of this method.

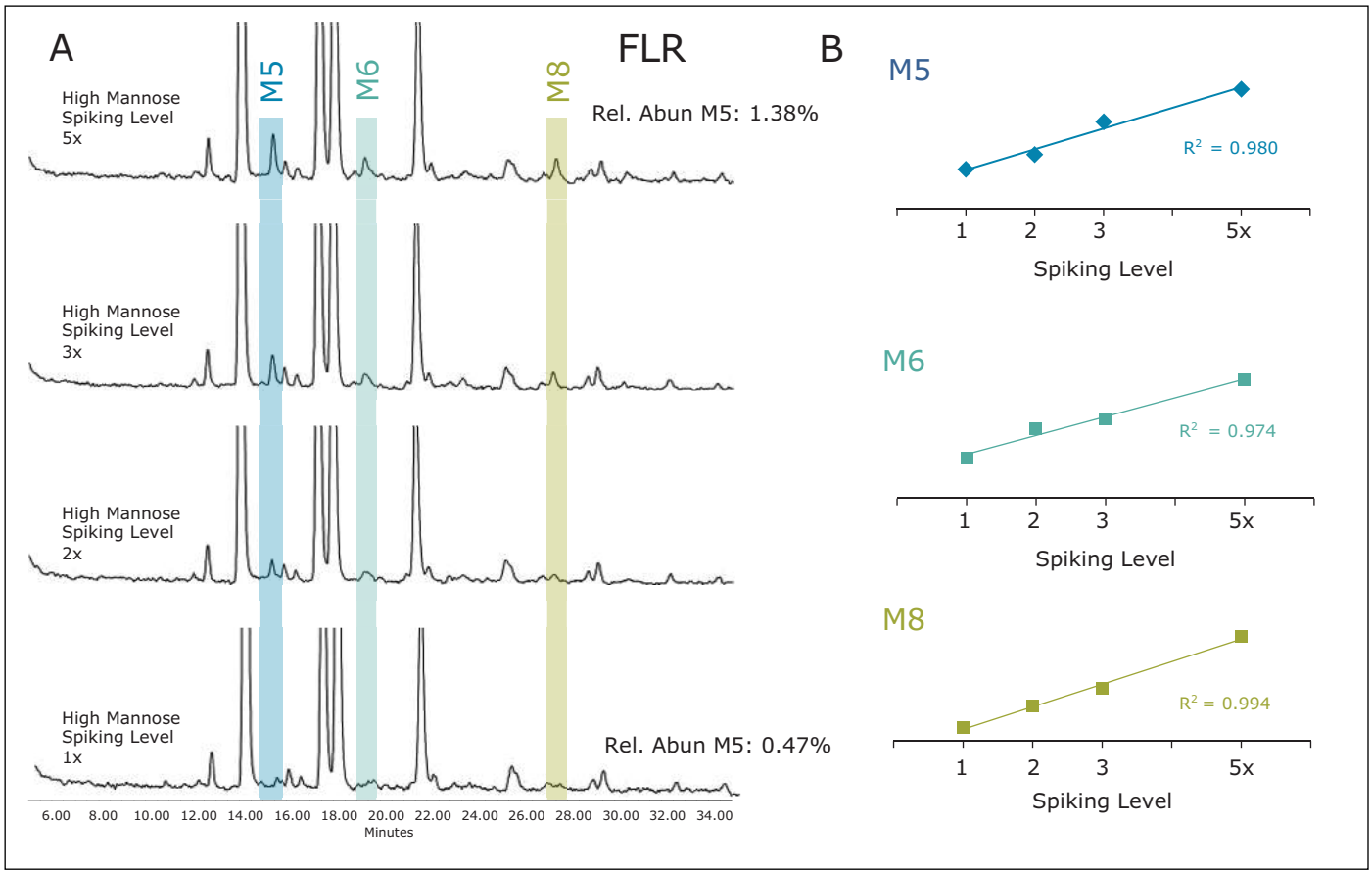


Figure 4. (A) Fluorescence (FLR) chromatogram obtained for RapiFluor-MS N-glycans prepared from Intact mAb Mass Check Standard spiked with varying concentrations of the RapiFluor-MS High Mannose Standard. Top to bottom: spiking levels are 5x, 3x, 2x, and 1x. (B) Peak area of high mannose structures at different spike concentrations. N-glycan samples corresponding to 0.34 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. Separations were performed using the mAb N-glycan profiling method and an ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column.

GU Values from the Universal N-Glycan Profiling Method Versus the mAb N-Glycan Profiling Method

As mentioned earlier, the universal N-glycan profiling method is a generic tool for all N-glycan sample types. It will also be the method recommended for workflows involving assignment of new glycan peaks based on matching GU values to data in an upcoming *Rapi*Fluor-MS GU database, which is currently being constructed in collaboration with the National Institute for Bioprocessing Research and Training (NIBRT).

With this in mind, it is important to recognize that GU values, regardless of labeling strategy, are method specific. So although the mAb N-glycan profiling method can be used with GU values, it will not generate GU values that are meaningful for searching a NIBRT database based on the universal N-glycan profiling method. GU values still have merit for glycan analyses, even if they are not used for database matching. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[3] To assign GU values, a dextran ladder, consisting of glucose multimers of increasing length, is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values. Chromatographic data collected from separations of the *Rapi*Fluor-MS Dextran Calibration Ladder are provided in Table 2, one set of data obtained with the universal N-glycan profiling method and the other with the new, mAb N-glycan profiling method. Not surprisingly, differences between the methods led to shifts in the retention times of the individual glucose multimers. Therefore, GU values derived for the mAb N-glycans are also shifted, as shown in (Table 2). For the most strongly retained species, the FA2G2Ga1Sg1 glycan, there is, in fact, a GU shift of +0.37. Clearly, it is important to give consideration to how GU values are generated and how they are to be used. The universal N-glycan profiling method is the appropriate method for GU database searching. Nevertheless, GU values can be used along with the mAb N-glycan profiling method as replacements to standard retention times to improve the robustness of data reporting.

		Universal N-Glycan Profiling	mAb N-Glycan Profiling
Component	Name	Glucose Units	Glucose Units
1	A2	5.49	5.54
2	FA2	5.82	5.91
3	M5	6.19	6.24
4	FA1G1 + A2G1	6.23	6.37
5	A2G1	6.38	6.49
6	FA2G1	6.69	6.72
7	FA2G1	6.85	6.86
8	FA2G2	7.43	7.69
9	FA2G1Ga1	7.55	7.81
10	FA2G2Ga1	8.25	8.57
11	FA2G2Ga1	8.30	8.60
12	FA2G2Sg1	9.06	9.39
13	FA2G2Ga2	9.11	9.49
14	FA2G2Ga1Sg1	9.88	10.25

Table 2. Glucose unit values for the RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard and the RapiFluor-MS High Mannose Standard, as obtained with the universal N-glycan profiling method versus the mAb N-glycan profiling method. Glucose unit (GU) values were assigned using cubic spline fitting and UNIFI 1.7 Software.

CONCLUSIONS

The N-linked glycosylation of mAbs can impact their circulation half-life and efficacy. Therefore, it is particularly important for the N-glycans of a mAb to be well characterized and routinely monitored. By labeling mAb N-glycans with *Rapi*Fluor-MS, high sensitivity detection by both fluorescence and MS is made possible. The sample loading condition, gradient steepness, flow rate, and separation temperature of the universal N-glycan profiling method were adjusted to create a mAb N-glycan profiling method that was able to better resolve the Man5/A2G1+FA1G1 and FA2G2Sg1/FA2G2Ga2 critical pairs. The mAb N-glycan profiling method yielded a half-height resolution of 1.61 for M5/A2G1+ N-glycans and FA2G2Sg1/FA2G2Ga2 of 1.13. By improving the resolution of these critical pairs of N-glycans, we have provided additional separation space for monitoring high mannose structures. To this end, the *Rapi*Fluor-MS High Mannose Standard was used in a series of spiking experiments to demonstrate the quantitative performance of this new gradient for analyzing high mannose N-glycan structures. Using the mAb N-glycan profiling method in conjunction with the new *Rapi*Fluor-MS High Mannose Standard and the *Rapi*Fluor-MS Dextran Ladder allows for the easier adoption of this system solution.

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HILIC Glycopeptide Mapping with a Wide-Pore Amide Stationary Phase

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APPLICATION BENEFITS

- Orthogonal selectivity to conventional reversed phase (RP) peptide mapping for enhanced characterization of hydrophilic protein modifications, such as glycosylation
- Class-leading HILIC separations of IgG glycopeptides to interrogate sites of modification
- MS compatible HILIC to enable detailed investigations of sample constituents
- Enhanced glycan information that complements *Rapi*Fluor-MS released N-glycan analyses
- Glycoprotein BEH Amide 300Å 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide 300Å Column (patent pending)

Glycoprotein Performance Test Standard

ACQUITY UPLC H-Class Bio System

Waters SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated proteins, glycosylation, HILIC, mab, glycopeptide, glycoprotein

INTRODUCTION

Peptide mapping of biopharmaceuticals has longed been used as a tool for identity tests and for monitoring residue-specific modifications.[1-2] In a traditional analysis, peptides resulting from the use of high fidelity proteases, like trypsin and Lys-C, are separated with very high peak capacities by reversed phase (RP) separations with C_{18} bonded stationary phases using ion-pairing reagents. Separations such as these are able to resolve peptides with single amino acid differences such as asparagine; and the two potential products of asparagine deamidation, aspartic acid and isoaspartic acid.[3-4]

Nevertheless, not all protein modifications are so easily resolved by RP separations. Glycosylated peptides, in comparison, are often separated with relatively poor selectivity, particularly if one considers that glycopeptide isoforms usually differ in their glycan mass by about 10 to 2,000 Da. So, while RP separations are advantageous for generic peptide mapping, they are limited in their ability to resolve hydrophilic modifications. Previous studies have demonstrated that hydrophilic interaction chromatography (HILIC) with an amide-bonded stationary phase can provide complementary and highly resolving separations of glycosylated peptides.[5-6] These studies have demonstrated that amide-bonded stationary phases are particularly effective for these separations, because they afford high retentivity as a consequence of their hydrophilicity and propensity for hydrogen bonding.[7]

Expanding upon this technology, we have developed an amide-bonded stationary phase with a nominally larger pore diameter, a so-called "wide-pore" material, such that amide HILIC separations can be universally applied to separating the glycoforms of both intact and digested glycoproteins. This stationary phase found in ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns ensures that glycopeptides, regardless of their size, will have access to the majority of the porous network and be less prone to restricted diffusion.[8-9] In previous work, we have demonstrated the use of this HILIC column to assay the glycan occupancy of an intact monoclonal antibody (mAb),[10] to map the domain-specific glycosylation of IgG subunits,[11] and to improve the resolution of tri- and tetra-antennary GlycoWorks,™ *Rapi*Fluor-MS™ labeled N-glycans.[12] Here, we explore the use of the Glycoprotein BEH Amide 300Å 1.7 µm Column to produce high resolution HILIC separations of glycopeptides from three different monoclonal antibodies: trastuzumab, cetuximab and an IgG1K candidate reference material from NIST.

EXPERIMENTAL

Sample description

Lys-C digest of trastuzumab and NIST candidate reference material

An adaptation of a previously published single reaction vial, overnight (16+ hours) procedure[4] was employed to prepare non-reduced Lys-C digests of trastuzumab and a IgG1K monoclonal antibody candidate reference material obtained from NIST (#8670, lot# 3F1b). TFA quenched digests were stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

Lys-C/tryptic digest of cetuximab

Reduced and alkylated cetuximab was digested with a combination of Achromobacter protease I (Lys-C) and trypsin. Formulated cetuximab was concentrated to 10 mg/mL and buffer exchanged with a 10 kDa MWCO centrifugal filter (Millipore, Billerica, MA) into a solution of 6 M GuHCl, 50 mM DTT, and 0.2 M phosphate (pH 8.1), then incubated at 37 °C for 2 hours. Thereafter, the sample was diluted with a solution of iodoacetamide, bringing the antibody concentration to 8 mg/mL and the buffer composition to 4.8 M GuHCl, 40 mM DTT, 50 mM iodoacetamide, and 0.17 M phosphate (pH 8.1). Alkylation with iodoacetamide was allowed to proceed under these conditions for 10 min in the dark at 37 °C, before being quenched by the addition of cysteine, diluted with a urea-containing buffer, and mixed with Achromobacter protease I (Lys-C) at a 4:1 w/w ratio. The resulting digest solution of 0.8 mg/mL cetuximab, 0.5 M GuHCl, 3 M Urea, 40 mM NH_2OH, 4 mM DTT, 5 mM iodoacetamide, 6 mM cysteine, and 0.1 M phosphate (pH ~7.1) was incubated at 37 °C. After 2 hours of incubation, this digest solution was diluted two fold with water and an aliquot of trypsin (Sigma T6567), such that the protein:trypsin ratio was 4:1 (w/w). After incubation at 37 °C for another 2 hours, the digest solution was again diluted two fold with water and a fresh aliquot of trypsin. With a total protein:trypsin ratio of 2:1 (w/w), the digest was left to incubate at 37 °C for 16 hours. Following this incubation, the digest was quenched by acidification with TFA and stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

Method conditions

(unless otherwise noted):

Column conditioning

ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns (as well as other amide columns intended for glycoprotein or glycopeptide separations) should be conditioned via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (p/n 186008010; 10 µL injections of 4 mg/mL in 0.1% TFA, 80% ACN) or with equivalent loads of a test sample for which the column has been acquired. The separation outlined by the following method can be employed for conditioning with the Glycoprotein Performance Test Standard.

Column conditioning gradient

2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, H2O

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	33.0	67.0	6
21.0	40.0	60.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

LC conditions for LC-UV-MS of mAb glycopeptides (Figures 1–6):

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	30 °C (trastuzumab Lys-C digest HILIC separations) 60 °C (cetuximab Lys-C/tryptic digest HILIC separations) 60 °C (trastuzumab Lys-C reversed phase separations)

Flow Rate:	0.2 mL/min
Mobile phase A:	0.1% (v/v) TFA, H_2O
Mobile phase B:	0.1% (v/v) TFA, ACN
HILIC injection volume:	100–250 µL (Aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide to obtain a miscible, HILIC compatible diluent.)
Reversed phase injection volume:	24.2 µL (Aqueous digest)
Columns:	ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard)
	ACQUITY UPLC Peptide BEH C_{18} 300 Å 1.7 µm, 2.1 x 150 mm (p/n 186003687)
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient used for reversed phase separations of trastuzumab Lys-C digests (Figure 1A):

Time	%A	%B	Curve
0.0	98.0	2.0	6
96.0	50.0	50.0	6
99.0	20.0	80.0	6
101.0	20.0	80.0	6
102.0	98.0	2.0	6
113.0	98.0	2.0	6

Gradient used for HILIC separations of trastuzumab Lys-C digests and Lys-C/tryptic digests of cetuximab (Figures 1B-6):

Time	%A	%B	Curve
0.0	20.0	80.0	6
60.0	50.0	50.0	6
61.0	80.0	20.0	6
63.0	80.0	20.0	6
64.0	20.0	80.0	6
75.0	20.0	80.0	6

MS conditions for IgG subunit separations

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	Resolution (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	25 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 100–2000 *m/z*
Lockspray:	300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds
Acquisition:	50–2500 *m/z*, 0.1 sec scan rate
Data management:	MassLynx Software (V4.1) / UNIFI V1.7

LC Conditions for a Glycopeptide Mapping of an IgG1K with Fluorescence Detection (Figure 7):

LC system:	ACQUITY UPLC H-Class Bio System
Sampletemp.:	10 °C
Analytical column temp.:	45 °C
Fluorescence detection:	Ex 280/Em 320 nm (10 Hz scan rate, Gain =1)
Injection volume:	100 µL (DMF/ACN diluted sample)
Mobile phase A:	0.1% TFA in water
Mobile phase B:	0.1% TFA in ACN
Columns:	ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 50 mm (p/n 176003702, with Glycoprotein Performance Test Standard)
Other columns:	Column A: 2.6 µm, 2.1 x 150 mm
	Column B: 1.8 µm, 2.1 x 150 mm
Vials:	Polypropylene 12 x 32mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient (Figure 7):

Time (min)	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	30.0	70.0	6
21.0	37.0	63.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

Data management:	UNIFI v1.7

RESULTS AND DISCUSSION

Orthogonal and complementary glycopeptide mapping separations

To demonstrate a conventional approach to peptide mapping, we first performed LC-UV-MS analysis on a Lys-C digest of a mAb using a RP chromatographic separation with a wide-pore C_{18} bonded stationary phase (Peptide BEH C_{18} 300Å 1.7 µm). Trastuzumab was selected for this study, given its prominence as a first generation mAb drug product and a potential target for biosimilar development.[13] Figure 1A shows a UPLC chromatogram that is typical for a Lys-C digest of trastuzumab, wherein peptides are broadly resolved across a separation with a gradient corresponding to a change of 0.5% acetonitrile per minute. The non-glycosylated peptides of the digest spread across the extremes of the chromatogram while the glycopeptides elute in an approximately one minute wide window at a retention time of about 60 minutes. The conditions to produce this high resolution separation involve the use of mobile phases modified with trifluoroacetic acid (TFA); the same mobile phases that have proven to be optimal for HILIC of proteinaceous analytes.[10-11]

Accordingly, an orthogonal method to the RP separation can be achieved via HILIC by simply reversing the gradient and using the newly developed wide-pore amide bonded stationary phase (Glycoprotein BEH Amide 300Å 1.7 µm). An example of a chromatogram obtained from a column packed with this wide-pore amide material and a gradient ramp of 0.5% acetonitrile per minute is shown in Figure 1B. Here, the peptides from the Lys-C digested trastuzumab are very clearly segregated into early and late eluting species, corresponding to the non-glycosylated and glycosylated species, respectively. The use of TFA ion pairing facilitates obtaining this separation, as it masks the hydrophilicity of the peptide residues and provides improved selectivity for the hydrophilic modifications. Also note that the glycopeptides have not only been class separated with the amide column, but the selectivity of the peptide glycoforms is remarkably improved over the analogous RP separation.

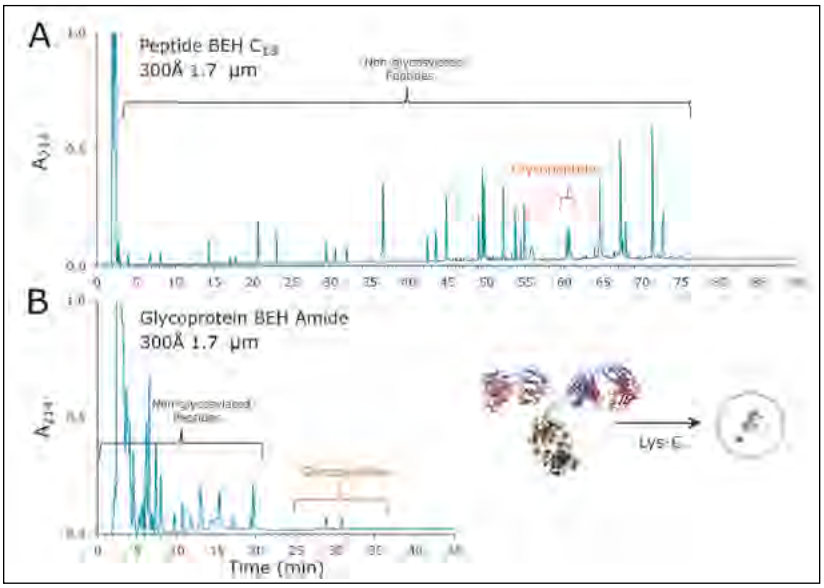

Figure 1. Lys-C glycopeptide mapping of trastuzumab. (A) A traditional reversed phase separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Peptide BEH C_{18} 300Å 1.7 µm Column. (B) A HILIC separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. In each analysis, 9.2 µg of the Lys-C digest was separated using the same gradient slope and injecting sample from a diluent comprised of either approximately 0.2% TFA in 80:20 ACN/water (HILIC) or 100% water (reversed phase).

By focusing on the strongly retained peaks, one can begin to interrogate the glycosylation of the trastuzumab molecule (Figure 2A). In particular, MS data acquired from online mass detection and a total ion chromatogram (TIC) can be applied to identify the peptide species and its corresponding glycoforms, as shown in Figure 2B. This Lys-C glycopeptide map presents a 29 amino acid residue peptide (K16) from the Fc domain of trastuzumab. From an analysis of the MS data, many biantennary structures typical found on mAbs in relatively high abundance can be readily identified (Figure 3A). Further interrogation of the MS data, also shows that low abundance N-glycan species can likewise be detected. Figure 3B, for instance, provides MS data supporting the identification of monosialylated and disialylated glycoforms at retention times of approximately 34.7 minutes and 36.4 minutes, respectively. These identifications correlate extremely well with the released N-glycan profiles of trastuzumab that have been previously reported.[14-15]

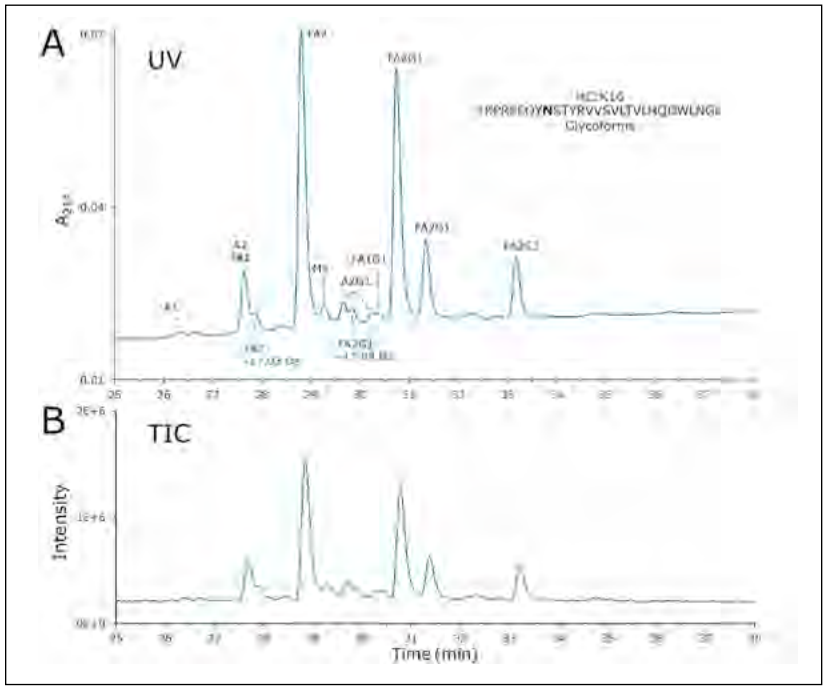

Figure 2. Lys-C glycopeptide mapping of trastuzumab with HILIC and an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. (A) UV chromatogram for the Lys-C glycopeptide retention window. (B) Total ion chromatogram (TIC) for the same retention window.

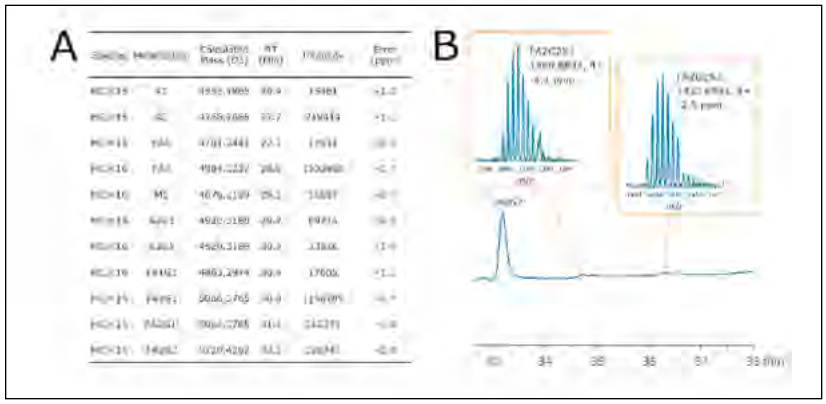

Figure 3. Mass spectrometric data supporting trastuzumab Lys-C glycopeptide identifications. (A) Retention times, MS intensities and mass errors for assignments labeled in Figure 2. (B) MS spectra supporting the identification of low abundance Lys-C glycopeptides modified with mono and di-sialylated N-glycans.

Lot-to-lot analysis of trastuzumab glycosylation via HILIC-UV glycopeptide mapping

HILIC-MS based glycopeptide mapping clearly yields information-rich data. However, these HILIC glycopeptide mapping separations also lend themselves to methods based only on optical detection. We have, for example, applied a HILIC-UV method to perform lot-to-lot analysis of trastuzumab glycosylation for two drug product samples. Representative HILIC chromatograms for glycopeptide K16 obtained from two different lots of trastuzumab are shown in Figure 4A. Previous released glycan analyses on these lots have shown there to be differences in glycosylation.[14] Through comparison of peak areas across the glycopeptide profile, we have found that these two lots of trastuzumab indeed differ with respect to their glycosylation. Specifically, these lots of trastuzumab appear to have different extents of terminal galactosylation, as can be seen in the differing abundances of FA2, FA2G1 and FA2G2 glycoforms (Figure 4B). This observation was consistent with data obtained from previous released glycan analyses and previous HILIC based profiling of trastuzumab subunits.[11]

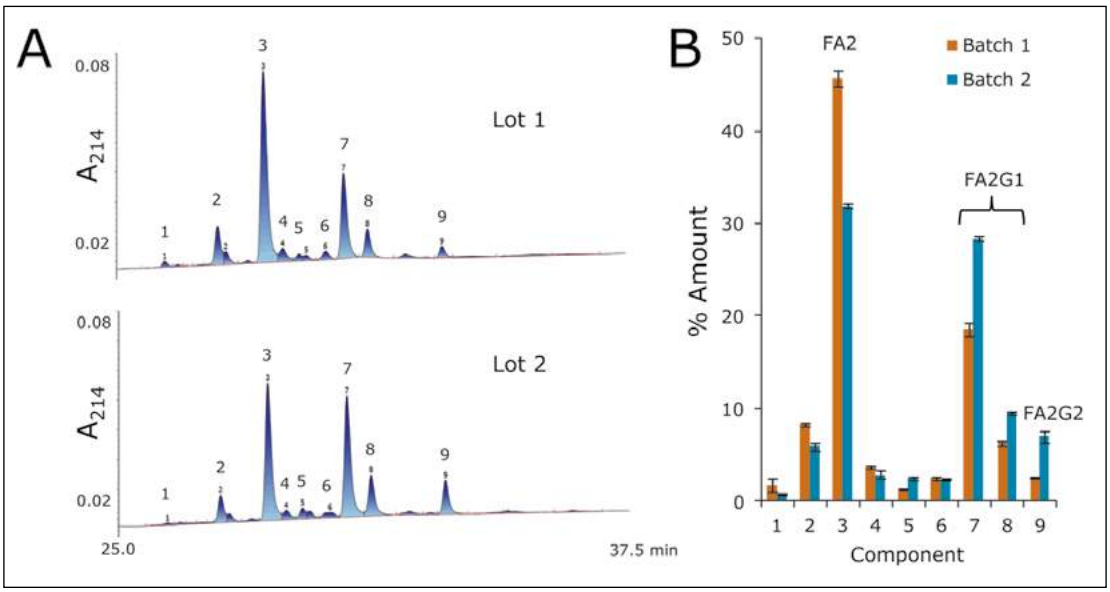

Figure 4. Lot-to-lot profiling of trastuzumab Lys-C peptide glycoforms. (A) HILIC chromatograms of trastuzumab Lys-C glycopeptides from two different lots of drug product. (B) Relative abundances of the major sample components. Analyses were performed in triplicate using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column.

Complementing GlycoWorks *Rapi*Fluor-MS N-glycan analyses with domain and peptide specific information about mAb glycosylation

An appealing aspect of glycopeptide mapping is that it can be applied to the elucidation of domain and peptide specific information. By inference or ETD fragmentation analyses, or both, glycopeptide mapping can also be used to detail the exact sites of glycosylation.[16] As we have noted before,[11] IgGs contain one conserved N-glycosylation site at Asn297 of the heavy chain, meaning they will be modified with two glycans in their Fc subunit. In addition, some IgGs and even some mAb IgG therapeutics exhibit multi-domain glycosylation. Cetuximab, for instance, is glycosylated in both its Fc and Fab domains,[17] making it a very interesting case study for this work.

A HILIC glycopeptide map of a Lys-C/tryptic digest of cetuximab provides a clear indication of the complicated glycan profile of this molecule (Figure 5). In the presented chromatogram, approximately thirty chromatographic peaks are observed. Furthermore, a cursory analysis of the MS data has shown there to be at a minimum twenty five different glycoform species with rather high relative abundances of greater than 1–2%. Figure 6 provides the MS data supporting these assignments. As can be seen, 9 unique glycoforms could be assigned to tryptic peptide T22 from the Fc domain, while the other 16 glycoforms could be assigned to tryptic peptide T8 from the Fab domain of cetuximab. It is interesting to note that the majority of the Fab domain (T8) glycans contain immunogenic epitopes, such as non-human alpa-1,3-galactose or non-human N-glycolyl-neuraminic acid moieties.[18] In previous work, these glycan species were identified through complementary subunit mapping and *Rapi*Fluor-MS released N-glycan analyses.[11] With these results on glycopeptide mapping, we show yet another complementary technique for assessing protein glycosylation.

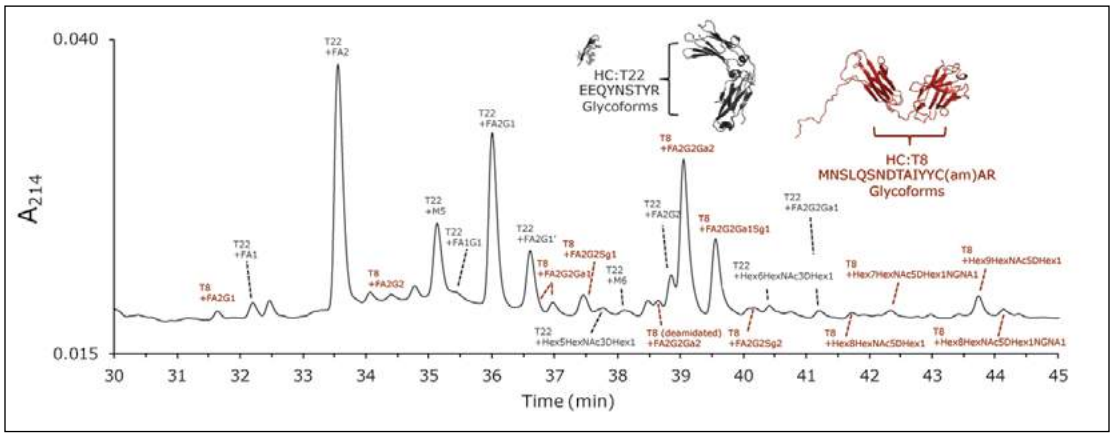

Figure 5. Assaying the N-linked glycan sites of cetuximab and their microheterogeneity using a combined Lys-C/tryptic digest. Assignments for glycoforms of peptide T22 from the Fc domain of cetuximab are shown in dark gray, while assignments to the glycoforms of peptide T8 from the Fab domain are shown in red. Analyses were performed on 9.2 µg of Lys-C/tryptic digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column and a sample diluent of approximately 0.2% TFA in 80:20 ACN/water.

Species	Modification	Calculated Mass (Da)	RT (Min)	Intensity	Error (ppm)
HC:T22	FA1	2429.9592	32.2	9047	1.5
HC:T22	FA2	2633.0386	33.6	263523	1.4
HC:T22	M5	2404.9275	35.2	78988	0.0
HC:T22	FA2G1	2795.0913	36.1	186156	1.2
HC:T22	FA2G1'	2795.0913	36.7	57185	0.7
HC:T22	[Hex5HexNAc3DHex1]	2754.0647	37.8	7783	1.0
HC:T22	M6	2566.9805	38.1	5432	-1.8
HC:T22	FA2G2	2957.1443	38.9	38257	1.1
HC:T22	[Hex6HexNAc3DHex1]	2916.1177	40.5	12246	-1.3
HC:T8	FA2	3350.3689	29.4	5688	4.9
HC:T8	M5	3122.2578	30.4	6437	2.7
HC:T8	FA2G1	3512.4216	31.7	8925	3.2
HC:T8	FA2G2	3674.4746	34.1	11695	8.2
HC:T8	FA2G2Ga1	3836.5273	36.7	9657	-0.6
HC:T8	FA2G2Ga1'	3836.5273	37.0	15605	0.3
HC:T8	FA2G2Sg1	3981.5649	37.5	25532	1.9
HC:T8	FA2G2Ga2	3998.5801	39.1	212644	-0.7
HC:T8	A2G2	3471.3950	39.1	21542	2.0
HC:T8	[Hex7HexNAc5DHex1]	4144.6377	39.2	6944	-10.8
HC:T8	FA2G2Ga1Sg1	4143.6177	39.6	90905	-0.7
HC:T8	FA2G2Sg2	4288.6553	40.2	7428	-2.5
HC:T8	[Hex8HexNAc5DHex1]	4363.7124	41.8	5280	1.7
HC:T8	[Hex7HexNAc5DHex1NGNA1]	4508.7498	42.4	7114	-3.6
HC:T8	[Hex9HexNAc5DHex1]	4525.7651	43.8	20104	4.3
HC:T8	[Hex8HexNAc5DHex1NGNA1]	4670.8027	44.2	7640	1.2

*Hex: hexose, HexNAc: N-acetylate hexosamine, DHex: deoxyhexose, NGNA: N-glycoyl neuraminic acid / (') structural isomer

Figure 6. Mass spectrometic data supporting glycopeptide assignments from the HILIC-UV-MS analysis of Lys-C/trypsin digested cetuximab. From a cursory analysis of MS data, 9 unique glycoforms could be assigned to the Fc domain, and 16 unique glycoforms could be assigned to the Fab domain.

Benchmarking the Capabilities of the Glycoprotein BEH Amide 300Å 1.7 µm Column

The peak capacities obtained in these example glycopeptide separations are particularly noteworthy when a comparison is made to otherwise available column technologies. To benchmark the performance of the Glycoprotein BEH Amide 300Å 1.7 µm Column, we have analyzed a Lys-C digest of a NIST candidate reference material, an IgG1K mAb. In this testing, a focused gradient was used along with intrinsic peptide fluorescence instead of low wavelength UV detection so that higher signal-to-noise could be achieved in the obtained chromatograms. The glycopeptide that originates from the Fc domain of a mAb will contain a tryptophan residue upon Lys-C cleavage, which in large part makes this detection mechanism feasible. Three fluorescence chromatograms obtained for the Lys-C glycopeptides from the NIST IgG1K are presented in Figure 7. These three chromatograms were obtained from the use of the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm as well as two commercially available alternatives (Columns A and B). Peak capacities have been measured for each specific column using the retention windows demarcated by the most extreme glycopeptide retention times (*) and the half-height peak widths of the K16+FA2, K16+FA2G1, K16+FA2G1', K16+FA2G2, and K16+FA2G2Ga1 peaks. This analysis shows that these columns exhibit strikingly different resolving power. With an effective peak capacity of 72.8, the Glycoprotein BEH Amide column shows a superior peak capacity and performance increases over the alternative amide column technologies of 40 and 96%.

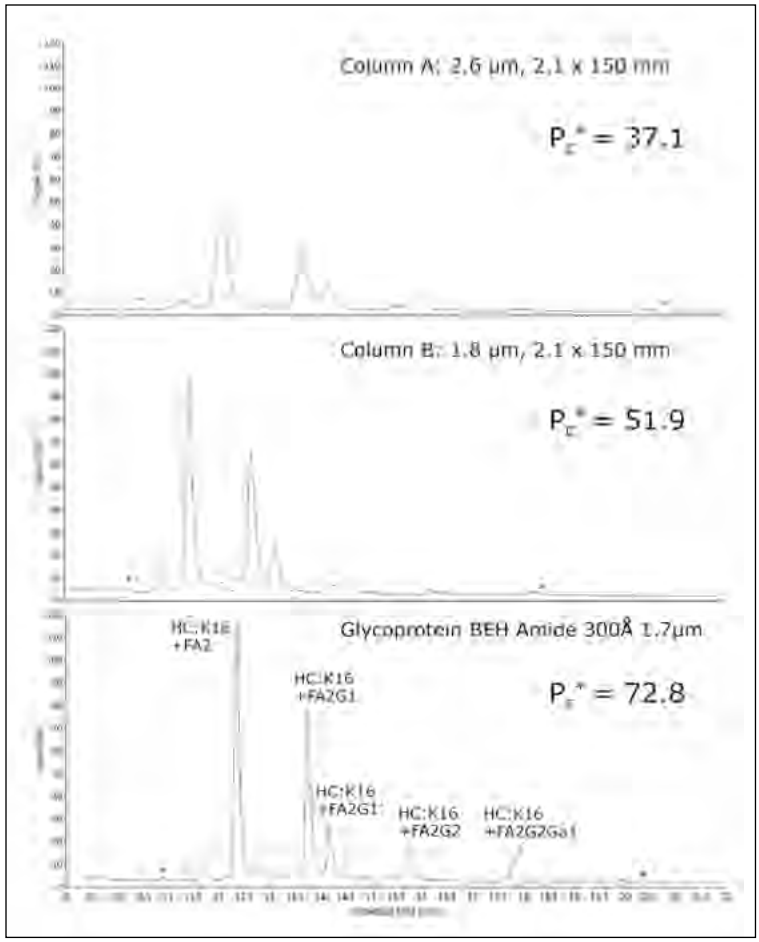

Figure 7. Lys-C glycopeptide mapping of an IgG1K using fluorescence detection and various 2.1 x 150 mm columns packed with amide bonded stationary phase: a Competitor Column A: 150Å 2.6 µm, 2.1 x 150 mm (Top), a Competitor Column B: 1.8 µm, 2.1 x 150 mm (Middle), and on an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm column (Bottom). Peak capacities were calculated based on the half-height peak widths of the labeled glycopeptides and the retention window established by the earliest and latest eluting glycopeptide species, marked with asterisks(*). Comparative separations may not be representative in all applications.

CONCLUSIONS

Glycopeptide mapping of glycoproteins presents a highly effective technique that can be used to elucidate both domain and peptide-specific glycosylation. In this work, we have demonstrated the use of an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column to obtain HILIC separations of glycopeptides that complement the chromatographic information afforded by a reversed phase separation. In addition, our results indicate that these HILIC separations provide exemplary peak capacity in comparison to other commercially available amide column technologies. That the HILIC separation is MS-compatible means that information-rich data can be readily acquired to characterize a glycopeptide map. For instance, this work shows that it can be a relatively straightforward exercise to characterize multidomain protein glycosylation, such as the Fc and Fab domain glycosylation of cetuximab. Combined with other recently developed strategies, such as HILIC subunit mapping and GlycoWorks *Rapi*Fluor-MS released N-glycan analyses, glycopeptide mapping with the ACQUITY UPLC Glycoprotein BEH Amide Column shows significant promise for facilitating the characterization of protein glycosylation to unprecedented levels of detail.

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Quality Control and Automation-Friendly GlycoWorks *Rapi*Fluor-MS N-Glycan Sample Preparation

Stephan M Koza, Scott A McCall, Matthew A Lauber, and Erin E Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- *Rapi*Fluor-MS™ glycan labeling procedure with larger volume, simplified liquid transfer to improve ease of use and automatability.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Intact mAb Mass Check Standard

ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm, Column

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

MassLynx® 4.1 Software

GlycoWorks Rapid Buffer, 5 mL

KEY WORDS

HILIC Chromatography, UPLC,® HPLC, method transfer, N-glycans, *Rapi*Fluor-MS, GlycoWorks

INTRODUCTION

Recently, Waters® introduced a novel labeling reagent, *Rapi*Fluor-MS (RFMS), that provides a fast, efficient, and reproducible sample preparation workflow and unsurpassed fluorescent and MS sensitivity for released N-glycan profiling.[1,2] This initial methodology was designed to accommodate the lowest possible glycoprotein sample concentration and, as result, calls for several low volume (1.2 to 7 µL) liquid transfers. Looking to minimize the impact of pipetting volume inaccuracies, we have redesigned this sample preparation to make pipetting volumes larger (≥10 µL) and thereby reduce the variation in the absolute quantities of analytes and reagents that get delivered during the denaturation, PNGase F deglycosylation, and RFMS labeling steps of the procedure.

In the following work, we demonstrate that RFMS labeled glycan samples prepared using this alternative sample preparation scheme are comparable to those produced by the previously published flexible volume procedure. By virtue of its simplification and use of larger volumes, this protocol should be an excellent fit for adoption of RFMS into Quality Control (QC) environments and automated platforms.

EXPERIMENTAL

Method conditions

LC system:	ACQUITY UPLC H-Class Bio System
Detection:	ACQUITY UPLC FLR Detector with analytical flow cell
Wavelength:	265 nm Excitation, 425 nm Emission
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	10.0 µL
Mobile phase A:	50 mM ammonium formate (pH 4.4) LC-MS grade water, from a 100 x concentrate (p/n 186007081)
Mobile phase B:	LC-MS grade acetonitrile

Gradient:

Time	Flow Rate (mL/min.)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

Sample vials:	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL (p/n 186002640)
Data management:	MassLynx 4.1 Software

MS Conditions for *Rapi*Fluor-MS Released N-Glycans

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~ 40,000)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray:	300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds
Data management:	MassLynx 4.1 Software

RESULTS AND DISCUSSION

Comparison of GlycoWorks *Rapi*Fluor-MS Protocols

The GlycoWorks *Rapi*Fluor-MS sample preparation procedure (Table 1) was developed to allow for maximum flexibility with respect to the concentration of the sample being prepared for released N-glycan analysis.[3] By altering the addition of water, this method is capable of preparing samples with concentrations as low as 0.66 mg/mL. While this procedure was designed with significant molar excesses of the critical reagents, such as denaturant, enzyme, and the RFMS label, to produce reproducible results,[4] a potential drawback of this procedure is that several of the aliquoted volumes are well below 10 µL. As such, the methodology is not as amenable for adoption into certain QC laboratories, depending on their internally imposed method requirements, or for use in specific robotic platforms. Pipetted volume accuracy and precision increases with volume and, based on the International Organization for Standardization (ISO) requirements for mechanical pipette accuracy and precision, it is at 10 µL or more that the lowest permissible systematic and random errors are obtained (Figure 1, Adapted from Reference 5). It should be noted that these maximum permissible errors are doubled for the use of multi-channel pipettes. For this reason, some laboratories prefer to avoid procedures with pipetted volumes lower than 10 µL.

Component	Flexible Volume Standard Tube (1 mL tube)	Flexible Volume PCR Tube (200 µL tube)
2.0 mg/mL sample	7.5 µL	7.5 µL
5% *Rapi*Gest[1]	6.0 µL	3.0 µL
Water	15.3 µL	3.3 µL
PNGaseF	1.2 µL	1.2 µL
Total volume of released N-glycan sample	30 µL	15 µL
RFMS[2]	12.0 µL	6.0 µL
Total volume of the labeled N-glycan sample	42 µL aliquot	21 µL aliquot
ACN dilution	358 µL	179 µL
Total volume of HILIC SPE Load	400 µL	200 µL

Table 1. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit flexible-volume protocols for 1 mL and 200 µL tubes.

RapiGest™ reconstitution: 10 mg with mg w/200 µL buffer or 3 mg w/60 µL buffer

RFMS reconstitution: 23 mg in 335 µL DMF and 9 mg in 131 µL DMF (68.7 µg/µL) for Standard Protocol or 23 mg in 168 µL DMF and 9 mg in 66 µL DMF (136.4 µg/µL) for PCR Protocol

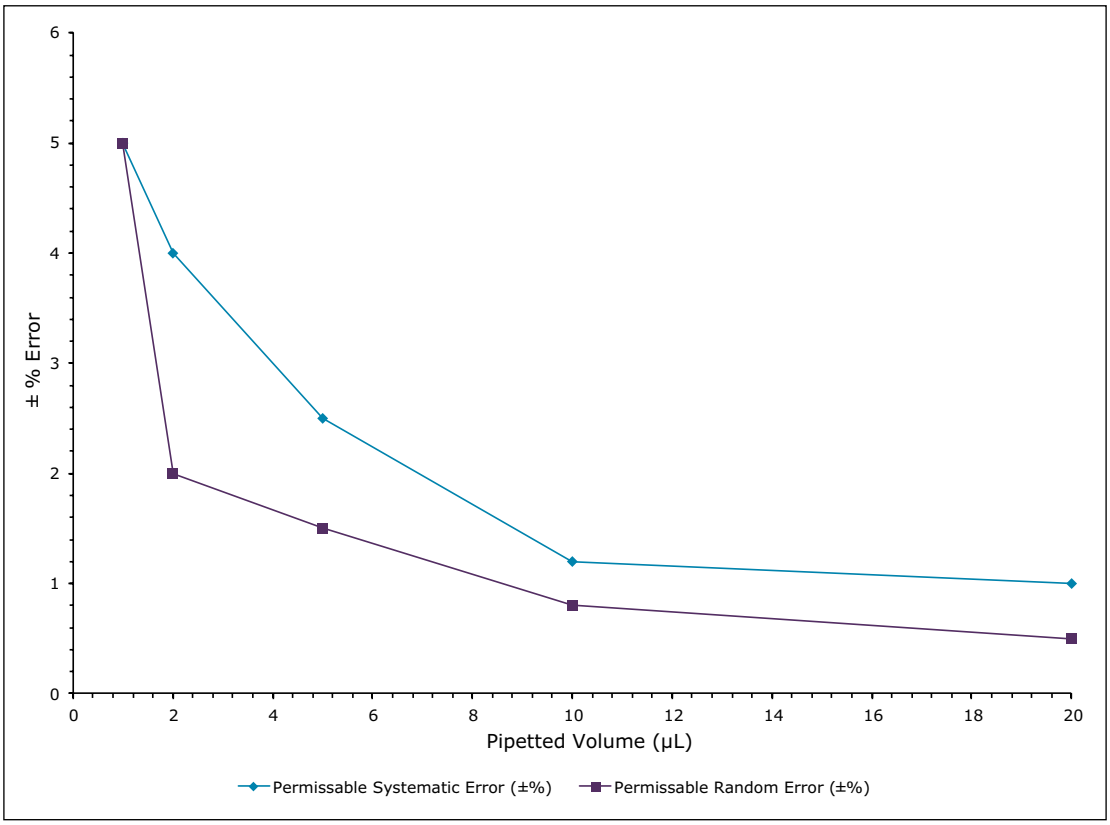

Figure 1. Trends in the maximum permissible pipette volume errors based on ISO 8655.

To provide a protocol with transfer volumes of 10 µL or more, the standard RFMS sample preparation procedure was reconsidered. The primary changes made to the deglycosylation procedure were that the addition of water was removed, and more dilute solutions of *Rapi*Gest™ SF surfactant and PNGase F were used. The final recommended conditions are presented in Table 2. This revised procedure is designed to give optimal results using a 10 µL sample of glycoprotein at a concentration of 1.5 mg/mL (15 µg of glycoprotein), however, samples that are more or less concentrated can still potentially produce quality results. It should be noted that at significant deviations from the optimal sample quantity, i.e. <5 µg and >30 µg, the labeling reaction can produce undesirable side reactions or low yields, so it is recommended to assess these situations on a case-by-case basis.

The only fundamental difference in this revised procedure is that the concentration of *Rapi*Gest SF is slightly higher (1.5%) during the denaturation step versus the previous procedure (1.0%). This increase is not predicted to cause any deleterious effects and may provide some benefit for certain glycoprotein samples that are particularly resistant to denaturation. In the following step, PNGase F deglycosylation, concentrations of the principal components (glycoprotein, *Rapi*Gest SF, and PNGase F) are equivalent to the standard, flexible volume procedure.

In modifying the labeling step, the aliquoted amount of the RFMS label solution was decreased from 12 to 10 µL to be consistent with the other lowest pipetted volumes of the procedure. To account for this volume change, the concentration of the RFMS reagent was increased proportionally such that the final ratio of RFMS to glycoprotein remains equivalent. In addition to the protocol using the 1 mL reaction tubes provided in the kit, this revised procedure, like the previous procedure, has also been adapted for use with a 200 µL thermocycler tube. If using a thermocycler with this new QC and automation friendly protocol, it is necessary to divide the final released and labeled glycan sample into two aliquots, or to transfer sample to a larger tube, prior to dilution and SPE clean-up.

Component	Automated /QC Standard Tube (1 mL tube)	Automated/QC PCR Tube (200 uL tube)
1.5 mg/mL sample	10 µL	10 µL
3% *Rapi*Gest[1]	10 µL	10 µL
Water	0 µL	0 µL
PNGaseF (diluted)[2]	10 µL	10 µL
Total volume of released N-glycan sample	30 µL	30 µL
RFMS[3]	10 µL	10 µL
Total volume of the labeled N-glycan sample	40 µL aliquot	Divide into 2 x 20 µL aliquots
ACN dilution	360 µL	2 x 180 µL
Total volume of HILIC SPE Load	400 µL	200 + 200 = 400 µL

1. *RapiGest reconstitution: 10 mg with mg w/ 200 µL buffer + 135 µL water or 3 mg w/60 µL buffer + 40 µL water*
2. *PNGase F dilution (contents of vial 30 µL + 170 µL water)*
3. *RFMS reconstitution: 23 mg in 280 µL DMF or 9 mg in 110 µL DMF (82.5 µg/µL)*

Table 2. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit automation and QC volume protocols for 1 mL and 200 µL tubes.

Comparing *Rapi*Fluor-MS Labeled N-Glycan Profiles

To compare the standard, flexible volume procedure with its newly designed, QC and automation-friendly analog, analyses of the Waters Intact mAb Mass Check Standard were performed. Samples from this murine monoclonal antibody were prepared and analyzed following the two different protocols along with 1 mL sample tubes and single-channel pipettes. A comparison of representative chromatograms for each of these sample preparations are presented in Figure 2. The labeled peaks were integrated and the quantitative results are presented in Figure 3. The glycan species observed in this profile were assigned using online ESI-MS detection with a Xevo G2-XS QTof Mass Spectrometer (Table 3). As can be clearly seen, the two procedures produce both qualitative and quantitative results that are comparable and reproducible for peaks with relative abundances as low as 0.06%.

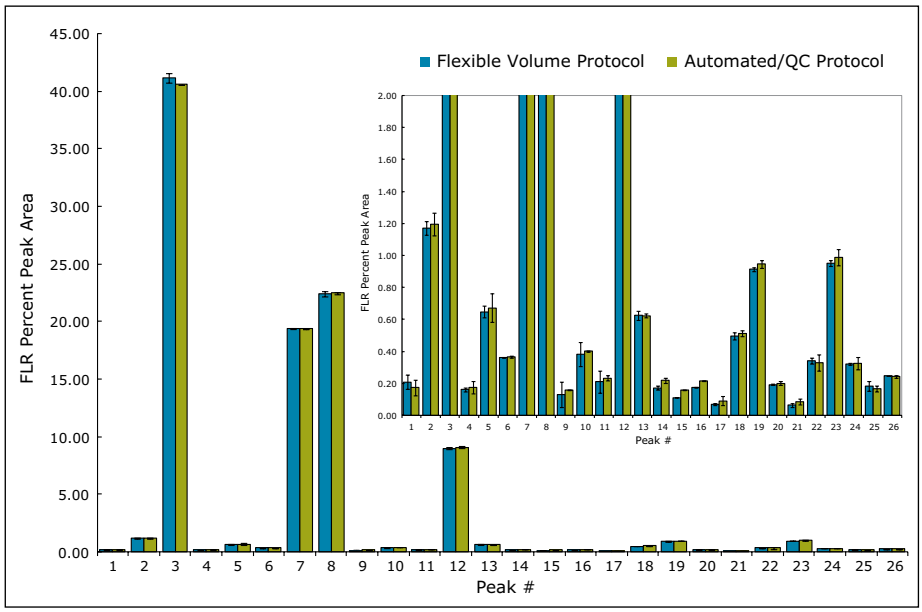

Figure 2. Quantitative comparison of RFMS labeled glycans prepared using GlycoWorks RapiFluor-MS Kit flexible volume protocol and proposed automation and QC volume protocol. Inset shows zoomed view of results.

Peak #	Peak ID
1	FA1
2	A2
3	FA2
4	M5
5	FA1G1 A2G1
6	A2G1 (iso)
7	FA2G1
8	FA2G1 (iso)
10	FA2G1B
11	FA2G1B (iso)
12	FA2G2
13	FA2G1Ga1
14	FA2BG2
18	FA2G2Ga1
19	FA2G2Ga1 (iso)
22	FA2G2Sg1
23	FA2G2Ga2
24	Fa2G2GaSg1
25	Fa2G2GaSg1 (iso)
9, 15, 16, 17, 20, 21, 26	unidentified

Table 3. Figure 1 peak identifications based on mass (Xevo G2-S QTof).

CONCLUSIONS

The GlycoWorks *Rapi*Fluor-MS N-Glycan sample preparation procedure has been successfully adapted to be more amenable to automation and QC use by adjusting pipetted volumes to ≥10 µL. This supplemental procedure requires a glycoprotein sample concentration of 1.5 mg/mL to obtain optimal results. As an added benefit, the dispensed aliquots of the sample and principal reagents are equivalent in volume (10 µL), thereby providing greater assurance that the relative amounts of these components will be equivalent regardless of the systematic accuracy of the pipetting device that is used, which should result in greater intra-laboratory and inter-laboratory reproducibility.

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Comprehensive Characterization of the N- and O-Linked Glycosylation of a Recombinant Human EPO

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Two facile strategies to elucidate information about both the N and O-linked glycosylation of EPO
- Unprecedented HILIC separations of high antennarity released N-glycans and intact protein glycoforms
- MS compatible HILIC to enable detailed investigations of sample constituents
- ACQUITY UPLC® Glycoprotein BEH Amide Column (300Å, 1.7 µm stationary phase) is QC tested via a glycoprotein separation to ensure consistent batch-to-batch reproducibility

WATERS SOLUTIONS

ACQUITY UPLC Glycoprotein BEH Amide, 300Å Column (patent pending)

Glycoprotein Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof
Mass Spectrometer

SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated protein, glycosylation, O-Linked, N-Linked, HILIC, *Rapi*Fluor-MS Labeling

INTRODUCTION

The immunoglobulin G (IgG) modality has paved the way for many efficacious protein-based therapies.[1] At the same time, numerous highly effective patient therapies have also been made possible by the production of recombinant, human hormones and enzymes. For example, erythropoesis stimulating therapeutics, like epoetin (EPO) alpha, have long been available for the treatment of anemia. Such a therapy for increasing patient red blood cell counts was first made possible by the commercialization of Epogen,® which has been available in the US market since its approval by the FDA in 1989.[2] And now, because the landscape of the biopharmaceutical industry continues to evolve and Epogen patents expired in 2013,[3] EPO drug products are targets for being developed into both international and domestic-market biosimilars.

Epoetin alpha has a relatively small primary structure, yet it has 3 sites of N-glycosylation and 1 site of O-glycosylation (Figure 1).[4] Because of its glycosylation, epoetin alpha has a molecular weight between 30 and 40 kDa even though its protein mass amounts to only 18 kDa. Interestingly, the glycosylation of epoetin is very much tied to its potency and serum half life. Two attributes of its glycan profile that are known to show positive correlations with *in vivo* activity include antennarity and sialylation.[5-7] As a result, it is critical for the glycosylation of an epoetin therapeutic to be well characterized. In addition, the significance of epoetin glycosylation suggests that detailed glycan profiling would be a path toward establishing a viable epoetin biosimilar.

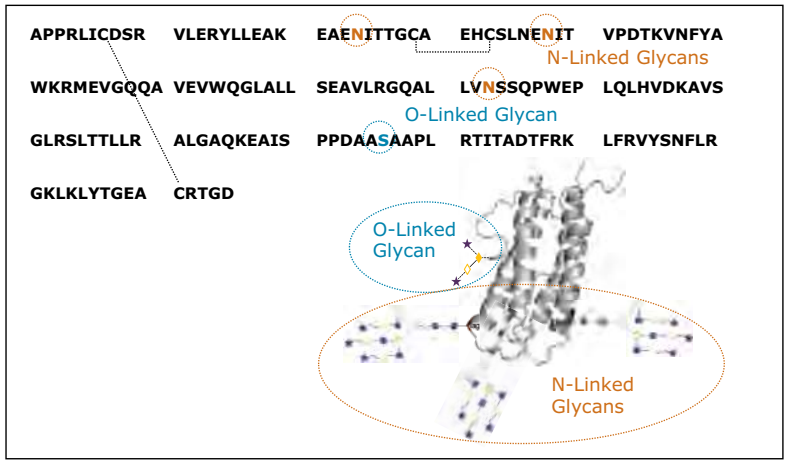

Figure 1. Sequence and structural information for recombinant, human epoetin alpha (rhEPO).

EXPERIMENTAL

Sample description

A recombinant, human epoetin alpha expressed from CHO cells (PeproTech, Rocky Hill, NJ) was reconstituted in 50 mM HEPES NaOH pH 7.9 buffer to a concentration of 2 mg/mL.

N-glycans were released from rhEPO and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and the instructions provided in its care and use manual (p/n 715004793). *Rapi*Fluor-MS labeled N-glycans were injected as a mixture of 90 µL SPE eluate, 100 µL dimethylformamide, and 210 µL acetonitrile.

To facilitate analysis of O-glycosylation, rhEPO was N-deglycosylated using the rapid deglycosylation technique outlined in the care and use manual of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 715004793).

Method conditions (unless otherwise noted)

Column Conditioning

New (previously unused) ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns should be conditioned via two or more sequential injections and separations until a consistent profile is achieved. The care and use manual of the column can be referred to for more information (p/n 720005408EN).

LC conditions for *Rapi*Fluor-MS Released N-Glycans

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Column:	ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard)
Fluorescence detection:	Ex 265 nm / Em 425 nm, 2 Hz
Sample collection/ Vials:	Sample Collection Module (p/n 186007988) Polypropylene 12 x 32 mm Screw Neck vial, 300 µL volume (p/n 186002640)
Mobile phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

MS conditions for *Rapi*Fluor-MS Released N-Glycans

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray:	300 fmol/µL Human glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/ acetonitrile every 90 seconds
Data management:	MassLynx® Software v4.1

LC conditions for Intact Protein HILIC of N-Deglycosylated rhEPO

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	45 °C
Flow rate:	0.2 mL/min
Fluorescence detection:	Ex 280 nm/Em 320 nm (Intrinsic fluorescence), 10 Hz

Mobile phase A:	0.1% (v/v) TFA, H_2O
Mobile phase B:	0.1% (v/v) TFA, ACN
HILIC injection volume:	1.3 µL (A 2.1 mm I.D. HILIC column can accommodate up to an ~1 µL aqueous injection before chromatographic performance is negatively affected)
Columns:	ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column (p/n 176003702, with Glycoprotein Performance Test Standard)
Vials:	Polypropylene 12 x 32 mm Screw Neck, 300 µL volume (p/n 186002640)

Gradient:

Time	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	25.0	75.0	6
21.0	35.0	65.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

MS conditions for for Intact Protein HILIC of N-Deglycosylated rhEPO

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	Resolution (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source offset:	50 V
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–5000 *m/z*
Acquisition:	700–4800 *m/z*, 1 sec scan rate
Data management:	MassLynx Software v4.1

In this application note, we demonstrate the use of two facile strategies that can be used to detail the N and O-linked glycosylation of a recombinant, human epoetin (rhEPO). In this work, rhEPO N-glycans were rapidly released, labeled with GlycoWorks *Rapi*Fluor-MS and profiled by hydrophilic interaction chromatography (HILIC) using sensitive fluorescence and mass spectrometric detection. Then, in a second, parallel analysis, N-deglycosylated rhEPO was interrogated by intact protein HILIC to elucidate information on O-glycosylation.

RESULTS AND DISCUSSION

Released N-Glycan analysis of rhEPO using *Rapi*Fluor-MS labeling and HILIC profiling

The glycosylation of recombinant, human epoetin (rhEPO) has been investigated many times before.[4-5, 8-13] In large part, these previous studies have required relatively involved techniques. With this work, it was our objective to establish two facile and complementary, LC based approaches for the analysis of EPO, one capable of providing information about N-glycosylation and the other information about O-glycosylation.

A profile of the N-glycans from rhEPO can be readily obtained with a new sample preparation strategy involving the novel glycan labeling reagent, *Rapi*Fluor-MS. This sample preparation, based on the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, allows an analyst to rapidly release N-glycans and label them with a tag that provides enhanced sensitivity for fluorescence and electrospray ionization mass spectrometric (ESI-MS) detection.[14] In previous applications, *Rapi*Fluor-MS has been predominately used in the analysis of different IgG samples.[14-16] Nevertheless, using the protocol from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, an analyst can successfully prepare samples from even heavily glycosylated proteins, such as rhEPO.

*Rapi*Fluor-MS labeled N-glycans have proven to be amenable to hydrophilic interaction chromatography (HILIC). Accordingly, HILIC-fluorescence-MS of *Rapi*Fluor-MS has emerged as a very powerful tool for detailing the N-glycosylation of proteins.[14]

To this end, a sample of *Rapi*Fluor-MS N-glycans derived from rhEPO was profiled using HILIC. A recently introduced widepore amide column, the ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7µm Column, was selected for this work to obtain high resolution N-glycan separations. This column was purposefully designed to facilitate HILIC separations of large molecules, such as glycopeptide and glycoproteins. However, the widepore particle architecture has also been shown to increase the peak capacity of highly branched, tri- and tetra-antennary N-glycans by 10–20%,[17] making it an ideal choice for the HILIC profiling of EPO N-glycans, which typically exhibit high antennarity. Figure 2A shows the HILIC fluorescence and base peak intensity (BPI) MS chromatograms of the *Rapi*Fluor-MS N-glycans resulting from 0.4 µg of rhEPO. Even with this relatively limited amount of sample, high signal-to-noise chromatograms are obtained. The sensitivity of the fluorescence trace allows for accurate, relative quantitation across the profile. The signal-to-noise of the MS chromatogram is also particularly noteworthy, though it should be noted that MS sensitivity decreases as N-glycan structures become larger. Nevertheless, the quality of these particular data is made possible by use of the *Rapi*Fluor-MS reagent in combination with the Xevo G2-XS QTof, a new generation MS instrument with improved transmission efficiency and sensitivity. This QTof technology provides unprecedented sensitivity as well as high mass resolution, as can be observed in the collection of mass spectra in Figure 2B that have been used to support the assignment of various N-glycan species.

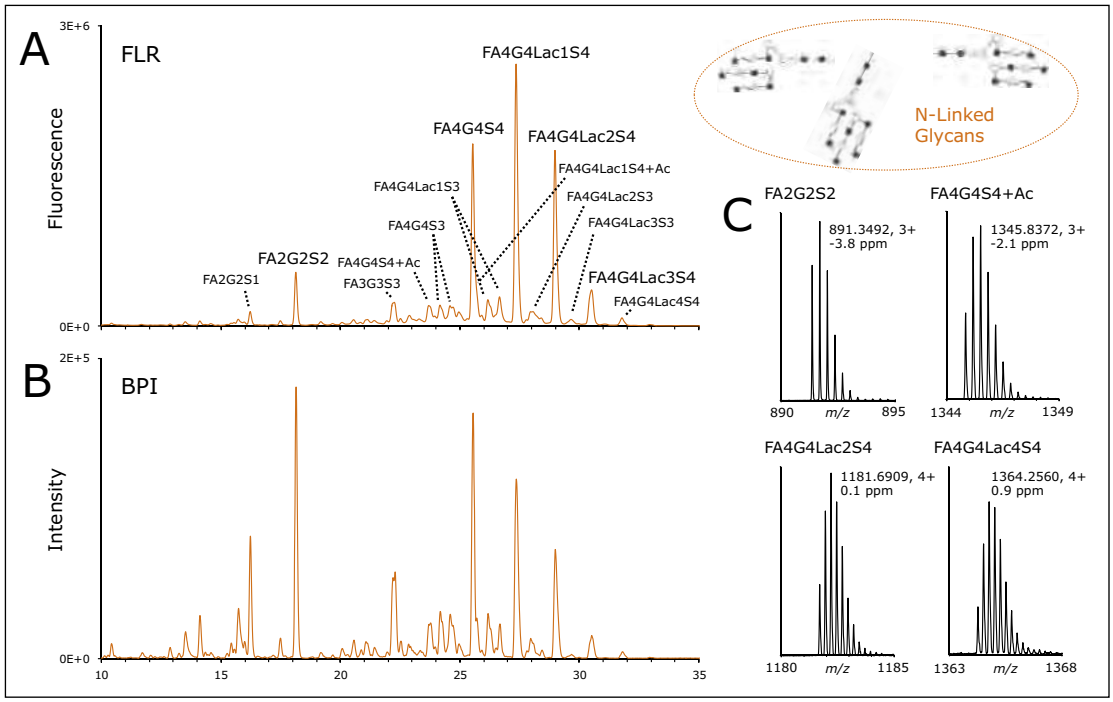

Figure 2. HILIC profiling of released N-glycans from rhEPO. (A) Fluorescence and (B) base peak intensity (BPI) chromatograms for RapiFluor-MS labeled N-glycans from rhEPO. Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (C) MS spectra for four example N-glycan species. N-glycan assignments are listed according to Oxford notation. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

The chromatographic and MS-level selectivity afforded by this analysis simplifies making N-glycan assignments such that the species of the rhEPO N-glycan profile were easily mapped (Figure 3).

The rhEPO analyzed in this study exhibits an N-glycan profile comprised primarily of tetra-antennary, tetrasialylated N-glycans (FA4G4S4) with varying N-acetyl lactosamine extensions. However, the profile also shows a highly abundant peak that corresponds to a disialylated, biantennary N-glycan (FA2G2S2). Given that the ratio of tetra-antennary to biantennary N-glycans has a positive correlation with the *in vivo* activity of an EPO,[6] this analysis has clearly produced valuable information. Other information that can be readily obtained from this N-glycan analysis includes the degree of sialylation and the extent to which structures are modified with lactosylamine extensions. Overall, these results demonstrate that a very-information rich N-glycan profile can indeed be obtained from a comparatively simple *Rapi*Fluor-MS N-glycan preparation and a corresponding HILIC-fluorescence-MS analysis.

RT (min)	Species	$MW_{Mono, Theo}$ (Da)	Observed *m/z*	*z*	$MW_{Mono, Obs}$ (Da)	Mass error (ppm)
16.21	FA2G2S1	2388.9201	1195.4659	2	2388.9172	1.2
18.12	FA2G2S2	2680.0155	894.3492	3	2680.0258	-3.8
22.24	FA3G3S3	3336.2432	1113.0924	3	3336.2554	-3.7
23.68	FA4G4S4 + Ac	4034.4813	1345.8372	3	4034.4898	-2.1
24.15/24.60	FA4G4S3	3701.3754	1234.7966	3	3701.368	2.0
25.52	FA4G4S4	3992.4708	1331.8309	3	3992.4709	0.0
25.7	FA4G4Lac1S4 + Ac	4399.6135	1467.5425	3	4399.6057	1.8
26.16/26.66	FA4G4Lac1S3	4066.5076	1356.5104	3	4066.5094	-0.4
27.34	FA4G4Lac1S4	4357.6030	1090.4097	4	4357.6097	-1.5
27.95	FA4G4Lac2S3	4431.6397	1108.9143	4	4431.6281	2.6
28.97	FA4G4Lac2S4	4722.7352	1181.6909	4	4722.7345	0.1
29.66	FA4G4Lac3S3	4796.7719	1200.2004	4	4796.7725	-0.1
30.50	FA4G4Lac3S4	5087.8674	1272.976	4	5087.8749	-1.5
31.77	FA4G4Lac4S4	5452.9996	1364.256	4	5452.9949	0.9

Figure 3. LC-MS data supporting the identification of various released N-glycan species. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

Profiling the O-Glycosylation of Intact rhEPO using a Widepore Amide HILIC Separation

O-linked glycans can be challenging to characterize due to the paucity of high fidelity mechanisms to release them from their counterpart proteins. Released glycan analysis is an attractive approach for the characterization of N-glycans because of the simplicity and effectiveness of PNGase F deglycosylation. In place of using an analogous, universal glycosidase, analysts have resorted to releasing O-linked glycans by chemical means, such as alkaline beta elimination[18] or hydrazinolysis.[19] These release mechanisms can be challenging to implement and can very often produce artifacts, known as peeling products.

Rather than attempt a released O-glycan analysis of rhEPO, we looked to develop an alternative characterization strategy. A novel workflow was devised that first involved subjecting the rhEPO to rapid deglycosylation using GlycoWorks Rapid PNGase F and 1% *Rapi*Gest™ SF surfactant. In a 10-minute preparation, a sample of N-deglycosylated intact rhEPO was obtained that could then be profiled via a HILIC separation with an ACQUITY UPLC Glycoprotein BEH Amide Column. Figure 4 presents the chromatogram obtained in this analysis using intrinsic fluorescence detection and intact protein HILIC techniques that have been described in previous work.[20] The N-deglycosylated rhEPO analyzed in this study resolved into a series of approximately 10 peaks. Online ESI-MS provided highly detailed information, allowing for proteoforms of rhEPO to be assigned to the various chromatographic peaks. The two most abundant LC peaks were found to be represented by deconvoluted masses of 18893.8 and 19185.3 Da, which are consistent with N-deglycosylated rhEPO that has a C-terminal arginine truncation as well as trisaccharide and tetrasaccharide O-linked glycan modifications, respectively. More specifically, the mass shift observed for the lighter species is indicative of a glycan modification comprised of 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. Meanwhile, the mass shift observed for the heavier species suggests a glycan modification comprised of the same structure with an additional N-acetyl neuraminic acid.

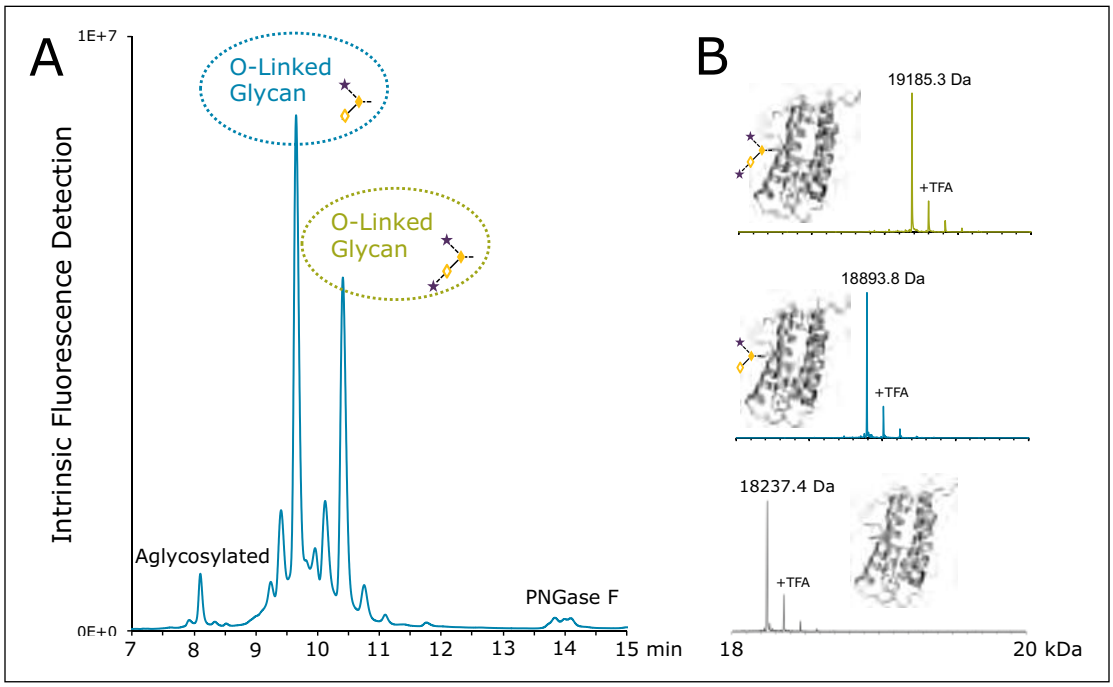

Figure 4. HILIC-fluorescence-MS analysis of N-deglycosylated, intact rhEPO. (A) Fluorescence chromatogram demonstrating O-linked glycan heterogeneity and occupancy. Chromatograms obtained from 0.7 µg protein using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Deconvoluted mass spectra corresponding to three of the major rhEPO proteoforms. Peak identifications, in addition to those denoted here, are tabulated in Figure 5.

Further investigation of the LC-MS data also showed that the proteoform of rhEPO that is aglycosylated with respect to the O-linked glycan eluted with a retention time of approximately 8.2 min. Moreover, these LC-MS data indicated there to be at least two additional O-linked glycoforms and even more C-terminal truncation proteoforms (Figure 5). Here, it is seen that this workflow can indeed be used to rapidly profile the O-linked glycosylation of an rhEPO, such that information is gained about both occupancy and heterogeneity.

RT (min)	Species	$MW_{Avg, Theo}$ (Da)	$MW_{Avg, Obs}$ (Da)	Mass Error (Da)
8.0	N-deglycosylated, –C-term GDR	18066.5	18065.2	-1.3
	N-deglycosylated, –C-term DR	18123.6	18122.4	-1.2
8.2	**N-deglycosylated, –C-term R**	**18238.7**	**18237.4**	**-1.3**
9.3	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1+Ac**	**18937.3**	**18936.2**	**-1.1**
9.5	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac1	18723.1	18722.3	-0.8
	N-deglycosylated, –C-term DR +Hex1HexNAc1Neu5Ac1	18780.1	18779.1	-1.0
9.7	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1**	**18895.2**	**18893.8**	**-1.4**
9.9	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2+Ac**	**19228.5**	**19227.3**	**-1.2**
10.0	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac1 + O**	**18911.2**	**18910.0**	**-1.2**
10.2	N-deglycosylated, –C-term GDR +Hex1HexNAc1Neu5Ac2	19014.3	19013.7	-0.6
10.5	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2**	**19186.5**	**19185.3**	**-1.2**
10.8	**N-deglycosylated, –C-term R +Hex1HexNAc1Neu5Ac2 + O**	**19202.5**	**19201.2**	**-1.3**

Figure 5. LC-MS data supporting the identification of various N-deglycosylated rhEPO proteoforms. "–C-term" denotes the C-terminal truncation of the rhEPO; losses of different residues are noted. Hex, HexNAc, and Neu5Ac stand for hexose, n-acetylhexosamine, and N-acetylneuraminic acid. For example, Hex1HexNAc1NeuN5Ac1 corresponds to O-glycosylation involving 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. "+O" denotes a mass shift indicative of the addition of an oxygen atom, such as an oxidation or an exchange of Neu5Ac for Neu5Gc.[8] Data supporting identifications of the most abundant rhEPO sequence variant (–C-term R) and its glycoforms are highlighted with bold text. "+Ac" denotes an acetylation, such as the previously reported O-acetylation of sialic acid residues (Neu5Ac).[8]

CONCLUSIONS

Several powerful tools have recently emerged for the analysis of glycans that are built upon LC-MS compatible hydrophilic interaction chromatography (HILIC). At the heart of these new glycan analysis workflows is a HILIC column that has been purposefully designed for large molecule separations. With this ACQUITY UPLC Glycoprotein BEH Amide Column, an analyst can achieve higher resolution separations of large, released N-glycans. And when this analysis is paired with *Rapi*Fluor-MS labeling, a technique is established that affords not only high resolution but also unprecedented sensitivity. This approach has been successfully applied to obtain highly detailed information about the N-glycosylation of a recombinant, human epoetin alpha (rhEPO). Given that N-glycosylation correlates with the half life and activity of an EPO, such information, with its unparalleled quality, would be invaluable in developing a new EPO therapeutic. EPO is also O-glycosylated; the occupancy and heterogeneity of which could also be critical to demonstrate comparability among different drug substances. Using the ACQUITY UPLC Glycoprotein BEH Amide Column, we have outlined a simple sample preparation and subsequent HILIC separation that is capable of profiling these O-glycan attributes on intact rhEPO. In summary, we have demonstrated the use of two facile strategies that can be used to detail both the N and O-linked glycosylation of recombinant, human epoetin (rhEPO), a molecule which has been perceived to be challenging to characterize due to its relatively complicated glycosylation. Collectively, these tools could be used to accelerate the development of new biosimilars.

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Rapidly Monitoring Released N-Glycan Profiles During Process Development Using *Rapi*Fluor-MS and the ACQUITY QDa Detector

Eoin F.J. Cosgrave, Robert Birdsall, and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapid feedback on glycoprofiles during production to ensure product quality
- Reduced sample preparation times for released N-glycans
- Increased throughput for N-glycan analysis
- Specificity for N-glycan species by incorporating mass detection

WATERS SOLUTIONS

*Rapi*Fluor-MS™ Glycan Performance Test Standard (p/n 186007983)

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class Bio System (FTN)

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Columns

Empower®3 Chromatography Data Software

Waters® Fraction Manager – Analytical

KEY WORDS

Glycans, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS, IgG

INTRODUCTION

As glycosylated biotherapeutics move through the development pipeline, the glycoprofile and N-glycan species present are characterized. In addition, as new protein therapeutics progress through development, manufacturing conditions are carefully studied and evaluated during scale-up to ensure consistent safety and efficacy in preparation for clinical studies, and eventual commercialization. As part of this process, the critical quality attributes are often monitored closely to ensure production batches remain within defined acceptance criteria, and to identify those parameters that are critical, often as part of a quality-by-design (QbD) approach. In particular, the N-glycan profile is often monitored closely due to the importance of glycans on the safety and efficacy of protein biotherapeutics.

Monitoring of released N-glycan profiles has historically been burdened with labor intensive sample preparation, which often takes several hours to days. This makes monitoring of the impact of manufacturing conditions on N-glycan profiles challenging. In addition, the analysis of released and labeled N-glycans frequently requires long analysis times. When monitoring of specific structures is desired, users often rely on optical detection for identification and quantification.

In this application note we present the use of *Rapi*Fluor-MS, a novel reagent for rapidly labeling released N-glycans. *Rapi*Fluor-MS dramatically reduces overall released N-glycan sample preparation times to 30 minutes, while improving fluorescence signal by up to 14x and MS signal by up to 1000x, compared to traditional labeling techniques. In conjunction with reduced sample preparation times, we geometrically scaled a highly resolving chromatographic method to one having a total cycle time of 10 minutes. Finally, we incorporated the ACQUITY QDa Detector to monitor specific glycan species using selected ion recording (SIR), which provides a selective means of monitoring species, even if they co-elute. We will discuss how the combination of *Rapi*Fluor-MS and the ACQUITY QDa Detector provides a powerful solution for obtaining meaningful data rapidly and efficiently.

EXPERIMENTAL

Released N-glycans were prepared from commercially available trastuzumab following the protocol provided within the *Rapi*Fluor-MS sample preparation kit. High mannose species used in spiking studies were isolated from RNase-B following release and labeling with *Rapi*Fluor-MS. Mannose species were chromatographically separated and collected using the Waters Fraction Manager – Analytical. Collected samples were dried down using a CentriVap and reconstituted in water. For each analysis the mass load was approximately 32 pmol of released and labeled N-glycan on column. LC-MS grade acetonitrile and water were purchased from Pierce. Ammonium formate was prepared using Ammonium Formate Solution-Glycan Analysis (p/n 186007081) by pouring the entire contents of the solution into 1 L of water and mixed. The UPLC® system used was dedicated for applications which do not require non-volatile salts to reduce the likelihood of adduct formation in the mass detector.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR ACQUITY QDa
Columns:	High resolving method: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742) High throughput method: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004740)
Column temp.:	60 °C
Sample temp.:	10 °C

FLR settings

Data rate:	5 points/sec
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500 – 1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	400 °C
Mode:	Positive ion
Mobile phase A:	Acetonitrile (Pierce, LC-MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC-MS Grade, ammonium formate concentrate)
Mobile phase C:	Acetonitrile (LC-MS Grade)
Mobile phase D:	Acetonitrile (LC-MS Grade)

Gradient table high resolution method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

Gradient table high throughput method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.800	75	25	0	0
5.8	0.800	54	46	0	0
6.1	0.400	0	100	0	0
6.6	0.400	0	100	0	0
7.1	0.400	75	25	0	0
8.0	0.800	75	25	0	0
10.0	0.800	75	25	0	0

Data management

Empower 3 Chromatography Data Software (CDS)

RESULTS AND DISCUSSION

During characterization of released N-glycans, a highly resolving method is often used to provide accurate identification and quantification of the species present in samples. While these methods can be effectively scaled, there is a corresponding loss in resolution as overall run time decreases when using the same chromatograph and particle size column. Often, some loss in resolution will be tolerated if the benefit of speed is achieved, however critical structures must remain clearly identifiable. As shown in Figure 1, moving from a higher resolving 55 min method to a 10 min high throughput method preserves much of the resolution between N-glycan species, however there is loss of resolution between the indicated peaks when moving to the shorter method. This loss of resolution complicates accurate monitoring by optical detection as there is no ability to discriminate between two species.

Due to the improved MS response, we introduced the use of the ACQUITY QDa Detector as part of the detector stream to selectively monitor each of the species present in the sample. By using the SIR function of the ACQUITY QDa Detector we were able to collect independent chromatographic traces for each of the components to overcome the challenge of using optical only detection. As shown in Figure 2, we can clearly discriminate between different glycoforms by using selected ion recording. For each species the corresponding peak, or peaks for species with resolved positional isomers, can be clearly identified and integrated for quantification.

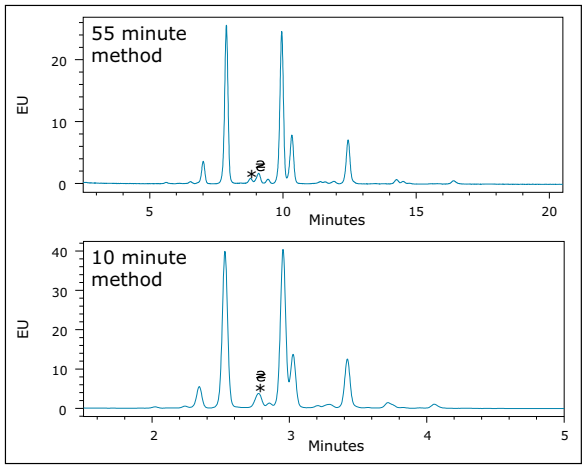

Figure 1. A high resolution separation (top chromatogram) was scaled to a high throughput method by scaling the gradient geometrically while reducing column length and flow rate. While resolution is reduced, selectivity remains constant.

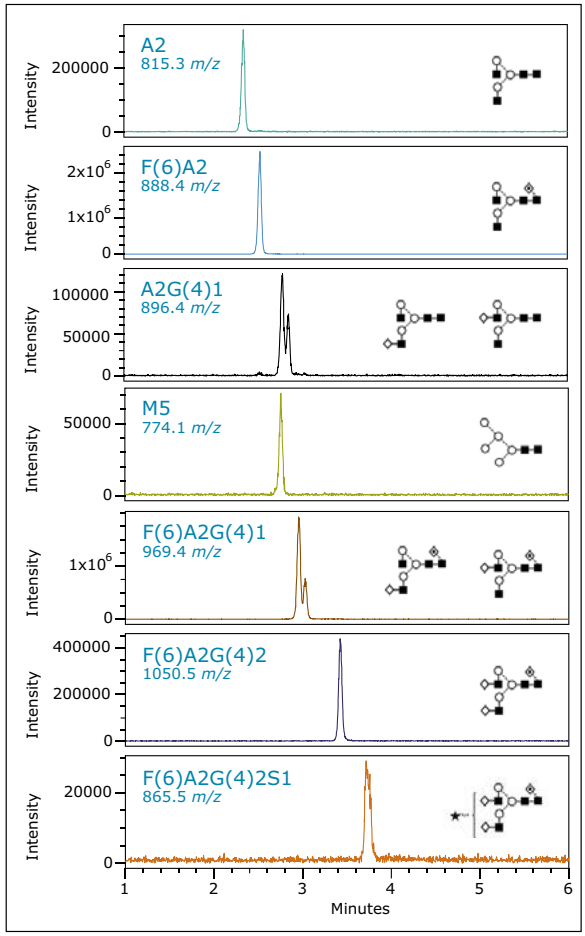

Figure 2. Selected ion chromatograms for N-Glycan species separated using high throughput method. Co-eluting M5 and A2G(4)1are easily discriminated by mass detection.

With a high throughput method developed and the ability to selectively monitor particular species with mass detection, we investigated the linearity of response for the target N-glycan species Mannose 5 (M5). As described in the experimental section, the *Rapi*Fluor-MS labeled M5 species was isolated from the labeled N-glycan pool of RNAse-B. After collection, the collected material was dried and reconstituted in water. The reconstituted sample was added to a *Rapi*Fluor-MS labeled released N-Glycan sample from trastuzumab at various levels. We investigated the linearity of the response by selectively monitoring the peak area of M5 in relation to the volume added to the sample. As shown in Figure 3, the chromatographic reproducibility was quite good. In addition, the peak area for each volume added was highly linear (Figure 4), strongly indicating that the mass detector provides a response suitable for quantification.

After determining the linearity of response for spiked M5 species, we simulated a bioreactor process in which the relative amount of M5 was increasing. For this study we selected the A2G1 species as the reference for relative quantification and spiked in increasing amounts of M5. As shown in Figure 5, the abundance of A2G1 (right column) remains largely constant over the course of the study while the M5 species (middle column) increases as expected with increased spiking levels. In addition, the FLR trace (left column) demonstrates an increase in peak area for these two species (labeled peak), however in the absence of mass information the precise cause of this increase cannot be determined.

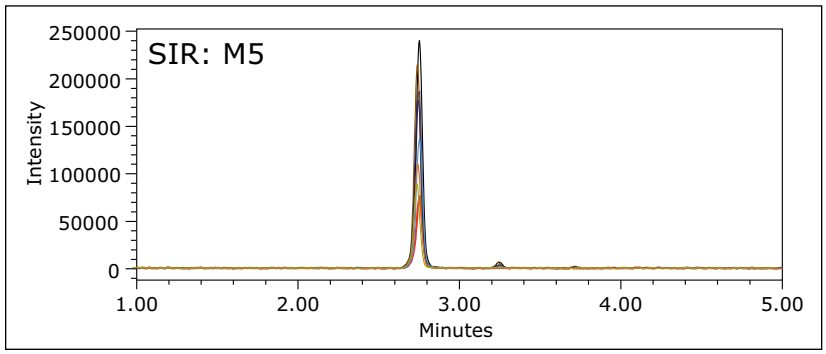

Figure 3. Overlay of chromatograms over a range of M5 spike levels. Spiked amounts ranged from 1-6 µL of reconstituted M5. Absolute concentrations were not determined.

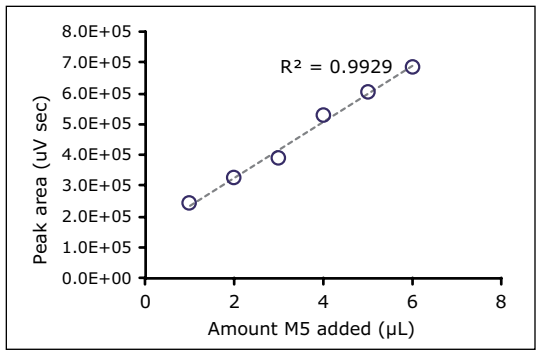

Figure 4. Plot of amount of M5 added vs. peak area for spike M5 samples (data shown in Figure 3).

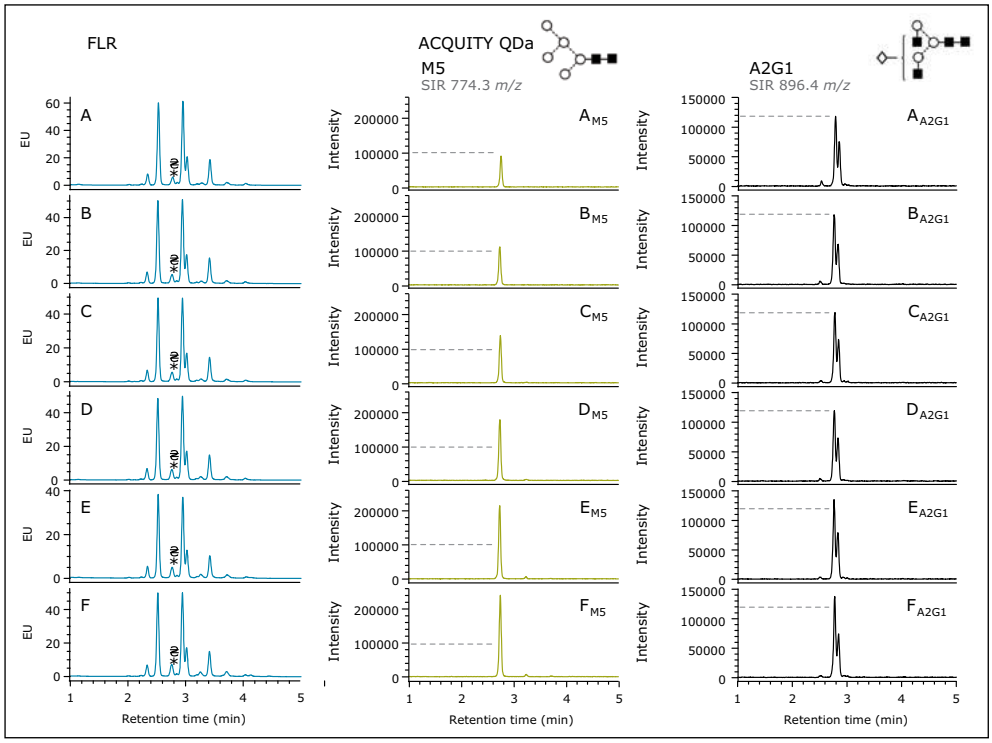

Figure 5. SIR for co-eluting glycan structures. Left: fluorescence profiles of trastuzumab N-glycans with increasing M5 (A to F). The indicators denote the retention times for co-eluting glycans M5 and A2G1. Middle: SIR of M5 for each of the glycan samples A to F. Right: SIR for the co-eluting structure, A2G1. Use of ACQUITY QDa SIR enables the quick determination of glycan structure responsible for changing peak area in fluorescence profiles.

CONCLUSIONS

For routine high throughput assays, *Rapi*Fluor-MS with the ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Detector provides a novel approach for accurately monitoring released N-glycan species. Reduced sample preparation times and greatly improved MS response when coupled with the ACQUITY UPLC and ACQUITY QDa enable the ability to more closely monitor released N-glycan profiles, something which has previously not been possible. While FLR detection was used in this example, for high throughput methods requiring only relative quantification this may not be needed as each species can be monitored with the ACQUITY QDa Detector. As discussed here, complete sample preparation and analysis can be completed in 40 minutes. In addition to reproducible sample preparation, separation and quantification are reproducible and quantitative, allowing scientists to make meaningful decisions rapidly.

Reference

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MAIN MENU TABLE OF CONTENTS

Characterization of EPO N-Glycans Using *Rapi*Fluor-MS and HILIC Profiling

Rajasekar R. Prasanna, Rajiv Bharadwaj, Matthew A. Lauber, Stephan M. Koza, and Erin E. Chambers
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Waters Corporation, Milford, MA, USA

GOAL

To elucidate the complex N-glycans from erythropoietin using the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit and hydrophilic interaction chromatography (HILIC).

BACKGROUND

Erythropoietin (EPO) is a highly glycosylated protein hormone that stimulates the production of red blood cells. EPO exhibits significant heterogeneity due to its multiple sites of glycosylation (three N-glycosylation sites at Asn 24, 38 and 83 and one O-glycosylation site at Ser 126) and the fact that each of these sites can bear various highly branched sialylated N-glycan structures.[1-2] As a consequence of these post-translational modifications, an EPO will have an apparent SDS-PAGE molecular weight between 30 and 40 kDa, 40% of which corresponds to glycan content. Not surprisingly, the glycosylation of an EPO has been found to impact its therapeutic characteristics, namely its stability, efficacy and potency.[1, 3-5] *In vivo* studies using glyco-engineered EPO have shown links between the safety and efficacy of a therapeutic EPO and several glycosylation associated critical quality attributes (CQA), perhaps with sialic acid content being the most important. Sialylated oligosaccharides have been shown to be associated with increased half-life in plasma as compared to desialylated forms which tend to be cleared within minutes.[1, 3-5]

The analysis of EPO N-glycans is facilitated by the high fluorescence and MS sensitivity afforded by *Rapi*Fluor-MS labeling.

Recombinant human EPO (rhEPO) expressed using Chinese hamster ovary (CHO) cells has been used efficiently in the treatment of anemia, since Epogen was approved by the FDA in 1989.[6] As patents for EPO therapeutics approach expiration, the market for biosimilar rhEPO is expected to increase exponentially. Accordingly, there is a need for efficient and accurate methods that can be used for the characterization of EPO glycosylation.

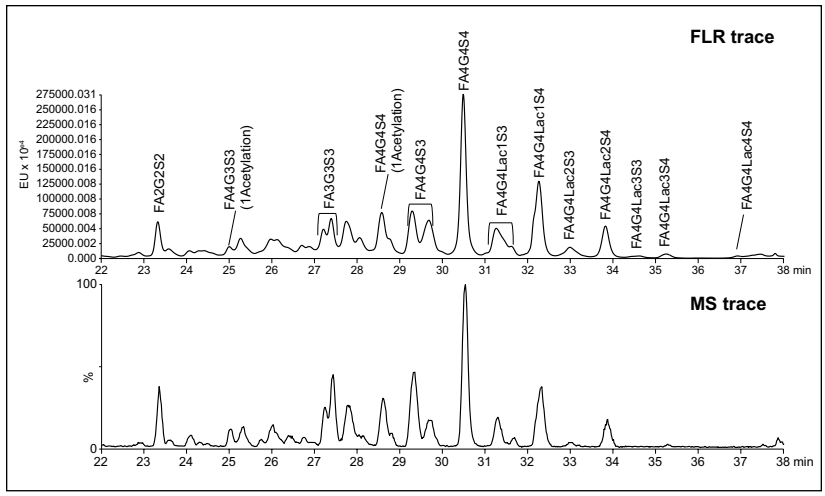

Figure 1. HILIC-FLR-MS of RapiFluor-MS labeled N-glycans. N-glycans from 0.5 µg of an rhEPO were separated using a 2.1 x 150 mm, ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm Column, mobile phases comprised of 50 mM ammonium formate (pH 4.4) (A) and acetontrile (B), and a column temperature of 60 °C. The separation was performed using a 35 minute gradient from 25% A to 46% A. Additional details on the method can be found in the GlycoWorks RapiFluor-MS Care and Use Manual (p/n 715004793).

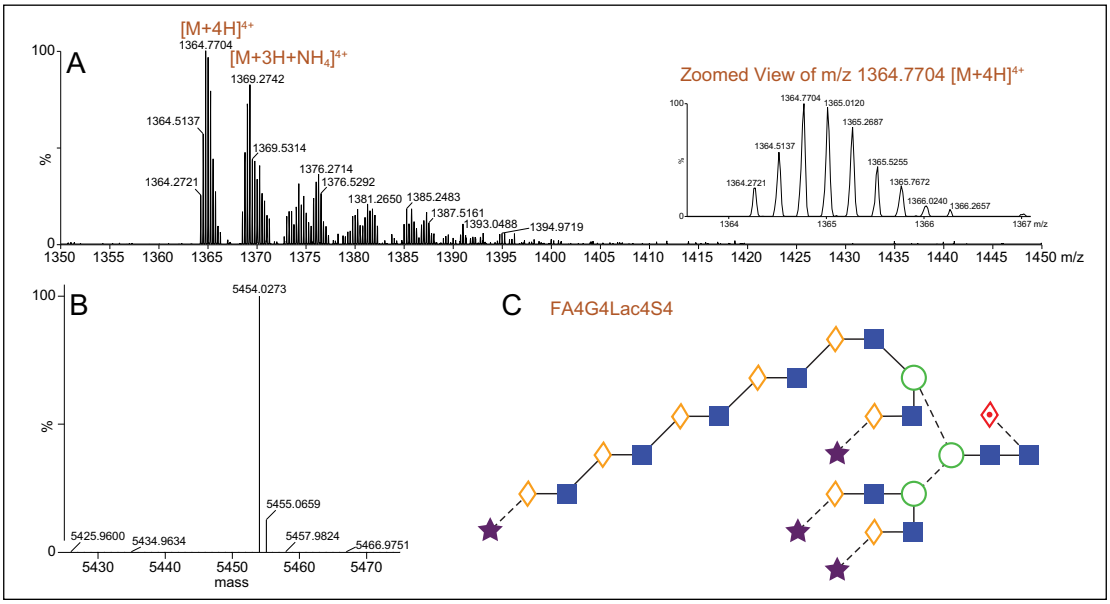

Figure 2. Mass spectrometric analysis of the RapiFluor-MS labeled FA4G4Lac4S4 glycan eluting at retention time 36.94 minutes. (A) Background subtracted ESI-MS mass spectrum showing an $[M+4H]^{4+}$ ion of FA4G4Lac4S4. (B) Charge deconvoluted and deisotoped mass spectrum. (C) Diagram of the identified FA4G4Lac4S4 glycan. (Proposed structure based on a previous study of Lac repeats.[7])

THE SOLUTION

N-glycans from rhEPO were released through fast enzymatic deglycosylation and rapidly labeled using a Waters GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003635).[7] The new *Rapi*Fluor-MS reagent has been designed to facilitate rapid labeling, improve fluorescence quantum yields and greatly enhance MS sensitivity.[7] In this sample preparation, the complex N-glycans of EPO were first made accessible for enzymatic deglycosylation by the use of *Rapi*Gest™ SF, an anionic surfactant. Subsequently, its N-glycans were released in approximately 5 minutes using Rapid PNGase F and an elevated incubation temperature of 50 °C. The resulting deglycosylation mixture, containing free N-glycans (glycosylamines), was then subjected to a 5 minute labeling reaction with *Rapi*Fluor-MS. Labeled N-glycans were thereafter efficiently extracted from the reaction mixture using a GlycoWorks HILIC µElution plate (p/n 186002780) and GlycoWorks SPE Elution Buffer (p/n 186007992). This process of going from glycoprotein to extracted, labeled N-glycans was accomplished in 30 minutes. In addition, this sample preparation allowed for the immediate analysis of the *Rapi*Fluor-MS labeled N-glycans via a HILIC separation with a 2.1 mm x 150 mm, ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column (p/n 186004742) and an ACQUITY UPLC I-Class System. *Rapi*Fluor-MS N-glycan species eluting during these separations were serially detected by their fluorescence (FLR) and by positive ion-mode ESI-MS with a Xevo® G2-S QTof Mass Spectrometer.

Figure 1 presents chromatograms from the HILIC-FLR-MS analysis of EPO N-glycans as labeled with *Rapi*Fluor-MS. Notably, both the fluorescence and base peak intensity (BPI) MS chromatograms showed high signal-to-noise such that the presence of different N-glycan species could be readily confirmed. The major N-glycans species in this profile were identified using the accurate mass information in combination with data from previous observations of EPO N-glycans.[7] Previously, multidimensional chromatography strategies combining anion exchange chromatography and HILIC had been required to comprehensively characterize the N-glycans of EPO.[7] In this work, we have been successful in identifying EPO N-glycans by employing a one dimensional HILIC separation along with online ESI-Q-Tof MS detection. This is an approach that is facilitated by the improved fluorescence and MS sensitivity afforded by *Rapi*Fluor-MS labeling.[8] These new developments in N-glycan analysis aided in identifying tetra-antennary glycans with multiple sialic acids (three and four) as the most abundant species present on the analyzed rhEPO. The GlycoWorks

*Rapi*Fluor-MS approach also helped in determining that tetra-antennary glycans with poly-N-acetyl lactosamine extensions were also present in relatively high abundance. Relative quantification from the fluorescence profile, in fact, showed that that the tetra-sialylated, tetra-antennary glycan species (FA4G4S4) represents approximately 20% of the total N-glycan pool, while tetra-antennary glycans carrying one (FA4G4Lac1S4) and two (FA4G4Lac2S4) lactosamine extensions constitute approximately 12 and 4.5% of the total N-glycans, respectively. More interestingly, GlycoWorks *Rapi*Fluor-MS approach has yielded identifications of tetra-antennary structures with three (FA4G4Lac3S4) and four (FA4G4Lac4S4) lactosamine extensions at levels of 0.75% and 0.25%, respectively. Although some previous studies on EPO have reported one and two N-acetyl lactosamine extensions, few studies have reported detailed information on species containing four or more lactosamine repeats.[7] In this work, we have been able to successfully identify up to four repeats of poly-N-acetyl lactosamine using only a single dimension of separation and a gradient time of just 35 minutes (Figure 2). Moreover, it was possible to make confident identifications throughout this HILIC profile because of the enhanced fluorescence yields and the improvements in the ionization efficiencies of complex N-glycans that result from the use of the novel *Rapi*Fluor-MS labeling reagent.

SUMMARY

An approach combining the advantages of GlycoWorks *Rapi*Fluor-MS N-glycan sample preparation with the separation capabilities of UPLC HILIC has enabled us to perform a comprehensive analysis of the complex N-glycans present on a recombinant human erythropoietin (rhEPO). With the GlycoWorks *Rapi*Fluor-MS workflow, N-glycan samples were prepared in just 30 minutes. Most importantly, the samples were amenable to direct analysis by HILIC-ESI-QTof-MS analysis. *Rapi*Fluor-MS labeling not only reduced the burden of the sample preparation, but also enhanced the sensitivity of N-glycan detection, making it possible to obtain information-rich data and to elucidate the complicated N-glycan profile of an rhRPO. Because of these benefits, this new approach to N-glycan analysis could be used to hasten the development of EPO biosimilars.

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Measuring the Glycan Occupancy of Intact mAbs Using HILIC and Detection by Intrinsic Fluorescence

Authors: Matthew A. Lauber and Stephan M. Koza

GOAL

To demonstrate the use of HILIC with an ACQUITY UPLC® Glycoprotein BEH Amide 300Å Column to assay the glycan occupancy of intact mAbs.

BACKGROUND

Monoclonal antibodies (mAbs) have emerged as some of the most important therapeutics on the market. These mAbs that are prescribed for therapeutic use are most often expressed from eukaryotic cell lines, such as CHO, and, as a result, are N-glycosylated at two consensus site asparagine residues in the Fc portion of their heavy chains. Since glycosylation can be a measure of efficacy, safety and manufacturing conditions, it is often critical to characterize and routinely monitor the N-glycan profile of a mAb drug substance.[1-2] The recent introduction of the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit has made it significantly easier to perform highly detailed HILIC-based, released N-glycan analyses and to thereby elucidate the heterogeneity of *N*-glycosylation.[3-4] However, it is also critical to determine the extent to which the asparagine linkage sites are occupied with N-glycans, particularly since incomplete glycosylation dramatically changes the effector functions of a mAb.[5] Traditionally, this assessment has been performed by a sized-based Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS) separation of the heavy chains resulting from reduction of a mAb.[6,7] To instead directly assess glycan

Unprecedented Hydrophilic Interaction Chromatography (HILIC) separations of intact mAb glycan occupancy variants using the ACQUITY UPLC Glycoprotein BEH Amide 300A 1.7µm Column.

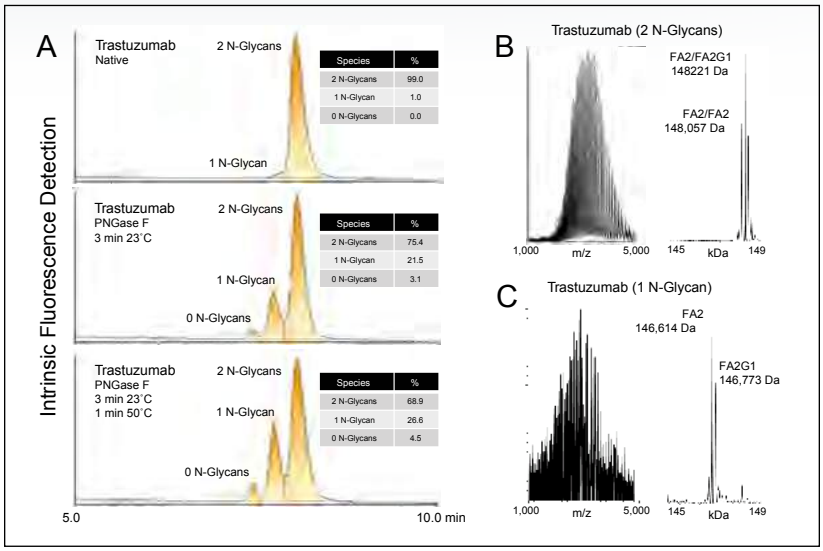

Figure 1. HILIC analysis of intact trastuzumab. (A) HILIC fluorescence chromatograms for native and partially deglycosylated trastuzumab. (B) Raw and deconvoluted ESI mass spectra for the major species resolved in the native trastuzumab sample. (C) Raw and deconvoluted ESI mass spectra for the low abundance species resolved in the native trastuzumab sample. HILIC was performed on 1 µL aqueous injections of 5 mg/mL trastuzumab using an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column heated to 80°C. Sample was separated at a flow rate of 0.4 mL/min with aqueous (A) and acetonitrile (B) mobile phases containing 0.1% (v/v) TFA and 0.3% (v/v) HFIP. A linear gradient was applied as follows: hold at 20% A for 0.5 min, 20 to 25% in 0.5 min, 25% to 40% A in 9 min, 40% to 100% A in 0.5 min, hold at 100% A for 0.5 min, 100% to 20% A in 0.5 min, and hold at 20% A for 3.5 min for re-equilibration. Eluting proteins were detected by intrinsic fluorescence (Ex 280 nm/Em 320 nm).

occupancy variants for intact mAbs, we have developed an LC separation based on HILIC. To achieve these unprecedented separations, a new, purposefully designed HILIC column was employed. This new column, the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column, contains a wide-pore amide bonded stationary phase that ensures that mAb species have access to the porous network of the stationary phase and are not significantly impaired by restricted diffusion.[8]

THE SOLUTION

A high-throughput, high resolution HILIC separation was established for intact mAbs using a 2.1 x 150 mm wide-pore BEH amide column, a 0.4 mL/min flow rate, and a column temperature of 80 °C. In addition, two mobile phase additives, 0.1% trifluoroacetic acid (TFA) and 0.3% hexafluoroisopropanol (HFIP) were employed to improve the solubility of intact mAbs in the high organic, initial mobile phase conditions of the HILIC gradient. To enhance the sensitivity of this LC method, the intact proteins were detected by means of their intrinsic fluorescence. Excitation and emission wavelengths of 280 and 320 nm were found to provide optimal signal-to-noise and consistently flat chromatographic baselines that are desirable for peak integration. A representative set of chromatograms resulting from this 15 minute LC method is shown in Figure 1A. Three chromatograms are displayed. The top chromatogram shows trastuzumab as injected from a dilution of its formulation, while the other two chromatograms show samples of trastuzumab after being subjected to partial PNGase F deglycosylation. Deglycosylated samples of trastuzumab clearly showed three distinct peaks in their HILIC profiles, as was predicted if the glycan occupancy variants of a mAb were to be resolved. The unadulterated sample of trastuzumab contained measurable levels of only the fully occupied and singly occupied forms (1%) as confirmed by online mass analysis. The deconvoluted mass spectrum corresponding to the main LC peak exhibited several masses, such as 148,057 Da and 148,221 Da, that are within 2 Da of

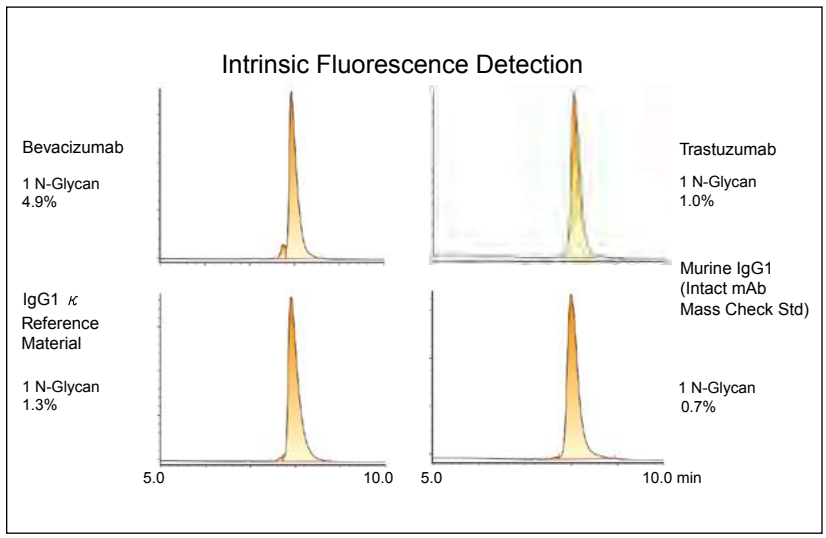

Figure 2. HILIC analysis of four intact mAbs. Bevacizumab, NIST IgG1κ candidate reference material, and trastuzumab were injected without any sample preparation except for dilution to 5 mg/mL from their respective formulations. Prior to HILIC analysis, Intact mAb Mass Check Standard (1 mg) was reconstituted in 500 µL of 6M guanidine HCl, filtered via 3 passes with a 100KDa MWCO polyethersulfone membrane (GE Healthcare Life Sciences, Vivaspin 500), and concentrated to 5 mg/mL.

the theoretical molecular weights for the predominant glycoforms of trastuzumab (Figure 1B).[9] In contrast, the deconvoluted mass spectrum for the smaller, less strongly retained peak showed reduced heterogeneity and masses that were lighter by approximately 1000 to 2000 Da, consistent with the single occupancy form (Figure 2B). Fully aglycosylated species were not detected in this sample. An interesting observation from these data is that the levels of fully deglycosylated forms are lower than would be predicted if both sites were deglycosylated at the same rate. In these examples, the observed level of fully deglycosylated forms is approximately one-third lower than would be predicted. This may indicate that either the digestion rate for one of the N-glycans is slower than the other, or that upon removal of the first N-glycan, the digestion rate of the remaining N-glycan is reduced.

To assess the applicability of this technique to other mAbs, we analyzed three additional IgG samples. Results on trastuzumab, bevacizumab, a candidate IgG1κ reference material, and a murine IgG1 (Intact mAb Mass Check Standard, p/n 186006552) are shown in Figure 2. Integrations on the HILIC-fluorescence chromatograms indicated that these samples contain 1 N-glycan (incomplete glycosylation) variants at relative abundances ranging from 0.7 to 4.9%. These observations suggest that this HILIC method could be an attractive technique for assaying incomplete glycosylation of mAbs down to levels well below 1% for the 1 N-glycan form. Detection limits for the 0 N-glycan form may perhaps be even lower. Of these samples, bevacizumab might be predicted to have the highest abundance of the 0 N-glycan form. Indeed, the bevacizumab profile presented a peak, albeit very minor, with a retention time consistent with a 0 N-glycan species, the area of which would contribute to the overall profile

at a level of only 0.05%. Such an observation suggests that the 0 N-glycan level is lower than would be statistically predicted; however, rigorous determination of the quantitative limits of this analysis and the identity of this putative 0 N-glycan peak would need to be evaluated to confirm that this is indeed the case.

This strategy for measuring mAb glycan occupancy is most appealing in that it requires minimal, if any, sample preparation. We have observed that some interferences can be encountered that are due to partially fragmented and/or reduced mAb species. In which case, as with the Intact mAb Mass Check Standard, a simple, centrifugal filtration clean-up step with a 100kDa MWCO polyethersulfone membrane was sufficient to minimize such interferences. Given the unique selectivity of the HILIC separation, it should also be possible to address potential interferences by performing offline or online 2D-LC, wherein a size exclusion or reversed phase separation could be coupled to the wide-pore amide HILIC separation. Future investigations could also include reducing glycan heterogeneity (via a sialidase or other exoglycosidase) to allow the mAb glycan occupancy variants to be more discretely resolved. Similarly, there is an opportunity to use this separation in combination with enzymes that generate Fc subunits.

SUMMARY

In addition to profiling the heterogeneity of glycosylation, it is also critical to assay glycan occupancy. Here, we demonstrate that a Glycoprotein BEH Amide column, purposefully designed for large molecule HILIC separations, can be used to directly quantify incomplete N-glycan occupancy in intact mAb samples. Unlike a conventional CE-SDS separation of reduced mAbs, this technique provides a non-inferred assessment on the nature (i.e. 1 N-glycan versus 0 N-glycans) of glycan occupancy for the intact mAb. The proposed HILIC methodology is also MS-compatible, making it possible to readily confirm the assignments of observed peaks.

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Robustness of *Rapi*Fluor-MS N-Glycan Sample Preparations and Glycan BEH Amide HILIC Chromatographic Separations

Matthew A. Lauber,[1] Michael F. Morris,[1] Darryl W. Brousmiche,[1] and Stephan M. Koza[1]
[1] Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- High yield sample preparation with quantitative recovery to ensure accurate and repeatable profiling of N-glycans
- Comparability to historical 2-AB based released glycan analysis approaches
- *Rapi*Fluor-MS Glycan Performance Test Standard for method familiarization, troubleshooting, and benchmarking
- Robust Glycan BEH Amide HILIC separations supported by GU calibration with the novel *Rapi*Fluor-MS Dextran Calibration Ladder

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

GlycoWorks HILIC µElution Plate

*Rapi*Fluor-MS Glycan Performance Test Standard

*Rapi*Fluor-MS Dextran Calibration Ladder

ACQUITY UPLC® Glycan BEH Amide, 130Å Column

XBridge® Glycan BEH Amide, 130Å Column

ACQUITY UPLC H-Class Bio System

ACQUITY® QDa® Mass Detector

Xevo® G2-XS QTof MS

SYNAPT® G2-S*i* HDMS

KEY WORDS

GlycoWorks, *Rapi*Fluor-MS, *Rapi*Gest™ SF, Rapid Tagging, PNGase F, Deglycosylation, ACQUITY UPLC H-Class Bio System, BEH Amide 130Å, Glycans, Glycoproteins, Glycosylation, HILIC, Fluorescence

INTRODUCTION

N-glycosylation of proteins is routinely characterized and monitored because of its significance to the detection of disease states[1-3] and the manufacturing of biopharmaceuticals.[4-5] Glycosylation profiles are most often assessed by means of released glycan analyses, wherein samples are often prepared by techniques that are notoriously time-consuming or lead to compromises in MS sensitivity.[6-7] With the development of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, we have addressed these shortcomings by enabling unprecedented sensitivity for glycan detection while also improving the throughput of N-glycan sample preparation.[8] Using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, glycoproteins are deglycosylated in 10 minutes to produce N-glycosylamines that are then rapidly reacted with the novel *Rapi*Fluor-MS labeling reagent (Figure 1). In a final step, the resulting labeled glycans are extracted from reaction byproducts by means of an SPE method that facilitates immediate analysis of samples. As a result, an analyst can now complete an N-glycan sample preparation, from glycoprotein to ready-to-analyze sample, in just 30 minutes and be poised to perform high sensitivity N-glycan profiling using hydrophilic interaction chromatography (HILIC) and mass spectrometric (MS) or fluorescence (FLR) detection.

Equally important as the efficiency and sensitivity gains afforded by this new sample preparation approach is its robustness and its ability to produce results consistent with historical N-glycan profiling. Within this application note, we will discuss these attributes of the *Rapi*Fluor-MS based sample preparation and the corresponding HILIC-based LC analyses.

EXPERIMENTAL

Method conditions

(unless otherwise noted)

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Fluorescence detection:	Ex 265/Em 425 nm (*Rapi*Fluor-MS)
	Ex 330 / Em 420 nm (2-AB)
	(2 Hz scan rate [150 mm column], Gain =1)
	(5 Hz scan rate [50 mm column], Gain=1)
Injection volume:	≤1 µL (aqueous diluents with 2.1 mm I.D. columns)
	≤30 µL (DMF/ACN diluted samples with 2.1 mm I.D. columns)
Columns:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740)
	XBridge Glycan BEH Amide XP, 130Å, 2.5 µm, 2.1 x 150 mm (p/n 186007265)
	Agilent AdvanceBio Glycan Mapping Rapid Resolution HD, 1.8 µm, 2.1 x 150 mm
	Thermo Scientific Accucore™ 150 Amide HILIC, 2.6 µm, 2.1 x 150 mm
Sample collection/vials:	Sample Collection Module (p/n 186007988)
	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

Gradient used with 2.1 x 50 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

Gradient used with 2.1 x 150 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

Intact mass analysis was performed by LC-MS with a Xevo G2-QTof

MS conditions

MS system:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–5000 *m/z*
Acquisition:	500–5000 *m/z*, 1 Hz scan rate
Data management:	UNIFI® 1.7/MassLynx® Software (v4.1)

Sample description

*Rapi*Fluor-MS labeled N-glycans were prepared from glycoproteins, including Intact mAb Mass Check Standard (p/n: 186006552), using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n: 176003606) according to the guidelines provided in its Care and Use Manual (715004793).

2-AB labeled N-glycans were prepared using a Prozyme GlykoPrep® Rapid N-Glycan Preparation with 2-AB Kit according to the manufacturer's recommended protocol. In addition, 2-AB labeled N-glycans were also prepared using an approach combining the use of a Prozyme GlykoPrep Digestion Module, an in-house optimized 2-AB labeling protocol, and a GlykoPrep Cleanup Module (Prozyme, Hayward, CA).

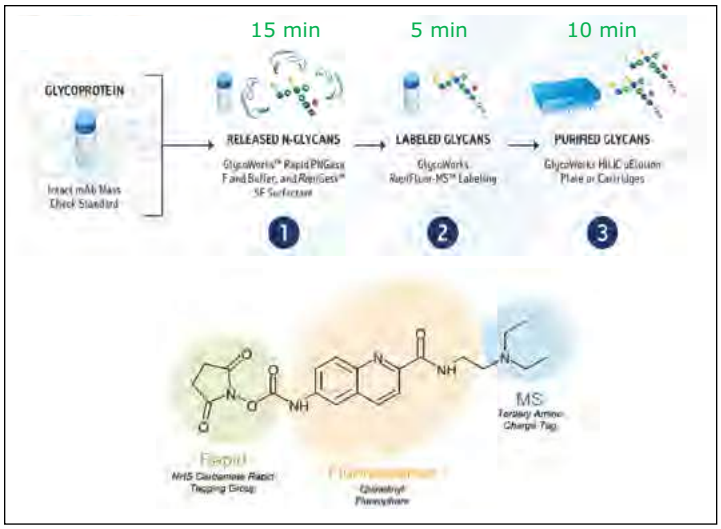


Figure 1. GlycoWorks RapiFluor-MS N-Glycan Kit sample preparation workflow and the chemical structure of the RapiFluor-MS Reagent.

*Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983) was reconstituted in 50 µL of water and injected as a 1 µL volume for chromatographic benchmarking and lifetime testing experiments. *Rapi*Fluor-MS Dextran Calibration Ladder (p/n: 186007982) was reconstituted in 100 µL of water and injected as a 1 µL volume for retention time calibrations.

Percent yields for the sample preparation workflows were determined by means of quantitative analyses. Column loads were calibrated using external quantitative standards of 2-AB labeled triacetyl chitotriose and *Rapi*Fluor-MS derivatized propylamine obtained in high purity (confirmed by HPLC and 1H NMR).

To determine percent yields, the measured quantities of FA2 glycan from Intact mAb Mass Check Standard (p/n: 186006552) were compared to theoretical yields calculated for the preparation. For example, the theoretical yield for the FA2 glycan resulting from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit was calculated as follows:

$$1.5 \times 10^7 \text{ pg IgG} \times \frac{1 \text{ pmol}}{150{,}000 \text{ pg}} \times \frac{2 \text{ pmol glycan}}{1 \text{ pmol IgG}} \times \frac{0.45 \text{ pmol FA2}}{1 \text{ pmol total glycan pool}} \times \frac{10 \text{ µL injection}}{400 \text{ µL sample prepared}} = 2.3 \text{ pmol}$$

**This calculation is based on the assumption that the sample of Intact mAb Mass Check Standard was 15 µg, that the mAb has a molecular weight of 150 kDa, that there are only 2 N-glycans per one mAb, that the N-glycan profile of the mAb contains the FA2 glycan at a relative abundance of 45%, and that only 2.5% of the sample was analyzed.*

RESULTS AND DISCUSSION

Robust sample preparation: Deglycosylation

Each procedural step in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit has been optimized to be high yielding and to minimize the introduction of bias to an N-glycan profile. Previous work based on SDS PAGE gel shift assays has demonstrated that the rapid deglycosylation procedure developed for this kit produces complete deglycosylation of a diverse set of glycoproteins.[8] This completeness of deglycosylation is also supported by intact mass analysis using LC-MS, where the deglycosylation of a monoclonal antibody (mAb) can be readily tracked. Figure 2 presents deconvoluted ESI mass spectra for Intact mAb Mass Check Standard, a murine IgG1 mAb. The top spectrum shows the mAb before it had been subjected to rapid deglycosylation (Figure 2A). The bottom spectrum meanwhile presents the mAb after it was processed according to the approach specified in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, wherein glycoproteins are subjected to 1% (w/v) *Rapi*Gest SF Surfactant-assisted heat denaturation followed by incubation with Rapid PNGase F at 50 °C for 5 minutes (Figure 2B). The masses observed in these spectra confirm that these samples differ in terms of glycan occupancy. The control sample contains the mAb in its doubly glycosylated, native form (one glycan on each heavy chain). In contrast, the sample subjected to the proposed 2-step rapid deglycosylation procedure is homogenous with an observed molecular weight that is in agreement with the predicted molecular weight of the fully deglycosylated mAb (145.3 kDa). And although high temperatures are employed in this method for the purpose of heat denaturation, no detrimental effects on an N-glycan profile have been observed. To this point, notice that there are no differences in an N-glycan profile prepared from pooled human IgG when using an excessive 20 minute heat denaturation at 90 °C versus the rapid 3 minute procedure (Figure 3).

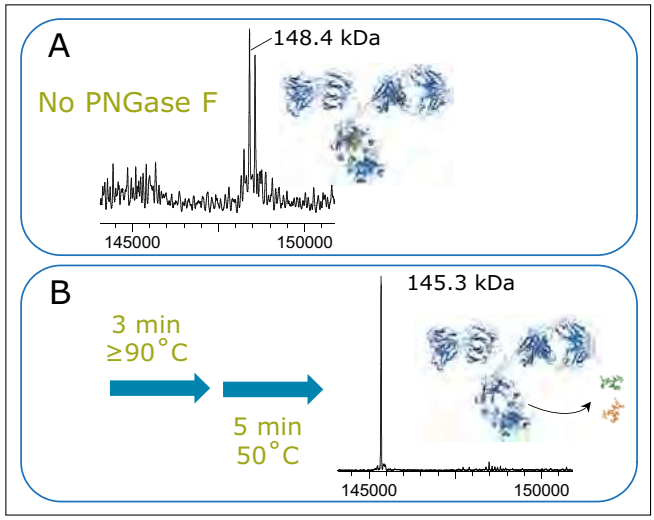


Figure 2. Intact mass analysis of Intact mAb Mass Check Standard (A) before and (B) after rapid deglycosylation with the GlycoWorks RapiFluor-MS N-Glycan Kit.

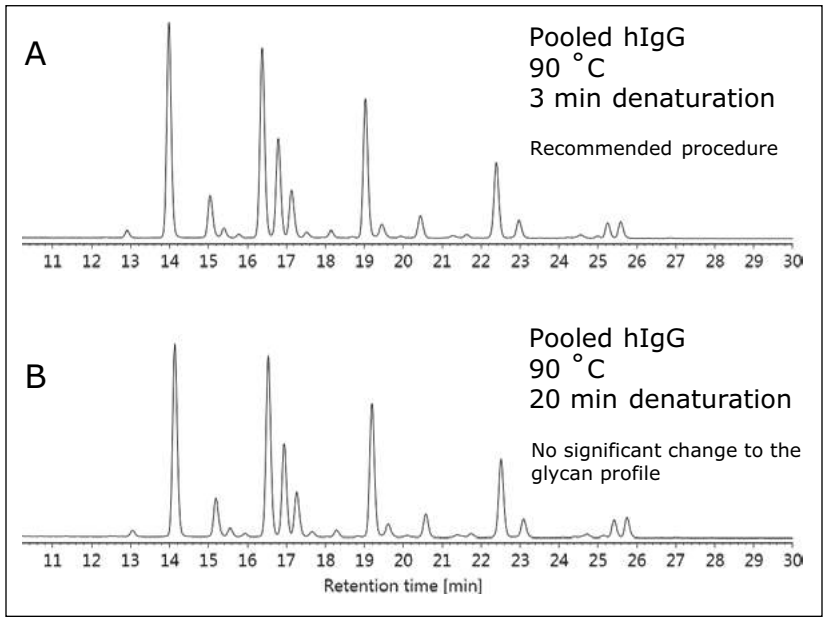
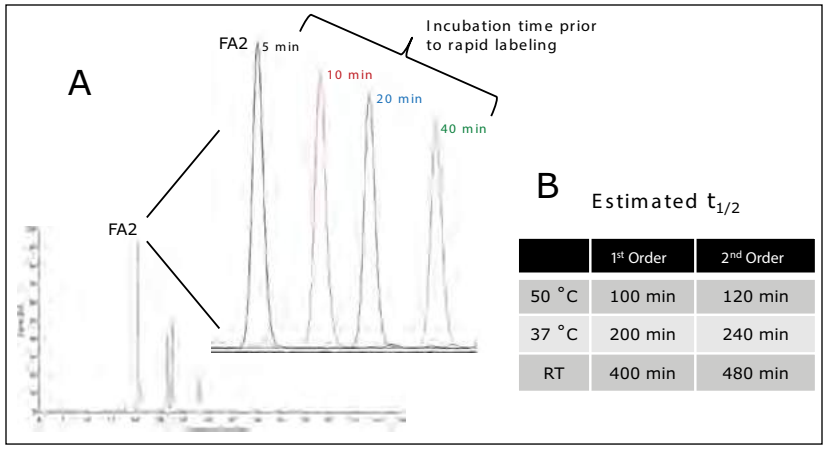


Figure 3. Testing the effects of subjecting human IgG and its N-glycans to heat denaturation. (A) The RapiFluor-MS N-glycan profile as observed using the recommended 3-minute heat denaturation versus (B) the RapiFluor-MS N-glycan profile as observed using a 20-minute heat denaturation.

Robust sample preparation: Rapid labeling

The efficiency of the sample preparation carries over from deglycosylation to *Rapi*Fluor-MS labeling. A primary concern in this step is the relative stability of the PNGase F released N-glycosylamines, which are required for *Rapi*Fluor-MS labeling, in the pH 7.9 GlycoWorks Rapid Buffer. A time-course study involving varying delays between deglycosylation and *Rapi*Fluor-MS labeling steps has shown that the N-glycosylamines have a relatively long half-life of approximately 2 hours at 50 °C (Figure 4). That is, with our 5 minute deglycosylation step, there should be little concern over sample loss (< 3% loss) due to hydrolysis of the glycosylamine. In addition, sample losses from the labeling reaction are minimal. Many experimental parameters were explored during the development of the rapid labeling reaction specified in the *Rapi*Fluor-MS N-Glycan Kit, including pH, temperature, ionic strength, time, buffer components, and reagent molar excess. Figure 5 shows an example of optimizing the reagent molar excess as needed to maximize labeling yield. Fluorescence chromatograms for labeled, released N-glycans from Intact mAb Mass Check Standard are stacked on the left (Figure 5A). Note that with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit proteins are purposely not depleted from the sample after deglycosylation to save time and to give better control over the labeling. The *Rapi*Fluor-MS Reagent is therefore used in a molar excess over all of the nucleophiles from the glycoprotein, which for an IgG corresponds to approximately seventy five protein amines and two N-glycosylamines. Each of the corresponding samples was obtained from labeling a fixed glycoprotein concentration of 0.36 mg/mL with *Rapi*Fluor-MS Reagent at concentrations varying from 18 to 108 mM. As shown in Figure 5B, plotting of the fluorescence peak areas for the resulting N-glycan profile indicates that labeling is maximized near a *Rapi*Fluor-MS Reagent concentration of 36 mM, the conditions designed into the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Moreover, molar excess conditions both higher and lower than the 36 mM reagent condition produced comparable fluorescence profiles, underscoring the robustness of *Rapi*Fluor-MS labeling.



	1st Order	2nd Order
50 °C	100 min	120 min
37 °C	200 min	240 min
RT	400 min	480 min

Figure 4. Estimating the half-life of N-glycosylamine hydrolysis through a time-course on deglycosylation incubation. (A) Fluorescence traces for RapiFluor-MS labeled FA2 from Intact mAb Mass Check Standard observed after implementing varying incubation times for deglycosylation (50°C incubations). (B) Approximation of the N-glycosylamine half-life assuming 1st or 2nd order reaction kinetics.

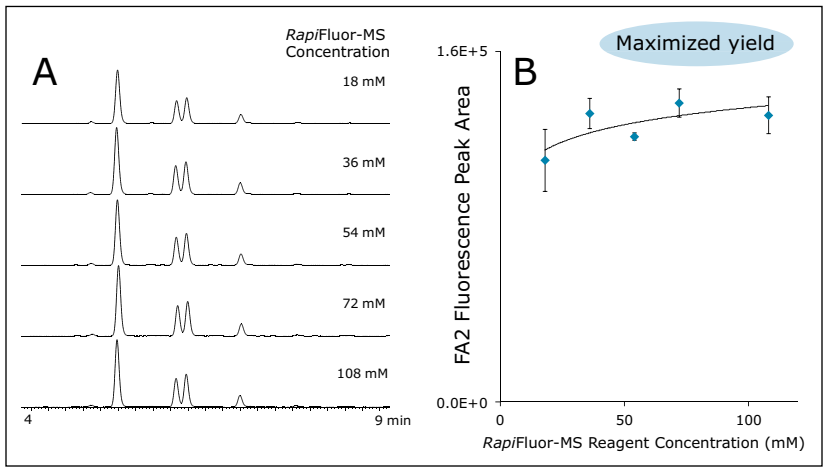


Figure 5. Optimization of labeling reagent molar excess for the GlycoWorks RapiFluor-MS N-Glycan Kit. (A) Fluorescence chromatograms for labeled glycans obtained by titration of 0.36 mg/mL deglycosylated Intact mAb Mass Check Standard with varying concentrations of RapiFluor-MS Reagent. Separations were performed with labeled glycans from 0.4 µg of glycoprotein and an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm Column. (B) Fluorescence peak area as a function of RapiFluor-MS Reagent concentration.

Robust sample preparation: µElution HILIC SPE

The last step in the sample preparation involves extraction of the *Rapi*Fluor-MS labeled glycans from reaction byproducts using HILIC SPE. This technique has been routinely used for preparations of 2-AB labeled N-glycans and has now been optimized for *Rapi*Fluor-MS labeled species.[8-9] Previous studies have shown that *Rapi*Fluor-MS labeled glycans are obtained through this SPE processing at relatively high yields of approximately 74%.[8] Nearly all of the observed sample losses in this step are non-specific. Figure 6 plots fluorescence peak areas for preparations of N-glycans from Intact mAb Mass Checked Standard, in which the final SPE elution volume was either 30, 90 or 180 µL. This plot shows that SPE recovery is a function of elution volume and that highest recoveries are achieved when employing large elution volumes. To facilitate direct analyses, however, a compromise is made such that a 90 µL elution volume is used in order to obtain a relatively concentrated glycan eluate. Regardless of the elution volume and absolute yield of glycans from the SPE sorbent, the most important characteristic of this clean-up is that the observed sample losses have been determined to be non-specific with no significant bias being introduced to a glycan profile for a wide range of glycans with diverse chemical properties, including small, neutral glycans up to large, tetrasialylated glycans (see Reference 8 for more details about GlycoWorks HILIC SPE).

Yield of *Rapi*Fluor-MS labeled N-glycans

In another measurement of robustness, it is worth looking at the yield of N-glycans through the entire workflow. This was evaluated in order to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of *Rapi*Fluor-MS labeled glycans (Figure 7). *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were prepared, analyzed by HILIC-FLR, and quantified by means of an external calibration. Based on a calculated theoretical yield (see experimental) and duplicate analyses, it was determined that the percent yield through the entire *Rapi*Fluor-MS N-Glycan Kit sample preparation was approximately 73%. To provide perspective, we evaluated the yield of 2-AB labeled N-glycans from an alternative sample preparation workflow involving the use of a GlykoPrep Rapid N-Glycan Preparation with 2-AB Kit.

Quantitative analyses showed that 2-AB labeled N-glycans are prepared using this kit with a relatively low yield of approximately 35%, though it has been found that the yield of this kit can be dramatically improved by optimization and lengthening of the labeling step. Comparatively speaking, though, these results show that not only does the *Rapi*Fluor-MS approach quicken a historically time-consuming sample preparation, it also exhibits reasonably high yields.

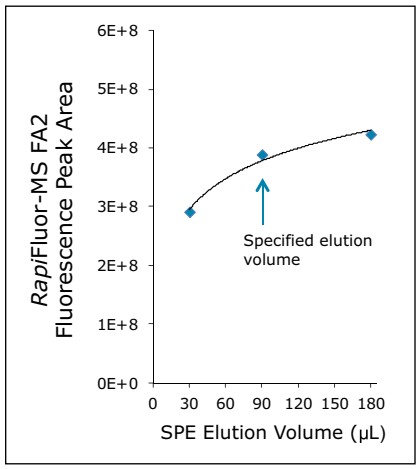


Figure 6. Fluorescence peak area as a function of SPE elution volume. The specified elution volume in the GlycoWorks RapiFluor-MS N-Glycan Kit is 90 µL.

Step	Yield	Testing to confirm minimal bias
Deglycosylation	Complete	▪ Intact mass analysis/subunit LC-MS ▪ Gel shift assays
Labeling	>95%	▪ Released glycan profile vs. subunit derived glycan information
SPE	~74%	▪ Recovery measurements ▪ Glycan profile before vs. after SPE
GlycoWorks RapiFluor-MS N-Glycan Kit	~73% Yield	
GlykoPrep® Rapid N-Glycan Preparation with 2-AB	~35% Yield	

Figure 7. Percent yield for the preparation of RapiFluor-MS labeled N-glycans with the GlycoWorks RapiFluor-MS N-Glycan Kit. Testing that has been performed to confirm minimal sample loss and quantitative recovery is listed for each procedural step. The percent yield that has been measured for the preparation of 2-AB labeled N-glycans with a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit is also provided. These results may not be representative of all applications.

Minimal impact to glycan profiling with reagent batch variation

Lastly, sample preparations with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit have proven to be robust with respect to reagent manufacturing. A robustness study was performed to test the impact of changing the batches of each reagent that plays a critical role in the preparation of *Rapi*Fluor-MS labeled N-glycans, namely *Rapi*Gest SF, GlycoWorks Rapid Buffer, GlycoWorks Rapid PNGase F, *Rapi*Fluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE Plate, and the SPE Elution Buffer. Three sets of these reagents, each varying by batch, were tested in their application to profiling the N-glycans from Intact mAb Mass Check Standard. Average relative abundances observed for the glycan species in this standard with the three different reaction sets are presented in Figure 8. Relative abundances of N-glycans were observed to be largely comparable across the different preparations with an average RSD for the labeled N-glycan species being 2.3%.

Percent Abundance (%)

Species	Reaction set 1	2	3	Avg	Std Dev	RSD
A2	1.23	1.25	1.21	1.23	0.021	1.70
FA2	44.54	43.95	43.29	43.93	0.622	1.42
FA2G1	19.81	20.01	19.95	19.92	0.101	0.51
FA2G1'	22.54	22.86	22.91	22.77	0.199	0.87
FA2G2	8.93	9.02	9.42	9.12	0.264	2.90
FA2G2Sg1/FA2G2Ga2	0.92	0.82	0.94	0.89	0.060	6.71

For each reaction set: unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer.

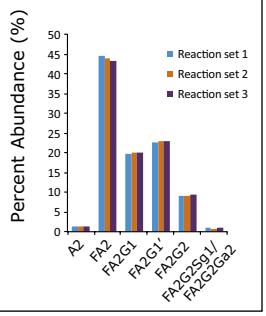


Figure 8. Characterization of batch-to-batch variation in the RapiFluor-MS sample preparation. Percent abundances were measured for the preparation of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard using three different sets of materials. Each reaction set was represented by unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer. Testing was performed in triplicate. FA2G1' denotes the structural isomer of FA2G1.

Comparability to 2-AB N-glycan profiling

Another critical aspect to the *Rapi*Fluor-MS N-glycan sample preparation is that it can be used in place of legacy 2-AB methods without requiring significant adaptations to existing analytical techniques. With the speed of the sample preparation and the enhanced method sensitivity afforded by the *Rapi*Fluor-MS tag,[8] the task of analyzing N-glycosylation is in fact made significantly easier.

Just like 2-AB labeled glycans, *Rapi*Fluor-MS labeled glycans are ideally suited for HILIC separations with an amide bonded stationary phase, such as that found in the Waters Glycan BEH Amide Columns. Figure 9 shows example separations for 2-AB and *Rapi*Fluor-MS labeled glycans obtained from Intact mAb Mass Check Standard. The 2-AB labeled glycans, in this case, were prepared using the previously mentioned GlykoPrep Kit and an approximately 3.5 hour protocol, whereas the *Rapi*Fluor-MS labeled glycans were prepared in less than 30 minutes using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit.

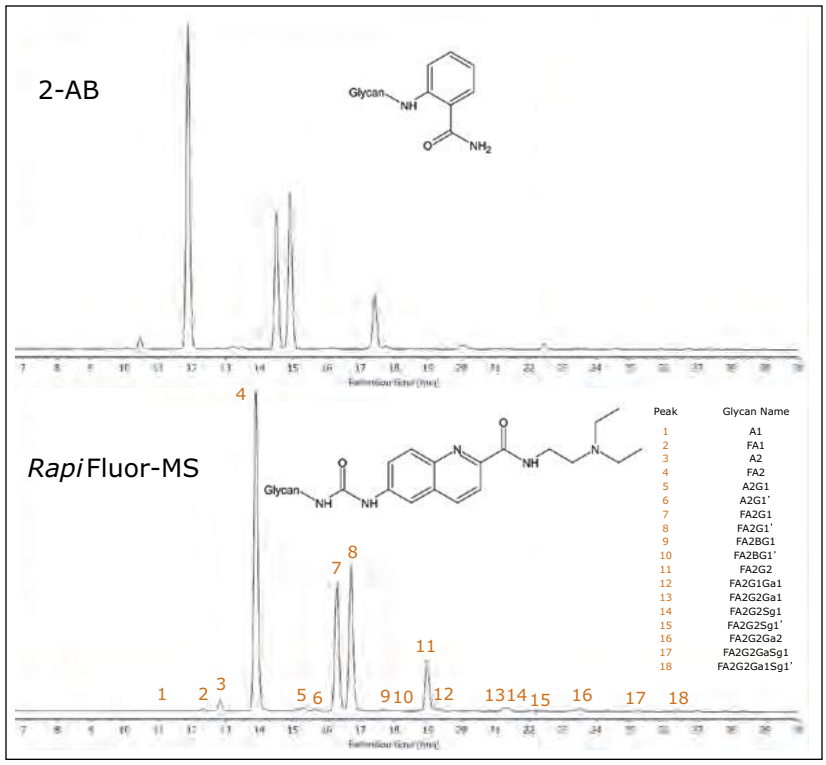


Figure 9. Similarity between 2-AB and RapiFluor-MS N-glycan HILIC profiles for a typical mAb. Fluorescence chromatograms for labeled glycans from Intact mAb Mass Check Standard using an ACQUITY UPLC BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications for the RapiFluor-MS labeled N-glycans are provided. 2-AB labeled N-glycans were prepared using a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit. (') denotes a structural isomer.

So that chromatograms exhibiting equivalent signal-to-noise could be compared, the *Rapi*Fluor-MS sample was analyzed in this study at a significantly lower mass load than the 2-AB labeled sample. Despite being prepared by different approaches, it can be seen that the labeled N-glycans are resolved by the HILIC separation into very similar profiles. For a typical mAb profile, *Rapi*Fluor-MS and 2-AB labeling both yield HILIC glycan separations with similar selectivity. However, as a result of its additional hydrogen bonding donors/acceptors, the *Rapi*Fluor-MS label introduces a slight shift of the mAb N-glycan profile to higher retention times. This change in the absolute retention window of an N-glycan profile is predictable and can therefore be easily accounted for when transitioning from 2-AB to *Rapi*Fluor-MS based methods.

Consistency in results observed for the *Rapi*Fluor-MS-based approach compared to historical 2-AB techniques was also evaluated. N-glycan profiling of the same monoclonal IgG1 reference sample has been studied between these different methodologies. Figure 10 displays N-glycan information obtained for this mAb sample throughout 160 different profiling experiments involving 2-AB labeling and HPLC chromatography. Likewise, Figure 10 provides data from 12 recent experiments using *Rapi*Fluor-MS labeling and UPLC® chromatography. Comparable relative abundances are observed for this sample in a direct comparison (Figure 10, center panel) and a control chart demonstrates the ability to transition between these two methods (Figure 10, right panel). This consistency in N-glycan profiling makes it possible to replace time-consuming 2-AB/HPLC methods with *Rapi*Fluor-MS/UPLC techniques.

N-Glycan	2-AB with HPLC (n=160)	*Rapi*Fluor-MS with UPLC (n=12)
	Avg (%)	Avg (%)
A1	0.33	0.28
FA1	0.23	0.42
A2	5.98	5.76
FA2	45.44	45.88
Man5	1.20	1.08
A2G1	1.91	1.86
A2G1'	0.95	0.79
FA2G1	27.36	26.29
FA2G1'	9.66	9.23
A2G2	0.29	0.32
FA2G2	6.00	5.58
FA2G2S1	0.31	0.51

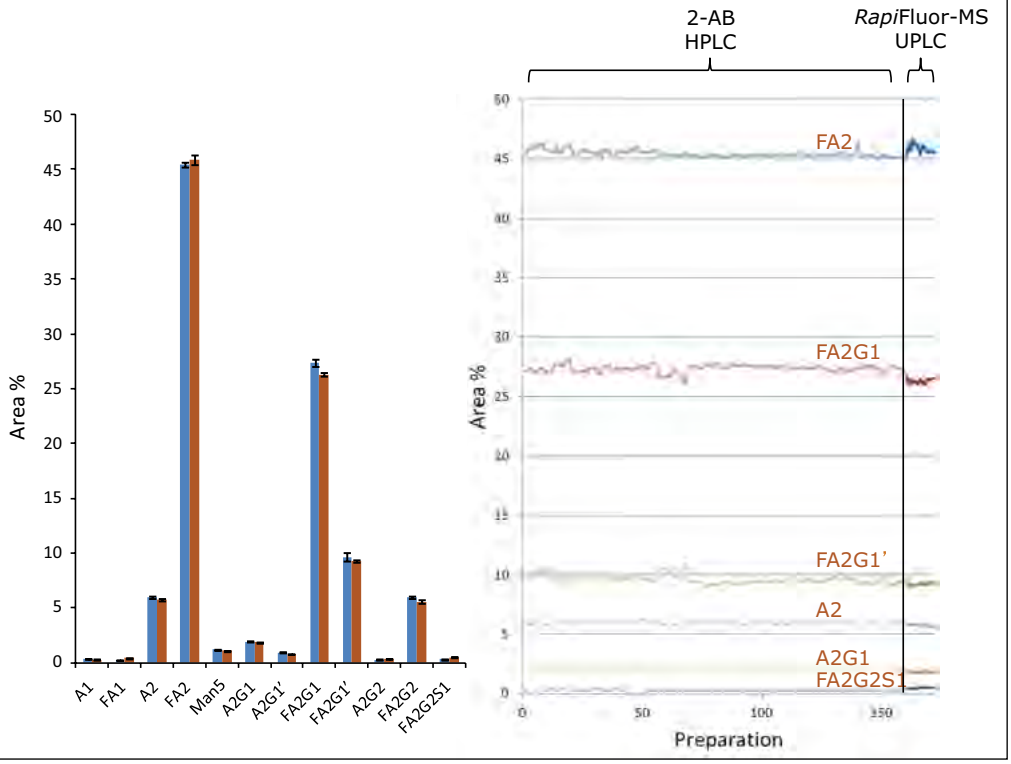
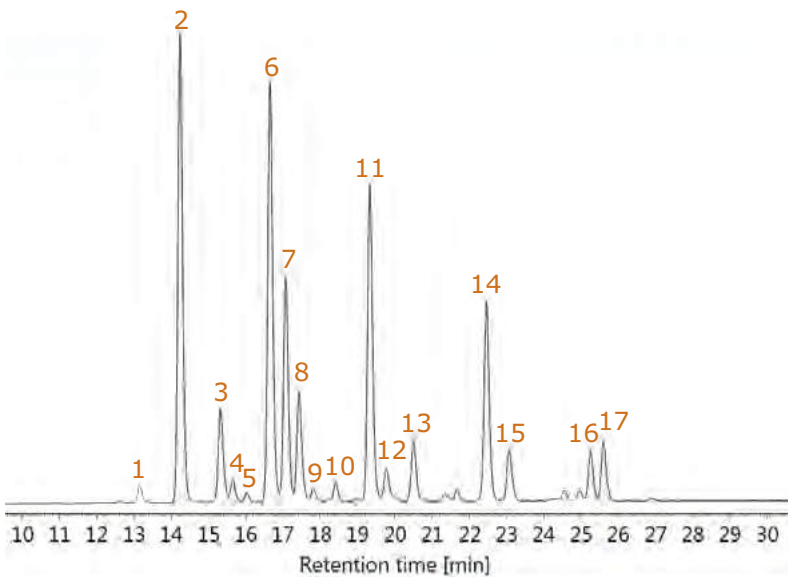


Figure 10. Consistency between UPLC-based RapiFluor-MS N-glycan profiling and HPLC-based 2-AB N-glycan profiling of a humanized monoclonal IgG1. Comparison of relative abundances for N-glycans detected using a method combining the GlycoWorks RapiFluor-MS N-Glycan Kit with a UPLC separation (n=12) versus a historical 2-AB sample preparation combined with an HPLC separation (n=160). Trending data for the N-glycans from the human monoclonal IgG1 (light colored lines = 2-AB/HPLC, dark colored lines = RapiFluor-MS/UPLC). FA2G1' and A2G1' denote the structural isomers of FA2G1 and A2G1, respectively.

Robustness of *Rapi*Fluor-MS N-glycan separations with glycan BEH amide columns

The robustness and resolving power of the HILIC column chromatography is critically important to successfully implementing this methodology. To this end, a test standard called *Rapi*Fluor-MS Glycan Performance Test Standard is available for method familiarization, system suitability, troubleshooting, and benchmarking studies. This standard contains a complex mixture of *Rapi*Fluor-MS N-glycans from human IgG that has been isolated from pooled human serum. Its composition of approximately 20 different major constituents makes it useful for evaluating the resolving power of a separation and the sensitivity of detection methods (Figure 11).

*Rapi*Fluor-MS Glycan Performance Test Standard



	Glycan	*Rapi*Fluor-MS Labeled Glycan Composition	M_i (Da)	M_{avg} (Da)
1	A2	C67H105O37N9	1627.6611	1628.5887
2	FA2	C73H115O41N9	1773.7190	1774.7299
3	FA2B	C81H128O46N10	1976.7984	1977.9224
4	A2G1	C73H115O42N9	1789.7140	1790.7293
5	A2G1'	C73H115O42N9	1789.7140	1790.7293
6	FA2G1	C79H125O46N9	1935.7719	1936.8705
7	FA2G1'	C79H125O46N9	1935.7719	1936.8705
8	FA2BG1	C87H138O51N10	2138.8512	2140.0630
9	FA2BG1'	C87H138O51N10	2138.8512	2140.0630
10	A2G2	C79H125O47N9	1951.7668	1952.8699
11	FA2G2	C85H135O51N9	2097.8247	2099.0111
12	FA2BG2	C93H148O56N10	2300.9041	2302.2036
13	FA2G1S1	C90H142O54N10	2226.8673	2228.1251
14	FA2G2S1	C96H152O59N10	2388.9201	2390.2657
15	FA2BG2S1	C104H165O64N11	2591.9995	2593.4582
16	FA2G2S2	C107H169O67N11	2680.0150	2681.5203
17	FA2BG2S2	C115H182O72N12	2883.0949	2884.7128

Figure 11. RapiFluor-MS Glycan Performance Test Standard. An example fluorescence chromatogram obtained from an 8 pmole load of the standard and a separation with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications are provided. FA2G1', A2G1', and FA2BG1' denote the structural isomers of FA2G1, A2G1, and FA2BG1 respectively.

In line with its intended purpose, we have used the *Rapi*Fluor-MS Glycan Performance Test Standard to benchmark the chromatographic performance of four different columns containing amide bonded stationary phases designed for glycan separations. Two of the columns were UPLC-based and contained sub-2-µm particles while the remaining two were intended for use on HPLC instrumentation and contained 2.5 µm and 2.6 µm particles. Figure 12 shows representative chromatograms obtained with each of these columns run under equivalent conditions and linear velocities. Four glycan species spread across these separations were monitored to measure retention windows, average peak widths, and peak capacities. Notice that whether performing a separation with a phase intended for UPLC or HPLC chromatography, Glycan BEH Amide Columns provide exemplary resolving power and comparable selectivities thereby enabling the seamless transfer of this glycan separation between HPLC and UPLC platforms.[10]

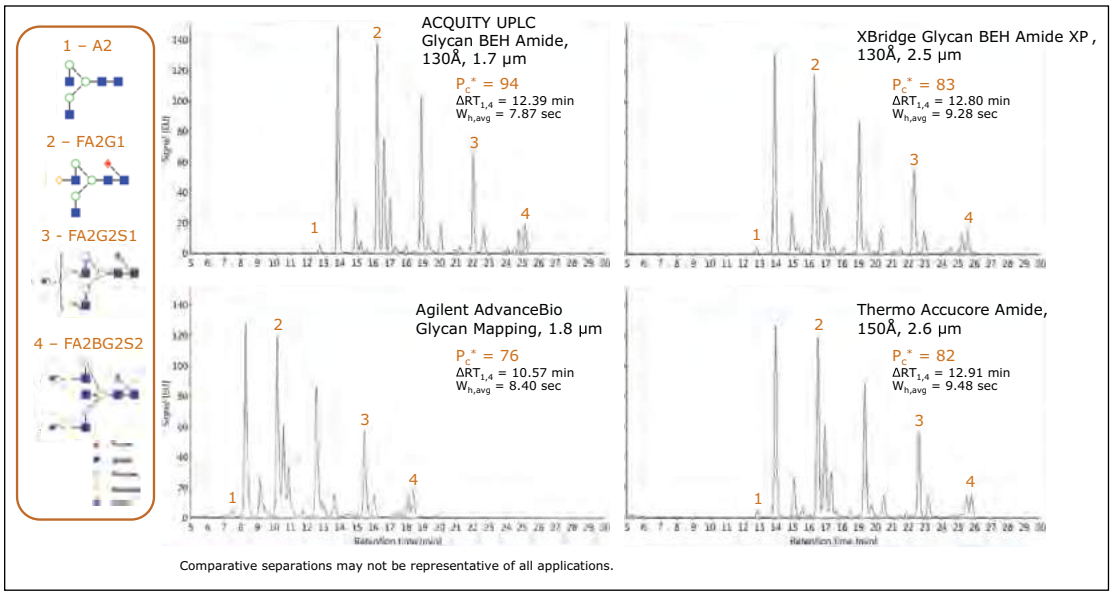


Figure 12. Chromatographic benchmarking of HILIC columns containing amide bonded stationary phases designed for glycan separations. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained from an 8 pmole load of the standard and separations with 2.1 x 150 mm columns. All separations were performed at the same linear velocity on an ACQUITY UPLC H-Class Bio System. Four glycan species spread across the separations were monitored to measure retention windows, average peak widths, and peak capacities.

Separations of *Rapi*Fluor-MS labeled glycans with glycan BEH amide columns have also proven to be very robust. In demonstration of this, a single Glycan BEH Amide, 130Å, 1.7 µm Column was subjected to lifetime testing and 300 sequential runs. At every 20th run, *Rapi*Fluor-MS Glycan Performance Test Standard was separated in order to track any changes in the retentivity and selectivity of the column.

Chromatograms corresponding to the 1st and 300th runs are provided in Figures 13A and 13B, respectively. Quite clearly, near identical separations were obtained at the onset as well as at the end of this approximately 2-week constant use scenario, with no significant shifts in retention times of the labeled N-glycans having been observed throughout the testing (Figure 13C).

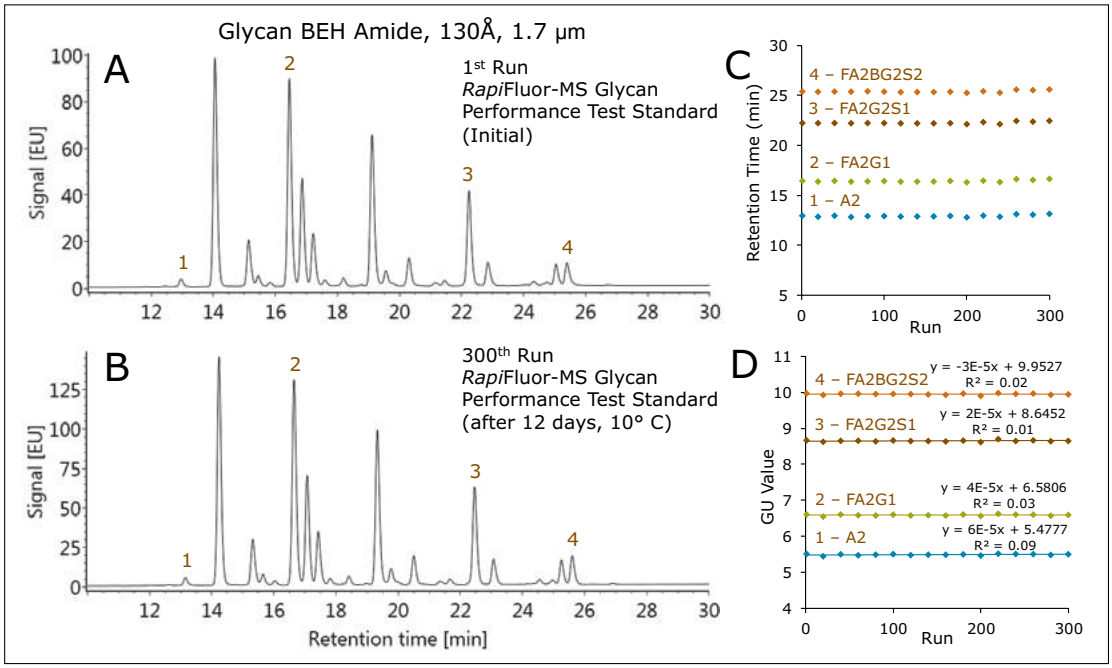


Figure 13. Robustness testing of an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm 2.1 x 150 mm Column for separations of RapiFluor-MS labeled N-glycans. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained at every 20th run from an 8 pmole load of the standard. Four glycan species spread across the separations were monitored to track the retentivity of the stationary phase and column. Fluorescence chromatograms are shown for the (A) 1st run and the (B) 300th run with the column. (C) Retention times as a function of run. (D) Glucose unit (GU) values as a function of run.

In this testing, LC calibrations were performed after every separation of the glycan mixture through application of a dextran ladder and assignment of glucose unit (GU) values. Separations with glycan BEH amide columns can be used in conjunction with glucose unit (GU) values as a means to calibrate HILIC-based glycan separations. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[11] To assign GU values, a dextran ladder (comprised of glucose multimers of increasing length) is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values.

The development of a dextran calibration ladder suitable for use with *Rapi*Fluor-MS labeled glycans was essential yet technically challenging. Given that dextran is a reducing sugar without a strong nucleophile, it cannot, unlike N-glycosylamines, be readily labeled with *Rapi*Fluor-MS Reagent. Because of the distinctive urea linkage imparted to N-glycans upon their derivatization with rapid tagging reagents, *Rapi*Fluor-MS labeled N-glycans have very unique fluorescence maxima at approximately 265 nm (excitation) and 425 nm (emission) (Figure 14A). In a novel labeling approach, we have prepared a *Rapi*Fluor-MS Dextran Calibration Ladder by first reductively aminating dextran with ethanolamine and then labeling it with *Rapi*Fluor-MS.

The resulting urea-linked dextran derivatives exhibit identical fluorescence properties to those of *Rapi*Fluor-MS labeled N-glycans. Furthermore, the obtained dextran is tuned for desired HILIC retention because of the hydroxyl group being incorporated through ethanolamine. A representative fluorescence chromatogram for this novel dextran ladder is provided in Figure 14B, and an example cubic spline fit of the retention data is shown in Figure 14C.

The impact of implementing GU value calibration is exemplified in Figure 13D, where the retention time data throughout the Glycan BEH Amide lifetime testing are reported in GU values. In comparing the retention time data shown in Figure 13C to the GU data in Figure 13D, one can see that the subtle fluctuations in retention times across the 2-week lifetime testing are compensated for by the GU calibration. In fact, RSDs for the GU value data are reduced by a factor of 2 compared to the RSDs in the retention time data.

Also, linear regression analysis of the GU value data shows that there is essentially no drifting in the HILIC retention data once calibrated using a dextran ladder. This analysis therefore clearly demonstrates the value of GU calibration with respect to improving the quality of reported data.

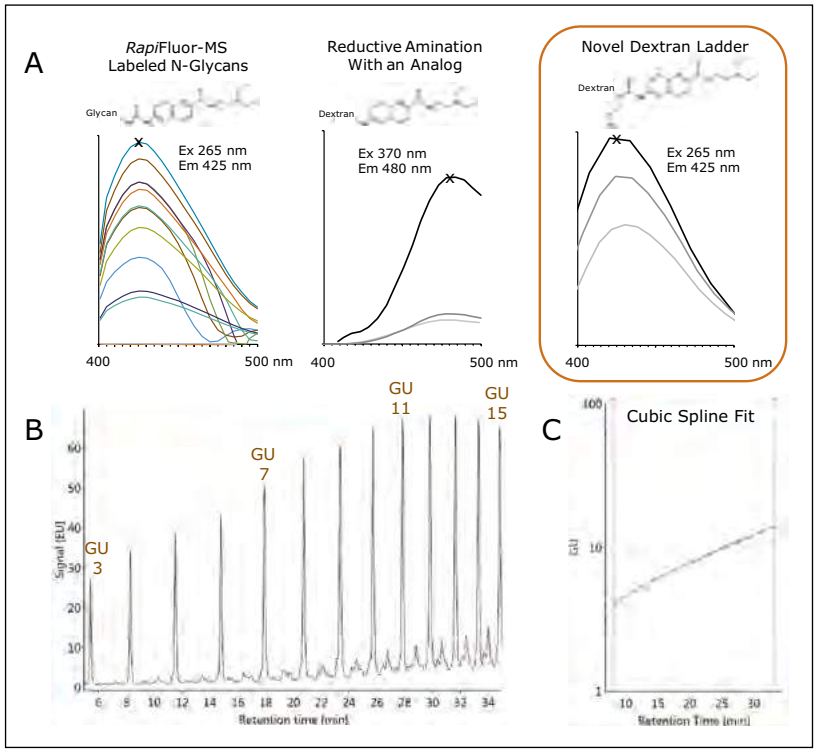


Figure 14. Assignment of Glucose Unit (GU) values with RapiFluor-MS labeling. (A) Chemical structures and fluorescence spectra of RapiFluor-MS labeled N-glycans versus dextrans derivatized with RapiFluor-MS-like labels. The novel dextran ladder that has been commercialized as the RapiFluor-MS Dextran Calibration Ladder is highlighted. (B) An example fluorescence chromatogram for the RapiFluor-MS Dextran Calibration obtained for a 0.5 µg mass load with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. (C) Calibration curve resulting from cubic spline fitting.

CONCLUSIONS

In this application note, we have demonstrated the robustness of *Rapi*Fluor-MS N-glycan preparations and Glycan BEH Amide HILIC Column chromatography. The *Rapi*Fluor-MS N-Glycan Kit enables analysts to perform a high yielding sample preparation with quantitative recovery that ensures accurate and repeatable profiling of N-glycans that is highly comparable to HPLC, 2-AB based methodologies. Moreover, it has been demonstrated that Glycan BEH Amide Columns afford exemplary resolving power and ruggedness for separations of *Rapi*Fluor-MS labeled N-glycans. Additionally, this separation can be readily transferred between UPLC and HPLC platforms. To further ensure success with these new methodologies, two standards have been commercialized, and their use to facilitate *Rapi*Fluor-MS analyses has been demonstrated. The *Rapi*Fluor-MS Glycan Performance Test Standard has been used for benchmarking studies, while the novel *Rapi*Fluor-MS Dextran Calibration Ladder has been employed to enhance the reproducibility of chromatographic retention time data. In summary, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and supporting standards and columns can significantly reduce the burdens associated with N-glycan profiling while providing accurate, reproducible, and sensitive analyses.

Acknowledgement

We would like to thank Peter De Vreugd and Mark Eggink from Synthon Biopharmaceuticals BV for providing data on the comparability of released N-glycan analyses with *Rapi*Fluor-MS and UPLC versus 2-AB and HPLC.

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Applying a Novel Glycan Tagging Reagent, *Rapi*Fluor-MS, and an Integrated UPLC-FLR/QTof MS System for Low Abundant N-Glycan Analysis

Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A novel glycan labeling reagent, *Rapi*Fluor-MS,™ significantly enhances both FLR and MS signals. Improvement from MS detection allows better detection for minor glycan forms.
- The Xevo® G2-XS QTof Mass Spectrometer combines an off-axis ion guide, StepWave,™ with a novel collision cell design to provide significant increases in sensitivity for *Rapi*Fluor-MS labeled glycans.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Biopharmaceutical Platform Solution with UNIFI®

ACQUITY UPLC® H-Class System

ACQUITY UPLC Glycan BEH Amide Column

ACQUITY UPLC FLR Detector

Xevo G2-XS Mass Spectrometer

KEY WORDS

Automated N-Glycan analysis

INTRODUCTION

UPLC-FLR/MS(MS) analysis of released N-glycans labeled with a fluorescent tag has become routine with high-performance LC and MS instrumentations. Glycans labeled with commonly used fluorescent tags, such as 2-AB and 2-AA, can be detected by fluorescent (FLR) detection with ultra-high sensitivity. Unlike an FLR detector, mass spectrometry is known to be less sensitive to detect native or tagged glycans, especially low abundant ones, due to their poor ESI performance. The limited dynamic range of this approach has restricted the use of this combined workflow for glycan characterization.

To overcome the low MS ionization efficiency associated with conventional labels and confidently assign lower-level glycans, a novel tag, *Rapi*Fluor-MS has been developed by Waters. *Rapi*Fluor-MS contains a rapid tagging reactive group, an efficient fluorophore, and a functional group that imparts high ionization efficiency.[1] Complete tagging of glycans can be achieved in less than 5 minutes using this novel reagent.

Initial results with this glycan label show significant enhancement in both FLR and MS(MS) signals compared to 2-AB.[1] The increased sensitivity enables the detection and identification of very low level glycans, at 0.1%, with sufficient MS signal. In this study, we demonstrate the benefits of combining *Rapi*Fluor-MS with an integrated UPLC-FLR/QTof MS system for detailed characterization of the minor glycoforms from the human IgG and mouse IgG1 samples.

EXPERIMENTAL

Sample preparation

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use manual (p/n 715004793en) contains a detailed sample preparation procedure for the deglycosylation of N-glycans from biotherapeutics, followed by the *Rapi*Fluor-MS labeling step and glycan extraction using an SPE device. The entire sample preparation procedure took 30 minutes.

LC conditions

All chromatographic mobile phases are prepared using LC/MS compatible solvents and reagents.

System:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC FLR
Column:	ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)
Column temp.:	60 °C
Mobile phase A:	50 mM ammonium formate (pH 4.4)
Mobile phase B:	100% acetonitrile

UPLC HILIC LC gradient table:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
40.0	0.4	49	51	6
41.5	0.2	100	0	6
44.5	0.2	100	0	6
48.1	0.2	25	75	6
52.6	0.4	25	75	6
60.0	0.4	25	75	6

FLR settings:

MS conditions

System:	Xevo G2-XS QTof MS: ESI+ in sensitivity mode (resolution ~ 30,000)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	300 °C
Desolvation gas flow:	800 L/h

LockSpray

Capillary voltage:	3.0 V
Cone voltage:	40 V
Scan time:	0.5 s
Interval:	20 s

GFP solubilized in 0.1% formic acid with 50:50 (MeCN: H_2O) at 200 fmol/µL was infused, *m/z* = 785.8421 (z = 2) was used for lock mass calibration.

Collision induced dissociation

MS/MS analyses were performed in continuum mode from 100–2000 *m/z* with collision induced dissociation (CID) to generate glycan fragmentation data. Ions with 2+ and 3+ charge states were selected for fragmentation. Customized collision energy tables that were charge state and mass specific were used for optimized fragmentations; the approximated CE range was between 15 to 40 eV. Data Dependent Acquisition (DDA) was used with duty cycle times of 1.6 sec and 0.5 sec for MS and MS/MS modes. The two most abundant precursors were selected for fragmentation.

Data management

UNIFI Scientific Information System v1.7.1

RESULTS

Previous work showed that the *Rapi*Fluor-MS labeling reagent improves N-glycan MS ionization in positive ion mode. More than two order of magnitude MS sensitivity increase was observed when compared to 2-AB label.[1] Combined with highly sensitive Xevo G2-XS QTof Mass Spectrometer, we are now able to detect minor glycoforms with high confidence.

Figure 2 shows an example of analyzing the *Rapi*Fluor-MS labeled N-Glycans released from 0.5 µg of human IgG on UPLC/FLR/QTof MS system. Comparable FLR and MS response across a broad range of glycans was easily achieved.

The MS and MS/MS fragmentation spectra were also shown as an example in Figure 2 for a minor glycoform, A2G2S1, which is present at 0.1% level. The MS spectrum shows doubly charged ions with minor sodium adduct ions in the raw MS spectrum.

We observed a similar fragmentation pathway for the *Rapi*Fluor-MS labeled glycans compared to the 2-AB labeled glycans. The MS/MS fragmentation of A2G2S1 showed that glycosidic bond cleavage from both reducing and non-reducing end was the dominant fragmentation pathway. The observed sequential neutral losses from the non-reducing end stops at the first GlcNAc residue at the reducing end with the *Rapi*Fluor-MS label attached. Also, the counter fragment ions from the non-reducing end, oxonium ions, were readily observed.

In addition to human IgG, we also tested the *Rapi*Fluor-MS labeled glycans released from a mouse IgG1 sample. It is well known that N-glycolyneuraminic acid and alpha (1-3) galactose containing N-glycans on mAbs generated from murine cell lines are glycans with immunogenic epitopes. These glycans present analytical challenges, due to 1) their low abundance in the glycan mixture, and 2) difficulty to characterize them structurally due to poor MS and MS/MS signals from using the conventional labels.

Figure 1. Biopharmaceutical System Solution with UNIFI for glycan analysis.

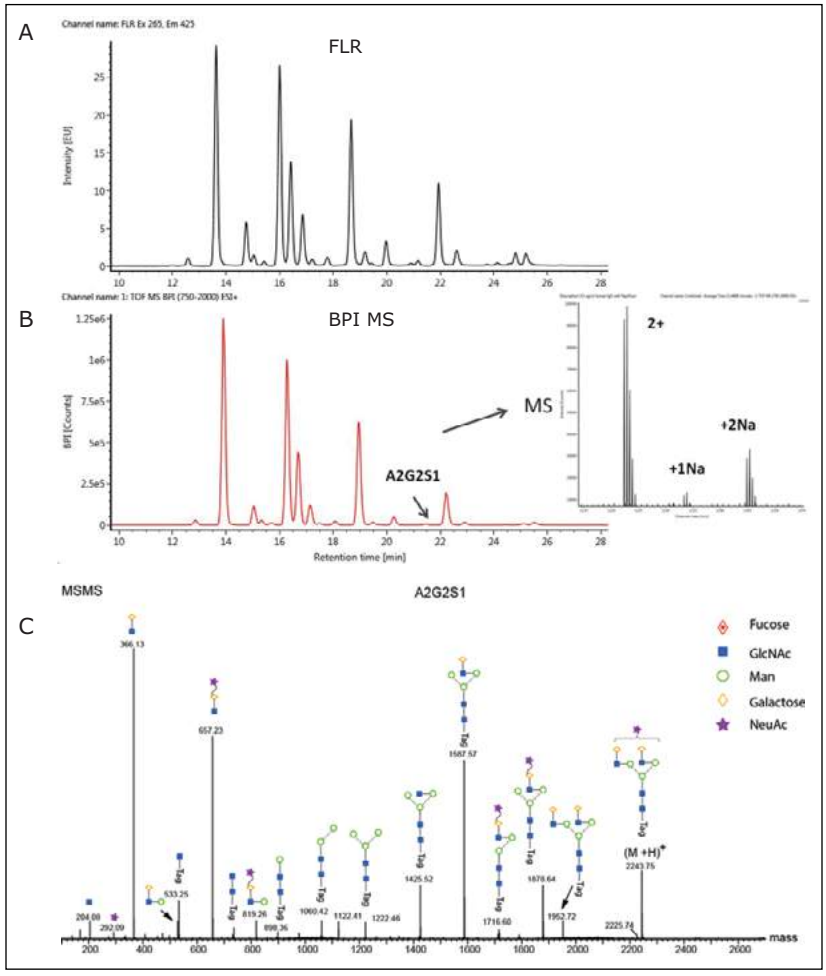

Figure 2. UPLC/FLR/MSMS analysis of RapiFluor labeled human IgG N-glycans. A) FLR data channel. B) BPI MS data channel. The MS spectrum of a low intensity ion was inserted (A2G2S1). The dominant ions were doubly charged with minor sodium adduct ions. C) Deconvoluted MS/MS spectrum of A2G2S1 was displayed.

Figure 3 shows an example of a UPLC/FLR/QTof MS analysis of the mouse IgG1 glycans that contain these immunogenic epitopes. Structural informative fragments (with asterisks) are observed for a low abundant immunogenic glycan, FA2Gal1Sg1, which is present at about 0.1% level. The fragment ion at *m/z* of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at *m/z* of 2260.8 was generated from losing one NeuGc from the precursor ion. This glycan was also observed in FLR chromatogram of 2-AB labeled glycans without sufficient MS signals to obtain good quality CID fragmentation (data not shown). With *Rapi*Fluor-MS labeling chemistry, sufficient amount of precursor ions were obtained for subsequent MS/MS fragmentation.

Overall, we demonstrated that *Rapi*Fluor-MS labeling chemistry enhances MS and MS/MS sensitivity to obtain high quality precursor and fragmentation ion spectra. Therefore, rich structural information for low abundant glycan species are achieved using this approach.

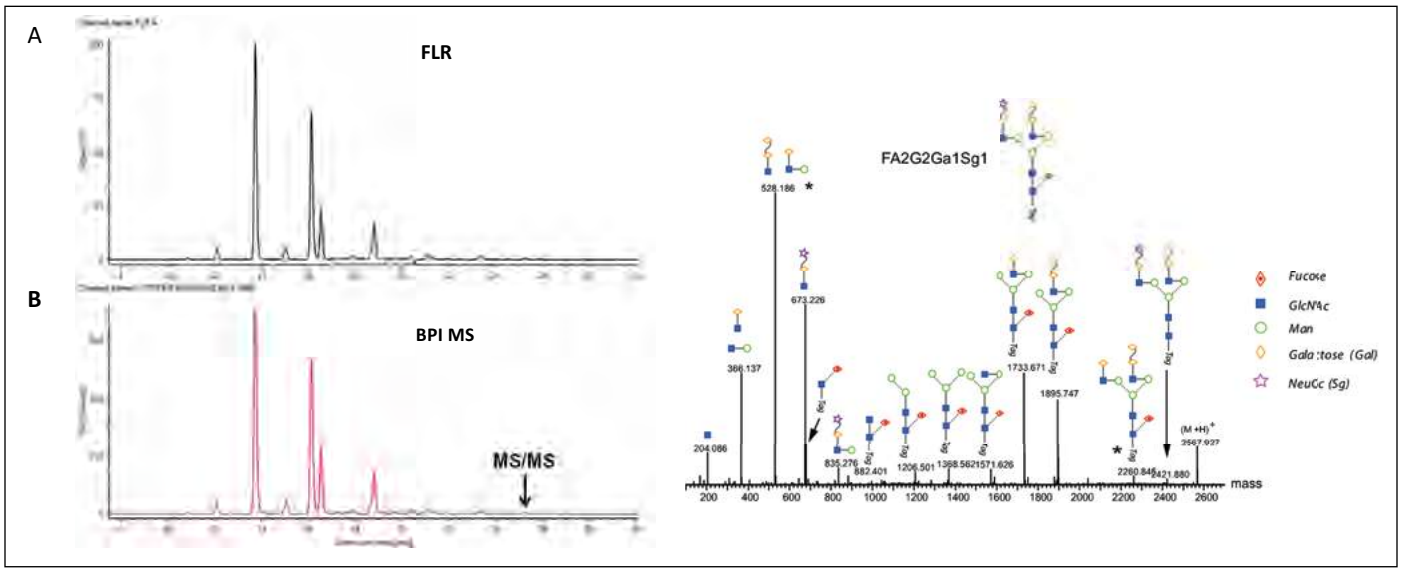

Figure 3. UPLC/FLR/MSMS analysis of RapiFluor-MS labeled mouse IgG1 N-glycans. A) FLR data channel. B) BPI MS data channel. One of the last eluting glycans were selected for MS/MS fragmentation. The deconvoluted fragmentation data from FA2Ga1Sg1 was displayed in C); "Ga" stands for galatose and "Sg" stands for NeuGc. Structurally informative fragments (with asterisks) are observed for this low abundant ion (< 0.1% relative abundance). Fragment ion at m/z of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at m/z of 2260.8 was generated from the loss of one NeuGc from the precursor ion.

CONCLUSIONS

LC/FLR analysis of N-glycans released from protein therapeutics is performed routinely in analytical laboratories around the world. For scientists who want to add MS characterization capability to their glycan analysis, they often struggle with low MS signals and poor quality MS/MS fragmentation for mass confirmation and structure elucidation using conventional FLR labels such as 2-AB and 2-AA. To address these challenges, Waters offers enabling technologies that include the novel *Rapi*Fluor-MS labeling chemistry for rapid glycan sample preparation, and a UPLC/FLR/QTof MS system controlled by UNIFI Scientific Information System. The improved FLR and MS sensitivity from the *Rapi*Fluor-MS label and the QTof MS with StepWave Technology allow confident identification and characterization of minor but critical glycoforms from mAbs.

References

1. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. Waters and New England Biolabs application note (p/n 720005275en.)
2. GlycoWorks *Rapi*Fluor-MS Kit Care and Use Manual (p/n 715004793en.)

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Developing High Resolution HILIC Separations of Intact Glycosylated Proteins Using a Wide-Pore Amide-Bonded Stationary Phase

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APPLICATION BENEFITS

- Improved HILIC separations of intact protein glycoforms through optimization of stationary phase (bonded phase and pore size), ion pairing, column pressurization, and injection approaches.
- MS-compatible HILIC to enable detailed investigations of sample constituents.
- Orthogonal selectivity to conventional reversed-phase (RP) separations for enhanced characterization of glycoprotein samples.
- Glycoprotein BEH Amide, 300Å, 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility.

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide, 300Å Column

Glycoprotein Performance Test Standard

ACQUITY UPLC H-Class Bio System

Xevo® G2 QTof Mass Spectrometer

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated proteins, glycosylation, HILIC

INTRODUCTION

Hydrophilic interaction chromatography (HILIC) has been widely adopted as a tool for separating highly polar compounds. In fact, it has become a relatively widespread technique for small molecule separations. By comparison, the application of HILIC to large biomolecules has been comparatively limited even though there are instances in which the separation selectivity of HILIC would be highly valuable, for example during the characterization of protein glycosylation. A standard approach to the analysis of glycans involves their enzymatic or chemical release from their counterpart protein followed by their chromatographic separation using HILIC. UPLC®-based separations founded upon an optimized, sub-2-µm amide-bonded stationary phase has transformed HILIC separations of released glycans by facilitating faster, higher resolution separations.[1-2] Although released glycan analysis is a gold-standard approach, it has historically required lengthy and at times cumbersome sample preparation techniques. And while the recent introduction of the GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit alleviates many of these shortcomings,[3] alternative means of characterizing protein glycosylation must sometimes be investigated,[4-6] for instance when it is of interest to elucidate sites of modification.[7]

To enable the complementary analysis of glycans as they are still attached to their counterpart proteins, we present an optimized HILIC stationary phase and corresponding methods for resolving the glycoforms of intact and digested glycoproteins. A wide-pore (300Å) amide-bonded, organosilica (ethylene bridged hybrid; BEH)[8] stationary phase is employed along with rigorously developed methods to achieve unprecedented separations of the glycoforms of intact proteins ranging in mass from 10 to 150 kDa.

EXPERIMENTAL

Sample description

Glycoprotein Performance Test Standard (a formulation of bovine RNase A and RNase B, p/n 186008010) and RNase B (Sigma R7884) were reconstituted in 18.2 MΩ water to a concentration of 2 mg/mL. Trastuzumab was diluted with water from its formulated concentration of 21 mg/mL to a concentration of 2 mg/mL.

For column conditioning, the components of a vial of Glycoprotein Performance Test Standard (100 µg) were dissolved in 25 µL of 0.1% trifluoroacetic acid (TFA), 80% acetonitrile (ACN) to create a 4 mg/mL protein solution.

To investigate the resolution of glycan occupancy isoforms, Intact mAb Mass Check Standard (p/n 186006552) was deglycosylated using the following techniques. The glycoprotein (15 µg) was reconstituted to a concentration of 0.52 mg/mL into a 28.2 µL solution of 1% (w/v) *Rapi*Gest™ SF Surfactant and 50 mM HEPES (pH 7.9). This solution was heated to 90 °C over 3 minutes, allowed to cool to 50 °C, and mixed with 1.2 µL of GlycoWorks Rapid PNGase F solution. Deglycosylation was completed by incubating the samples at 50 °C for 5 minutes. To produce partial deglycosylation, Intact mAb Mass Check Standard was deglycosylated using only a 5 minute, 50 °C incubation with PNGase F without a heat-assisted pre-denaturation.

Method conditions

(unless otherwise noted)

Column conditioning

New (previously unused) ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Columns should be conditioned, before actual test sample analyses, via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (10 µL injections of 4 mg/mL in 0.1% TFA, 80% ACN) or with equivalent loads of a test sample for which the column has been acquired. The separation outlined in Figure 2 can be employed for conditioning with the Glycoprotein Performance Test Standard.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	5 °C
Analytical column temp.:	30 °C (unless noted otherwise in the caption)
UV detection:	214/280 nm, 2 Hz
Fluorescence detection:	Ex 280/Em 320 nm, 10 Hz
Flow rate:	0.2 mL/min
Injection volume:	≤1 µL (aqueous diluents). Note: It might be necessary to avoid high organic diluents for some samples due to the propensity for proteins to precipitate under ambient conditions. A 2.1 mm I.D. column can accommodate up to a 1.2 µL aqueous injection before chromatographic performance is negatively affected.
Columns:	ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard); ACQUITY UPLC Glycoprotein BEH Amide 300Å, 1.7 µm, 2.1 x 100 mm (p/n 176003701, with Glycoprotein Performance Test Standard); ACQUITY UPLC BEH HILIC, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186003462) XBridge BEH HILIC, 130Å, 5 µm, 2.1 x 150 mm (p/n 186004446) ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742); ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 100 mm (p/n 186004741);

Competitor columns: PolyHYDROXYETHYL A™, 300Å, 3 µm, 2.1 x 100 mm;
Glycoplex® A, 3 µm, 2.1 x 100 mm;
ZORBAX® RRHD 300-HILIC, 300Å, 1.8 µm, 2.1 x 100 mm;
Halo® PentaHILIC, 90Å, 2.7 µm, 2.1 x 100 mm;
SeQuant® ZIC-HILIC, 200Å, 3.5 µm, 2.1 x 100 mm;
Accucore™ Amide, 150Å, 2.6 µm, 2.1 x 100 mm;
TSKgel® Amide-80, 80Å, 3 µm, 2.0 x 100 mm

Column connector (for coupling 150 mm columns): 0.005 x 1.75 mm UPLC SEC Connection Tubing (p/n 186006613)

Vials: Polypropylene 12 x 32 mm , 300 µL Screw Neck Vial, (p/n 186002640)

Gradient used to demonstrate the progression of HILIC separation technologies (Figure 1):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
20	80.0	20.0	6
21	20.0	80.0	6
30	20.0	80.0	6

Focused gradient for RNase B HILIC separations (Figures 2 and 5)

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
1	34.0	66.0	6
21	41.0	59.0	6
22	100.0	0.0	6
24	100.0	0.0	6
25	20.0	80.0	6
35	20.0	80.0	6

Gradient for benchmarking/evaluations (Figure 3)

Column dimension: 2.1 x 100 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0.0	20.0	80.0	6
0.7	30.0	70.0	6
29.3	45.0	55.0	6
30.0	80.0	20.0	6
31.3	80.0	20.0	6
32.0	20.0	80.0	6
40.0	20.0	80.0	6

Gradient employed to select a mobile phase additive (Figure 4):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water or 50 mM ammonium formate, pH 4.4 or 0.5% (w/v) formic acid, water

Mobile phase B: ACN

Time	%A	%B	Curve
0	20.0	80.0	6
20	80.0	20.0	6
21	20.0	80.0	6
30	20.0	80.0	6

Focused gradient for reversed phase of RNase B (Figure 6):

Column dimension: 2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, water

Mobile phase B: 0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	95.0	5.0	6
1	74.5	25.5	6
21	67.5	32.5	6
22	10.0	90.0	6
24	10.0	90.0	6
25	95.0	5.0	6
35	95.0	5.0	6

Focused gradient for intact trastuzumab (Figures 7 and 8)

Column dimension:	2.1 x 150 mm, with varying lengths 25 µm I.D. PEEK post-column tubing Or two coupled 2.1 x 150 mm columns
Mobile phase A:	0.1% (v/v) TFA, water
Mobile phase B:	0.1% (v/v) TFA, ACN

Time	%A	%B	Curve
0	20.0	80.0	6
1	30.0	70.0	6
21	37.0	63.0	6
22	70.0	30.0	6
24	70.0	30.0	6
25	20.0	80.0	6
45	20.0	80.0	6

Conditions for resolving glycan occupancy isoforms of an IgG (Figure 9):

Column dimension:	Two coupled 2.1 x 150 mm or a single 2.1 x 150 mm
Column temp.:	80 °C
Mobile phase A:	0.1% TFA, 0.3% HFIP in water
Mobile phase B:	0.1% TFA, 0.3% HFIP in ACN

Time	%A	%B	Curve
0.0	20	80	6
10.0	50	50	6
11.0	100	0	6
14.0	100	0	6
15.0	20	80	6
25.0	20	80	6

MS conditions

MS system:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 2 µg/µL from 100–2000 *m/z*
Acquisition:	500–4000 *m/z*, 0.5 sec scan rate
Data management:	MassLynx® Software (v4.1)

RESULTS AND DISCUSSION

Progression of HILIC technology for glycoprotein separations

HILIC originated in the early 1990s as a separation technique to resolve highly polar molecules using mobile phases adapted from reversed phase chromatography.[9] The HILIC separation mechanism is largely believed to be dependent on a polar stationary phase that adopts an immobilized water layer.[9] Hydrophilic analytes partition into this immobilized water layer and undergo interaction with the phase via a combination of hydrogen bonding, dipole-dipole, and ionic interactions. In this way, hydrophilic analytes will be retained on the HILIC phase under apolar initial mobile phase conditions and later eluted by increasing polar mobile phase concentration via use of an LC gradient.[9]

Numerous HILIC or HILIC-like stationary phases have been developed in the last two decades. Many based solely on unbonded silica particles are widely available, so too are HILIC phases based on polyalcohol bondings or charge bearing surfaces, such as those with zwitterionic bondings. For the enhanced retention and selectivity of glycans, amide bonded phases have become increasingly popular. The ACQUITY UPLC Glycan BEH Amide stationary phase found in Waters Glycan Column has, for instance, found wide-spread use for high resolution released glycan separations.

As mentioned before, HILIC has, however, not seen wide-spread use in intact large molecule applications. Concerns that high organic solvent concentrations can result in protein precipitation have most likely discouraged many from attempting to develop HILIC-based, protein separation methods. Endeavoring beyond these perceptions, we have developed a new amide-bonded stationary phase based on a wide-pore, organosilica (ethylene bridged hybrid; BEH) particle that was specifically designed to facilitate large molecule separations. It exhibits a porous network accessible to most proteins and an average pore diameter that does not impart significant peak broadening due to restricted diffusion, which can occur when protein analytes are too close in size to the average pore diameter of a stationary phase (e.g. within a factor of 3).

The progression of HILIC technology culminating in this new stationary phase is remarkable. The emerging technology of large molecule HILIC can be captured by separations of bovine ribonuclease B (RNase B), a 13 kDa protein comprised of several high mannose (Man5 to Man9) glycoforms. Figure 1 shows RNase B separated by several different stationary phases. From bottom to top, increasingly better separations of RNase B were achieved as increasingly newer chromatographic technologies were adopted, from 5 µm to 1.7 µm particles, from unbonded to amide bonded particles, and from standard pore diameter (130Å) to wide-pore diameter (300Å) particles. It is with BEH Amide, 300Å, 1.7 µm particles that RNase B glycoforms are best separated. The use of a wide-pore stationary phase plays a significant role in achieving optimal resolution. This is highlighted in Figure 2 wherein benchmarking results are presented from the use of a newly developed test mixture, called Glycoprotein Performance Test Standard, which contains bovine RNase B, its corresponding glycoforms and its aglycosylated isoform (RNase A). Example separations are provided for this standard wherein a focused gradient has been used with the wide-pore (300Å) BEH Amide as well as the standard pore size (130Å) BEH Amide stationary phase. Notice that the widepore amide column affords a measurable (24%) increase in the resolution between the aglycosylated RNase A isoform and the Man5 glycoform of RNase B, in addition to sizeable increases in resolution throughout the separation.

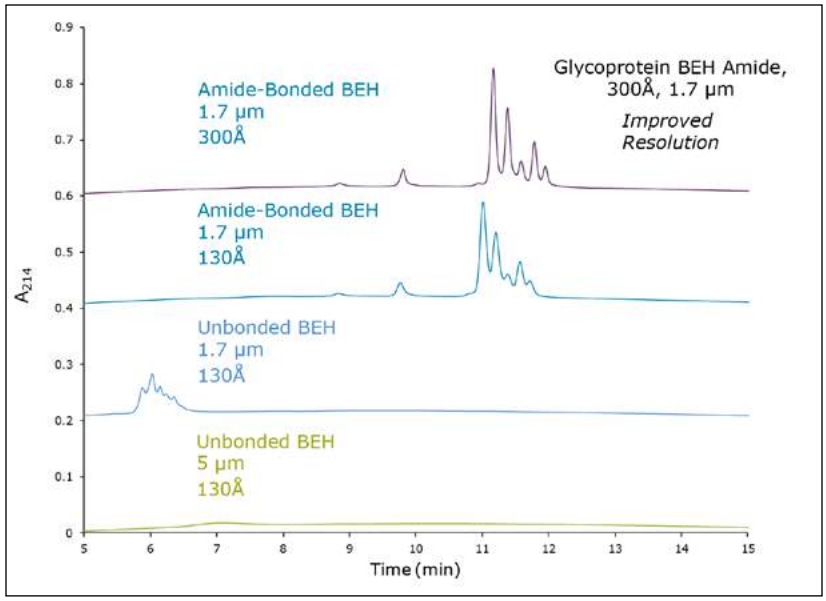
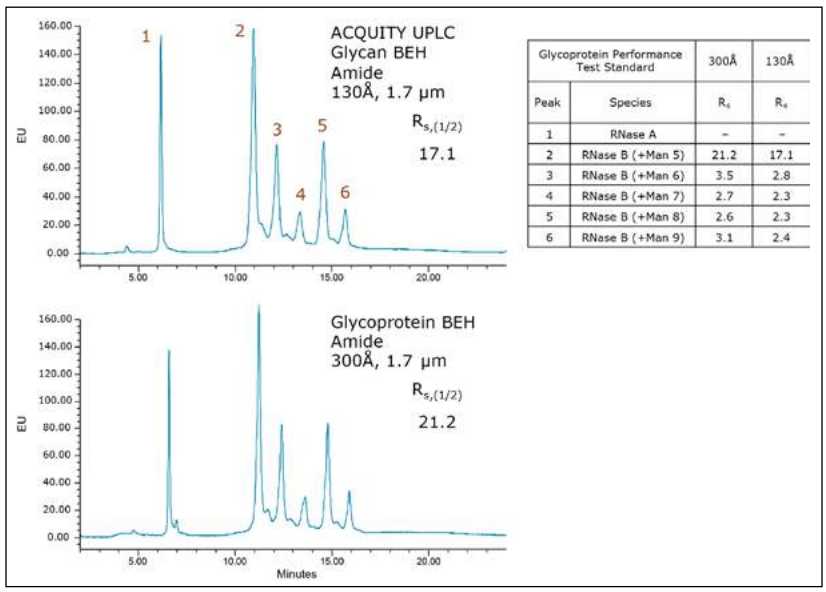


Figure 1. Progression of HILIC stationary-phase technologies for intact glycoprotein separations. Separation of 1 µg of RNase B using 2.1 x 150 mm columns packed with stationary phases ranging from HPLC-size unbonded organosilica (XBridge BEH HILIC, 130Å, 5 µm) to sub-2-µm amide-bonded organosilica 300Å, 1.7 µm particles (ACQUITY UPLC Glycoprotein BEH Amide 300Å, 1.7 µm).



Glycoprotein Performance Test Standard		300Å	130Å
Peak	Species	R_s	R_s
1	RNase A	-	-
2	RNase B (+Man 5)	21.2	17.1
3	RNase B (+Man 6)	3.5	2.8
4	RNase B (+Man 7)	2.7	2.3
5	RNase B (+Man 8)	2.6	2.3
6	RNase B (+Man 9)	3.1	2.4

Figure 2. Separations of the Glycoprotein Performance Test Standard (RNase A + RNase B glycoforms) using a Glycoprotein BEH Amide 300Å, 1.7 µm Column versus a BEH Amide, 130Å, 1.7 µm Column. The reported resolution values were calculated using the half-height peak widths of species 1 and 2 (RNase A and RNase B Man5 glycoforms, respectively). Fluorescence detection at Ex 280 nm and Em 320 nm and a column temperature of 45 °C were employed in this example.

The significance of these recent developments becomes more apparent when benchmarked against other commercially available HILIC phases. RNase B separations resulting from an evaluation of 10 different HILIC stationary phases are shown in Figure 3. It can be seen that 6 out of the 10 evaluated materials showed undesirable characteristics, including poor recovery and poor retention. It was only with the amide bonded stationary phases and particle technologies based on 100Å or greater pore diameters that reasonable separations of RNase B glycoforms could be achieved.

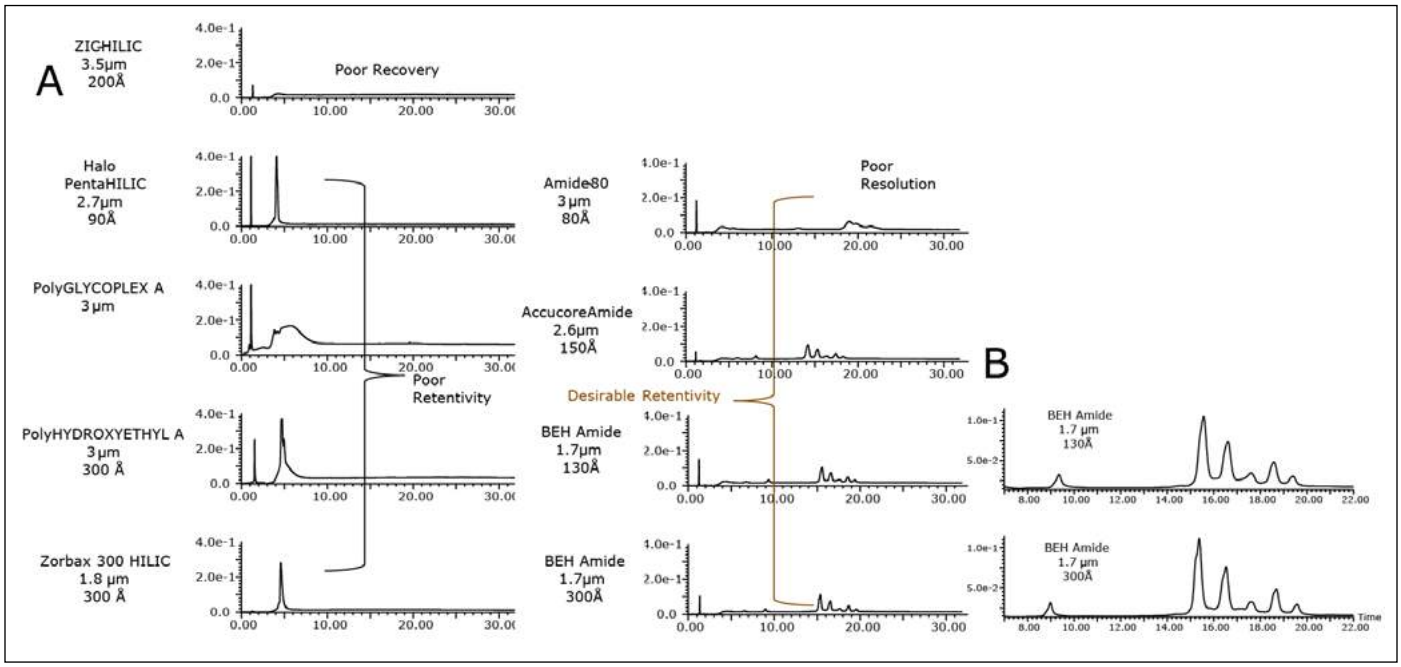


Figure 3. Evaluation of commercially available HILIC columns for intact glycoprotein separations. (A) UV chromatograms obtained for RNase B using 10 different stationary phases. (B) Zoomed HILIC UV chromatograms for the highest resolution separations.

Mobile phase optimization, MS compatibility, and orthogonality to reversed phase

High resolution HILIC separations of protein glycoforms require that mobile phase selection be given significant consideration. Most HILIC separations have been developed so as to rely on ammonium salts (formate or acetate) to mitigate significant ionic interactions and to control mobile phase pH. The suitability of such mobile phase systems to glycoproteins was evaluated using RNase B.

Figure 4 shows the corresponding RNase B chromatogram obtained when 0.1% TFA is used as the mobile phase modifier instead of 50 mM ammonium formate or 0.5% formic acid, two mobile phase compositions more frequently used for HILIC separations.[2,7] It is with 0.1% TFA that glycoforms are best resolved. Along with enhancing glycoform resolution, the TFA-modified mobile phase reduced the retention of RNase B. Together these observations highlight the significance of acidic, ion pairing mobile phases to being able to achieve high resolution glycoprotein separations using HILIC. It is proposed that the acidic condition imparted by the TFA ensures that acidic residues of the protein are fully protonated and thus present in their more hydrophobic state. In addition, the ion pairing of the TFA counter ion to basic residues, ensures that cationic residues will also be separated in a more hydrophobic form. In this way, retention of a glycoprotein onto a HILIC phase is driven primarily by the glycans and a separation more selective to resolving differences in the glycan modification is achieved.

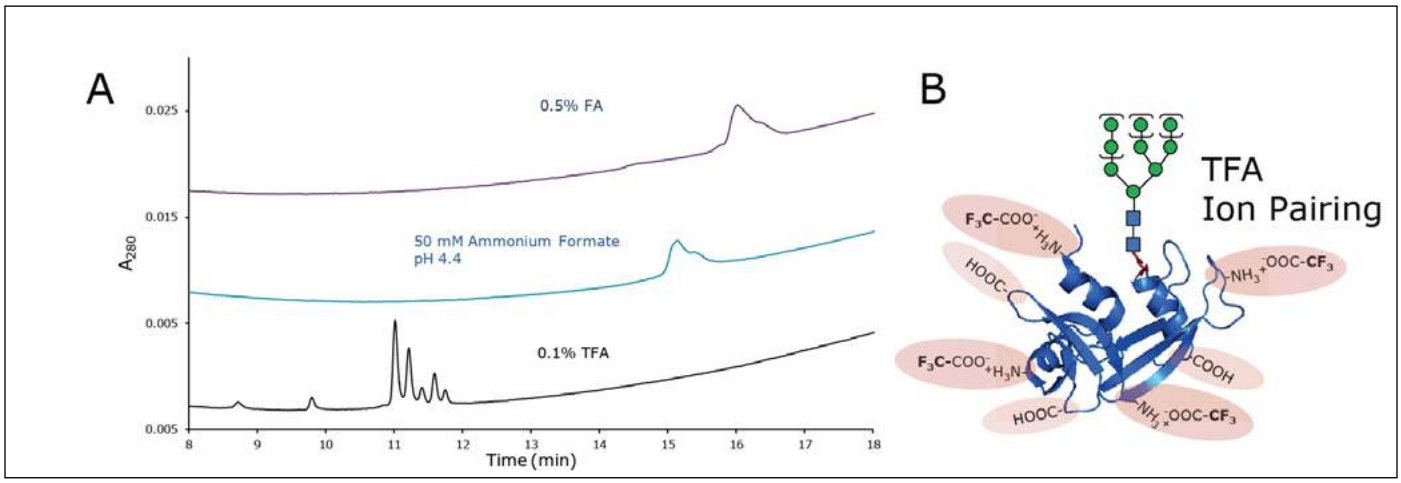
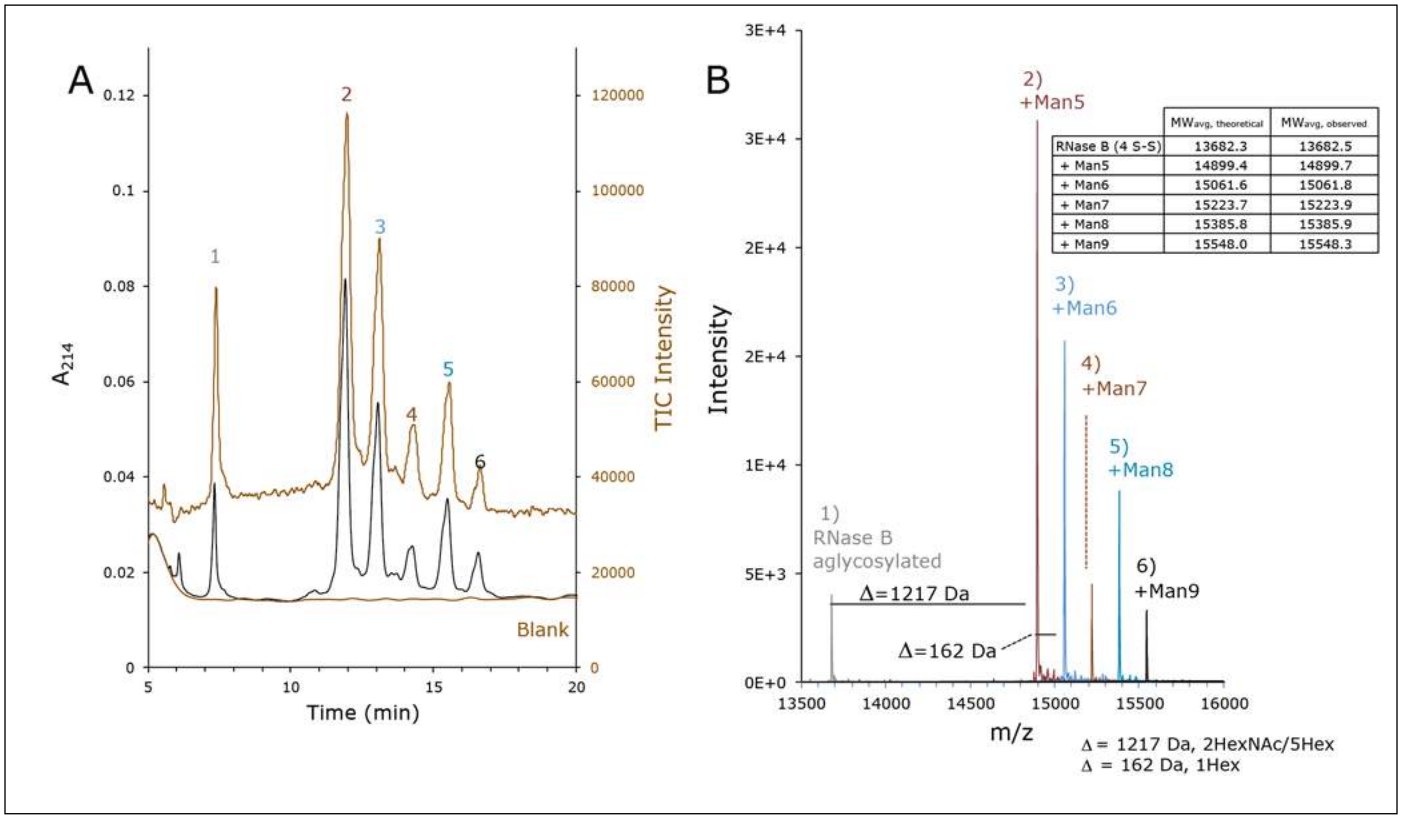


Figure 4. Optimization of mobile phase conditions for separations of intact and digested glycoproteins. (A) UV chromatograms obtained for RNase B when using various mobile phases and a Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Schematic portraying the utility of ion pairing for glycoprotein HILIC separations. Reduced hydrophilicity imparted via ion pairing with a hydrophobic, strong acid is displayed with shading. [PDB:1RBB]

Fortunately, TFA-modified mobile phases can be readily coupled to ESI-MS, due to their volatility. This aspect of the developed HILIC methods enables on-line characterization of the resolved glycoforms and presents a new option for profiling a sample containing glycosylated protein. To this end, the peaks resolved from RNase B using a BEH Amide, 300Å, 1.7 µm column were subjected to interrogation by ESI-MS.

Figure 5 shows both a UV chromatogram and a corresponding total ion chromatogram (TIC) obtained when separating RNase B. By summing and deconvoluting (MaxEnt™ 1) the mass spectra obtained for the six labeled peaks, it was confirmed that RNase B glycoforms were being detected. In fact, the observed deconvoluted masses support identifications of aglycosylated RNase B (RNase A) along with RNase B modified by Man5 through Man9.



	MW avg, theoretical	MW avg, observed
RNase B (4 S-S)	13682.3	13682.5
+ Man5	14899.4	14899.7
+ Man6	15061.6	15061.8
+ Man7	15223.7	15223.9
+ Man8	15385.8	15385.9
+ Man9	15548.0	15548.3

Figure 5. HILIC-MS of RNase B. (A) UV (bottom) and TIC (top) chromatograms obtained for RNase B when using a focused gradient and a Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Deconvoluted mass spectra obtained for each labeled peak along with corresponding glycoform identifications.

Finally, it should be pointed out that the newly developed stationary phase and the demonstrated methodologies provide new separation selectivity, one that is orthogonal and complementary to conventional reversed phase separations. Figure 6A shows that RNase B can, for instance, be separated by reversed-phase chromatography using a BEH C_4, 300Å, 1.7 µm column so as to produce a high resolution separation of aglyocosylated RNAse B (RNase B) from its glycosylated isoforms. By reversed phase, however, none of high mannose glycoforms of RNase B can be resolved from one another. In contrast, a BEH Amide, 300Å, 1.7 µm column yields baseline resolution of each major glycoform (Figure 6B).

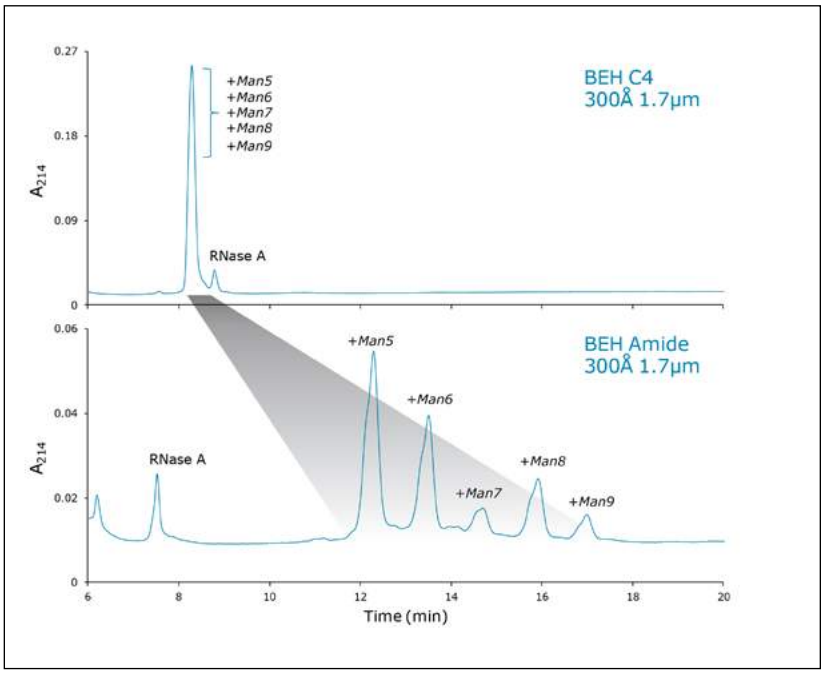


Figure 6. Orthogonality of reversed phase with BEH C_4, 300Å, 1.7 µm and HILIC with BEH Amide, 300Å, 1.7 µm Columns. (A) Separation of RNase B (1 µg) using an ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm, 2.1 x 150 mm Column. (B) Separation of RNase B (1 µg) using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

Separation of the heterogeneous glycoforms of an intact mAb

To explore the limits of this new technology, we have investigated the capabilities of resolving the glycoforms of intact mAbs. Specifically, separations of trastuzumab have been explored. These experiments required special considerations regarding sample injection, primarily because trastuzumab and numerous other glycoproteins are not readily soluble in high organic concentrations. In fact, 70–80% ACN is generally a solution condition that initiates the precipitation of proteins, such as an IgG. Accordingly, conditions for the optimal injection of aqueous diluents were developed. It has been found that a 2.1 mm I.D. column can accommodate an injection of aqueous sample up to 1 µL. From a 2 mg/mL aqueous sample of trastuzumab, appropriate sample mass loads could thus be injected and HILIC separations of the IgG could be performed. It should be mentioned that high ACN diluents can be used in intact protein HILIC, but care must be taken to enhance the solubility of the protein sample through either the use of TFA ion pairing at concentrations between 0.2–1.0%, the combined application of TFA and hexafluoroisopropanol (HFIP), or by use of co-solvents, such as dimethylsulfoxide (DMSO) (data not shown).

As shown in Figure 7, trastuzumab can indeed be separated into multiple chromatographic peaks using a BEH Amide, 300Å, 1.7 µm column and an injection from a simple 100% aqueous diluent. However, at the backpressures produced from just a 150 mm length column, a noticeably tailing profile was observed. MS analysis indicated that the first set of peaks could be accurately assigned as the G0F/G0F, G0F/G1F, G1F/G1F, and G1F/G2F glycoforms of intact trastuzumab. An intact IgG is a dimeric structure, with a minimum of two N-glycan sites on two heavy chains, explaining the observation of combinatorially formed glycoforms. This is consistent with observations by intact mass analysis of IgGs.[10] The tailing component of the chromatographic profile was in contrast found by MS to correspond to multiple, co-eluting trastuzumab glycoforms. With this result, we proposed that on-column aggregation was occurring and that increased column pressure could be a solution to HILIC of intact immunoglobulins, specifically since it had previously been reported that ultrahigh pressures can be beneficial to limiting carryover and ghosting during reversed phase of intact proteins.[11] The effects of introducing additional column pressure was investigated by means of introducing varying lengths of narrow I.D., post-column PEEK tubing. Figure 7 (darker traces) displays the effects of introducing increasingly higher column pressure. By doubling the column pressure so that trastuzumab would elute under conditions of approximately 7,500 psi, the putative, aggregate peaks in the chromatographic profile were eliminated. It is encouraging that under these conditions the resulting chromatographic profile is represented by 5 major glycoforms, which again is consistent with ESI-MS of intact trastuzumab.[10] It is interesting to additionally note that retention decreases as column pressure increases. This is a phenomenon that has been described previously for HILIC separations of monosaccharides.[12] It has been proposed that increasingly higher pressures result in less coordination of water to the analyte and in turn reduced retention, an opposite effect to that observed during reversed phase chromatography.[12]

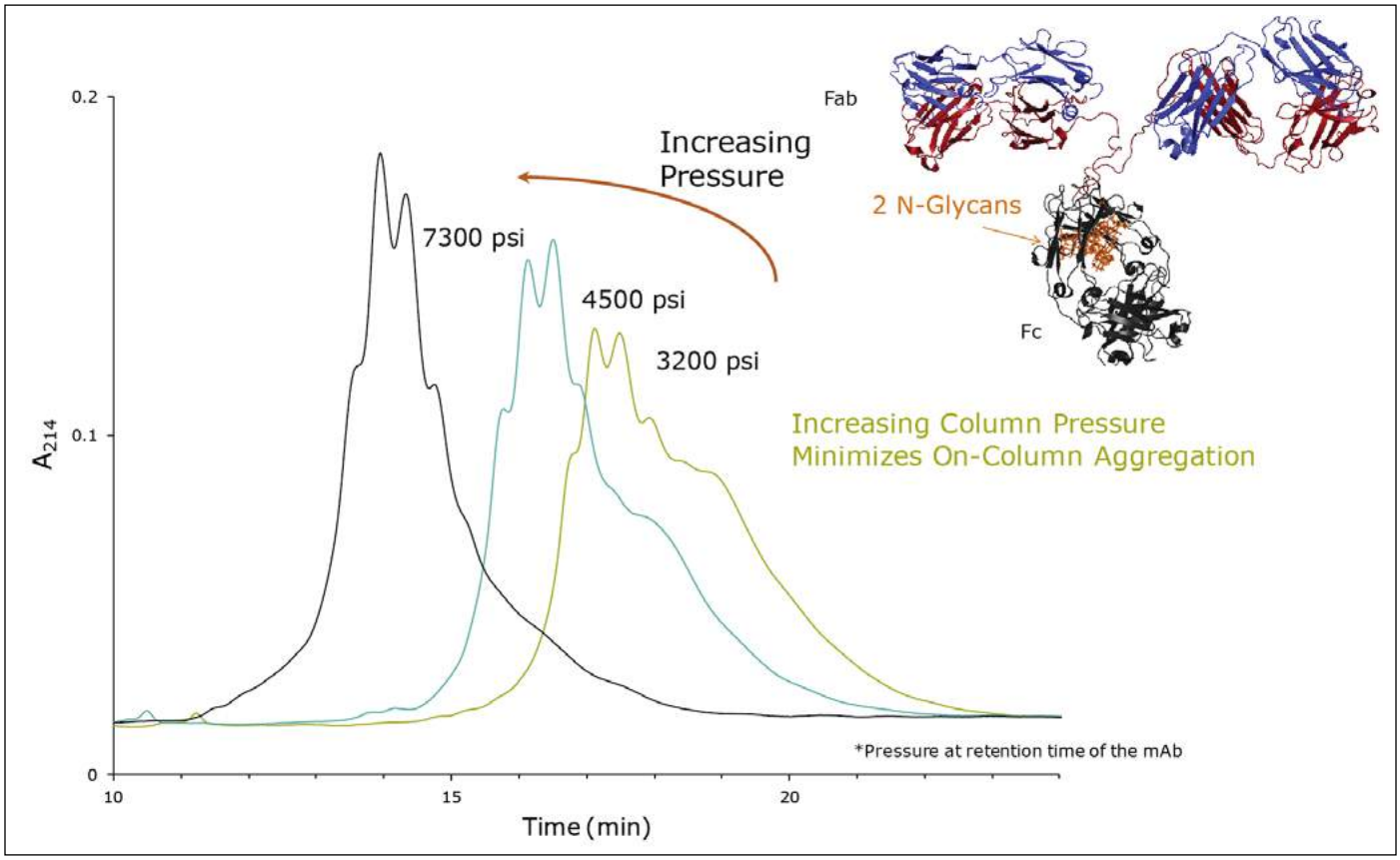


Figure 7. Effect of column pressure on the HILIC separation of an IgG. Trastuzumab (1 µg) was separated on Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column with and without flow restriction. [PDB:1IGT]

Given that intact IgGs benefit from separations at ultrahigh pressures, we pursued separations based on the use of two BEH amide 300Å, 1.7 µm, 2.1 x 150 mm columns coupled with a low volume, high pressure column connector. The separation for intact trastuzumab obtained with these coupled columns is displayed in Figure 8, along with extracted ion chromatograms that provide evidence to achieving separations of the glycoforms. This 300 mm configuration provided the requisite column pressures for an optimal HILIC separation and additionally produced greater resolution between glycoforms. Clearly, additional theoretical plates are therefore advantageous during HILIC of even very high molecular weight species, which supports the significance of partitioning for such separations.

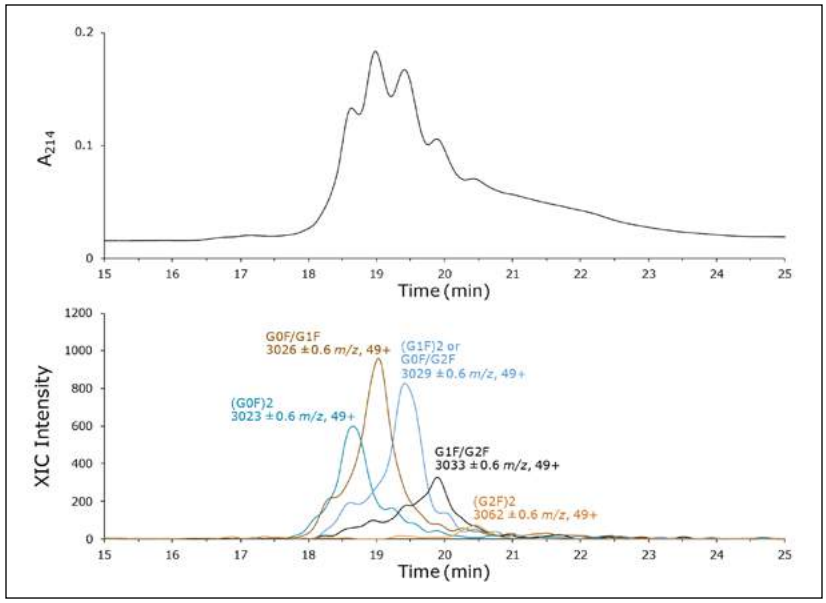


Figure 8. Separation of intact trastuzumab glycoforms using coupled ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Columns. A UV chromatogram and extracted ion chromatograms for each of the major heterogenous glycoforms of trastuzumab are displayed. The column pressure at the retention time of the mAb was approximately 7,000 psi.

An LC method for glycan occupancy

A UPLC HILIC separation of an intact IgG can be used for more than just an attempt to separate individual glycoforms. Equally interesting is the use of these new separation capabilities to resolve information about glycan occupancy. To this end, we evaluated the capabilities of the BEH Amide, 300Å column to assess the glycan occupancy of an IgG. This was exemplified by a study of reaction products resulting from various PNGase F deglycosylation treatments. Using an elevated 80 °C column temperature, TFA ion pairing, and an HFIP mobile phase additive, we have been successful in enhancing the solubility of IgGs and collapsing the fine structure otherwise captured for the individual, heterogenous intact IgG glycoforms (i.e. G0F/G0F versus G0F/G1F). Figure 9 presents HILIC fluorescence chromatograms resulting from such a separation of native Intact mAb Mass Check Standard (a murine IgG1 mAb) and its partially as well as completely deglycosylated isoforms. As can be seen, HILIC fluorescence profiles for these three samples are dramatically different. On-line mass spectrometric detection has confirmed that the peaks in these profiles correspond to different states of glycan occupancy.

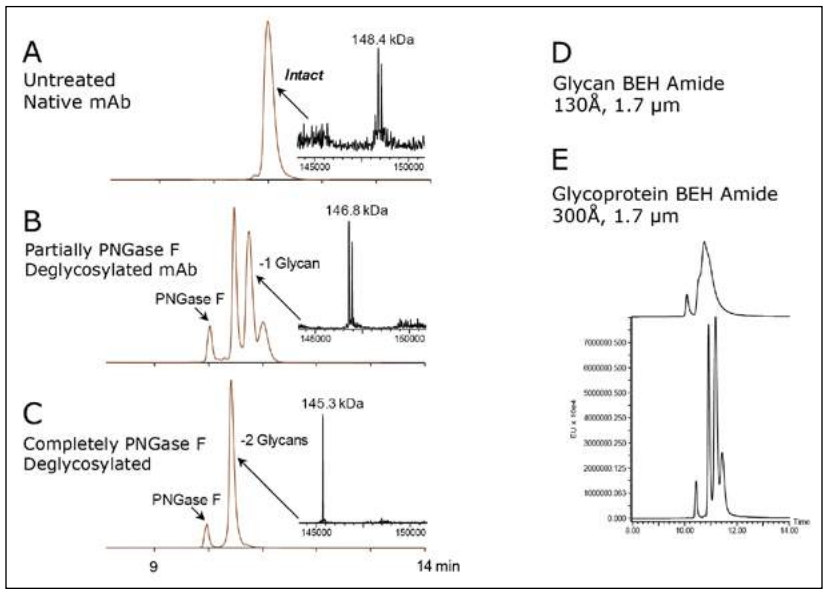


Figure 9. Assaying glycan occupancy and deglycosylation by intact protein HILIC-FLR-MS. HILIC fluorescence profiles obtained for three different samples are shown: (A) native, (B) partially deglycosylated, and (C) completely deglycosylated Intact mAb Mass Check Standard. Samples of this mAb (1.5 µg) were separated using two coupled Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Columns. HILIC fluorescence profiles of partially deglycosylated Intact mAb Mass Check Standard using a (D) ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column versus a (E) Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

The most strongly retained species, represented by the native mAb sample, corresponds to the doubly (fully) glycosylated form of the intact mAb. The partially deglycosylated mAb sample meanwhile yielded several additional peaks with lower HILIC retention, two of which with corresponding detected molecular weights that are indicative of once deglycosylated and fully deglycosylated mAb species and a third with a corresponding detected molecular weight consistent with PNGase F. In contrast, the completely deglycosylated mAb sample presented a homogenous fluorescence profile along with an observed molecular weight for the mAb that is in agreement with the predicted molecular weight of the deglycosylated mAb (145.3 kDa). It is worth noting that when attempting to use the BEH Amide, 130Å, 1.7 µm stationary phase, none of the above peaks could be resolved (Figures 9D and 9E). So indeed, the widepore phase facilitates the development of previously unobtainable separations.

In our hands, the above assay has been used to develop rapid enzymatic deglycosylation protocols.[3] However, it is natural to suggest that these same methods could be applied to measure the glycan occupancy of an intact therapeutic mAb, in which case the relative abundance of aglycosylated forms (-2 and -1 N-glycans) could potentially be monitored by fluorescence and corroborated by LC-MS.

CONCLUSIONS

HILIC of small molecules has garnered wide-spread attention and use. In contrast, the application of the technique to large biomolecule separations has been limited. With the development of the above mentioned amide-bonded, wide-pore HILIC stationary phase and corresponding methods, it is now possible to resolve the glycoforms of intact glycosylated proteins, as has been exemplified by the resolution of the heterogenous glycoforms on intact trastuzumab. Alternatively, the described techniques can be applied to studies of glycan occupancy. Just as reversed phase separations are employed for resolving protein isoforms that have varying hydrophobicities, HILIC separations with BEH Amide 300Å can be explored for resolving protein isoforms that exhibit varying hydrophilicities, such as isoforms differing with respect to glycan occupancy. With the availability of these new separation capabilities, it will be possible to perform more detailed characterization of intact glycoproteins, whether by means of combining HILIC with optical detection or with ESI-MS.

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MAIN MENU TABLE OF CONTENTS

Enhancing the Peak Capacity of High Molecular Weight N-Glycan HILIC Separations with a Wide-Pore Amide Bonded Stationary Phase

Matthew A. Lauber and Stephan M. Koza

GOAL

To demonstrate the enhanced resolving power of the ACQUITY UPLC® Glycoprotein BEH amide, 300Å, Column for separations of high molecular weight, *Rapi*Fluor-MS™ labeled *N*-glycans.

BACKGROUND

Protein glycosylation is frequently profiled by removing glycans from their counterpart glycoprotein and imparting them with a detectable chemical moiety, such as the fluorescence and MS-active *Rapi*Fluor-MS label.[1] High resolution separations of these released and labeled *N*-glycans can be obtained by UPLC® hydrophilic interaction chromatography (HILIC) with purposefully designed glycan BEH amide, 130Å columns.[2] Interestingly, glycosylation of proteins can be extremely diverse. While monoclonal antibodies tend to be modified with relatively low molecular weight (1 to 3 kDa) biantennary structures, numerous biotherapeutic proteins are expressed with comparatively high molecular weight (3 to 6 kDa) tri- and tetra-antennary structures. Such large and highly branched glycan structures exhibit large radii of hydration.

Wide-pore glycoprotein BEH amide, 300Å, 1.7 µm columns for enhancing the resolution of tri- and tetra-antennary, *Rapi*Fluor-MS labeled *N*-glycans.

Consequently, the application of chromatography columns containing particles with standard average pore diameters (80 to 150Å) can limit the resolution with which these species can be separated. It is therefore advantageous to employ a stationary phase with a wide average pore diameter, wherein large structures will have access to the majority of the porous network and the surface area of the stationary phase. In addition, the large labeled glycan structures are less likely to experience restricted diffusion while migrating through the pores of a wide-pore material.[3-4] In this technology brief, we demonstrate the utility of an amide bonded stationary phase with an average pore diameter of 300Å (glycoprotein BEH amide, 300Å, 1.7 µm) and its ability to enhance the resolution of *Rapi*Fluor-MS labeled tri and tetra-antennary *N*-glycans derived from recombinant human Factor IX.

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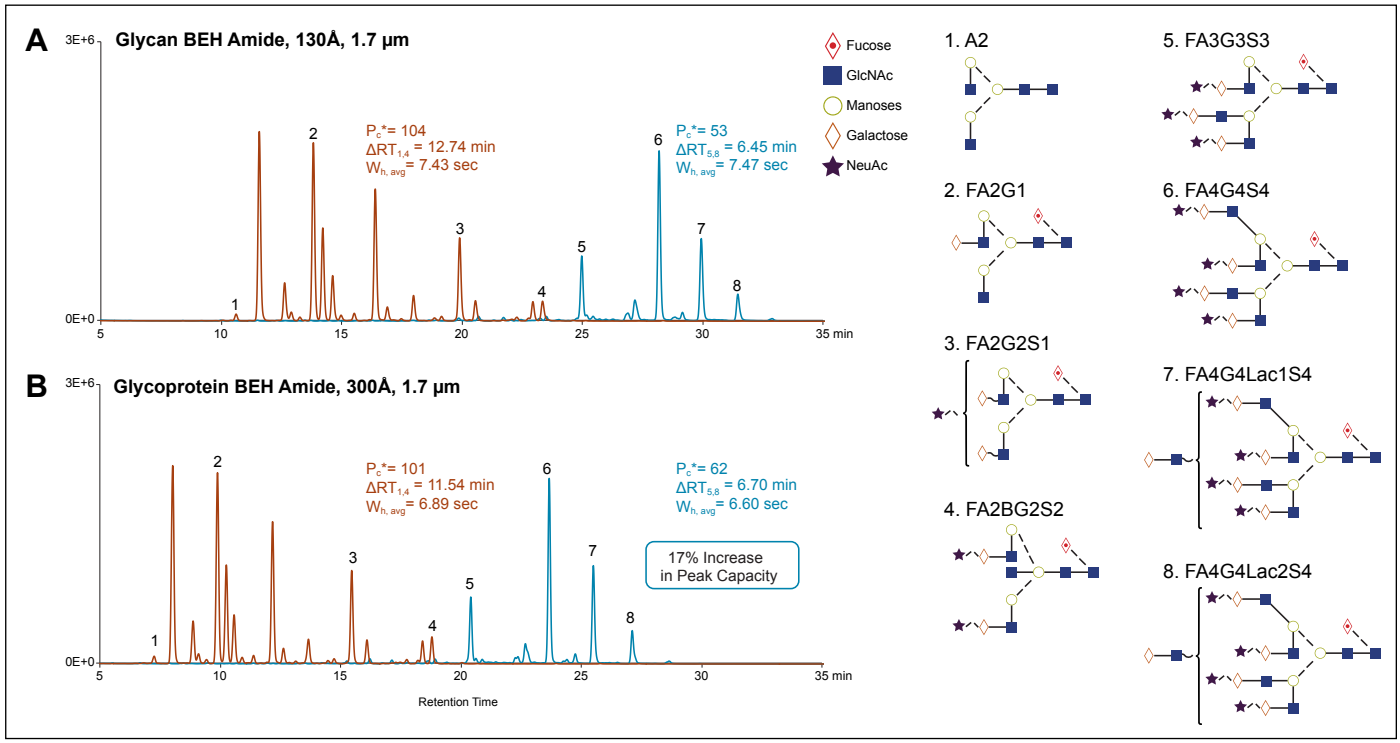

Figure 1. HILIC fluorescence chromatograms for RapiFluor-MS labeled N-glycans from pooled human IgG (orange) and recombinant Factor IX (blue). (A) Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. (B) Chromatograms obtained for glycans from 0.4 µg protein using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. Separations were performed according to the conditions in the GlycoWorks™ RapiFluor-MS Care and Use Manual (p/n 715004793). Peak capacities were calculated from half-height widths and retention windows derived from the labeled peaks.

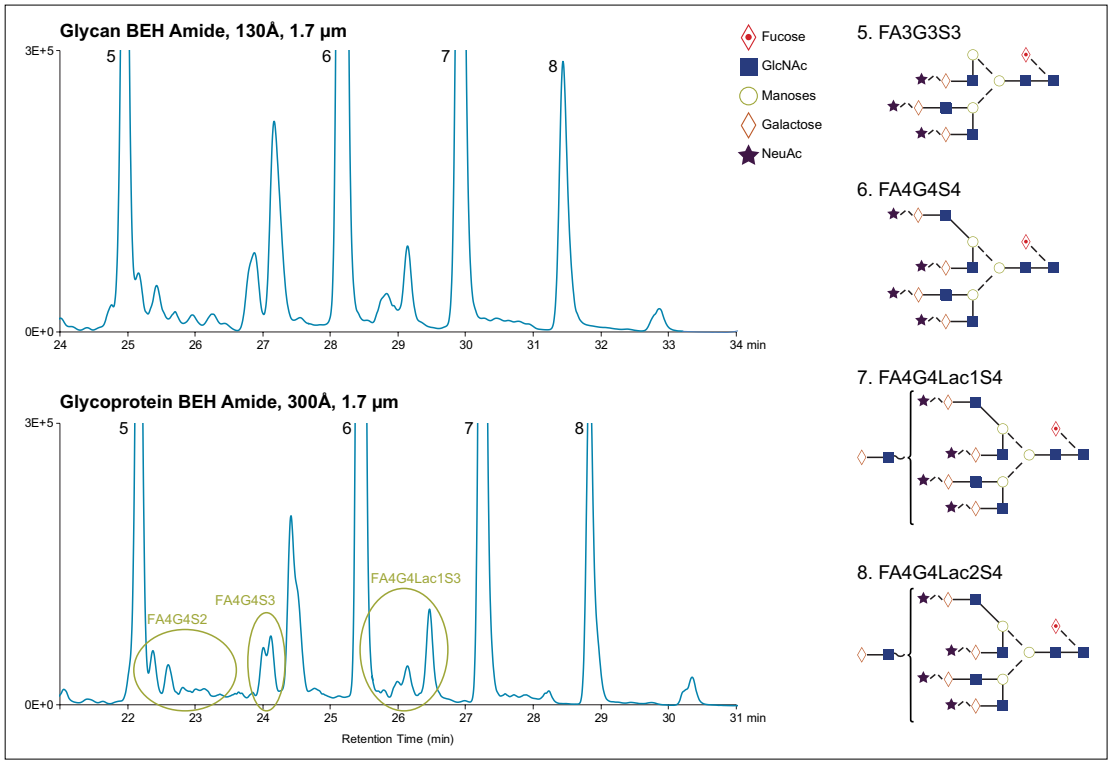

Figure 2. HILIC fluorescence chromatograms for RapiFluor-MS labeled N-glycans from recombinant Factor IX as obtained using 2.1 x 150 mm columns with various amide bonded stationary phases. Peak capacities were calculated from half-height widths and retention windows derived from the labeled peaks.

THE SOLUTION

N-glycans were prepared from both pooled human IgG as well as recombinant human Factor IX, a glycoprotein known to be modified with large, highly sialylated *N*-glycans.[5] Specifically, these samples were prepared using rapid deglycosylation, *Rapi*Fluor-MS labeling, and µElution HILIC SPE, as described in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use Manual.[1, 6] Glycan mapping of the resulting *Rapi*Fluor-MS labeled *N*-glycans was first performed with a glycan BEH amide, 130Å, 1.7 µm column, given that it is intended for general purpose glycan analyses and for use with GlycoBase database searching.[7] Figure 1A displays the fluorescence chromatograms obtained when HILIC-based chromatography is performed on a sample containing less hydrophilic IgG *N*-glycans (orange) versus a sample containing later eluting, more hydrophilic *N*-glycans from Factor IX (blue). In addition, Figure 1A displays the effective peak capacities for the retention windows of the two sample types. These results can be compared to Figure 1B, which presents the chromatograms and peak capacities obtained with a glycoprotein BEH amide column containing the 300Å, wide-pore stationary phase. While the effective peak capacities for the IgG *N*-glycans are comparable, a marked improvement in peak capacity of approximately 17% is apparent in the separations of the Factor IX *N*-glycans when the wide-pore glycoprotein BEH amide 300Å, 1.7 µm column is used. The resolving power of the wide-pore, BEH amide column for the large *N*-glycans is noteworthy, in that it facilitates resolving several low abundance species. Figure 2 highlights some of the impacted regions of the chromatogram where there are improvements in the resolution of FA4G4S2, FA4G4S3, and FA4G4Lac1S3 *N*-glycans. In summary, when smaller, biantennary *N*-glycans are to be separated, a glycan BEH amide, 130Å, 1.7 µm stationary phase is an ideal choice due to its high surface area and high retentivity. For the characterization of large, tri- and tetra-antennary *N*-glycans, it is, however, advantageous to use the wide-pore amide stationary phase. Moreover, the wide-pore amide column is intended specifically for large biomolecule separations: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm stationary phase is ensured to have consistent batch-to-batch performance through stringent quality control testing involving a separation of ribonuclease B (RNase B) glycoforms at the intact protein level (see reference 8 for an example of this chromatography).[8]

SUMMARY

High molecular weight, tri- and tetra-antennary *N*-glycans are highly branched structures that adopt relatively large radii of hydration in solution. To achieve optimal HILIC separations of these large structures, we propose a column with a wide-pore amide bonded stationary phase, a glycoprotein BEH amide, 300Å, 1.7 µm column. For large glycan species, this column provides increases in peak capacity over a conventional pore diameter column of approximately 17%. Improved resolving power is particularly useful in this separation space as it is typified by highly complex glycan profiles. Most notably, these improvements in resolution should be of significant utility in the characterization and routine monitoring of biopharmaceuticals that are expressed with large, highly complicated *N*-glycan structures, such as coagulation Factor IX, erythropoietin, and darbepoetin.

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Mapping IgG Subunit Glycoforms Using HILIC and a Wide-Pore Amide Stationary Phase

Matthew A. Lauber and Stephan M. Koza
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved HILIC separations of IgG subunit glycoforms.
- MS-compatible HILIC to enable detailed investigations of sample constituents.
- Orthogonal selectivity to conventional reversed-phase (RP) separations for enhanced characterization of hydrophilic protein modifications.
- Domain-specific glycan information that complements profiling glycosylation by *Rapi*Fluor-MS released N-glycan analyses.
- Glycoprotein BEH amide, 300Å, 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility.

WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide, 300Å Column

Glycoprotein Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY UPLC H-Class Bio System

Xevo® G2 QTof Mass Spectrometer

SYNAPT® G2-S HDMS

KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, Glycans, Glycosylated Proteins, Glycosylation, HILIC, IdeS

INTRODUCTION

Without question, the most successfully exploited protein modality for therapeutic applications has been monoclonal antibodies (mAbs), which currently account for nearly half of the biopharmaceutical market.[1] An intriguing characteristic of mAbs, in particular IgG-based mAbs, is that they are formed by the linking of two identical light chains and two identical heavy chains through disulfide bonding and non-covalent interactions. Moreover, the resulting mAb structure exhibits functionally significant subunits, for instance one crystallizable fragment (Fc domain) and two equivalent antigen binding fragments (Fab domains). In what is commonly referred to as a middle-up or middle-down analysis,[2-5] native mAbs can be proteolyzed into these and other related subunits enzymatically, as a means to perform cell-based studies and to facilitate characterization. One increasingly popular way to produce subunit digests of mAbs is via the IdeS protease (Immunoglobulin Degrading Enzyme of S. *pyogenes*).[2,6] IdeS cleaves with high fidelity at a conserved sequence motif in the hinge region of humanized mAbs to cleanly produce, upon reduction, three 25 kDa mAb fragments that are amenable to mass spectrometry and useful for localizing different attributes of therapeutic mAbs (Figure 1).[3] IdeS digestion combined with reversed-phase (RP) chromatography has, in fact, been proposed as a simple identity test for mAbs and fusion proteins, because IdeS produced subunits from different drug products will exhibit diagnostic RP retention times.[3] Additionally, RP techniques have been shown to be useful in assaying and obtaining domain specific information about oxidation, since RP retention can be dramatically affected by the oxidation of protein residues, such as methionine.[3]

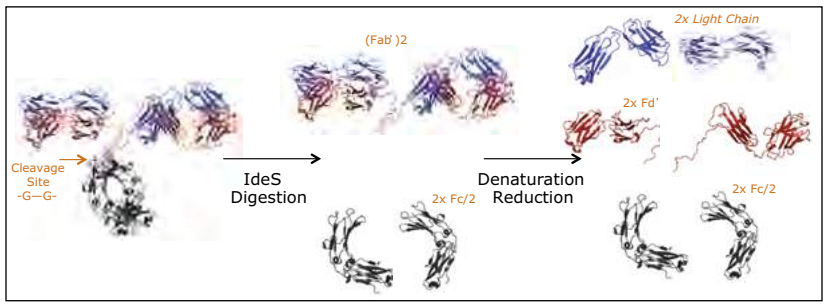


Figure 1. IdeS digestion and reduction scheme for preparing IgG LC, Fd', and Fc/2 subunits.

EXPERIMENTAL

Sample description

IdeS digestion and reduction of mAbs:

Formulated trastuzumab was diluted 7 fold into 20 mM phosphate (pH 7.1) and incubated at a concentration of 3 mg/mL with IdeS (Promega, Madison, WI) for 30 minutes at 37 °C at a 50:1 w/w ratio of trastuzumab to IdeS. The resulting IdeS-digested antibody was denatured and reduced by the addition of 1M TCEP (tris(2-carboxyethyl)phosphine) and solid GuHCl (guanidine hydrochloride). The final buffer composition for the denaturation/reduction step was approximately 6 M GuHCl, 80 mM TCEP, and 10 mM phosphate (pH 7.1). IdeS-digested trastuzumab (1.5 mg/mL) was incubated in this buffer at 37 °C for 1 hour. An IdeS digested, reduced sample of an IgG1K mAb obtained from NIST as candidate reference material #8670 (lot #3F1b) was prepared in the same manner.

Cetuximab IdeS/carboxypeptidase B digestion and reduction:

Prior to digestion with IdeS,[10] cetuximab was treated with carboxypeptidase B to complete the partial removal of the lysine-C-terminal residues that is typical of the antibody.[4] Formulated cetuximab was mixed with carboxypeptidase B (223 µ/mg, Worthington, Lakewood, NJ) at a ratio of 100:1 (w/w), diluted into 20 mM phosphate (pH 7.1), and incubated at a concentration of 1.8 mg/mL for 2 hours at 37 °C. The carboxypeptidase B treated cetuximab was then added to 100 units of IdeS and incubated for 30 minutes at 37 °C. The resulting IdeS digest was denatured and reduced by the addition of 1 M TCEP and solid GuHCl. The final buffer composition for the denaturation/reduction step was approximately 6 M GuHCl, 80 mM TCEP, and 10 mM phosphate (pH 7.1). IdeS-digested cetuximab (0.9 mg/mL) was incubated in this buffer at 37 °C for 1 hour.

Preparation of *Rapi*Fluor-MS Labeled N-Glycans from Cetuximab:

*Rapi*Fluor-MS labeled N-glycans were prepared from cetuximab using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606) according to the guidelines provided in its Care and Use Manual (715004793).

Method conditions

(unless otherwise noted)

Column conditioning

ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm columns (as well as other amide columns intended for glycoprotein separations) should be conditioned via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (p/n 186008010; 10 µL injections of 4 mg/mL in 0.1% trifluoroacetic acid [TFA], 80% acetonitrile [ACN]) or with equivalent loads of a sample for which the column has been acquired. The separation outlined by the following method can be employed for conditioning with the Glycoprotein Performance Test Standard.

Column conditioning gradient:

Column dimensions:	2.1 x 150 mm
Mobile phase A:	0.1% (v/v) TFA, water
Mobile phase B:	0.1% (v/v) TFA, ACN

Time (min)	%A	%B	Curve
0.0	15.0	85.0	6
0.5	15.0	85.0	6
1.0	33.0	67.0	6
21.0	40.0	60.0	6
22.0	100.0	0.0	6
24.0	100.0	0.0	6
25.0	15.0	85.0	6
35.0	15.0	85.0	6

LC conditions for IgG subunit separations

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	5 °C
Analytical column temp.:	45 °C (trastuzumab and NIST IgG1K subunit HILIC separations) 60 °C (cetuximab subunit HILIC separations) 80 °C (trastuzumab reversed phase (RP) subunit separations)
Flow rate:	0.2 mL/min
Mobile phase A:	0.1% (v/v) TFA, water
Mobile phase B:	0.1% (v/v) TFA, ACN
UV detection:	214 nm, 10 Hz

Injection volume:	≤1.2 µL (aqueous diluents). Note: It might be necessary to avoid high organic diluents for some samples due to the propensity for proteins to precipitate under ambient conditions. A 2.1 mm I.D. column can accommodate up to a 1.2 µL aqueous injection before chromatographic performance is negatively affected.
Waters columns:	ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard);
	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742);
	ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm, 2.1 x 150 mm (p/n 186004497)
Other columns:	Agilent® AdvanceBio Glycan Mapping, 1.8 µm, 2.1 x 150 mm;
	Thermo Scientific® Accucore™ Amide 150 HILIC, 150Å 2.6 µm, 2.1 x 150 mm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL volume (p/n 186002640)

Gradient used for reversed-phase (RP) separations of trastuzumab subunits (Figure 2A):

Time (min)	%A	%B	Curve
0.0	95.0	5.0	6
1.0	66.7	33.3	6
21.0	59.7	40.3	6
22.0	20.0	80.0	6
24.0	20.0	80.0	6
25.0	95.0	5.0	6
35.0	95.0	5.0	6

Gradient used for HILIC separations of IgG subunits (Figures 2–7):

Time (min)	%A	%B	Curve
0.0	20.0	80.0	6
1	30.0	70.0	6
21	37.0	63.0	6
22	100.0	0.0	6
24	100.0	0.0	6
25	20.0	80.0	6
35	20.0	80.0	6

MS conditions for IgG subunit separations

MS system:	Xevo G2 QTof or SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	Resolution (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 2 µg/µL from 500–5000 *m/z*
Acquisition:	500–4000 *m/z*, 0.5 sec scan rate
Data management:	MassLynx® Software (v4.1)/UNIFI V1.7

LC conditions for *Rapi*Fluor-MS Released N-Glycan HILIC separations:

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Fluorescence detection:	Ex 265/Em 425 nm (*Rapi*Fluor-MS) (5 Hz scan rate [50 mm column], Gain =1)
Injection volume:	10 µL (DMF/ACN diluted sample)
Mobile phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)
Columns:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740)
Vials:	Polypropylene 12 x 32mm, 300 µL, Screw Neck Vial, (p/n 186002640)

Gradient used for *Rapi*Fluor-MS N-Glycan HILIC Separations (Figure 7B):

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

MS conditions for *RapiFluor*-MS N-Glycan HILIC separations

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–2500 *m/z*
Lockspray (ASM B-side):	100 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water every 90 seconds
Acquisition:	500–2500 *m/z*, 1 Hz scan rate
Data management:	MassLynx Software (v4.1)

It should, however, be kept in mind that many IgG modifications more strongly elicit changes in the hydrophilicity of a mAb along with its capacity for hydrogen bonding. A very obvious example of this type of modification is glycosylation. Glycans released from a mAb are very often profiled by hydrophilic interaction chromatography (HILIC), in which case an amide bonded stationary phase has historically been used, because it affords high retentivity as a consequence of its hydrophilicity and propensity for hydrogen bonding.[7] Here, we propose that HILIC with an amide bonded stationary phase also be considered for IgG subunit separations. For such an application, a stationary phase with a wide average pore diameter is critical, so that large subunit structures will have access to the majority of the porous network and be less prone to restricted diffusion while eluting through a column.[8-9] Through the development of a sub-2-µm wide-pore amide stationary phase, we have facilitated a novel and complementary workflow to RP based subunit analyses. In this application note, we demonstrate the use of a glycoprotein BEH amide, 300Å, 1.7 µm column to develop LC-MS and LC-UV techniques that can be used to rapidly profile domain specific information about the N-linked glycosylation of IgG molecules.

RESULTS AND DISCUSSION

Orthogonal, complementary IgG subunit separations

To demonstrate a conventional approach to IgG subunit mapping, we first analyzed a reduced\IdeS digest of an IgG1 mAb using a RP chromatographic separation with a wide-pore C4 bonded stationary phase (Protein BEH C_4, 300Å, 1.7 µm). The IgG1 mAb selected for this work was trastuzumab, given its prominence as a first generation mAb drug product and a potential target for biosimilar development.[11] Figure 2A shows a UPLC chromatogram that is typical for reduced, IdeS-digested trastuzumab, wherein three peaks are near equally spaced with an elution order corresponding to the Fc/2, LC and Fd' subunits, respectively. The conditions to produce this high resolution separation entail the use of TFA for ion-pairing. Interestingly, the same mobile phases have proven to be optimal for protein HILIC, as they reduce the hydrophilicity of protein residues by masking them via a hydrophobic ion pair. This, in turn, leads to improved selectivity for hydrophilic modifications.[12] That is, an orthogonal method to the RP separation can be achieved via HILIC by simply reversing a gradient and using a newly developed wide-pore amide bonded stationary phase (glycoprotein BEH Amide, 300Å, 1.7 µm).

An example of a chromatogram obtained from a column packed with this wide-pore amide material is shown in Figure 2B. Here, the same reduced, IdeS digested trastuzumab is separated into approximately 10 peaks. The first two eluting peaks correspond to the Fd' and LC subunits, while the remaining, more strongly retained peaks correspond to the glycoforms of the Fc/2 subunit. By focusing on the more strongly retained peaks, an analyst can elucidate information about the heterogeneity of glycosylation (Figure 3A). Given that this is a method with volatile mobile phases, the glycoform peaks can be readily interrogated by ESI-MS. Deconvoluted mass spectra and molecular weights corresponding to species in the glycoform profile are presented in Figures 3B and 3C. In Figure 3, chromatographic peaks are labeled with the same color as their corresponding mass spectra. Notice that this HILIC separation facilitates producing deconvoluted mass spectra for individual glycoforms with limited interference between similar molecular weight species, for instance the Fc/2+A2G1 versus the Fc/2+FA2 species (orange versus blue spectrum). In a first pass analysis, all glycan species from trastuzumab that are known to be present at a relative abundance greater than 2% are readily detected.[13] It should be noted that lower abundance species, such as Fc/2+M5 (Man5), are also detected and can be observed by extracted ion chromatograms (XICs). This indicates there is a possibility to perform selected reaction monitoring (SRM) MS analyses when and if there is a need to monitor particular low abundance structures. While it is not resolved under these conditions, the M5 Fc/2 glycoform is resolved in a different example separation (see below, Figure 7A).

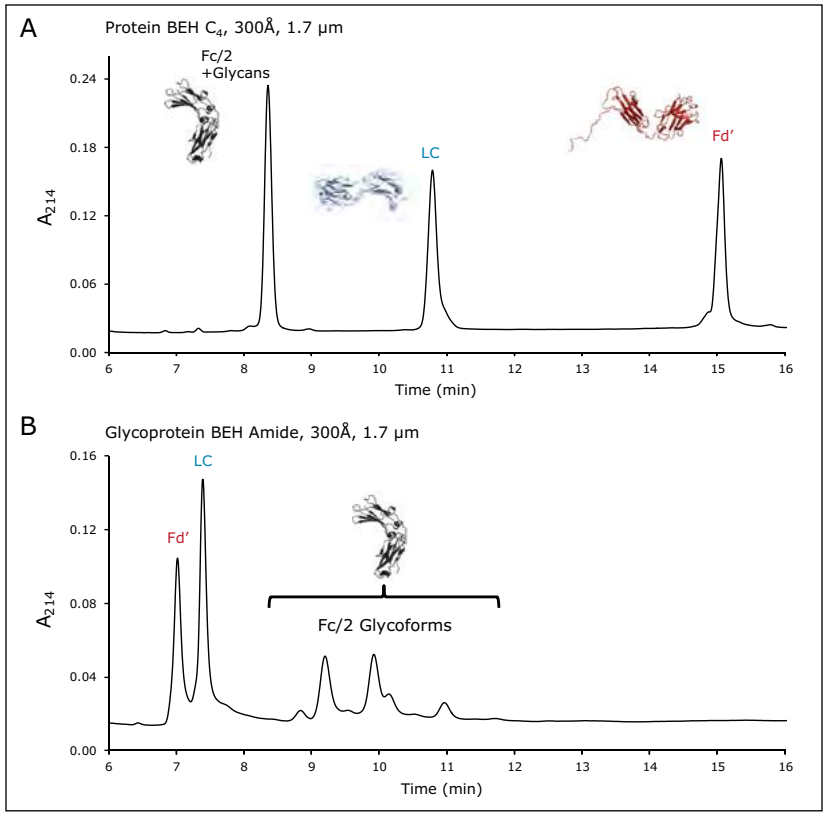
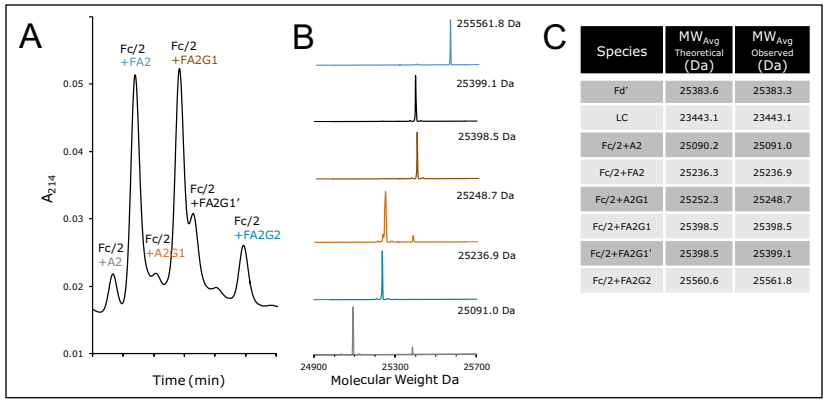


Figure 2. Trastuzumab subunit separations. (A) 1 µg of reduced, IdeS digested separated using an ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm Column (0.7 µL aqueous injection). (B) 1 µg of reduced, IdeS digested separated using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column (0.7 µL aqueous injection).



Species	MW_{Avg} Theoretical (Da)	MW_{Avg} Observed (Da)
Fd'	25383.6	25383.3
LC	23443.1	23443.1
Fc/2+A2	25090.2	25091.0
Fc/2+FA2	25236.3	25236.9
Fc/2+A2G1	25252.3	25248.7
Fc/2+FA2G1	25398.5	25398.5
Fc/2+FA2G1'	25398.5	25399.1
Fc/2+FA2G2	25560.6	25561.8

Figure 3. Profiling trastuzumab Fc/2 subunit glycoforms. (A) Retention window from Figure 2B corresponding to the glycoform separation space. (B) Deconvoluted ESI mass spectra for the HILIC chromatographic peaks. Chromatographic peaks are labeled with the same color as their corresponding mass spectra. (C) Molecular weights for the observed trastuzumab subunits.

Batch-to-batch analysis of trastuzumab Fc/2 glycosylation by HILIC-UV profiling

Clearly, data generated by subunit-level HILIC-MS are very information-rich. Optical detection based subunit HILIC separations can be equally informative. To this end, we have applied a HILIC-UV method to perform batch-to-batch analysis of trastuzumab Fc/2 glycosylation, as exemplified in Figure 4. Two example HILIC chromatograms for Fc/2 glycoforms obtained from two different lots of trastuzumab are shown in Figure 4A. Previous testing on these lots has demonstrated differences in glycosylation at the released glycan level.[14] Here, by integration of peaks across the profile, we have found that the two lots of trastuzumab indeed differ with respect to their Fc domain glycosylation profiles, in ways consistent with the mentioned released glycan assays. In particular, these lots of trastuzumab differ with respect to their extents of terminal galactosylation, as estimated from the abundances of FA2, FA2G1, and FA2G2 Fc/2 subunits (Figure 4B). This is an informative observation, since the extent of galactosylation can affect complement-dependent cytotoxicity (CDC).[15]

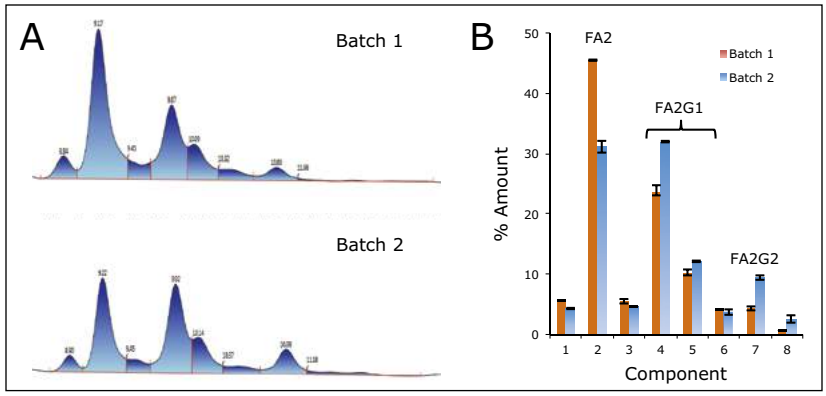


Figure 4. Batch-to-batch profiling of trastuzumab Fc/2 subunit glycoforms. (A) HILIC chromatograms of trastuzumab Fc/2 subunit glycoforms from two different lots of drug product. (B) Relative abundances of the major sample components. Analyses were performed in triplicate using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column.

Lifetime testing of glycoprotein BEH amide 300Å, 1.7µm columns for profiling IgG subunit glycoforms

The ability of a BEH amide, 300Å, 1.7 µm column to robustly deliver the above mentioned separations over time was tested by performing a series of experiments involving a single column being subjected to 300 sequential injections of a reduced, IdeS digested trastuzumab sample. This was a potentially challenging use scenario given that the reduced, IdeS digested mAb sample contains both high concentrations of guanidine denaturant and TCEP reducing agent. Total ion chromatograms corresponding to the 20th, 180th, and 300th injections of this experiment are displayed in Figure 5A. In these analyses, particular attention was paid to the half-height resolution of the Fc/2+A2 and Fc/2+FA2 species, which was assessed every 20th separation using extracted ion chromatograms (XICs).

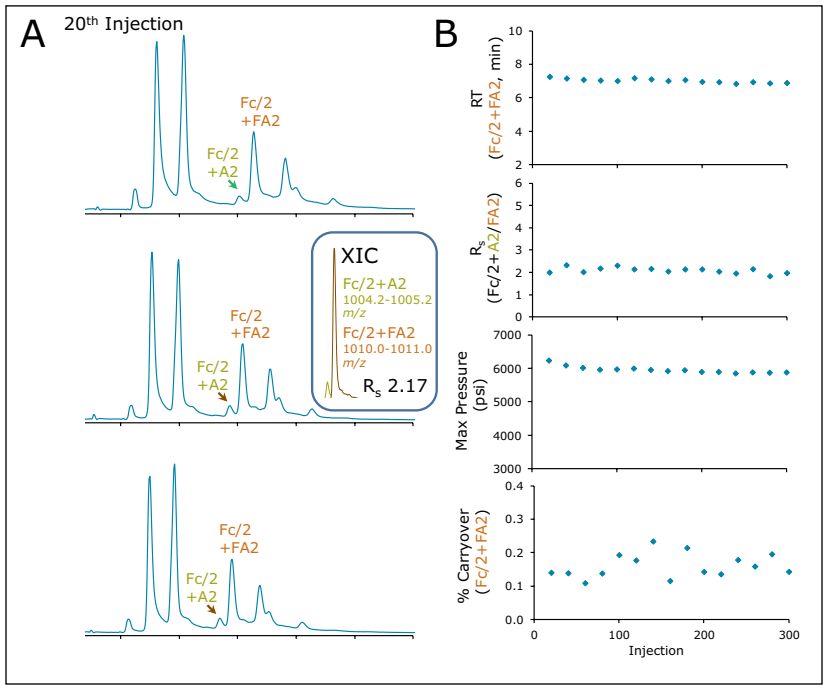


Figure 5. Lifetime testing of an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column for sequential injections of reduced, IdeS digested trastuzumab. (A) Total ion chromatograms (TICs) from the 20th, 180th, and 300th injections. Example extracted ion chromatograms (XICs) for Fc/2+A2 and Fc/2+FA2 that were used to measure resolution. (B) Chromatographic parameters observed across the 300 injection lifetime test. Each panel shows results for each 20th injection, including retention time (RT) of the FA2 glycoform, R_s between A2 and FA2 glycoforms, maximum pressure across the run, and % carryover as measured by a repeat gradient and XICs.

In this testing, several additional chromatographic parameters were also monitored, including the retention time of the Fc/2+FA2 species, the maximum system pressure observed during the chromatographic run, and the percent (%) carryover of the most abundant glycoform, the Fc/2+FA2 species (Figure 5B). Plots of these parameters underscore the consistency of the subunit separation across the lifetime of the column. With noteworthy consistency, the column produced relatively stable retention times, a consistent resolution of the A2 and FA2 glycoforms ($R_s \approx 2$), a maximum system pressure consistently at only ~6 Kpsi, and a remarkably low carryover between 0.1 and 0.2%. This latter aspect of the HILIC separations is particularly noteworthy since it indicates that carryover with these methods is almost an order of magnitude lower than analogous C4 based RP methods.

Benchmarking the capabilities of the glycoprotein BEH amide, 300Å, 1.7 µm column

We have benchmarked the performance of this new wide-pore column technology against not only its standard pore diameter analog but also its two most closely related, commercially-available alternatives. Figure 6 presents chromatograms obtained for a reduced, IdeS digested sample of an IgG1K mAb acquired from NIST using these different column technologies. In a visual comparison, it is clear that the glycoprotein BEH amide 300Å column significantly outperforms the other three columns. To quantify this assessment, peak-to-valley ratios were calculated for the separation of the FA2 glycoform away from the FA2G1 glycoform. The glycoprotein BEH amide 300Å column was found to demonstrate improvements of 48%, 152%, and 261% over the 130Å glycan BEH amide, the Accucore amide, and AdvanceBio glycan mapping columns. This mAb sample also has a particularly interesting attribute in that it has a reasonably high relative abundance of an immunogenic alpha-1,3-galactose containing glycan (an FA2G2Ga1 structure).[16-17] As shown in Figure 6, this Fc/2+FA2G2Ga1 species can be readily visualized with the wide-pore amide column. This represents a sizeable improvement in the peak capacity of large molecule HILIC separations for this emerging application.

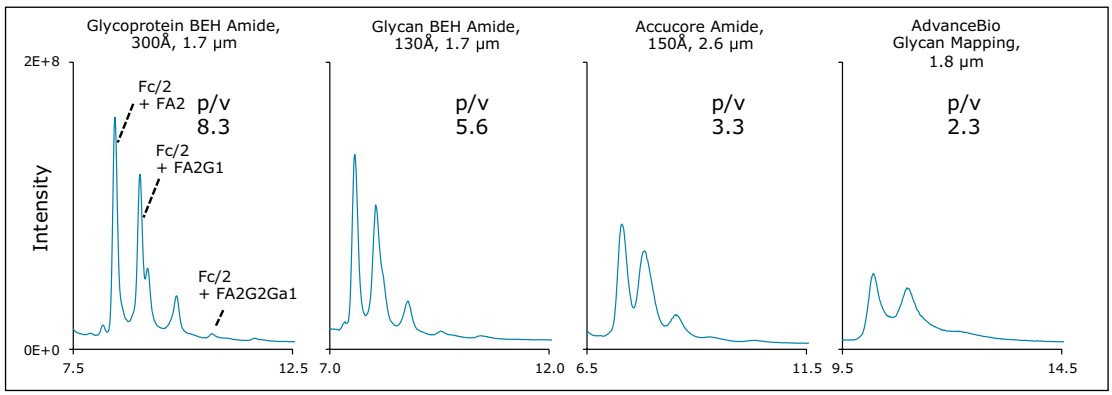
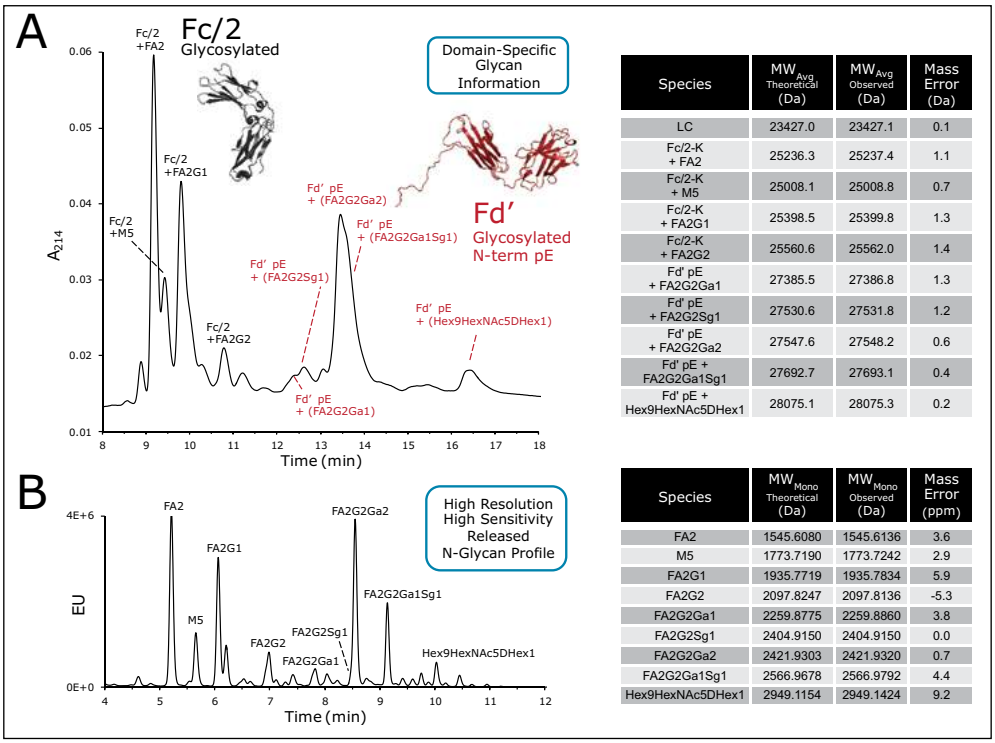


Figure 6. Subunit glycoform profiles of an IgG1K obtained with various 2.1 x 150 mm columns: ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm Column, ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Column, Thermo Scientific Accucore Amide, 150Å, 2.6 µm column, and Agilent AdvanceBio Glycan Mapping, 1.8 µm column. Peak-to-valley (p/v) ratios for the Fc/2+FA2 versus FA2G1 glycoforms are provided. An alpha gal containing Fc/2+FA2G2Ga1 is readily visualized with the glycoprotein BEH amide, 300Å, 1.7 µm column.

Complementing *Rapi*Fluor-MS N-glycan analyses with domain specific information about mAb glycosylation

One of the key advantages to profiling IgG subunits by HILIC is being able to elucidate domain specific information about glycosylation. In an IgG structure, there exists one conserved N-glycosylation site at Asn297 of the heavy chain. As a consequence, most IgGs will be modified with two glycans in the CH2 domains (constant heavy chain 2 domains) of the Fc subunit. However, it is estimated that 20% of human IgGs are also modified in their CH1 domains, which reside in the Fab subunits, and more specifically the IdeS generated Fd' subunit.[18-19] For example, it is known that cetuximab, a chimeric mAb expressed from a murine cell line, is glycosylated in both its CH1 and CH2 domains.[20] Characterization of this mAb has thus proven to be an interesting case study for the application of our newly developed techniques. HILIC separations obtained for a reduced, IdeS digested sample of carboxypeptidase B treated cetuximab showed only one weakly retained subunit species, which could be easily assigned to the LC subunit by online ESI-MS (data not shown). Furthermore, and as shown in Figure 7A, the glycoform retention window for cetuximab was populated with twice as many peaks as had been observed for trastuzumab and its glycosylated Fc/2 subunit. Deconvoluted ESI-MS data from these HILIC-MS separations confirmed that the first grouping of peaks (labeled in gray) corresponded to Fc/2 glycoforms and typical mAb glycan species, such as FA2, FA2G1, M5, and FA2G2. Meanwhile, the second grouping of peaks were found to be distinctively related to glycoforms of the Fd' subunit given their unique masses. Curiously, each of the identified Fd' glycoforms (labeled in red) are immunogenic in nature, containing either non-human alpha-1,3-galactose or non-human N-glycolyl-neuraminic acid epitopes.[21]

The identification of these glycan species has been confirmed through released N-glycan analyses. Using the newly developed GlycoWorks *Rapi*Fluor-MS N-Glycan Kit,[22] cetuximab N-glycans were rapidly prepared and labeled with the novel fluorescence and MS-active labeling reagent, *Rapi*Fluor-MS. The resulting labeled N-glycans were subsequently separated using a glycan BEH amide, 130Å, 1.7 µm column and detected by fluorescence and positive ion mode ESI-MS, as portrayed in Figure 7B. The sensitivity gains afforded by the *Rapi*Fluor-MS label facilitated making confident assignments of the released N-glycan structures. The species that have been assigned as a result of both this released glycan analysis as well as the subunit HILIC-UV-MS method are supported by previous reports on cetuximab glycosylation.[6,20]



Species	MW_{Avg} Theoretical (Da)	MW_{Avg} Observed (Da)	Mass Error (Da)
LC	23427.0	23427.1	0.1
Fc/2-K + FA2	25236.3	25237.4	1.1
Fc/2-K + M5	25008.1	25008.8	0.7
Fc/2-K + FA2G1	25398.5	25399.8	1.3
Fc/2-K + FA2G2	25560.6	25562.0	1.4
Fd' pE + FA2G2Ga1	27385.5	27386.8	1.3
Fd' pE + FA2G2Sg1	27530.6	27531.8	1.2
Fd' pE + FA2G2Ga2	27547.6	27548.2	0.6
Fd' pE + FA2G2Ga1Sg1	27692.7	27693.1	0.4
Fd' pE + Hex9HexNAc5DHex1	28075.1	28075.3	0.2

Species	MW_{Mono} Theoretical (Da)	MW_{Mono} Observed (Da)	Mass Error (ppm)
FA2	1545.6080	1545.6136	3.6
M5	1773.7190	1773.7242	2.9
FA2G1	1935.7719	1935.7834	5.9
FA2G2	2097.8247	2097.8136	-5.3
FA2G2Ga1	2259.8775	2259.8860	3.8
FA2G2Sg1	2404.9150	2404.9150	0.0
FA2G2Ga2	2421.9303	2421.9320	0.7
FA2G2Ga1Sg1	2566.9678	2566.9792	4.4
Hex9HexNAc5DHex1	2949.1154	2949.1424	9.2

Figure 7. HILIC Profiling of cetuximab glycosylation. (A) HILIC-UV chromatogram of reduced, IdeS/carboxy peptidase B-digested cetuximab obtained using an ACQUITY UPLC Glycoprotein BEH Amide, 300Å, 1.7 µm, 2.1 x 150 mm Column. Species corresponding to Fc/2 and Fd' subunits are labeled in gray and red, respectively. Subunit glycan assignments based on deconvoluted mass spectra are provided. (B) HILIC-fluorescence chromatograms of RapiFluor-MS labeled N-glycans from cetuximab obtained using an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm Column. Mass spectral data supporting the assignments of the RapiFluor-MS labeled N-glycans are provided.

With the combination of released glycan and subunit-derived glycan information, cetuximab glycosylation has been characterized with significant detail. With the *Rapi*Fluor-MS released glycan analysis, a very high resolution separation has been achieved with an LC-MS compatible method in which glycans can even be subjected to detailed MS/MS analyses. With an equally MS-compatible subunit HILIC separation, domain-specific glycan information has been readily obtained with minimal sample preparation. Each method has therefore provided complementary information on the glycosylation of the mAb. Nevertheless, the widepore amide HILIC method stands out as a useful technique for rapidly screening mAbs for multidomain glycosylation.

CONCLUSIONS

Subunit analyses of mAbs represent a useful strategy for rapidly investigating domain-specific modifications. The combination of high fidelity IdeS proteolysis with high resolution LC-UV-MS has presented a new approach to mAb identity testing and assaying oxidation.[3] The current subunit mapping strategies have exclusively relied upon reverse phase chromatography. However, since N-linked glycosylation of IgG proteins elicit dramatic changes in hydrophilicity and hydrogen bonding characteristics, a separation by hydrophilic interaction chromatography (HILIC) can be effectively used for this application or as a complementary method to reversed-phase separations since the same mobile phases can be employed. For this reason, we have proposed the use of HILIC with an amide bonded stationary phase that has been optimized for large molecule separations, the wide-pore glycoprotein BEH amide, 300Å, 1.7 µm stationary phase. Along with new developments in released N-glycan analysis afforded by *Rapi*Fluor-MS,[22] the glycoprotein BEH amide, 300Å, 1.7 µm column enables new possibilities for routine monitoring and detailed characterization of mAb glycosylation, including elucidation of domain-specific glycan information.

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Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection

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Supporting Information

ABSTRACT: *N*-glycosylation of proteins is now routinely characterized and monitored because of its significance to the detection of disease states and the manufacturing of biopharmaceuticals. At the same time, hydrophilic interaction chromatography (HILIC) has emerged as a powerful technology for *N*-glycan profiling. Sample preparation techniques for *N*-glycan HILIC analyses have however tended to be laborious or require compromises in sensitivity. To address these shortcomings, we have developed an *N*-glycan labeling reagent that provides enhanced fluorescence response and MS sensitivity for glycan detection and have also simplified the process of preparing a sample for analysis. The developed labeling reagent rapidly reacts with glycosylamines upon their release from glycoproteins. Within a 5 min reaction, enzymatically released *N*-glycans are labeled with this reagent comprised of an NHS-carbamate reactive group, a quinoline fluorophore, and a tertiary amine for enhancing ESI+ MS ionization. To further expedite the released *N*-glycan sample preparation, rapid tagging has been integrated with a fast PNGase F deglycosylation procedure that achieves complete deglycosylation of a diverse set of glycoproteins in approximately 10 min. Moreover, a technique for HILIC-SPE of the labeled glycans has been developed to provide quantitative recovery and facilitate immediate HILIC analysis of the prepared samples. The described approach makes it possible to quickly prepare *N*-glycan samples and to incorporate the use of a fluorescence and MS sensitivity enhancing labeling reagent. In demonstration of these new capabilities, we have combined the developed sample preparation techniques with UHPLC HILIC chromatography and high sensitivity mass spectrometry to thoroughly detail the *N*-glycan profile of a monoclonal antibody.

N-glycosylation of proteins is routinely characterized and monitored because of its significance to the detection of disease states[1−3] and the manufacturing of biopharmaceuticals.[4] In particular, the glycan profile of a biopharmaceutical is sometimes defined as a critical quality attribute,[5,6] since it can be a measure of efficacy, immunogenicity, and manufacturing conditions.[4,7] It is therefore beneficial for glycan analytical approaches to exhibit high sensitivity so that profiles can be thoroughly characterized. Additionally, decreasing analysis time and improving sample throughput would be valuable in situations where it is necessary to perform routine monitoring. Results from glycosylation assays are routinely used to guide the process development of protein-based drug candidates.[8,9] Accelerating *N*-glycan sample preparations and analyses might therefore hasten the commercialization of new protein-based therapeutics.

There are many diverse analytical approaches for the analysis of *N*-linked glycans. A commonality shared by most strategies for evaluating *N*-glycans is that they involve deglycosylation of glycoproteins with an enzyme, such as peptide-*N*-glycosidase F (PNGase F).[10] The resulting, free *N*-glycans can thereby be analyzed without interference from the heterogeneity of the protein. Numerous protein glycosylation studies have been performed without any further modification of these released *N*-glycans.[11,12] In an emerging approach, Bynum et al. characterized native *N*-glycans from monoclonal antibodies using an online PNGase F microreactor and an LC-MS technique based on porous graphitized carbon.[13] More often, released *N*-glycans are derivatized to enhance the sensitivity of an analysis. For example, permethylation of *N*-glycans has long been used to improve glycan MS detectability.[12,14−16] In yet another, highly effective approach, *N*-glycans can be labeled with a fluorescent tag.[12,17] Rudd and others have made pioneering contributions over the last two decades toward developing techniques wherein fluorescently labeled *N*-glycans are profiled using hydrophilic interaction chromatography (HILIC).[17,18] With recent advances in ultra high pressure liquid chromatography and sub-2 μm amide-bonded stationary

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phases, labeled *N*-glycans can now be separated with high resolution and detected by fluorescence (FLR) and potentially even MS.[19−23]

Unfortunately, approaches to the preparation of *N*-glycans for HILIC-FLR-MS have tended to be laborious or require compromises in MS sensitivity.[11,12] For instance, a traditional deglycosylation procedure requires that a glycoprotein sample be incubated for a minimum of 30 min, while many researchers conventionally employ an overnight incubation.[24,25] Combined with this process is a lengthy, 2 to 4 h labeling step based on reductive amination of aldehyde termini that form on *N*-glycans only after they hydrolyze from their glycosylamine forms.[11] Additionally, in the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans are readily detected by fluorescence but are challenging to detect by electrospray ionization (ESI)-MS becuase of their poor ionization efficiency.

Variations to conventional approaches for the preparation of *N*-glycan samples that are suitable for HILIC analysis have been explored, but have not, as of yet, presented a practical solution that combines the desired attributes of simplicity, speed and high sensitivity. Alternative labeling reagents that have functional groups to enhance electrospray ionization efficiency have been used,[26,27] but this does not address the time-consuming nature of reductive amination reactions. Rapid tagging procedures that yield labeled glycans in a matter of minutes have consequently been investigated. In this approach, glycosylamines are modified with electrophilic reactive groups before they hydrolyze to reducing, aldehyde terminated glycans. Cook and co-workers have presented the use of a rapid tagging analog of aminobenzamide (AB).[28] Although such a rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced ionization efficiency needed to simplify glycan characterization and peak identification. Gong and researchers have, in addition, demonstrated the use of a rapid tagging labeling reagent, constructed with a basic tertiary amine, for the rapid preparation of labeled glycosylamines that are amenable to MS analysis.[29] However, the proposed labeling reagent does not impart a chromophore onto the glycan, thus limiting its utility for many analyses, wherein fluorescence-based profiling is desirable.[22,30]

To address the above shortcomings, we have developed an approach that both decreases the time required for *N*-glycan sample preparation and enhances the sensitivity of glycan detection. A labeling reagent, named RapiFluor-MS (or RFMS), has been synthesized that rapidly reacts (in <5 min) with glycosylamines upon their release from glycoproteins. RFMS is comprised of an *N*-hydroxysuccinimide (NHS) carbamate reactive group, a quinoline fluorophore, and a basic tertiary amine. Consequently, RFMS-labeled glycans can be detected by both fluorescence and positive ion mode ESI-MS with high sensitivity. The entire *N*-glycan sample preparation procedure has also been accelerated by directly integrating rapid tagging with a fast deglycosylation procedure and a HILIC-solid phase extraction (SPE) cleanup step that provides quantitative recovery of glycans and allows for immediate analysis of samples. In demonstration of these capabilities, we have combined the new sample preparation techniques with HILIC chromatography and high sensitivity mass spectrometry to thoroughly characterize the *N*-glycan profile of a monoclonal antibody (mAb). Rapid preparation of RFMS-labeled *N*-glycans has, in particular, been combined with high sensitivity MS/MS, to yield valuable structural information for low abundance glycans.

EXPERIMENTAL SECTION

Deglycosylation of Glycoproteins. Samples of anticitrinin murine monoclonal IgG1, pooled human IgG (from serum), bovine fetuin, or combinations thereof were deglycosylated using the same technique. Glycoproteins (15 μg) were diluted or reconstituted to a concentration of 0.52 mg/mL into a 28.8 μL solution of 1% (w/v) RG surfactant (RapiGest SF, Waters, Milford, MA) and 50 mM HEPES (pH 7.9). These solutions were heated to approximately 95 °C over 2 min, allowed to cool to 50 °C, and mixed with 1.2 μL of PNGase F solution (GlycoWorks Rapid PNGase F, Waters, Milford, MA). Deglycosylation was completed by incubating the samples at 50 °C for 5 min. It is worth noting that some glycoproteins, other than IgGs, must be subjected to reducing conditions in order for complete deglycosylation to be achieved. In such cases, use of 4 mM TCEP along with 1% RG surfactant has proven to be effective (see Supporting Information). Moreover, heating of samples to ≥80 °C is critical to ensuring that glycoproteins are sufficiently denatured and that *N*-glycans are readily accessible for enzymatic deglycosylation.

An important feature of these deglycosylation conditions is that they are quick and relatively stabilizing for the *N*-glycosylamine structures. An analysis on the stability of *N*-glycosylamines shows that they have a half-life of approximately 2 h at a temperature of 50 °C in the described buffer conditions (Supporting Information Figure S1).

Labeling of *N*-Glycosylamines with Rapid Tagging Reagents. Deglycosylation mixtures were allowed to cool to room temperature following their incubation at 50 °C and were then reacted with RFMS (*Rapi*Fluor-MS, Waters, Milford, MA) or IAB (Instant AB, Prozyme, Hayward, CA) without a protein depletion step. RFMS or IAB was dissolved in anhydrous dimethylformamide (DMF) to a concentration of 127 mM then mixed with the deglycosylation mixture in a 1:2.5 volumetric ratio to produce a reaction comprised of 36 mM labeling reagent and 0.36 mg/mL of deglycosylated protein. The molar excess of reagent over modifiable amine in these reactions was estimated to be approximately 50−300-fold (assuming 1 mg of glycoprotein will produce 0.3−0.7 μmol of released *N*-glycosylamines and proteinaceous amines, in addition to any other free amines in the mixture). Labeling reactions were allowed to proceed at room temperature for 5 min then diluted 1:9 with acetonitrile (ACN) in preparation for HILIC SPE.

Extraction of Labeled *N*-Glycosylamines Using HILIC SPE. The ACN-diluted samples of labeled *N*-glycosylamines were thereafter subjected to SPE using vacuum aspiration and a silica based aminopropyl sorbent (GlycoWorks μElution Plate, Waters, Milford, MA). Wells containing 5 mg of sorbent were conditioned with water (200 μL), equilibrated with 85% (v/v) ACN (200 μL), and then loaded with sample. Adsorbed samples were subsequently washed with two, 600 μL volumes of a solution containing 1% formic acid in 90% acetonitrile (ACN). Lastly, enriched, labeled glycosylamines were eluted in three 30 μL elution volumes from the SPE sorbent using an eluent composed of 200 mM ammonium acetate (pH 7), 5% ACN. The obtained labeled *N*-glycosylamines were immediately analyzed in the form of the SPE eluate (diluted to 22:25:53 (v/v/v) eluate/DMF/ACN). A sample dry-down step

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was not needed with this preparation. Instead, samples were analyzed directly from this mixture. RFMS derivatized glycans were observed to be very stable in this sample diluent. Analysis of a sample before and after 3 days of storage at 10 °C showed there to be no changes in the glycan profile, as assessed by the techniques described below (Supporting Information Figure S2).

HILIC-Fluorescence-ESI-MS (MS/MS) Analysis of Labeled *N*-Glycans. To evaluate response factors, labeled *N*-glycans were analyzed via HILIC separations combined with fluorescence and mass spectrometric detection using a UHPLC chromatograph (ACQUITY UPLC H-Class Bio, Waters, Milford, MA). Either a 2.1 × 50 mm or a 2.1 × 150 mm column packed with 1.7 μm amide-bonded organosilica particles (ACQUITY UPLC Glycan BEH Amide 130 Å, Waters, Milford, MA) was employed along with an aqueous mobile phase comprised of 50 mM ammonium formate (pH 4.4) and another of ACN. Samples were injected as 1 μL aqueous volumes or 10 μL ACN/DMF volumes and separated at 60 °C according to the gradients shown in Supporting Information Tables S1 or S2. The quantities of *N*-glycans injected in each analysis are provided within the figure captions. Labeled *N*-glycans were detected using a fluorescence detector (5 Hz scan rate, Gain = 1, ACQUITY UPLC FLR, Waters, Milford, MA) using the excitation and emission wavelengths provided in Supporting Information Table S3. Eluting glycans were also detected by positive ion mode electrospray ionization mass spectrometry using an ion mobility capable QTof mass spectrometer (Synapt G2-S, Waters, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 120 °C, desolvation temperature of 350 °C, and sample cone voltage of 80 V. Mass spectra were acquired at a rate of 1 Hz with a resolution of approximately 20,000 over a range of 500−2500 *m*/*z*.

Detailed characterization of the anticitrinin murine IgG1 monoclonal antibody was performed with a QTof mass spectrometer outfitted with a high transmission efficiency collision cell (Xevo G2-XS QTof, Waters, Milford, MA). The mass spectrometer was operated with a capillary voltage of 3.0 kV, source temperature of 120 °C, desolvation temperature of 250 °C, and sample cone voltage of 80 V. Mass spectra were acquired at a rate of 2 Hz in MS and MS/MS modes with a resolution of approximately 30 000 over a range of 750−2000 *m*/*z*. MS/MS analyses were performed in continuum mode from 100−2000 *m*/*z* with collision induced dissociation (CID) to generate glycan fragmentation data. Ions with 2+ and 3+ charge states were selected for fragmentation. Customized collision energy (CE) tables that were charge state and mass specific were used for optimized fragmentations; the approximate CE range was between 15 and 40 eV. Data dependent acquisition (DDA) was used with duty cycle times of 1.6 and 0.5 s for MS and MS/MS modes. The two most abundant precursors were selected for fragmentation.

HILIC-Fluorescence-ESI-MS Analysis of Intact IgG. Deglycosylation of anticitrinin murine IgG1 was assayed via intact protein HILIC separations using columns packed with prototype sub-2 μm amide-bonded (300 Å average pore diameter) organosilica stationary phase and a UHPLC chromatograph (ACQUITY UPLC H-Class Bio, Waters, Milford, MA). Solutions of native or deglycosylated (see above) IgG were diluted to 0.14 mg/mL into solutions of 0.9% TFA, 73% ACN, and injected in 10 μL volumes onto two 2.1 × 150 mm columns coupled with a high pressure, low dead volume connector. Separations were performed at 80 °C using the mobile phases and gradient described in Supporting Information Table S4. Eluting species were detected serially via fluorescence detection (2 Hz, excitation 280 nm/emission 320 nm) followed by online ESI-MS with a QToF mass spectrometer (Waters Xevo G2 QTof, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 150 °C, desolvation temperature of 350 °C, and sample cone voltage of 45 V. Mass spectra were acquired at a rate of 2 Hz with a resolution of approximately 20 000 over a range of 500−5000 *m*/*z*.

RESULTS AND DISCUSSION

Rational Design of a New *N*-Glycan Labeling Reagent. A new labeling reagent (RFMS) for facilitating *N*-glycan analysis has been synthesized based on rational design considerations that would afford rapid labeling kinetics, high fluorescence quantum yields, and enhanced MS detectability (Figure 1). Conventional *N*-glycan sample preparation is dependent on reductive amination of aldehyde terminated saccharides, a process that requires glycans to undergo multiple chemical conversions and a lengthy high temperature incubation step.[12] Moreover, glycans must be reductively aminated in anhydrous conditions to minimize desialylation.[31] Sample preparations are therefore burdened with transitioning a sample from aqueous to anhydrous conditions. For these reasons, the newly designed labeling reagent foregoes reductive amination and instead takes advantage of an aqueous rapid tagging reaction. An NHS-carbamate reactive group was chosen for its rapid reaction kinetics (Figure 1, left-side). Electrophilic reactive groups activated with NHS leaving groups have long been used to modify proteinaceous amine residues.[32] The NHS-carbamate reactive group has, in particular, been used for decades to derivatize free amino acids.[33,34] Notably, NHS-carbamate reagents hydrolyze to form amine byproducts and thereby undergo self-quenching reactions (Figure 1, right-side), making reaction steps simple to implement. The NHS-carbamate reactive group of this reagent rapidly modifies glycosylamine-bearing *N*-glycans after their enzymatic release from glycoproteins. Within a 5 min reaction, *N*-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (Supporting Information Figure S2). In addition to rapid tagging

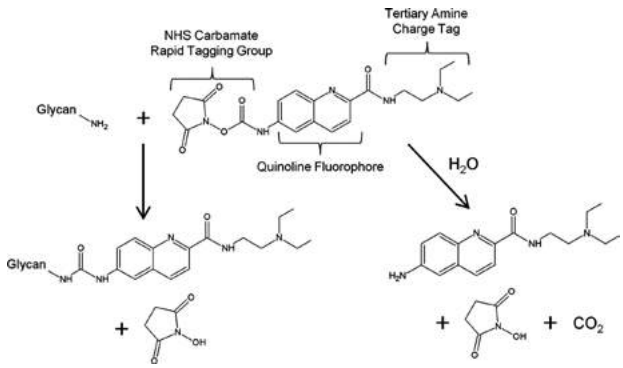

Figure 1. RFMS derivatization of an *N*-glycosylamine. The pathway on the left shows the derivatization of an *N*-glycosylamine, which produces an *N*-glycan with a urea (NH−CO−NH) linked RFMS label. Hydrolysis of RFMS is shown in the pathway on the right.

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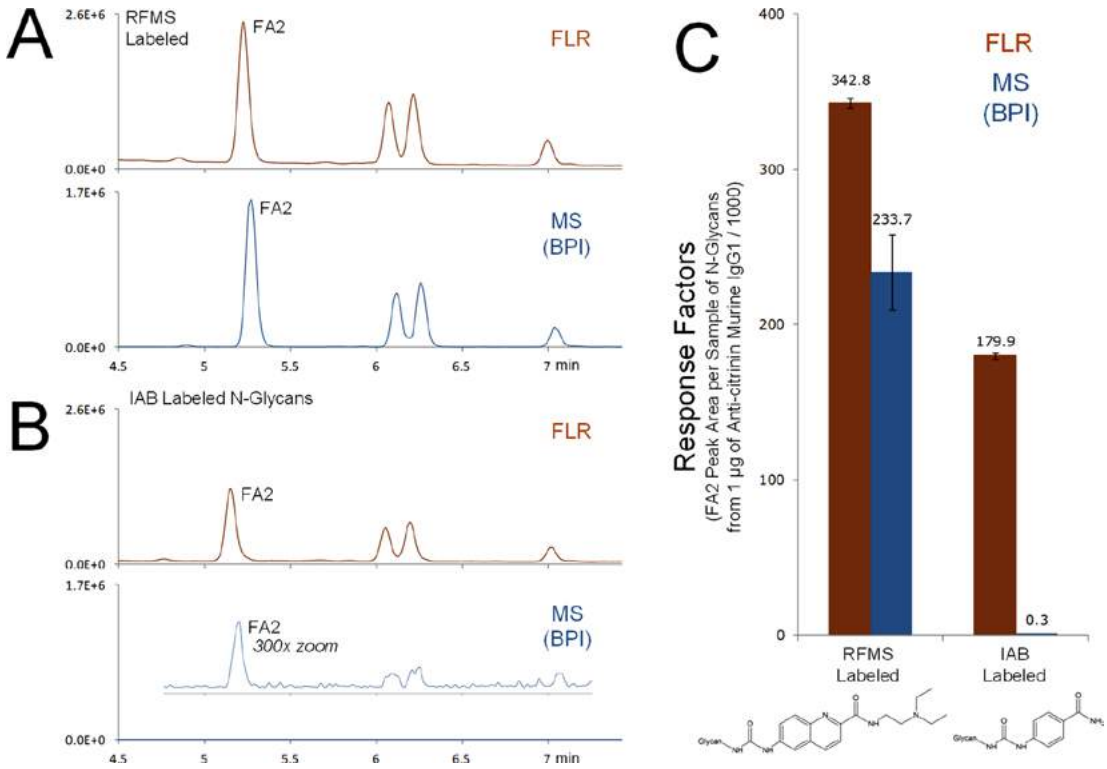

Figure 2. HILIC-FLR-MS of (A) RFMS- and (B) IAB-labeled *N*-glycans from anticitrinin murine IgG1. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (from 0.4 μg of glycoprotein, 1 μL of aqueous injection) were separated using a 2.1 × 50 mm column packed with 1.7 μm amide bonded organosilica (130 Å) stationary phase. (C) Response factors for RFMS and IAB labeled glycans (measured as the FA2 peak area per sample of *N*-glycans resulting from 1 μg of anticitrinin murine IgG1). Fluorescence (FLR) and MS (base peak intensity) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

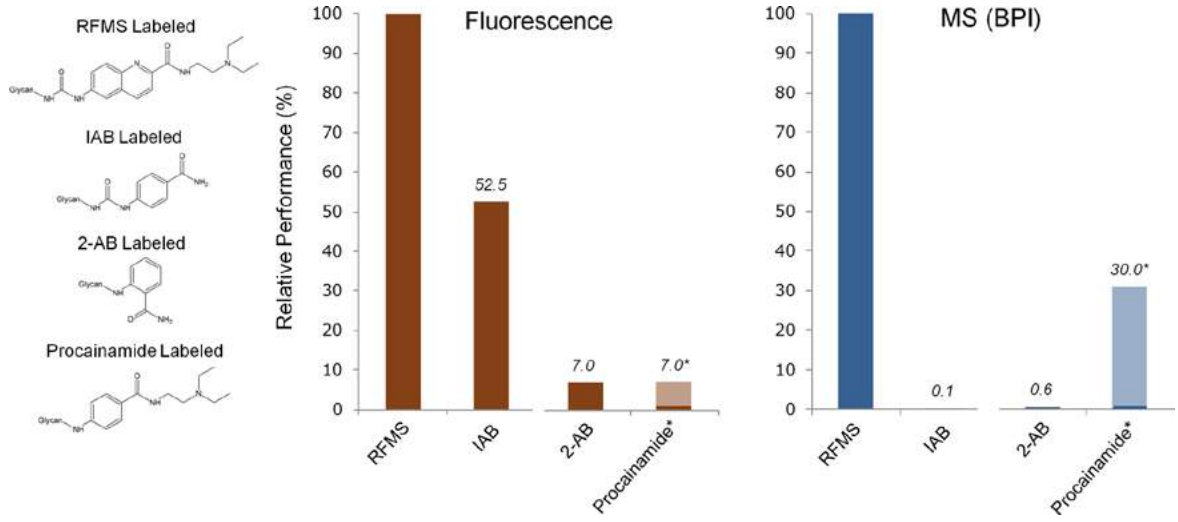

Figure 3. Relative (%) performance of glycan labels. Response factors shown as percentages versus the fluorescence and MS response factors of RFMS labeled *N*-glycans. (*) Comparative result extrapolated from a published comparison of *N*-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50-fold greater ESI-MS sensitivity when compared to 2-AB.[17]

capabilities, the new labeling reagent also supports high sensitivity fluorescence and MS detection. A highly efficient quinoline fluorophore[33,34] serves as the central functionality of the new reagent, though the quinoline group of RFMS has been derivatized to bear a tertiary amine side chain as a means to improve MS signal upon positive ion mode electrospray ionization (ESI+). RFMS has, in brief, been purposefully designed with three important chemical attributes, a rapid tagging reactive group, an efficient fluorophore, and a basic MS charge tag.

High Sensitivity Fluorescence and MS Detection. The sensitivity that RFMS labeling provides to *N*-glycan analyses

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has been evaluated. Specifically, the response factors of RFMS labeled glycans have been benchmarked against response factors for glycans labeled with alternative reagents. The most closely related, commercially available alternative to RFMS is an NHS carbamate analog of aminobenzamide that will be referred to as IAB.[28] Figures 2A and B present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of *N*-glycans released from a murine IgG1 monoclonal antibody and labeled with RFMS and IAB, respectively. On the basis of the observed chromatographic peak areas, response factors for fluorescence and MS detection were determined for the most abundant glycan in the IgG profile, the fucosylated, biantennary FA2 glycan (*Oxford* notation[35,36]) (Figure 2C). Our results for the FA2 glycan indicate that RFMS labeled glycans produce 2 times higher fluorescence signal and, more astoundingly, nearly 800 times greater MS signal than *N*-glycans labeled with IAB. In a similar fashion, RFMS labeling has also been compared to conventional 2-AB labeling. To draw such a comparison, *N*-glycans prepared from pooled human IgG with either RFMS or 2-AB were analyzed by HILIC-FLR-MS at equivalent mass loads (Supporting Information Figures S3A and S3B, respectively). Given that rapid tagging and reductive amination are performed by significantly different procedures, external calibrations were established using quantitative standards to determine the amounts of FA2 glycan loaded and eluted from the HILIC column (Supporting Information Figure S4 and S5). Response factors calculated using these calibrated amounts of FA2 glycan are provided in Supporting Information Figure S3C. It was determined that RFMS labeled glycans were detected with superior sensitivity, specifically with 14 times higher fluorescence and 160 times greater MS signal versus 2-AB labeled glycans.

To summarize the above observations, we have plotted the response factors of IAB and 2-AB as percentages against the response factors of RFMS (Figure 3). The gains in fluorescence and MS sensitivity are apparent in this plot, since it portrays response factors for IAB and 2-AB normalized to those for RFMS. In Figure 3, the relative performance of reductive amination with another alternative labeling reagent, procainamide, is also provided. Procainamide is a chemical analog to aminobenzamide that has recently been shown to enhance the ionization of reductively aminated glycans when they are analyzed by HILIC-ESI(+)-MS. Previous studies have shown that procainamide labeled glycans yield comparable fluorescence signal and 10 to 50 times greater MS signal when compared to 2-AB labeled glycans,[26] an observation corroborated by our own analyses of 2-AB and procainamide labeled *N*-glycans (Supporting Information Figure S6). Compared to procainamide, RFMS is thus predicted to provide, at a minimum, a 3-fold gain in MS sensitivity. Since both these labels contain a tertiary amine moiety, it is reasonable to suggest that the superior ionization of RFMS labeled glycans originates from the RFMS label being more hydrophobic than the procainamide label (Supporting Information Figure S7). Previous studies have shown that the addition of hydrophobic surface area to a glycan label leads to increased electrospray ionization.[37,38] The fact that permethylation enhances the MS detectability of glycans corroborates this observation.[12,14−16] It is therefore noteworthy that the RFMS label has a basic side chain in addition to a relatively hydrophobic core structure. These characteristics of the label give some explanation as to why RFMS labeled glycans exhibit high ionization efficiencies, though it should also be noted that the observed gains in MS sensitivity are likely to vary according to instrumentation. While reviewing previous studies, we have noticed that the MS gains reported for tertiary amine containing glycan labels have been highly variable. When using procainamide in place of 2-AB, Klapoetke et al. observed 10- to 50-fold increases in MS signals. When making the same comparison, Pabst and co-workers, in contrast, observed increases of less than 2-fold.[39] These observations suggest that gains in MS sensitivity are likely to vary as a function of the settings and designs of the employed mass spectrometers.

Rapid Deglycosylation. Since *N*-glycan sample preparation is equally dependent on deglycosylation as it is on a labeling step (Supporting Information Figure S8), we have also looked to optimize deglycosylation reactions. Although any mammalian *N*-glycan is a potential substrate for PNGase F, optimal enzymatic activity requires unrestricted access to the asparagine linkage site. Some protein *N*-glycan sites are sterically protected by protein structure, while others are generally accessible.[40] Also, because different *N*-glycan structures have varying rotational freedom, different glycan species can reshape local protein structure into distinct conformations.[41,42] Consequently, every *N*-glycan moiety-linkage site combination has a unique susceptibility or resistance to deglycosylation. Unless deglycosylation is complete, a biased *N*-glycan profile will be obtained. An optimized glycan sample preparation workflow benefits from a short PNGase F reaction that is both complete and compatible with fluorescent labeling reactions. Numerous approaches have been explored to reduce deglycosylation times.[29,43−47] In this work, we have investigated the combined use of 50 °C incubation temperatures and a high concentration of enzyme-friendly surfactant[46] in order to achieve complete deglycosylation in an approximately 10 min procedure.

To assay the completeness of glycan release, we have resolved deglycosylation products using intact protein HILIC separations and a prototype widepore, amide-bonded organo-silica stationary phase. Figure 4 presents HILIC-FLR chromatograms resulting from such an assay for a murine IgG1 mAb. Figure 4A specifically shows a chromatogram obtained for the mAb before it had been subjected to rapid deglycosylation (a negative control). Figure 4B meanwhile presents the chromatogram observed for this mAb after it had been subjected to 1% (w/v) RG surfactant at 95 °C for 2 min followed by incubation with PNGase F in the presence of the surfactant at 50 °C for 5 min. As can be seen, HILIC retention of these samples was found to be dramatically different. Online mass spectrometric detection has confirmed that the peaks in these profiles correspond to the mAb with different states of glycan occupancy. The control sample is represented by masses corresponding to the doubly glycosylated, native forms of the mAb. In contrast, the sample subjected to rapid deglycosylation is represented by a single mass that is in agreement with the molecular weight predicted for the mAb after complete loss of its *N*-glycans (145.3 kDa).

In addition to releasing *N*-glycans from IgG Fc domains, it has been confirmed that the proposed rapid deglycosylation approach produces complete release of Fab domain *N*-glycans, as is evidenced by a comparison of a HILIC profile for cetuximab subunits versus an *N*-glycan HILIC profile generated for cetuximab using rapid deglycosylation and RFMS labeling (Supporting Information Figure S9). Furthermore, using a gel shift assay,[48,49] we have confirmed that this approach produces

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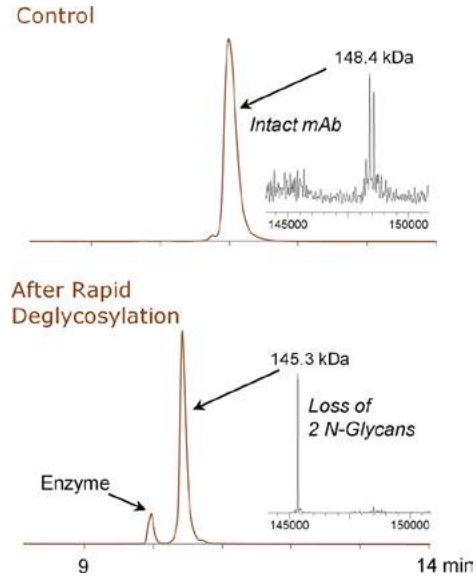

Figure 4. Assaying deglycosylation by intact protein HILIC-FLR-MS. HILIC fluorescence profiles obtained for two different conditions are shown: anticitrinin murine IgG1 mAb (A) before being subjected to rapid deglycosylation (a negative control) and (B) after being subjected to 1% (w/v) RG surfactant at 95 °C for 2 min followed by incubation with PNGase F and the surfactant at 50 °C for 5 min (a 2 step approach). Samples of the mAb (1.5 μg) were separated using two coupled 2.1 × 150 mm columns packed with prototype sub-2 μm (300 Å average pore diameter) amide bonded organosilica stationary phase.

deglycosylation of a diverse set of glycoproteins (Supporting Information Figure S10). As has been hypothesized before, it is believed that some surfactants, such as RG, enhance deglycosylation by relaxing protein structure and ensuring that *N*-glycans are accessible for efficient deglycosylation.[46] Here, use of such a surfactant is combined with elevated temperature incubations in a 2-step sample preparation that can be performed in approximately 10 min. And as described in the Experimental Section, this rapid deglycosylation procedure can be directly integrated with RFMS glycan labeling.

Quantitative HILIC SPE and Sample Preparation Recovery. The final step in an *N*-glycan sample preparation aims to extract the labeled glycans in preparation for their analysis. An effective approach for extraction of labeled glycans from reaction byproducts has been achieved using SPE designed to selectively extract RFMS labeled *N*-glycans from labeling reaction byproducts, which can otherwise interfere with analysis of the labeled glycans by HILIC column chromatography (Figure 5A). A highly polar, aminopropyl silica-based sorbent was selected for this application since it possesses useful hydrophilic interaction retentivity for glycans as well as a weakly basic surface that provides further selectivity advantages based on ion exchange and electrostatic repulsion. In the employed SPE process, glycans were adsorbed to the sorbent via a HILIC mechanism, then the sample was washed to remove sample matrix. An acidic wash solvent comprised of 1% formic acid in 90% acetonitrile was employed in this step to introduce electrostatic repulsion between the aminopropyl HILIC sorbent and labeling reaction byproducts and to enhance the solubility of the matrix components. After washing, the RFMS labeled *N*-glycans were eluted from the aminopropyl sorbent using an eluent comprised of a pH 7 solution of 200 mM ammonium acetate in 5% acetonitrile. Upon their elution, the RFMS labeled glycans were diluted with a mixture of organic solvents (ACN and DMF) and directly analyzed by HILIC column chromatography, as shown in Figure 5B.

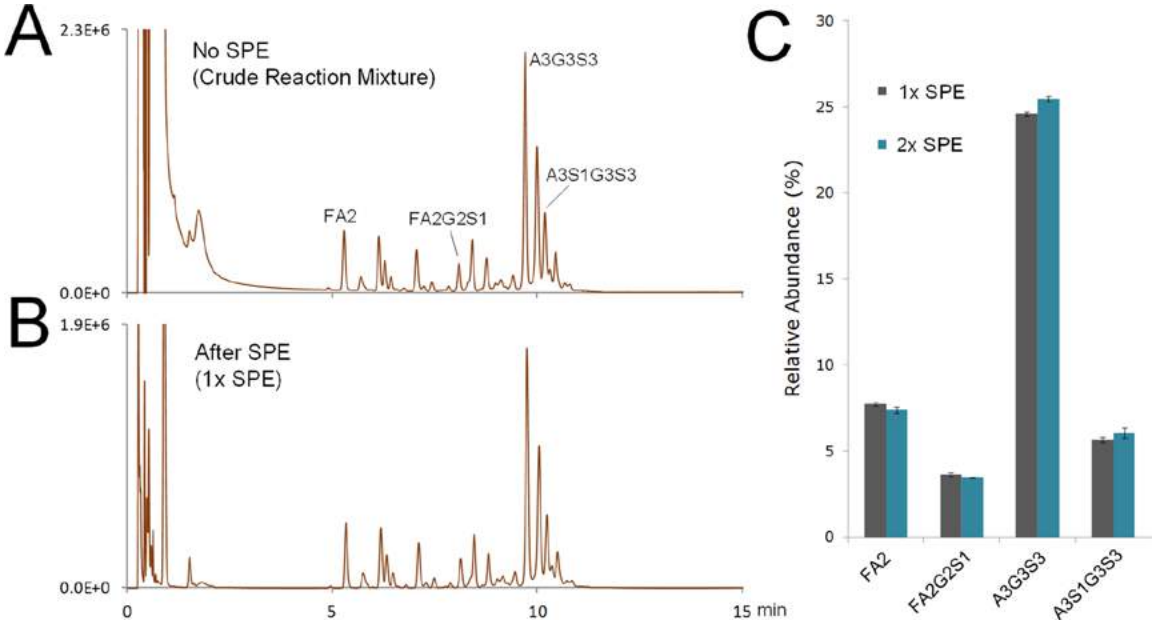

Figure 5. Extraction of RFMS labeled *N*-glycans by HILIC SPE. (A) A test mixture comprised of RFMS labeled glycans from pooled human IgG and bovine fetuin separated on a 2.1 × 50 mm column packed with 1.7 μm amide bonded organosilica (130 Å) stationary phase and detected via fluorescence (labeled *N*-glycans from 0.4 μg glycoprotein, 10 μL injection of ACN/DMF diluted sample). (B) The test mixture after extraction by HILIC SPE (one pass/1× SPE). (C) Relative abundances of four *N*-glycans from the test mixture as measured after 1 and 2 extractions by HILIC SPE (1× SPE versus 2× SPE). Samples were analyzed in triplicate.

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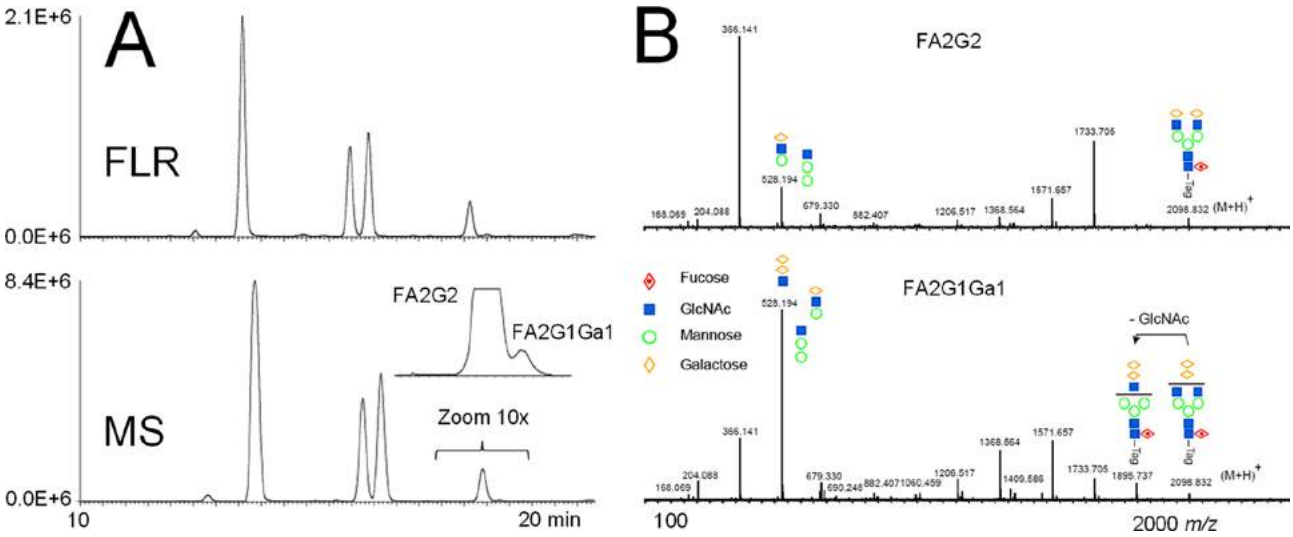

Figure 6. RFMS labeled *N*-glycans from 0.9 μg of anticitrinin murine IgG1 mAb. (A) Fluorescence (FLR) chromatogram and based peak intensity (BPI) MS chromatograms obtained using a 2.1 × 150 mm column packed with 1.7 μm amide bonded organosilica (130 Å) particles. The magnified peaks show the separation of two isobaric glycans, FA2G2 and FA2G1Ga1, that are present in the sample at significantly different abundances. (B) MS/MS spectra of FA2G2 and FA2G1Ga1: Structurally diagnostic ions for FA2G1Ga1 are displayed in the bottom spectrum. MS/MS spectra were charge deconvoluted and deisotoped using MaxEnt 3.

Absolute and relative recoveries for this novel HILIC SPE process have been studied. RFMS labeled *N*-glycans prepared from a mixture of pooled human IgG and bovine fetuin were processed by multiple passes through the HILIC SPE steps and recoveries across the serial processing were measured. Specifically, recoveries were measured for species representing extremes in glycan properties, including an asialo FA2 glycan and a glycan with a tetrasialylated, triantennary structure (A3S1G3S3). Absolute recovery through the SPE process was found to be approximately 74% (Supporting Information Table S5), which is a relatively high recovery for an oligosaccharide enrichment strategy. Zhang and co-workers recently studied oligosaccharide SPE and reported that recoveries between 60% and 80% are obtained with optimized protocols and the best performing sorbents.[50] With the SPE strategy we have proposed, the majority of the observed sample losses appear to be a consequence of elution volume. Increasingly higher SPE recoveries were achieved when increasingly larger elution volumes were employed (Supporting Information Figure S11). To facilitate direct analyses, however, a compromise was made with the technique such that a 90 μL elution volume was used to obtain a relatively concentrated glycan eluate.

Of greater significance is the fact that the observed sample losses appear to be nonspecific in nature, since this SPE has been found to exhibit highly accurate relative yields. Figure 5C shows the relative abundances for four glycans (FA2, FA2G2S1, A3G3S3, and A3S1G3S3) as determined after one pass or two passes of the SPE process, respectively. The largest deviation in relative abundance was observed for the tetrasialylated A3S1G3S3 glycan, in which case a relative abundance of 5.7% was determined after a single pass of SPE versus 6.1% after two passes of SPE. With these results, it is demonstrated that this SPE technique, combining HILIC and electrostatic repulsion, provides a mechanism to immediately analyze a sample of extracted RFMS labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of *N*-glycans.

The percent yield of the entire *N*-glycan sample preparation workflow also was evaluated to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of RFMS labeled glycans. RFMS labeled *N*-glycans from a murine IgG1 were prepared, analyzed by HILIC-FLR-MS, and quantified by means of an external calibration (Supporting Information Figures S12A and S12B). Using a theoretical yield (Supporting Information Figure S12C) and duplicate analyses, it was determined that the percent yield of the entire *N*-glycan sample preparation was approximately 73%, which indicates that the described approach is essentially lossless save for the SPE processing.

Detailing the *N*-Glycan Profile of a mAb. Because of the poor ionization efficiency of glycans labeled with conventional fluorescent labels, it is often difficult to detect, let alone characterize, low abundance glycans species during HILIC-ESI-MS analysis. To demonstrate the capability of RFMS glycan labeling to improve the quality of MS data, the *N*-glycan profile of the anticitrinin murine IgG1 mAb previously shown in Figure 2 was reanalyzed using a higher resolution HILIC separation and MS/MS analysis with a QTof mass spectrometer outfitted with a high transmission efficiency collision cell.

Figure 6A shows fluorescence and base peak intensity (BPI) MS chromatograms obtained in this study for RFMS labeled *N*-linked glycans resulting from only 0.9 μg of the mAb. Notably, the BPI MS chromatogram in this analysis presented signal-to-noise comparable to that of the fluorescence chromatogram, making assignment of glycan species via accurate mass measurement straightforward (Supporting Information Table S6). The magnified peaks in Figure 6A additionally show two isobaric glycans that are partially resolved by the HILIC separation. From historical data and abundances of glycans in typical mAb samples,[30,51] the first peak was assigned to a common FA2G2 glycan. A later yet closely eluting species was also detected with the same m/z value as the FA2G2 peak, albeit at a relative abundance of only 0.7%. MS/MS spectra of

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these RFMS labeled, isobaric glycans were thus contrasted to elucidate their structural differences.

With the enhanced MS signal afforded by the RFMS label, structurally diagnostic fragmentation data were obtained for the FA2G2 glycan and it isobaric analog. Figure 6B shows a side-by-side comparison of the fragment ions generated from the high abundance FA2G2 glycan versus its isobaric, low abundance counterpart that eluted in the shoulder peak. A 528 m/z ion was found to be prominent in the spectrum for the low abundance, isobaric species. High yields of such an ion have previously been reported as being diagnostic of an α-Gal configuration in glycans,[52] wherein two galactose residues are linked through an α-1,3 bond. Also observed in the spectrum for the low abundance, isobaric species was a very prominent loss of GlcNAc, which is observed when an *N*-glycan branch terminates with a GlcNAc residue. Collectively, these high signal-to-noise fragmentation data support the identification of the isobaric, low abundance species as an α-Gal containing FA2G1Gal glycan. By showing that low abundance glycans from a pool of released *N*-glycans can be readily detected and characterized with minimal ambiguity, this case study underscores the significant promise that RFMS labeling holds for *N*-glycan profiling and structure elucidation.

CONCLUSIONS

Hydrophilic interaction chromatography has matured over the last two decades into a powerful technology for *N*-glycan profiling. The methodology described in this work strengthens this approach to glycan characterization by addressing several shortcomings corresponding to sample preparation techniques. With the described developments, we have enhanced the sensitivity of glycan detection and have also decreased the time required to prepare *N*-glycans for analysis. In this approach, glycoproteins are deglycosylated in approximately 10 min to produce *N*-glycosylamines. These glycans are then rapidly reacted with the RFMS reagent and are thereby labeled with a tag comprised of an efficient fluorophore and a basic tertiary amine. In a final step, RFMS-labeled glycans are extracted from reaction byproducts by means of a robust HILIC SPE method that facilitates immediate analysis of samples. These techniques make it possible to quickly prepare *N*-glycan samples and to incorporate the use of a fluorescent labeling reagent that enhances the sensitivity of ESI-MS detection. To demonstrate these properties, the RFMS-labeled *N*-glycan profile of a monoclonal antibody was elucidated through the combined use of HILIC, accurate mass measurements and MS/MS fragmentation. As has been indicated with this work, it is believed that the described approach will facilitate future studies of *N*-glycosylation by allowing samples to be more quickly analyzed and to be more easily characterized to greater levels of detail. This should, at a minimum, help accelerate process development of biopharmaceuticals. With this work, we have clearly demonstrated the utility of the methodology for profiling the *N*-glycans of monoclonal antibodies, the most frequently exploited modality for therapeutic applications. Nevertheless, it is interesting to consider the use of these sample preparation techniques for the analysis of *N*-glycans from complex matrices, such as human blood serum,[53] though additional work will be required to investigate their applicability to such studies.

ASSOCIATED CONTENT

Supporting Information

Supplemental experimental information, supplemental results and discussion on the stability of RFMS glycan derivatives, the stability of *N*-glycosylamines, evaluation of response factors, deglycosylation, SPE recovery, SPE recovery as a function of elution volume, sample preparation yield, and IdeS-digested cetuximab, tables provides LC chromatographic conditions, a figure showing a comparison of HILIC-FLR-MS with RFMS labeled versus 2-AB labeled *N*-glycans, figures presenting the quantitative analysis of RFMS and 2-AB labeled *N*-glycans, a figure showing a comparison of HILIC-FLR-MS signal from procainamide versus 2-AB labeled glycans, a figure depicting the described *N*-glycan sample preparation workflow, a figure presenting the comparison of HILIC profiling of cetuximab subunits versus HILIC profiling of RFMS labeled *N*-glycans prepared from cetuximab using rapid deglycosylation, a figure showing a gel shift assay of a diverse set of glycoproteins deglycosylated using rapid deglycosylation, a figure outlining the yield calculation for the entire sample preparation technique, a table listing the fluorescence peak areas and relative abundances of RFMS labeled glycans as measured after one and two passes through HILIC SPE, a figure demonstrating the charge states of RFMS labeled glycans, a figure displaying an MS/MS spectrum for RFMS labeled FA2, a figure showing NMR spectra obtained for RFMS derivatized propylamine, and a figure presenting HPLC impurity assays obtained for RFMS derivatized propylamine. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00758.

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Notes

The authors declare the following competing financial interest(s):The authors of this manuscript are employed by Waters Corporation and New England Biolabs. Equipment and products from Waters and New England Biolabs were used for this research.

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Exploiting *Rapi*Fluor-MS Labeling to Monitor Diverse N-Glycan Structures via Fluorescence and Mass Detection

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APPLICATION BENEFITS

- Reduced sample preparation times for released N-glycan analyses
- Increased confidence in glycan monitoring by obtaining fluorescence and mass detection for every peak

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

ACQUITY® QDa® Mass Detector

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Column

Empower® 3 FR2 CDS

KEY WORDS

Glycan, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS

INTRODUCTION

Glycosylation is one of the most complex post-translational modifications of protein-based biotherapeutics. The efficacy of glycosylated therapeutics is directly related to the glycoprofile. The presence of undesired structures can lead to changes in PK/PD profiles, either positively or negatively, and have been associated with immunogenic responses. For these reasons glycosylation is often designated as a critical quality attribute (CQA). During the development process, the glycoprofile of candidate molecules is extensively studied and characterized. Characteristic profiles are then monitored through process development, commercialization, and post-approval studies to maintain product efficacy and safety.

In this application note, we present a streamlined approach to label released N-glycans with *Rapi*Fluor-MS and analyze the labeled N-glycans with the ACQUITY UPLC H-Class Bio System with fluorescent (FLR) and ACQUITY QDa Mass Detectors. This new monitoring workflow allows researchers to prepare samples from glycoprotein to UPLC-FLR/MS analysis in 30 minutes. In addition to reduced sample preparation times, *Rapi*Fluor-MS yields 14 times greater fluorescence response and 160 times greater MS response when compared to 2-AB. These improvements enable the use of FLR and mass detection with the ACQUITY QDa for routine analysis. In this application note we present the utility of *Rapi*Fluor-MS coupled with UPLC®-FLR-MS for monitoring labeled glycans ranging across a range of properties, masses, and relative abundance.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR and ACQUITY QDa Mass Detector
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	2 µL
Data management:	Empower 3 FR2 CDS

FLR settings

Data rate:	5 points/sec.
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500–1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C
Ionization mode:	ESI+
Mobile phase A:	Acetonitrile (Pierce, LC-MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC-MS Grade, Waters ammonium formate concentrate)
Mobile phase C:	Acetonitrile (LC-MS grade)
Mobile phase D:	Acetonitrile (LC-MS grade)

Time	Flow rate (mL/min)	% A	% B	%C	% D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

A sample of murine IgG1 mAb N-Glycans was prepared from Waters Intact mAb Mass Check Standard (p/n 186006552), which is included in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606). N-Glycans were also prepared from RNase B and bovine fetuin (Sigma Aldrich). Released and labeled N-glycan pools were generated using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit following the protocol provided in the Care and Use Manual (715004793). Following release and labeling, samples were dried using a CentriVap™ and reconstituted in 25 µl of a mixture of ACN/Water/DMF at a ratio of 22.5%:55.5%:22%, respectively. In each case the targeted mass load was 30 pmoles of released glycan. The ammonium formate mobile phase was prepared using Waters ammonium formate concentrate (p/n 186007081).

RESULTS AND DISCUSSION

N-glycosylation is a non-template driven process that generates a vast array of glycan structures that vary in size, charge, and extent of branching depending on the protein and expression system. To evaluate the capacity of the ACQUITY QDa to detect glycans both within and beyond its mass range, three glycoproteins (human IgG, RNAse B, and bovine fetuin) were selected to provide typically observed glycans ranging from neutral bi-antennary structures to tetra-sialylated structures. N-glycans from each protein were released using Rapid PNGase F and labeled with *Rapi*Fluor-MS following the provided sample preparation protocol. Labeled glycans were separated via UPLC-HILIC and detected using both an ACQUITY FLR and ACQUITY QDa.

As is evident in Figure 1, each glycan structure is chromatographically resolved using a single gradient method. In addition, each glycan structure observed in fluorescence (top panel) is also observed by the ACQUITY QDa Mass Detector (bottom panel), indicating the ability of the ACQUITY QDa to detect glycans across a range of possible structures and attributes when labeled with *Rapi*Fluor-MS. For traditional labeling technologies this is not possible due to poor ionization efficiency.

While it is useful that glycan structures can be observed by mass detection, it is important to understand the quality of the resulting spectra and the charge states of the glycan ions obtained within. To understand this aspect, we integrated peaks spanning a range of glycan properties and measured the relative abundances of species in each sample using FLR integrated data. The spectra shown in Figure 2 demonstrate the ability of the ACQUITY QDa to generate high quality spectra for glycan structures across a wide range of properties and masses. The data also demonstrate that both high and low abundance glycan structures can be readily detected. Our data indicates that high quality spectra are generated for structures present in the fluorescence profiles at abundances as low as 0.5% highlighting the sensitivity of ACQUITY QDa mass detection combined with the improved ionization efficiency afforded by *Rapi*Fluor-MS. Our data also demonstrate how the improved charging of glycan structures by the use of *Rapi*Fluor-MS allows small structures such as A2, as well as very large structures, such as the tetrasialylated A3G3S4, to be detected with the QDa.

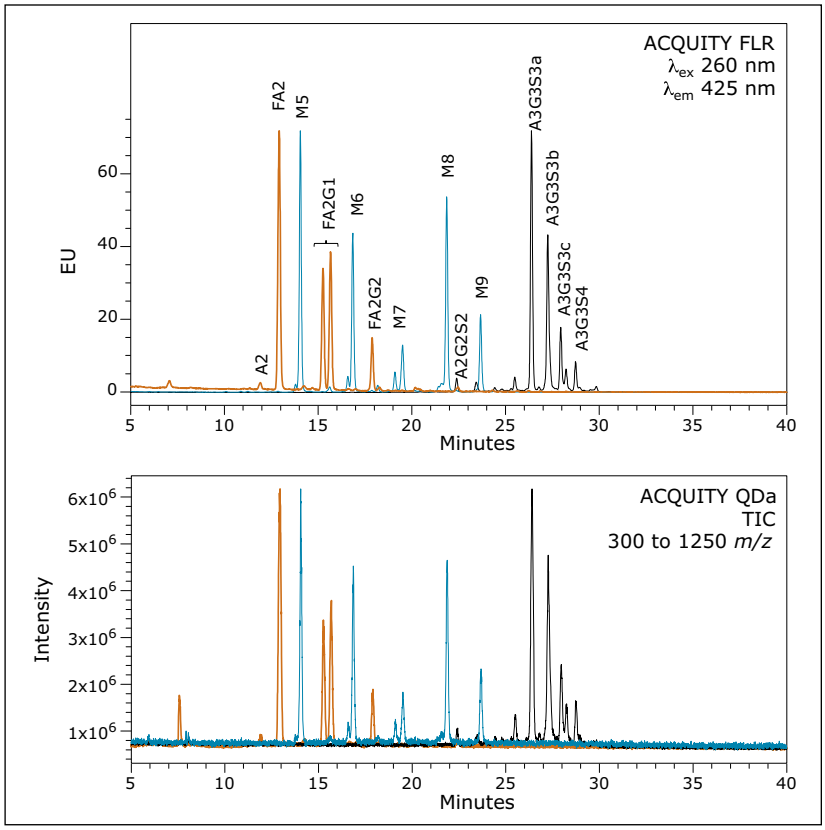

Figure 1. The ACQUITY QDa can detect an array of RapiFluor-MS labeled N-glycans. Glycans from human IgG (red trace), RNAse B (black trace), and bovine fetuin (blue trace) were released with Rapid PNGase F, labeled with RapiFluor-MS reagent. Individual glycan pools were then separated via HILIC and detected with both fluorescence (A) and mass detection (B).

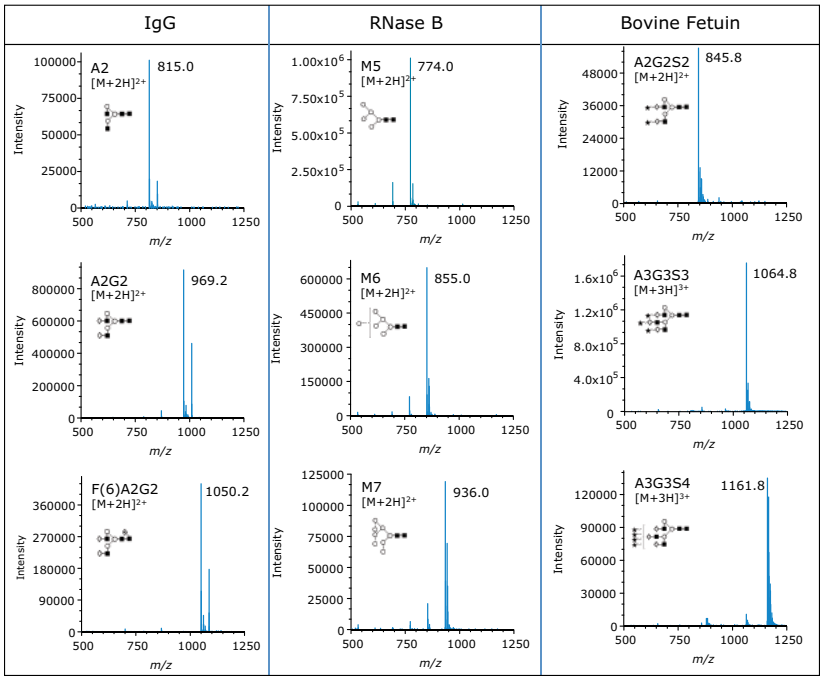

Figure 2. Spectra of selected RapiFluor-MS labeled glycans detected with an ACQUITY QDa Mass Detector. Glycans from murine IgG1 mAb, RNase B, and bovine fetuin were released with Rapid PNGase F and labeled with RapiFluor-MS. Shown are representative spectra for selected glycan structures separated in Figure 1.

CONCLUSIONS

Glycosylation of is a complex and critical aspect of most therapeutic proteins which must be well characterized. Often, the profile of N-glycans is identified as a critical quality attribute and as a result is monitored throughout the lifecycle of products. As discussed in this application note, preparation of samples with a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit can dramatically reduce sample preparation time and complexity. In addition, the use of *Rapi*Fluor-MS yields improved FLR sensitivity and dramatically improved MS sensitivity. Through improving glycan MS sensitivity *Rapi*Fluor-MS labeling permits the use of mass detection with the ACQUITY QDa and thereby affords greater confidence in peak monitoring across the range of structures encountered during biopharmaceutical development. Taken together, *Rapi*Fluor-MS labeling and HILIC-FLR-MS with ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Mass Detector offer an unparalleled solution for monitoring the N-glycan profiles of biotherapeutics.

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Future-Proofing the Biopharmaceutical QC Laboratory: Chromatographic Scaling of HPLC Monosaccharide Analyses Using the ACQUITY UPLC H-Class Bio System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Geometric scaling of an HPLC method for determining monosaccharide composition in glycoprotein samples to UPLC®
- Application of scalable column chemistries for monosaccharide analysis
- Guidance for scaling HPLC gradient methods to UPLC
- A high sample throughput and high resolution UPLC approach for determining monosaccharide analysis

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

Empower® 3 Chromatography Data Software

XBridge® HPLC Columns

ACQUITY UPLC BEH Column

KEY WORDS

Glycosylation, glycoproteins therapeutics, N-linked glycans, monosaccharide, 2AA-derivatized monosaccharides, glycan composition, HPLC, UPLC

INTRODUCTION

Evidence for reliable and consistent glycosylation of glycoprotein therapeutics is typically obtained through LC-based analysis of N-linked glycans. On occasion, regulatory agencies request information of monosaccharide content as an orthogonal technique for confirming the glycan composition. Beyond profiling changes in total glycan composition, monosaccharide analysis can also be used as an exploratory technique for identifying various monosaccharide modifications, including phosphorylation and sulfation, which can be particularly challenging to discern when analyzing at the released glycan level in the absence of mass spectrometry.

Initial investigation of monosaccharide analyses resulted in a reliable and informative HPLC-based approach using 2-aminobenzoic acid (2AA)-derivatized glycan hydrolysates where each of the individual monosaccharides were successfully separated using reversed-phase chromatography. Since this time, the analytical demands placed on development and QC labs that are tasked with characterizing and monitoring glycoprotein therapies have increased dramatically. Assays used to monitor changes in glycosylation therefore need to be updated in order to maximize productivity through improved analytical efficiency.

In this application note, we illustrate the transfer of a robust HPLC monosaccharide method to the ACQUITY UPLC H-Class Bio System running as an HPLC, using monosaccharide standards as well as bovine fetuin and the commercially available monoclonal antibody cetuximab as analytes. Geometric scaling of the presented method is then performed in a stepwise manner to demonstrate the advantages of transferring legacy HPLC monosaccharide analyses to UPLC technology as a means of decreasing assay time.

EXPERIMENTAL

LC conditions

Waters ACQUITY UPLC H-Class Bio System, comprised of:	ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM) ACQUITY UPLC H-Class Bio Sample Manager (FTN) ACQUITY UPLC H-Class Bio Column Heater (CH-A) ACQUITY UPLC FLR Detector Extension loop: 100 µL (p/n 430002625)
Waters columns:	XBridge C_{18} 5 µm, 130 Å, 4.6 x 100 mm (p/n 186003115) XBridge C_{18} 3.5 µm, 130 Å, 2.1 x 100 mm (p/n 186003033) XBridge C_{18} XP 2.5 µm, 130 Å, 2.1 x 100 mm (p/n 186006031) ACQUITY UPLC BEH C_{18} 1.7 µm, 130 Å, 2.1 mm x 100 mm (p/n 186002352)
Mobile phase A:	0.2% N-butylamine, 0.5% phosphoric acid, and 1% THF in H_2O
Mobile phase B:	50% mobile phase A in acetonitrile
Excitation wavelength:	360 nm
Emission wavelength:	425 nm
Column temp.:	30 °C
Injection vol.:	4.6 mm x 100 mm format, 4.8 µL, 2.1 mm x 100 mm format, 1 µL

Following the described approach, typical run times for HPLC-based monosaccharide analysis are reduced from 45 minutes to just 17 minutes. Importantly, chromatographic resolution between measured critical peak pairs is observed to improve with migration to smaller column particle sizes. Selectivity is unaffected due to the availability of reversed-phase column chemistries in a number of particle sizes and dimensions. The data presented here indicate migration to UPLC technology offers significant advantages for improving monosaccharide chromatographic quality.

Sample preparation

Derivatization of monosaccharides was performed as previously described,[1,2] with a number of minor modifications as recommended by Stepan and Staudacher.[3] Monosaccharides from bovine fetuin were released by acid hydrolysis using 2 M TFA with hydrolysis occurring for 3 h at 100 °C. Resulting hydrolysates were then dried by centrifugal evaporation followed by reconstitution in 5 µL of 80 mg/mL sodium acetate trihydrate. A 2AA labeling solution was prepared by dissolving 30 mg of 2AA in 1 mL of 2% (w/v) boric acid in methanol. This suspension was then used to dissolve 30 mg of sodium cyanoborohydride. Of this preparation, 10 µL was added to each of the monosaccharide mixtures. Monosaccharides were labeled at 80 °C for 60 min. Upon completion of labeling, serial dilutions were performed to generate a 1000-fold dilution of the labeled material. For preparation of monosaccharide standards, labeling was performed as outlined above with the omission of acid hydrolysis.

Step	%B[1]	Method details (flow rate and time)							
		5 µm		3.5 µm		2.5 µm		1.7 µm	
		Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)	Flow (mL min^{-1})	Time (min)
1	7	0.480	0.00	0.685	0.00	0.200	0.00	0.294	0.00
2	7	0.480	7.78	0.685	5.45	0.200	3.89	0.294	2.64
3	17	0.480	27.78	0.685	19.47	0.200	13.88	0.294	9.44
4	100	0.480	28.89	0.685	20.24	0.200	14.43	0.294	9.82
5	100	0.480	40.00	0.685	28.03	0.200	19.99	0.294	13.60
6	7	0.480	41.11	0.685	28.81	0.200	20.54	0.294	13.97
7	7	0.480	50.00	0.685	35.04	0.200	24.98	0.294	17.00

Calculations

Flow rate scaling: $$F_2 = F_1\left(\frac{d_2^{\,2}}{d_1^{\,2}}\right)\left(\frac{d_{p1}}{d_{p2}}\right)$$

F refers to flow rate, d refers to column I.D., and dp refers to particle diameter. In each case, 2 refers to the new column and 1 refers to the original column.

Injection volume scaling: $$Vi_2 = Vi_1\left(\frac{r_2^{\,2}L_2}{r_1^{\,2}L_1}\right)$$

Vi refers to injection volume, r refers to column internal radius, and L refers to column length. In each case, 2 refers to the new column and 1 refers to the original column.

RESULTS AND DISCUSSION

ACQUITY UPLC H-Class Bio System for HPLC monosaccharide analysis

To verify the ability of the ACQUITY UPLC H-Class Bio to run legacy analyses of 2AA-derivatized monosaccharides, we first established an HPLC separation using method conditions based on previously described chromatographic conditions[1,2] To evaluate the proposed method, a reference standard mix was prepared by combining individual monosaccharides into a common mix. This mix included the typical monosaccharides expected in biologically relevant samples, namely N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), glucose (Glc), mannose (Man), galactose (Gal), xylose (Xyl), and fucose (Fuc). In addition to these standards, two glycoprotein samples were also selected to determine the accuracy of this approach in determining monosaccharide composition. The first glycoprotein selected was bovine fetuin, a protein known to contain both N- and O-glycosylation sites. The second glycoprotein selected was the commercial monoclonal antibody cetumixab.

Following hydrolysis from the glycoprotein samples and 2AA derivatization, monosaccharides were separated using the aforementioned method. The resulting HPLC chromatogram (Figure 1) acquired on the ACQUITY UPLC H-Class Bio is consistent with previously published data.[2] In terms of chromatographic performance, peak capacity was measured together with selectivity and resolution between critical peak pairs. These data are summarized in Table 1. Consistent peak area was observed across separations using all particle sizes (Figure 2d). These data confirmed the separation of all relevant components and, therefore, established a suitable method for monosaccharide method scaling to UPLC technology.

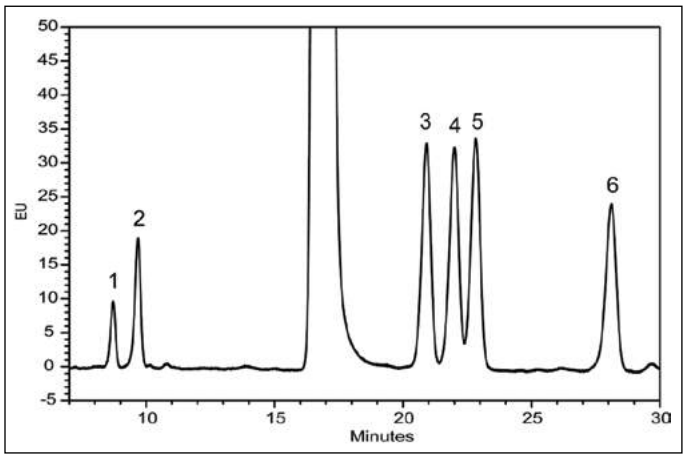

Figure 1. HPLC analysis of monosaccharides. A separation performed with a Waters XBridge 5 µm C_{18} Column using a previously described method1. Monosaccharides are identified as follows: 1) N-acetylglucosamine (GlcNAc), 2) N-acetylgalactosamine (GalNAc), 3) Galactose (Gal), 4) Mannose (Man), 5) Glucose (Glc), and 6) Fucose (Fuc).

Measurement		Particle size (µm)			
		5	3.5	2.5	1.7
Column ID (mm)		4.6	4.6	2.1	2.1
Column Length (mm)		100	100	100	100
Average $W_{1/2h}$		0.349	0.189	0.125	0.076
T_g		20.00	9.99	14.02	6.80
P_c		58	75	81	90
	GlcNAc	8.71	5.84	4.40	2.85
	GalNAc	9.69	6.49	4.87	3.15
	Gal	20.92	14.32	10.87	7.08
	Man	22.01	15.09	11.46	7.46
	Glc	22.83	15.66	11.92	7.78
	Fuc	28.12	19.39	14.76	9.74
	GlcNAc	3.65	3.83	3.69	3.67
	GalNAc	8.26	8.52	8.23	8.31
	Gal	22.17	22.48	22.45	22.06
	Man	23.04	23.07	23.05	22.72
	Glc	23.76	23.89	23.88	23.61
	Fuc	19.11	18.21	18.71	19.62
	GlcNAc, GalNAc	1.12	1.13	1.13	1.15
	GalNAc, Gal	2.26	2.38	2.47	2.67
	Gal, Man	1.05	1.06	1.06	1.06
	Man, Glc	1.04	1.04	1.04	1.05
	Glc, Fuc	1.24	1.25	1.26	1.28
	GlcNAc, GalNAc	2.52	3.10	3.16	3.32
	GalNAc, Gal	21.05	27.12	30.69	33.25
	Gal, Man	1.62	2.10	2.48	2.62
	Man, Glc	1.20	1.53	1.90	2.23
	Glc, Fuc	7.34	9.59	11.41	12.78

Table 1. Summary data for chromatographic analysis.

Migration of monosaccharide analysis from HPLC to UPLC improves resolution

In an effort to improve throughput of analyses and general chromatographic quality, the above described method was geometrically scaled in a stepwise manner to UPLC column technology. This involved scaling the flow rate to the new column dimensions and adjusting individual steps in the gradient method to deliver equivalent column volumes as itemized in the original recommended HPLC method. Details of the UPLC method can be found in the experimental section of this application note.

Several particle sizes of identical chemistry ranging from 1.7 to 5 µm were used in the scaling exercise. In the case of the 1.7 µm and 2.5 µm particles, a 2.1 mm x 100 mm column dimension was used while 4.6 mm x 100 mm column dimensions were used for the 3.5 µm and 5 µm particles. Flow rate and injection volume scaling calculations specific for individual column dimensions were determined using the appropriate equations defined in the experimental section of this application note. The duration for each step in the gradient table was subsequently modified based on the new flow rate and column volume to ensure consistent delivery of equivalent column volumes per change in organic composition when compared to the original method. The results of these calculations are summarized in the experimental section of this application note where flow rates and gradient step durations are itemized with respect to each column particle size.

To evaluate the results of method scaling, the monosaccharide mix was separated under the new gradient conditions for each particle size and column dimensions (Figure 2). Method scaling to UPLC column technology reduced the total required run time of the method from 50 min (in the case of the 5 µm column, Figure 2a) to just 17 min (in the case of the 1.7 µm column, Figure 2d), an improvement in efficiency of approximately 66%. Details of chromatographic performance are presented in Table 1.

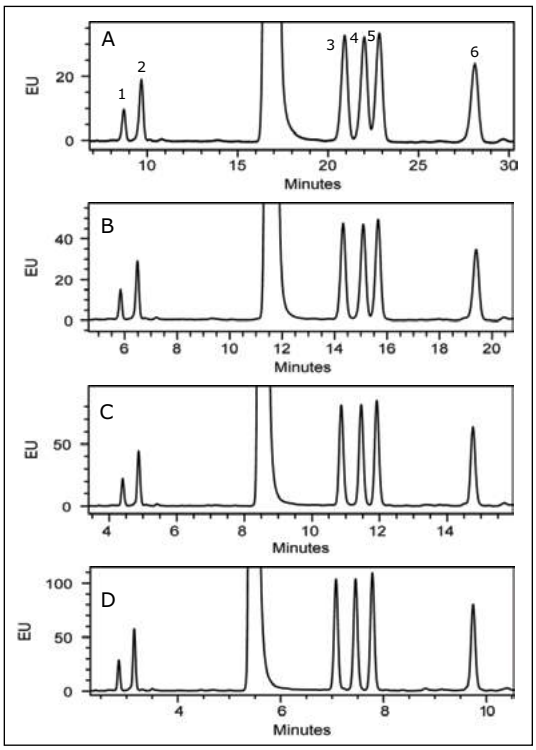

Figure 2. Geometric scaling of a monosaccharide separation. (A) 5 µm particle (B) 3.5 µm particle, (C) 2.5 µm particle, and (D) 1.7 µm particle. 1) GlcNAc, 2) GalNAc, 3) Gal, 4) Man, 5) Glc, and 6) Fuc.

In general, peak capacity was shown to increase with decreasing particle size (Figure 3a), an expected outcome based on the narrower peaks achieved with UPLC technology. The reduction in run time was not at the cost of resolution, where a general improvement was observed across all critical peak pairs as column particle size decreased (Figure 3b). Selectivity remained unaffected mainly due to the availability of identical column chemistry across multiple particle sizes (Figure 3c). Changes in column particle size did not impact relative peak area determination, evidenced by averaged peak areas for each monosaccharide across all column formats (Table 1 and Figure 3d). Taken together, scaling of the original monosaccharide method produced improved resolution in a shorter amount of time, with negligible impact to selectivity.

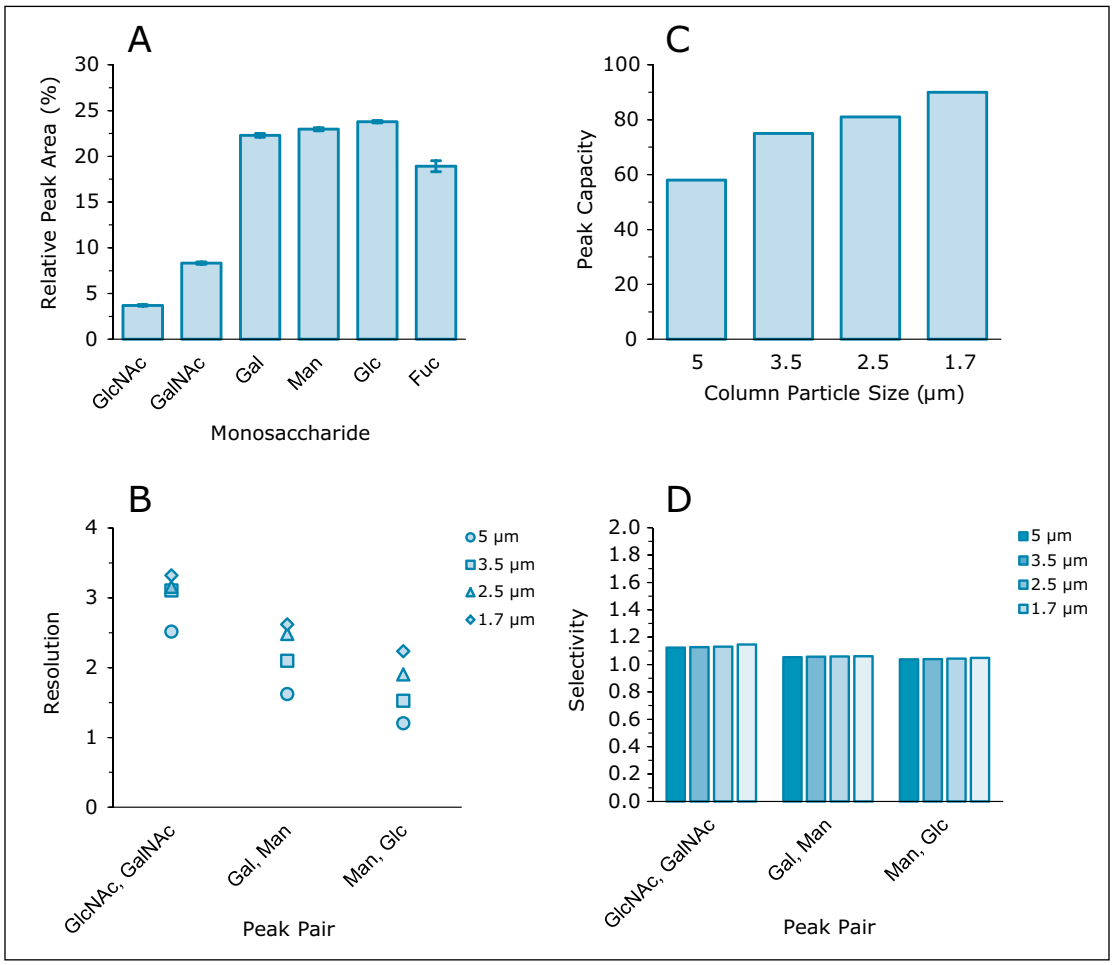

Figure 3. Quantitative analysis of geometric scaling. (A) Relative peak area measurements for individual monosaccharides, averaged across all columns with standard deviation denoted by error bars; (B) Resolution measurements from 3 monosaccharide peak pairs; (C) Peak capacity measurements of geometrically scaled methods across all column dimensions, and; (D) selectivity measured between 3 monosaccharide peak pairs.

Determination of monosaccharide content in glycoprotein samples

To verify this approach in determining monosaccharide composition, we analyzed both fetuin and cetuximab 2AA-labeled monosaccharides. Fetuin is known to contain O-glycosylation and therefore should demonstrate the presence of GalNAc within its profile. Cetuximab, on the other hand, contains no O-glycosylation and should therefore be absent of any GalNAc.

To perform the analysis, the 1.7-µm BEH C_{18} particle chemistry was used and results of the separations were compared to the monosaccharide standard (Figure 4a). GalNAc is clearly detected in the fetuin sample (Figure 4b), as evidenced by a chromatographic peak with similar retentivity to the GalNAc standard. Also consistent with literature is the absence of core fucosylation in fetuin, largely evidenced by the absence of a peak with similar retentivity to the fucose standard. This finding is consistent with previous reports of bovine fetuin N-glycosylation characterization.[4,5] Analysis of cetuximab reveals all individual monosaccharides with the exception of xylose, a monosaccharide not typically associated with mammalian N-linked glycosylation (Figure 4c). Mannose was found to be in higher quantity than galactose, a result consistent with previous literature indicating the presence of several high mannose structures in cetuximab.[6-8]

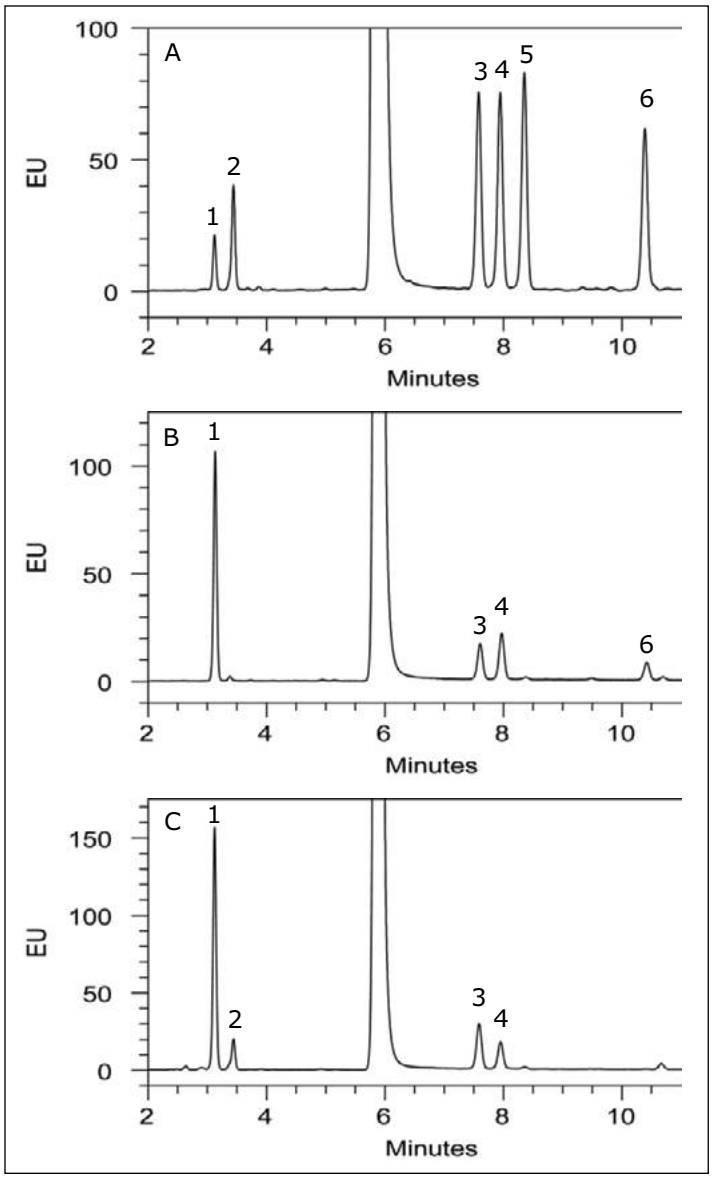

Figure 4. Monosaccharide analysis of fetuin and cetuximab using the developed UPLC separation. (A) monosaccharide standard, (B) cetuximab monosaccharides, (C) fetuin monosaccharides. In all chromatograms, monosaccharides are identified as: 1) GlcNAc, 2) GalNAc, 3) Gal, 4) Man, 5) Glc, and 6) Fuc.

CONCLUSIONS

As a complementary approach to released glycan analysis, monosaccharide profiling allows analysts to verify glycan composition determined in traditional HILIC-based separations. A well-established assay for monosaccharide analysis uses reversed-phase chromatography to separate 2AA-derivatized monosaccharides. In this application note, we have demonstrated the ability of the ACQUITY UPLC H-Class Bio System for running both HPLC and UPLC methods for monosaccharide analyses. This HPLC assay provided sufficient resolution of individual monosaccharides but was restricted in part by the time required to perform the separation. A significant reduction in runtime was obtained by transferring the legacy HPLC method to UPLC technology. With the modernized, UPLC-based separation, a higher throughput assay for monosaccharide analysis was thereby achieved.

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New Capabilities for Monitoring Released N-Glycans Through the Combined Use of *Rapi*Fluor-MS Labeling, ACQUITY UPLC H-Class Bio System, and Serial Fluorescence/ACQUITY QDa Mass Detection

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APPLICATION BENEFITS

- Reduce sample preparation times for released N-glycan analyses
- Increase confidence in glycan monitoring by routinely obtaining mass information and fluorescence for every peak

WATERS SOLUTIONS

*Rapi*Fluor-MS™ Glycan Performance Test Standard

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

ACQUITY® QDa® Mass Detector

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC FTN

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Column

Empower® 3 CDS Software

KEY WORDS

Glycans, glycoforms, labeled glycans, peak monitoring, mass detection, fluorescence detection, IgG

INTRODUCTION

During the development of biopharmaceuticals, it is important to characterize and monitor glycoprofiles as they are often implicated as a product critical quality attributes due to their impact on safety, efficacy, and potency among other factors. It is well accepted that structural characterization of the glycoforms present is necessary, and that mass spectrometry (MS) often plays a large role in the identification of glycans.

Often, once the profile has been established, methods are transferred downstream which incorporate fluorescence detection. In many cases, there is a desire to obtain mass information for each detected peak even after characterization. These data have been difficult to obtain for a number of reasons, including a scarcity of mass spectrometers due to their cost and the requirement that MS specialized analysts are needed to generate meaningful and useful data.

In this application note, we present the combined use of *Rapi*Fluor-MS labeling reagent, ACQUITY UPLC H-Class Bio System, and serial fluorescence/ACQUITY QDa Mass Detector for the monitoring of released N-glycan profiles from IgGs. Overall, this new workflow allows scientists to rapidly prepare samples, from glycoprotein to analysis in 30 minutes.

In addition, *Rapi*Fluor-MS labeling yields unprecendented MS response,[1] which enables the use of the ACQUITY QDa for mass detection. We will discuss the improved sensitivity and charge state profile afforded by *Rapi*Fluor-MS, its general utility for fluorescence and mass detection, and the quality of ACQUITY QDa mass spectra obtained for a range of IgG glycan structures.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR and ACQUITY QDa Mass Detector
Column:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	2 µL

FLR settings

Data rate:	5 points/sec
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500–1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C
Ionization mode:	ESI+
Mobile phase A:	Acetonitrile (Pierce, LC/MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC/MS grade, Waters Ammonium Formate Concentrate)
Mobile phase C:	Acetonitrile (LC/MS grade)
Mobile phase D:	Acetonitrile (LC/MS grade)

Time	Flow rate (mL/min)	%A	%B	%C	%D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

SYNAPT® G2-S was used for assessment of *Rapi* Fluor-MS versus 2-AB N-glycan charge states. See Reference 1 for experimental details.

The *Rapi* Fluor-MS Glycan Performance Test Standard (p/n 186007983) was reconstituted in 25 µL of a mixture of DMF/acetonitrile/water at a ratio of 22.5%:55.5%:22%, respectively and used directly. For each analysis the injection volume was 2 µL, which corresponds to 32 pmol of released and labeled N-glycan on column. LC/MS-grade acetonitrile and water were purchased from Pierce. Ammonium formate was prepared using Waters Ammonium Formate Solution-Glycan Analysis (p/n 18600708) by pouring the entire contents of the solution into 1 L of water and mixed. The UPLC® System used was dedicated for applications which do not require non-volatile salts to reduce the likelihood of adduct formation in the mass detector.

RESULTS AND DISCUSSION

Addition of mass detection to an existing analytical workflow permits rapid and unambiguous identification of glycans. Historically, this has been a difficult task due to the need for high resolution instruments with appropriate sensitivity to obtain meaningful mass data. To overcome this issue, the novel labeling reagent, *Rapi* Fluor-MS, can been used. *Rapi* Fluor-MS dramatically increases both the MS sensitivity and charging of released N-glycans.

To demonstrate this, we compared the mass spectra of *Rapi* Fluor-MS labeled glycans to those of glycans labeled with a more traditional fluorescent label, 2-AB. This analysis was performed using time-of-flight mass spectrometry, which characteristically has a very wide mass range. The charge state characteristics of the different labeling technologies could thereby be objectively observed.

As shown in Figure 1, signal intensity improves dramatically when using *Rapi* Fluor-MS. Equally interesting is the shift in the charge states of the detected glycan ions that results from use of *Rapi* Fluor-MS labeling. As shown, *Rapi* Fluor-MS labeled FA2 near exclusively adopts an $[M+2H]^{2+}$ charge state, while more complex structures begin to adopt even higher $[M+3H]^{3+}$ charge states. In each case, at least one highly populated charge state falls well within the mass range of the ACQUITY QDa. Accordingly, *Rapi* Fluor-MS makes it feasible to use the cost effective, user-friendly ACQUITY QDa Mass Detector for N-glycan monitoring experiments.

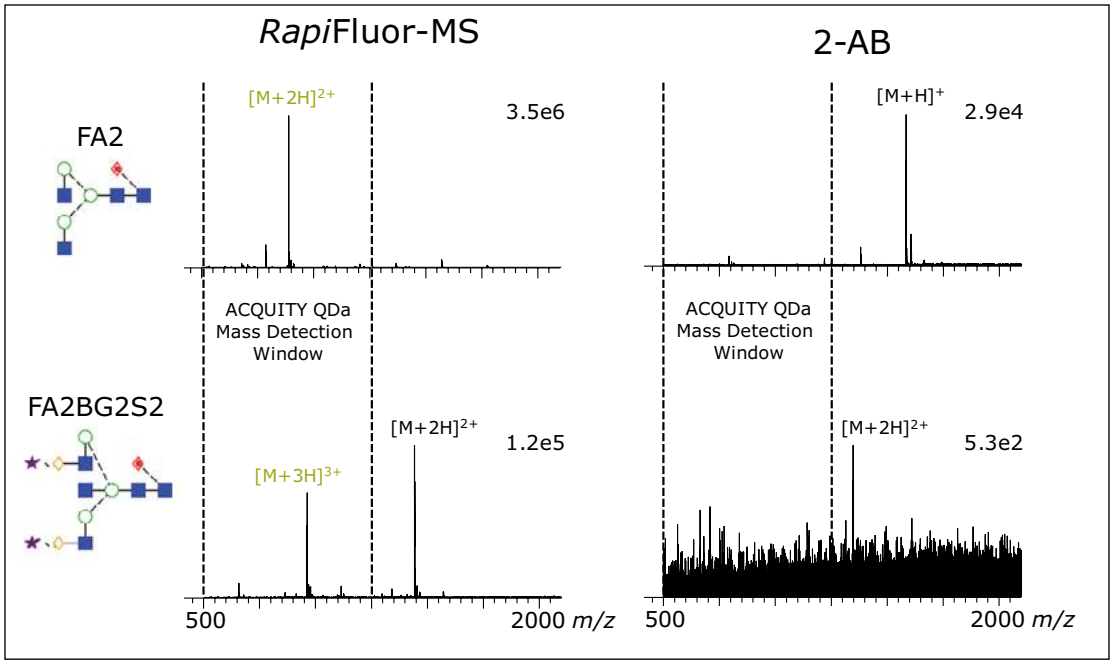

Figure 1. Charge States of RapiFluor-MS Labeled N-Glycans. Time-of-flight ESI+ mass spectra for two N-glycans labeled with RapiFluor-MS and 2-AB, respectively. The detected, protonated charge states that are within the ACQUITY QDa acquisition window are highlighted in green. The upper mass range of the ACQUITY QDa is indicated by the dashed line in each spectrum.

As discussed above, routine detection of N-glycans with the ACQUITY QDa is made possible by *Rapi* Fluor-MS labeling. Importantly, ACQUITY QDa mass detection can be paired with fluorescence detection to facilitate obtaining optical-based quantification along with corroborating data on peak homogeneity and mass information. To enable this data to be collected routinely, the design characteristics of the ACQUITY QDa are such that users without extensive mass spectrometry training are able to generate meaningful mass data easily.

To demonstrate this ability, we separated a sample of IgG released N-glycans labeled with *Rapi* Fluor-MS and monitored the eluting glycans with both FLR and ACQUITY QDa detectors. As shown in Figure 2, high quality data were obtained for both detector channels, with each species identified in the FLR also represented with ACQUITY QDa MS data such that peak assignments can be readily confirmed. Within Empower Software, it is possible to annotate peaks with component and mass information, which makes reviewing data simple, as exemplified in Figure 2.

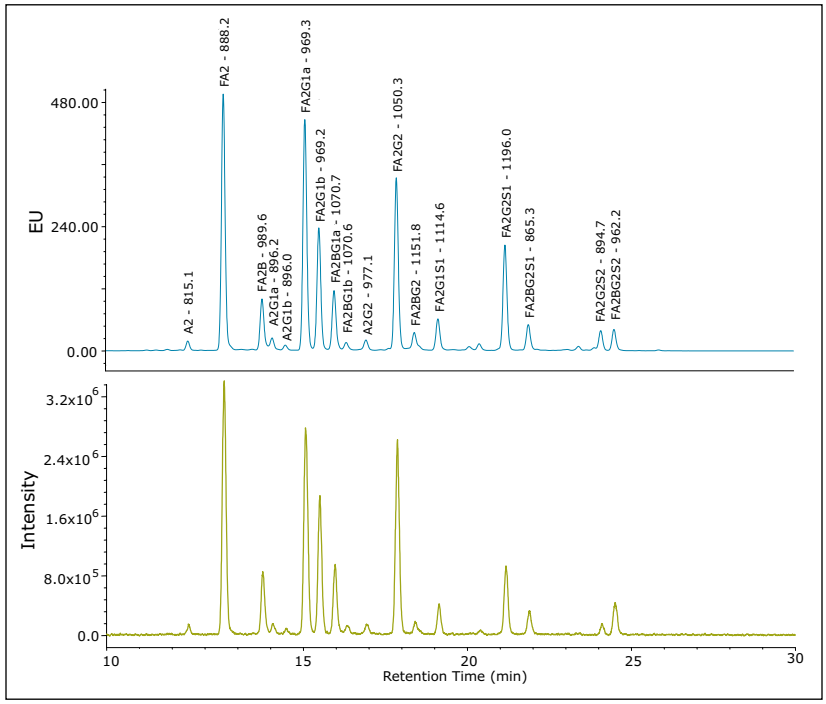

Figure 2. Fluorescence (top trace) and smoothed total ion chromatograms (bottom trace, 5 point mean smooth) of IgG glycans. Each peak is labeled with component name and base peak mass natively in Empower Data Management Software.

While the ability to detect N-glycan structures with the ACQUITY QDa is impressive, spectral quality is paramount for N-glycan monitoring, particularly when there is a need to interrogate the data in detail. We therefore reviewed the quality of MS data associated with peaks observed in the previously shown chromatograms. Figure 3 illustrates the spectra for each assigned peak in Figure 2. Notice that the ACQUITY QDa produced clean, easily interpretable mass spectra for the *Rapi*Fluor-MS labeled glycans, regardless of their relative abundance, molecular weight, or sialic acid content. Clearly, the ACQUITY QDa together with *Rapi*Fluor-MS can provide highly informative data that can be used to increase the confidence of assignments made during routine detection of N-glycans.

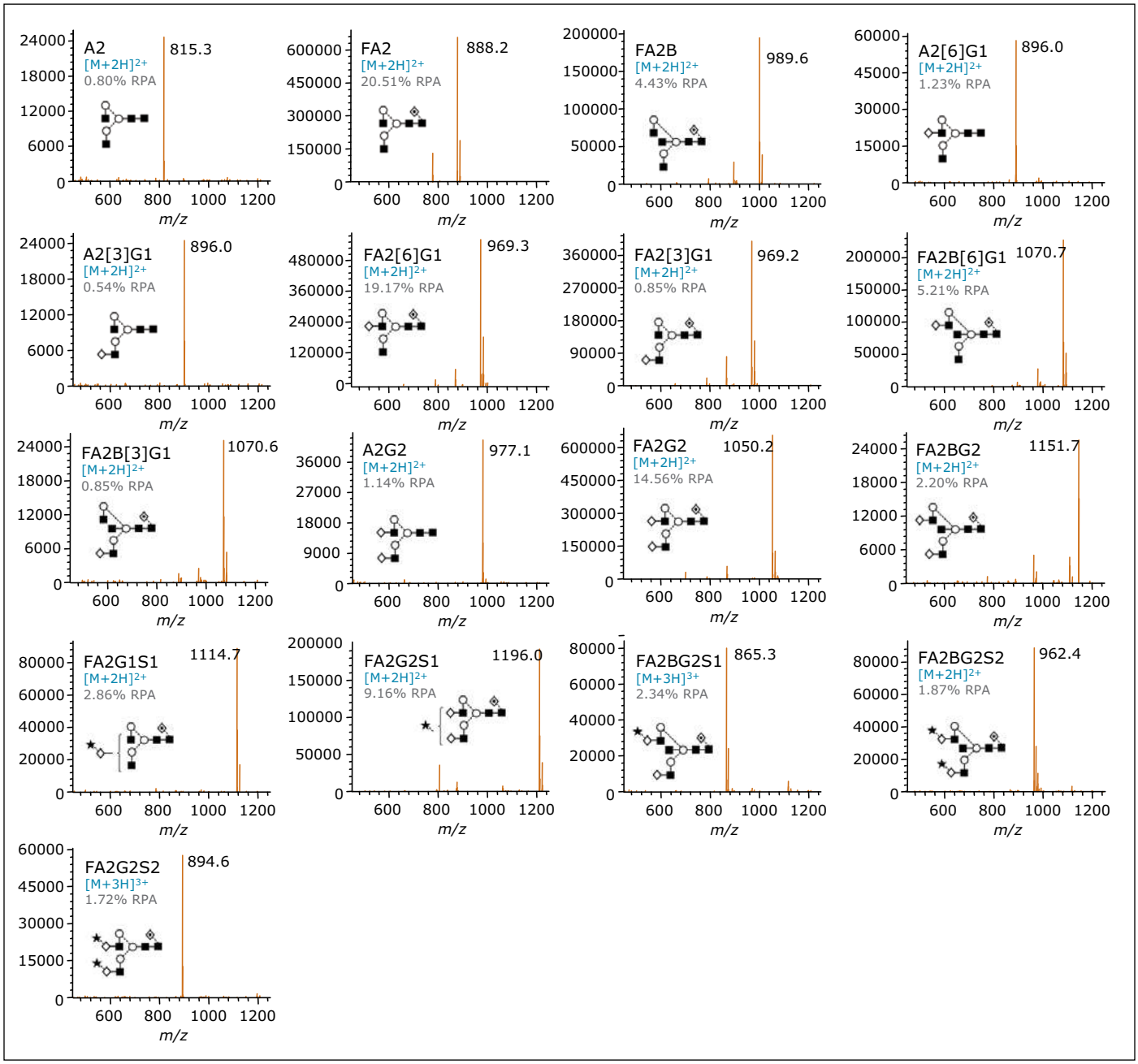

Figure 3. Combined spectra for each glycan structure identified in the chromatograms shown in Figure 2. Identified structures span from simple structures, such as A2, to more complex structures present in IgG samples, such as FA2G2S2. RPA = Relative Peak Area based on FLR integration.

CONCLUSIONS

Glycosylation is a complex and critical aspect of most therapeutic proteins that must be well characterized and monitored throughout product development and commercialization. As discussed in this application note, *Rapi*Fluor-MS can be used to dramatically reduce sample preparation times and complexity, to enhance FLR sensitivity, and to dramatically improve MS sensitivity. By improving glycan MS sensitivity, *Rapi*Fluor-MS labeling permits the use of mass detection with the ACQUITY QDa and thereby affords greater confidence in peak monitoring across the range of structures encountered during biopharmaceutical development.

Taken together, *Rapi*Fluor-MS labeling and HILIC-FLR-MS with the ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Mass Detector offer an unparalleled solution for monitoring the N-glycan profiles of biotherapeutics.

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Transferring *Rapi*Fluor-MS Labeled N-Glycan HILIC Separations Between UPLC and HPLC

Stephan M Koza, Matthew A Lauber, and Kenneth J Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Seamless scalability and transfer of *Rapi*Fluor-MS™ labeled glycan separations between UPLC and HPLC instrumentation
- ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm and XBridge® Glycan BEH Amide ***XP***, 130Å, 2.5 µm Columns provide high resolution UPLC and HPLC glycan separations

WATERS SOLUTIONS

ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Columns

XBridge® Glycan BEH Amide ***XP***, 130Å, 2.5 µm Columns

Alliance® HPLC System

ACQUITY UPLC H-Class Bio System

Empower® 3 and MassLynx® (v4.1) software

Waters *Rapi*Fluor-MS Glycan Performance Test Standard

KEY WORDS

HILIC Chromatography, UPLC, HPLC, method transfer, N-glycans, *Rapi*Fluor-MS

INTRODUCTION

In 2009, Waters introduced a revolutionary UPLC® HILIC Column designed specifically for achieving high resolution glycan separations. This column technology was based on stationary phase constructed from 1.7 µm diameter, 130Å pore size, ethylene bridged hybrid (BEH) particles with an optimized amide ligand bonding that has exhibited exceptional resolution for a broad range of N-glycans ranging from small neutral structures to highly sialylated extended structures.[1] In addition to this UPLC-based column, Waters has also introduced HPLC-based XBridge Glycan BEH Amide columns based on 2.5 µm particles and has demonstrated that these columns provide selectivity for 2-AB labeled N-glycans comparable to that observed in UPLC separations.[2] Most recently, Waters has introduced a novel labeling reagent, *Rapi*Fluor-MS, that provides both a fast and efficient sample preparation workflow and unsurpassed fluorescent and MS sensitivity.[3]

In the following work, we demonstrate that Glycan BEH Amide Columns packed with 1.7 µm and 2.5 µm particle sizes afford scalability between *Rapi*Fluor-MS labeled glycan separations performed under UPLC and HPLC-compatible conditions. Using standard LC method transfer principles to account for differences in particle diameter (dp), column length, and column internal diameter, we show that comparable chromatographic profiles and relative quantitation can be achieved with the larger particle size column at HPLC-compatible pressures, albeit with an increase in sample load, mobile phase use, and most importantly, analysis time.

EXPERIMENTAL

Method conditions

LC system:	Waters Alliance HPLC or ACQUITY UPLC H-Class Bio System
Detection:	Alliance HPLC 2475 Fluorescence (FLR) Detector ACQUITY UPLC FLR Detector with Analytical flow cell Wavelength: 265 nm excitation, 425 nm emission
Columns:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm, (p/n 186004742) XBridge Glycan BEH Amide ***XP***, 130Å, 2.5 µm, 3.0 x 150 mm (p/n 186008039) and 3.0 mm x 75 mm (p/n 186008038) in series. Columns connected by 0.005 x 1.75 UPLC SEC Connection Tubing (p/n 186006613)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	1.2 µL UPLC, 3.7 µL HPLC
Mobile phase A:	50 mM ammonium formate, pH 4.4 (LC/MS-grade water, from a 100X concentrate (p/n 186007081)
Mobile phase B:	LC/MS-grade acetonitrile
Gradients:	

Time UPLC/HPLC (minutes)	Flow Rate UPLC/HPLC (mL/minute)	% A (50 mM amm. formate, pH 4.4)	%B (Acetonitrile)
0.0/0.0	0.40/0.56	25	75
35.0/77.2	0.40/0.56	46	54
36.5/80.5	0.20/0.28	100	0
39.5/87.1	0.20/0.28	100	0
43.1/95.1	0.20/0.28	25	75
47.6/105.0	0.40/0.56	25	75
55.0/121.3	0.40/0.56	25	75

Table 1.

Sample vials:	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL (p/n 186002640)
Data management:	MassLynx (v4.1) UPLC, Empower Pro (v3) HPLC

Sample description

The Waters *Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983 400 pmole/vial) was diluted in water to a concentration of 20 pmole/µL.

RESULTS AND DISCUSSION

Calculating the transfer of glycan HILIC methods between UPLC and HPLC columns

There are two primary considerations to be made when transferring a HILIC-based N-glycan separation method from one LC system and column to another. Most importantly, the surface chemistry and pore size of the particles in the two columns must be comparable. Once appropriate columns have been chosen, the separation must then be appropriately scaled with respect to particle size. Generally this can be accomplished by maintaining a comparable ratio between the length of the column and the size of the particle, L/dp. Once determined, alterations to the gradient can be calculated. In this example, the transfer between a 1.7 µm particle size, 2.1 x 150 mm Glycan BEH Amide column to an XBridge Glycan BEH Amide column with a 2.5 µm particle size required a column approximately 50% greater in length (225 mm) since the ratio of the particle sizes is 1.47 (i.e. 2.5/1.7). In practice, a 225 mm length can be easily constructed by combining 150 mm and 75 mm length columns with a suitable column connector. In addition to column length, it is also important to consider the optimal column I.D.. HPLC systems invariably exhibit higher dispersion than UPLC systems (bandspread ~30 µL versus ~10 µL), so it is advisable to perform separations with relatively larger I.D. columns to ensure that the effect of extra-column band broadening is minimized. With a 3.0 mm HPLC column I.D. format, near optimal resolution separations can be achieved on an HPLC system, without the high mobile phase consumption rates typical of 4.6 mm I.D. column formats.

Having selected a 3.0 x 225 mm effective column dimension, we next calculated the appropriate gradient for the HPLC separation using general method transfer principals (refer back to Table 1 for the gradient).[4] Table 2 outlines column lengths, analysis times, and mobile phase plus sample consumption corresponding to the use of various scaled methods and potential Glycan BEH Amide column configurations. Clearly, this exercise highlights two of the significant advantages that UPLC separations provide: shorter analysis times (≥55% decrease) and decreased mobile phase usage (≥68% decrease). The UPLC separation also benefits from lower required sample loads (≥68% decrease), which can prove useful in cases where an analyst is sample limited. For these comparisons, mobile phase use was determined based on the gradient shown in Table 1. Based on these calculated results, the advantages of the UPLC format is evident as is the use of the XBridge 2.5 µm particle size, 3.0 mm I.D. columns on an low band spread (29 µL) HPLC.

Particle Size (µm)	Column Length (mm)	Column I.D. (mm)	Flow Rate (mL/min.)	Run Time (min.)	Mobile Phase (mL)	Sample (µL)
1.7	150	2.1	0.4	55	20	1.2
2.5	225	3.0	0.56	121.3	62	3.7
2.5	225	4.6	1.32	121.3	146	8.8
3.5	300	4.6	0.93	229.2	194	11.6

Table 1.

Comparing UPLC and HPLC *Rapi*Fluor-MS labeled N-glycan profiles

The effectiveness of scaling from a 2.1 x 150 mm, 1.7 µm particle size, Glycan BEH Amide column using an ACQUITY H-Class UPLC System to a total 225 cm length (150 mm + 75 mm) 2.5 µm particle size, 3.0 mm I.D., XBridge Column run on an Alliance HPLC System is demonstrated qualitatively in Figure 1. Both pairs of chromatograms show comparable profiles over normalized time ranges for the *Rapi*Fluor-MS Glycan Performance Test Standard, which represents the N-glycans released from a pooled human IgG sample. In this example, the analysis time difference is approximately 2.2-fold.

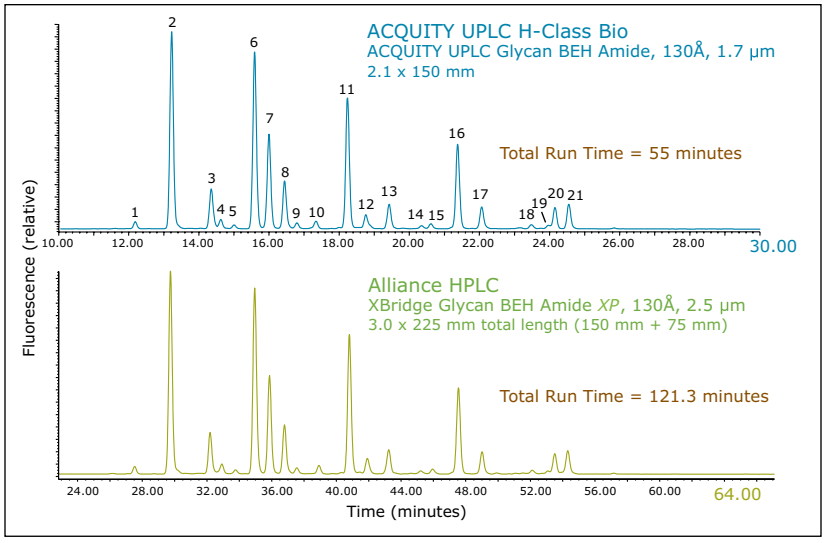

Figure 1. Comparison of UPLC and HPLC HILIC separations of RapiFluor-MS labeled N-glycans from the Waters Glycan Performance Test Standard (p/n 186007983, 400 pmole/vial) diluted in water to a concentration of 20 pmole/µL. Injection volumes of 1.2 and 3.7 µL for the UPLC and HPLC analyses.

These separations were also compared quantitatively. Shown in Figure 2 is a comparison of the relative retention times for 21 of the most abundant N-linked oligosaccharides observed. Relative retention times were calculated off of Peak 1 (Figure 1) and corrected for the increased system and column dwell volumes of the HPLC separation (~1.2 minutes). Figure 3 illustrates the general comparability of the two separations with respect to the relative quantitation for the same 21 peaks evaluated for retention time. The majority of these values are well within 5% of each other with the most significant difference (~35%) being observed for Peak 19, which has a relative abundance of ~0.2% as determined by the HPLC analysis. If more precise quantitation of these low abundance species is required it would be advantageous to report these results relative to a reference material. Overall, these data demonstrate that the HILIC-based separation of *Rapi*Fluor-MS labeled glycans can be readily transferred between UPLC and HPLC formats. The comparability of the observed chromatographic profiles underscores the chemical comparability of the particle surfaces, as well as the comparability in pore characteristics.

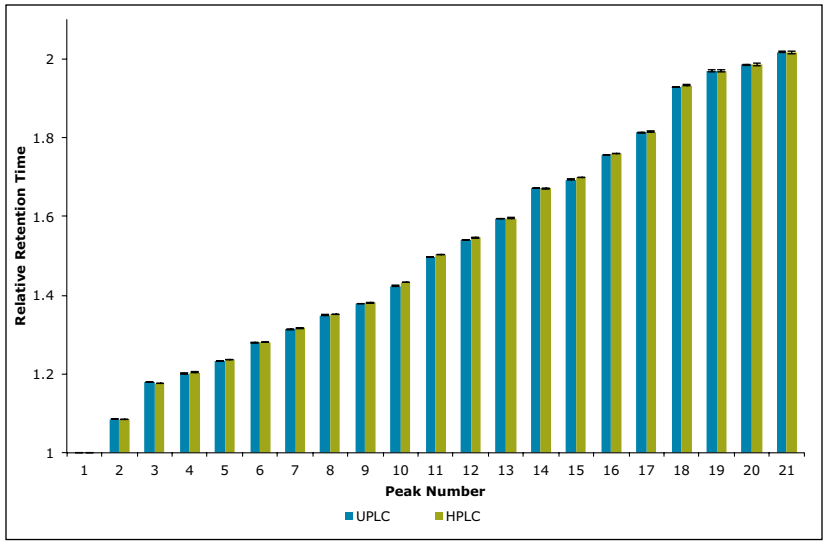

Figure 2. Comparison of UPLC and HPLC HILIC relative retention times (n=2) of RapiFluor-MS labeled N-glycans. Peak numbers are as labeled in Figure 1. Relative retention times were determined based on Peak 1.

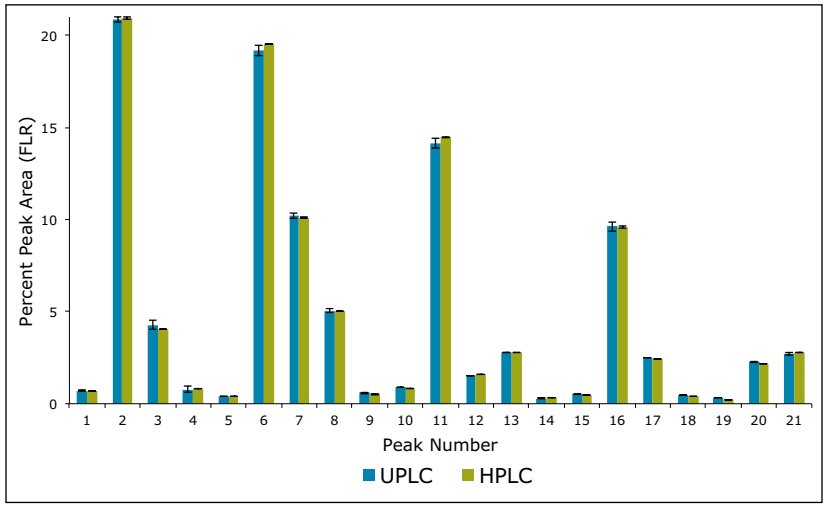

Figure 3. Comparison of UPLC and HPLC HILIC relative peak areas (n=2) of RapiFluor-MS labeled N-glycans. Peak numbers are as labeled in Figure 1.

CONCLUSIONS

These results demonstrate that a HILIC separation of *Rapi*Fluor-MS labeled N-linked oligosaccharides can be seamlessly transferred between UPLC and HPLC platforms when using the appropriate Glycan BEH Amide Columns. The advantage in using the UPLC-based separation is the capability to dramatically improve sample throughput while decreasing mobile phase use. Sample load requirements are also lowered. However, in the event that a laboratory encounters instrumentation limitations, it is beneficial to be able to easily transfer between UPLC and HPLC separations. Additionally, scaling from a UPLC to an HPLC platform can be useful if glycans must be fractionated and purified for structural analysis or to generate materials for method validation spiking studies.

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Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent

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[1] Waters Corporation, Milford, MA, USA
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APPLICATION BENEFITS

- Preparation of labeled *N*-glycans (from glycoprotein to analysis ready sample) in 30 minutes
- Complete deglycosylation to produce unbiased results
- Simple, streamlined protocol provided with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit
- Unprecedented sensitivity for labeled *N*-glycans with at least 2 and 100 fold increases to fluorescence and MS detection, respectively
- Accurate profiling based on robust SPE for neutral to tetrasialylated *N*-glycans

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GlycoWorks™ *Rapi*Fluor-MS™ *N*-Glycan Kit

GlycoWorks HILIC µElution Plate

ACQUITY UPLC® Glycan BEH Amide 130Å Column

ACQUITY UPLC H-Class Bio System

ACQUITY® QDa® Mass Detector

Xevo® G2-XS QTof MS

SYNAPT® G2-S HDMS

KEY WORDS

GlycoWorks, *Rapi*Fluor-MS, *Rapi*Gest™ SF, Rapid Tagging, PNGase F, Deglycosylation, ACQUITY UPLC H-Class Bio System, BEH Amide 130Å, Glycans, Glycoproteins, Glycosylation, HILIC, Fluorescence

INTRODUCTION

The *N*-glycan profile of a biopharmaceutical is commonly defined as a critical quality attribute, since it can be a measure of efficacy, safety, and manufacturing conditions.[1-2] Therefore, it is important that approaches for the glycan analysis of clinical and commercial biotherapeutic formulations exhibit high sensitivity and facilitate detailed characterization. Additionally, it would be highly advantageous if such an analysis could also be performed with rapid turnaround times and high throughput capacity to expedite product development. Most analytical strategies for evaluating *N*-glycans from glycoproteins involve deglycosylation via PNGase F and the labeling of the resulting *N*-glycans with a chemical moiety that imparts a detectable attribute. In one, highly effective approach, labeled glycans are separated by hydrophilic interaction chromatography (HILIC) and detected by fluorescence (FLR) and sometimes mass spectrometry (MS).[3-10]

Unfortunately, conventional approaches to the preparation of *N*-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity.[11] For instance, a conventional deglycosylation procedure requires that a glycoprotein sample be incubated for about 1 hour, while many analysts generically employ an overnight (16 hour) incubation. Combined with this process is a lengthy, 2 to 3 hour labeling step that relies on reductive amination of reducing, aldehyde termini that form on *N*-glycans only after they hydrolyze from their glycosylamine forms. And in the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans can be readily detected by fluorescence but are rather challenging to detect by electrospray ionization mass spectrometry (ESI-MS).

Variations to conventional approaches for *N*-glycan sample preparation have been explored, but have not, as of yet, presented a solution that combines the desired attributes of simplicity, high MS sensitivity, and high throughput. Alternative labeling reagents, for example procainamide, that have functional groups to enhance electrospray ionization efficiency have been used,[12] but this does not address the cumbersome, time consuming nature of relying on a reductive amination labeling step. Rapid tagging procedures that yield labeled glycans in a matter of minutes have consequently been investigated. In fact, two rapid tagging glycan labels were recently introduced, including a rapid tagging analog

EXPERIMENTAL

Method conditions (unless otherwise noted):

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	5 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Fluorescence detection:	Ex 265/Em 425 nm (*Rapi*Fluor-MS) Ex 278/Em 344 nm (Instant AB) Ex 330/Em 420 nm (2-AB) (2 Hz scan rate [150 mm column]/5 Hz scan rate [50 mm column], Gain =1)
Injection volume:	≤1 µL (aqueous diluents with 2.1 mm I.D. columns) ≤30 µL (DMF/ACN diluted samples with 2.1 mm I.D. columns)
Columns:	ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740) ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Sample collection/ vials:	Sample Collection Module (p/n 186007988) Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL volume (p/n 186002640)

Gradient used with 2.1 x 50 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

Gradient used with 2.1 x 150 mm columns:

Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

Mobile phase B: ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

MS conditions

MS system:	SYNAPT G2-S HDMS
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–2500 *m/z*
Lockspray (ASM B-side):	100 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds
Acquisition:	500–2500 *m/z*, 1 Hz scan rate
Data management:	MassLynx Software (V4.1)

of aminobenzamide (AB).[13] In a rapid reaction, the precursor glycosylamines of reducing, aldehyde terminated glycans are modified via a urea linked aminobenzamide. Although such a rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced ionization efficiencies needed in modern *N*-glycan analyses.

To address the above shortcomings, we have developed a sample preparation solution that enables unprecedented FLR and MS sensitivity for glycan detection while also improving the throughput of *N*-glycan sample preparation. A novel labeling reagent has been synthesized that rapidly reacts with glycosylamines upon their release from glycoproteins. Within a 5 minute reaction, *N*-glycans are labeled with *Rapi*Fluor-MS, a reagent comprised of an *N*-hydroxysuccinimide (NHS) carbamate rapid tagging group, an efficient quinoline fluorophore, and a highly basic tertiary amine for enhancing ionization. To further accelerate the preparation of *N*-glycans, rapid tagging has been directly integrated with a Rapid PNGase F deglycosylation procedure involving *Rapi*Gest SF surfactant and a HILIC µElution SPE clean-up step that provides highly quantitative recovery of the released and labeled glycans with the added benefit of not requiring a solvent dry-down step prior to the LC-FLR-MS analysis of samples.

SAMPLE DESCRIPTION

N-glycans from Intact mAb Mass Check Standard (p/n 186006552), bovine fetuin (Sigma F3004), and pooled human IgG (Sigma I4506) were prepared according to the guidelines provided in the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit Care and Use Manual (715004793).

To compare the response factors of Instant AB™ and *Rapi*Fluor-MS labeled glycans, labeling reactions were performed with equivalent molar excesses of reagent, and crude reaction mixtures were directly analyzed by HILIC-FLR-MS in order to avoid potential biases from SPE clean-up procedures. Response factors were determined as ratios of the FA2 *N*-glycan (Oxford notation) chromatographic peak area to the mass of glycoprotein from which the glycan originated.

To compare the response factors of 2-AB labeled versus *Rapi*Fluor-MS labeled glycans, equivalent quantities of labeled *N*-glycans from pooled human IgG were analyzed by HILIC-FLR-MS. Column loads were calibrated using external quantitative standards of 2-AB labeled triacetyl chitotriose and *Rapi*Fluor-MS derivatized propylamine (obtained in high purity; confirmed by HPLC and 1H NMR). Response factors were determined as ratios of the FA2 chromatographic peak area to the mole quantity of glycan.

The procedure for extracting labeled *Rapi*Fluor-MS glycans after derivatization was evaluated using a test mixture containing *N*-glycans released and labeled from a 1:1 mixture (by weight) of pooled human IgG and bovine fetuin. The test mixture was prepared and then reconstituted in a solution equivalent in

composition to the solution glycans are subjected to when following the protocol of the *Rapi*Fluor-MS *N*-Glycan Kit. All other sample preparation techniques are described in the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit Care and Use Manual (715004793).

RESULTS AND DISCUSSION

Rational design of a new *N*-Glycan labeling reagent

A new labeling reagent for facilitating *N*-glycan analysis has been synthesized based on rational design considerations (Figure 1) that would afford rapid labeling kinetics, high fluorescence quantum yield, and significantly enhanced MS detectability. Conventional *N*-glycan sample preparation is dependent on reductive amination of aldehyde terminated saccharides, a process that requires glycans to undergo multiple chemical conversions and a lengthy high temperature incubation step.[11] Moreover, glycans must be reductively aminated in anhydrous conditions in order to minimize desialylation. Sample preparations are therefore burdened with transitioning a sample from aqueous to anhydrous conditions. For these reasons, the newly designed labeling reagent foregoes reductive amination and instead takes advantage of an aqueous rapid tagging reaction. To this end, Waters has drawn upon its experience with rapid fluorescence labeling of amino acids to develop a new reagent that meets the needs of modern, *N*-glycan analysis. More than 20 years ago, Waters introduced a rapid tagging labeling reagent, known as AccQ•Fluor™, that is now widely use to accurately profile the amino acid composition of protein samples via fluorescence detection.[14-15]

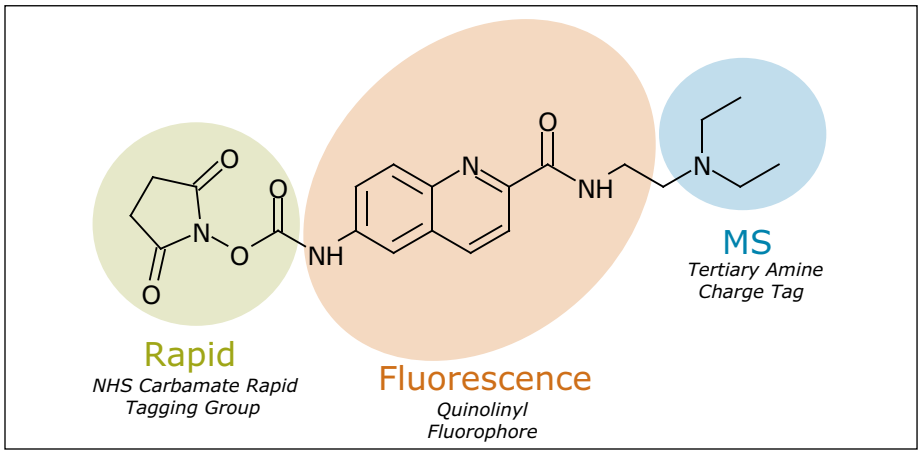


Figure 1. RapiFluor-MS Molecular Structure. Features of the chemical structure that enable rapid tagging, efficient fluorescence, and enhanced ionization efficiency are highlighted.

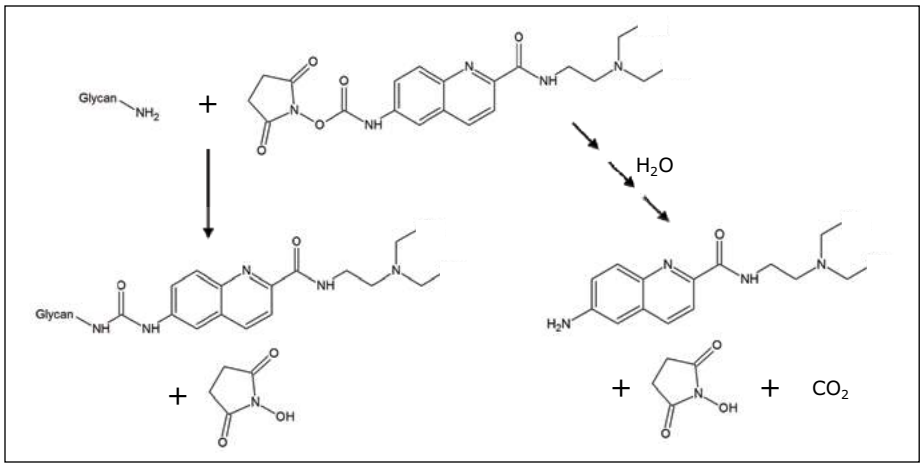


Figure 2. Reaction Schematic for RapiFluor-MS Derivatization of an N-glycosylamine. The pathway on the left shows the derivatization of a glycosylamine, which produces an N-glycan with a urea (NH-CO-NH) linked RapiFluor-MS label. Hydrolysis of RapiFluor-MS is shown in the pathway on the right.

AccQ•Fluor possesses two important chemical characteristics: an NHS-carbamate rapid tagging reactive group and a highly efficient quinolinyl fluorophore. These features of AccQ•Fluor form the basis of the new glycan labeling reagent. The NHS-carbamate reactive group of this reagent enables glycosylamine bearing *N*-glycans to be rapidly labeled following their enzymatic release from glycoproteins. Within a 5 minute reaction, *N*-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (Figure 2). In addition to rapid tagging capabilities, the new labeling reagent also supports high sensitivity for both MS and flourescence detection. A quinoline fluorophore serves as the central functionality of the new reagent that, as with AccQ•Fluor, facilitates high sensitivity fluorescence detection. In addition to AccQ•Fluor, however, the new reagent has been synthesized with a tertiary amine side chain as a means to enhance MS signal upon positive ion mode electrospray ionization (ESI+). In summary, the resulting *N*-glycan labeling reagent is built upon our expertise in chemical reagents and three important chemical attributes, a rapid tagging reactive group, an efficient fluorophore, and a highly basic MS active group. To describe these noteworthy attributes, the new labeling reagent has accordingly been named *Rapi*Fluor-MS.

*Rapi*Fluor-MS enables high sensitivity detection

*Rapi*Fluor-MS *N*-glycan labeling has been extensively studied. In particular, the response factors of *Rapi*Fluor-MS labeled glycans have been benchmarked against those observed for glycans labeled with alternative reagents. The most closely related, commercially available alternative to *Rapi*Fluor-MS is an NHS carbamate analog of aminobenzamide, known as Instant AB.[13] Figures 3A and 3B present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of *N*-glycans released from a murine monoclonal antibody (Intact mAb Mass Check Standard, p/n 186006552) and labeled with *Rapi*Fluor-MS and Instant AB, respectively. Based on the observed chromatographic peak areas, response factors for fluorescence and MS detection were determined for the most abundant glycan in the IgG profile, the fucosylated, biantennary FA2 glycan (Figure 3C). Our results for the FA2 glycan indicate that *Rapi*Fluor-MS labeled glycans produce 2 times higher fluorescence signal and, more astoundingly, 780 times greater MS signal than *N*-glycans labeled with Instant AB.

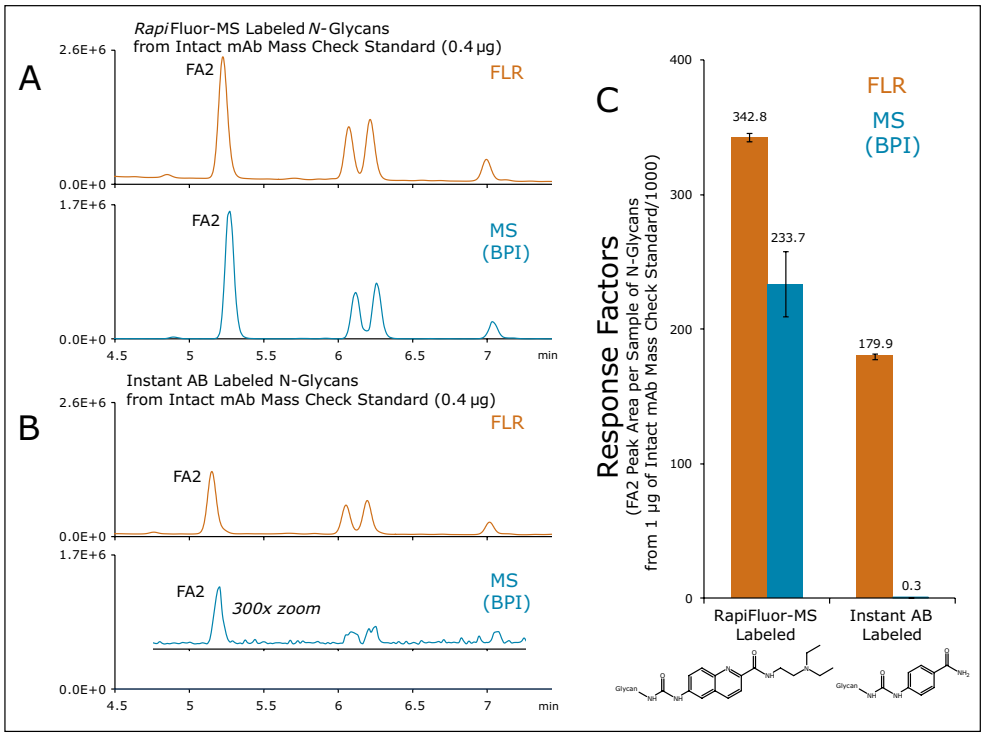


Figure 3. HILIC-FLR-MS of (A) RapiFluor-MS and (B) Instant AB Labeled N-Glycans from Intact mAb Mass Check Standard. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (from 0.4 µg of glycoprotein, 1 µL aqueous inection) were separated using a ACQUITY UPLC BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column. (C) Response factors for RapiFluor-MS and Instant AB labeled glycans (measured as the FA2 peak area per sample of N-glycans resulting from 1 µg of Intact mAb Mass Check Standard). Fluorescence (FLR) and MS (base peak intensity) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

In a similar fashion, *Rapi*Fluor-MS labeling has also been compared to conventional 2-AB labeling. To draw such a comparison, *N*-glycans released from pooled human IgG and labeled with either *Rapi*Fluor-MS or 2-AB were analyzed by HILIC-FLR-MS at equivalent mass loads (Figures 4A and 4B, respectively). Given that rapid tagging and reductive amination are performed by significantly different procedures, external calibrations were established using quantitative standards in order to determine the amounts of FA2 glycan loaded and eluted from the HILIC column. Response factors determined using these calibrated amounts of FA2 glycan are provided in Figure 4C. Again, it is found that *Rapi*Fluor-MS labeled glycans are detected with significantly higher signal (14 times higher fluorescence and 160 times greater MS signal versus 2-AB labeled glycans.)

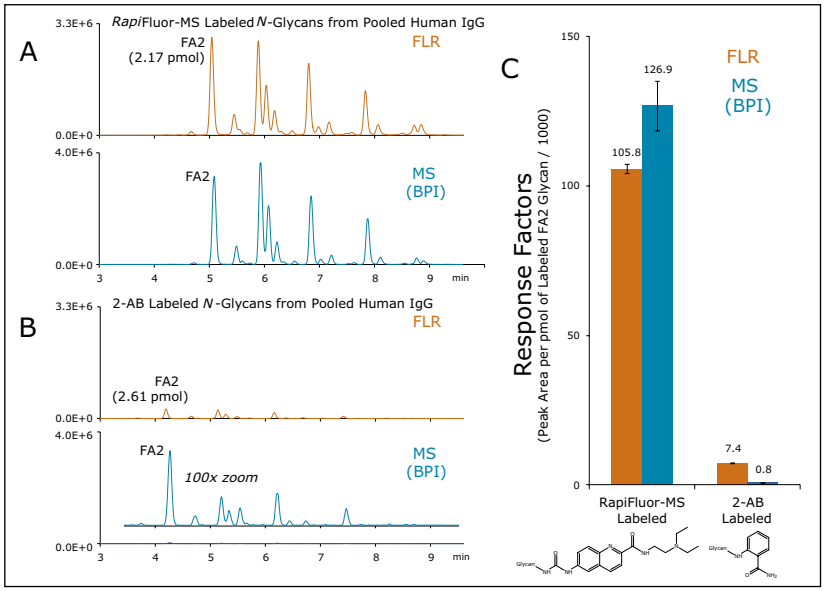


Figure 4. HILIC-FLR-MS of (A) RapiFluor-MS and (B) 2-AB Labeled N-Glycans from Pooled Human IgG. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. Labeled glycans (~14 pmol total glycan, 1 µL aqueous injection) were separated using a ACQUITY UPLC Glycan BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column. The quantities of FA2 glycan were calibrated via two-point external calibrations with quantitative standards. (C) Response factors for RapiFluor-MS and 2-AB labeled glycans (measured as the FA2 peak area per picomole of FA2 determined by the external calibration). Fluorescence (FLR) and MS (BPI) response factors are shown in orange and blue, respectively. Analyses were performed in duplicate.

To summarize the above observations, we have plotted the response factors of Instant AB and 2-AB as percentages of the response factors of *Rapi*Fluor-MS (Figure 5). The gains in fluorescence and MS sensitivity are apparent in this plot, since it portrays response factors for Instant AB and 2-AB normalized to those for *Rapi*Fluor-MS. In this plot, the relative performance of reductive amination with another alternative labeling reagent, procainamide, is also provided. Procainamide is a chemical analog to aminobenzamide that has recently been exploited to enhance the ionization of reductively aminated glycans when they are analyzed by HILIC-ESI(+)-MS. Previous studies have shown that procainamide labeled glycans yield comparable fluorescence signal and up to 50 times greater MS signal when compared to 2-AB labeled glycans.[12,16] Compared to procainamide, *Rapi*Fluor-MS will therefore provide sizeable gains in MS sensitivity. That is to say, the novel *Rapi*Fluor-MS labeling reagent not only supports rapid tagging of glycans but it also provides analysts with unmatched sensitivity for fluorescence and MS based detection.

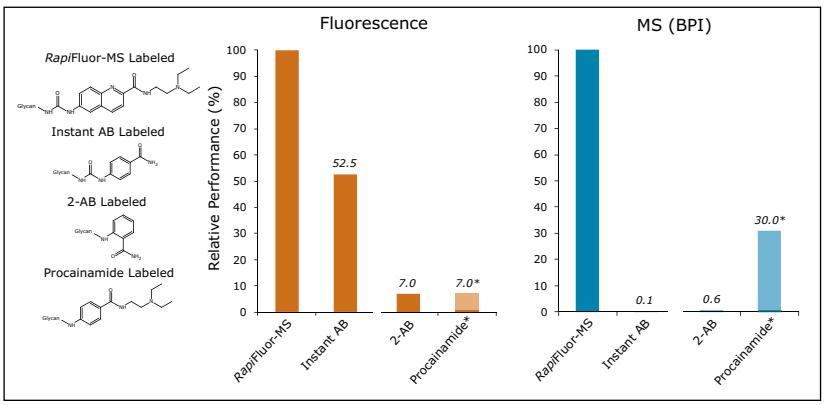


Figure 5. Relative Performance of Glycan Labels. Response factors normalized to the fluorescence and MS response factors of RapiFluor-MS labeled N-glycans. (*) Comparative result extrapolated from a published comparison of N-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50 fold greater ESI-MS sensitivity when compared to 2-AB (Klapoetke et al. 2010).

Rapid deglycosylation with a novel formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant

*Rapi*Fluor-MS labeling revolutionizes *N*-glycan sample preparation and can be readily adopted in the laboratory with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit. This complete solution from Waters and New England BioLabs was purposefully designed to remove the bottlenecks from all aspects of *N*-glycan sample preparation. The optimized *N*-glycan sample preparation workflow requires a minimum of three steps, including deglycosylation (to release glycans from a glycoprotein), labeling (to impart a detectable chemical entity to glycans), and a clean-up step (to remove potential interferences from the sample) (Figure 6). Conventional approaches to *N*-glycan sample preparation can be very time consuming due to not only lengthy labeling procedures but also lengthy deglycosylation steps that range from 1 to 16 hours. To ensure rapid labeling with *Rapi*Fluor-MS was not encumbered by a time-consuming deglycosylation process, Waters partnered with New England BioLabs to co-develop a Rapid PNGase F deglycosylation procedure specifically designed for integration with rapid tagging labeling reagents.

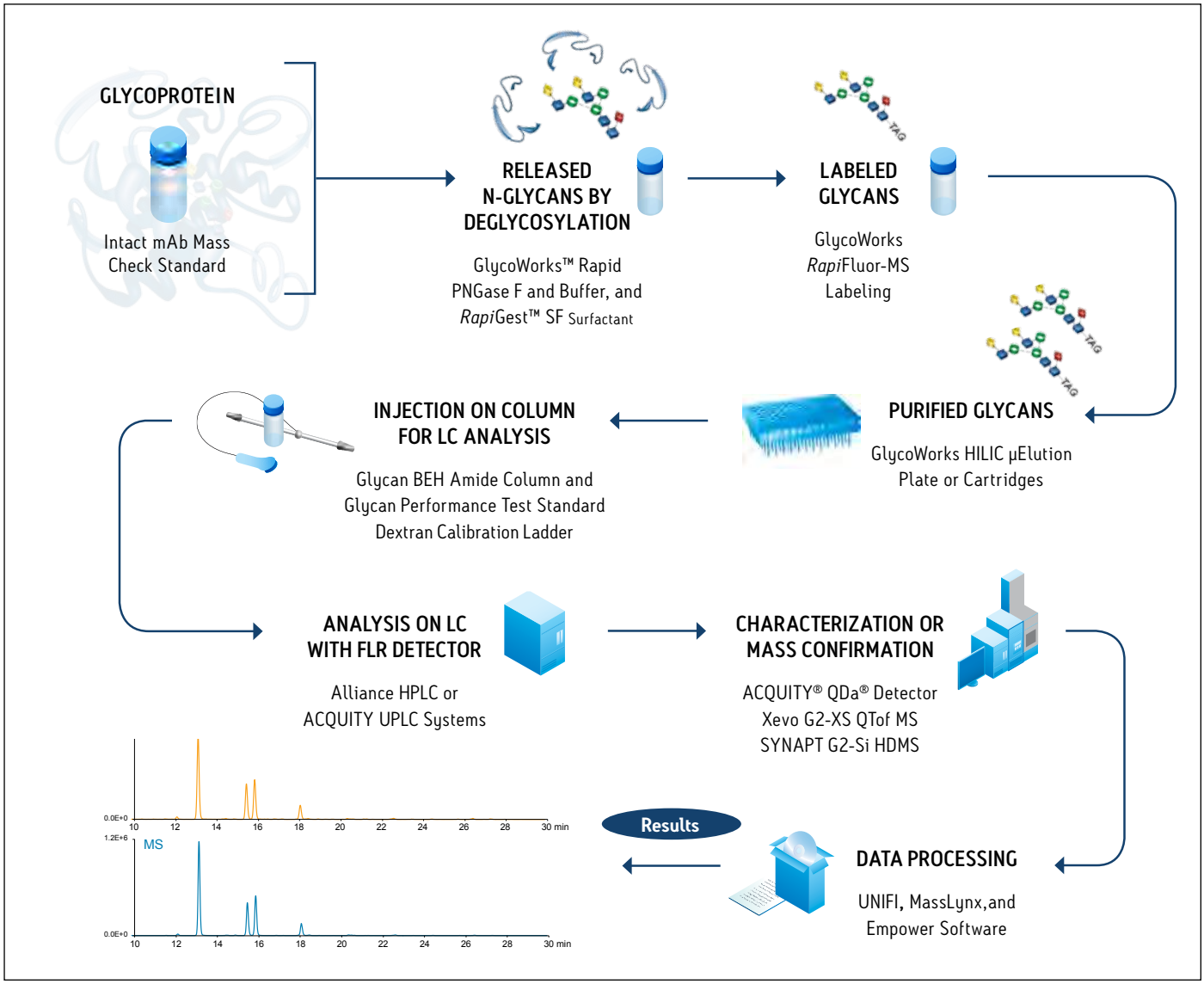
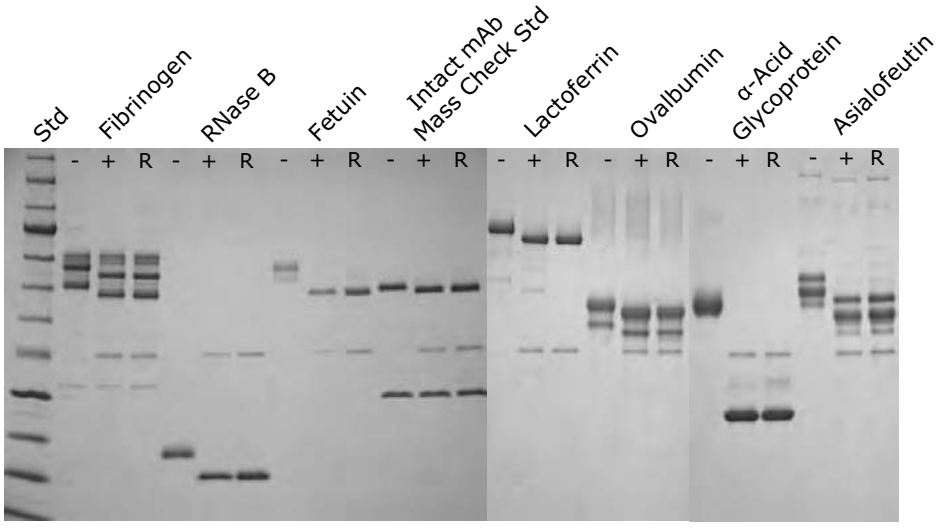


Figure 6. Workflow for the Rapid Preparation of N-glycans Using the RapiFluor-MS N-Glycan Kit.

The GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit provides a novel formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant that can be used to completely deglycosylate a diverse set of glycoproteins in an approximately 10 minute procedure. This fast deglycosylation procedure is facilitated by the use of *Rapi*Gest, an anionic surfactant, that is used to ensure that *N*-glycans are accessible to Rapid PNGase F and that glycoproteins remain soluble upon heat denaturation. Most importantly, *Rapi*Gest is an enzyme-friendly reagent and can therefore be used at high concentrations without hindering the activity of Rapid PNGase F. In the developed fast deglycosylation technique, a glycoprotein is subjected to a high concentration of *Rapi*Gest (1%) and heated to ≥80°C for 2 minutes. Subsequently and without any additional sample handling, Rapid PNGase F is added to the solution and the mixture is incubated at an elevated, 50°C temperature for 5 minutes to achieve complete, unbiased deglycosylation.

The effectiveness of this rapid deglycosylation process has been evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is an effective technique for separating proteins based on their size in solution and can often be used to separate the glycosylated and de-glycosylated forms of proteins.[17-18] A diverse set of glycoproteins were deglycosylated according to the rapid deglycosylation procedure and analyzed by SDS-PAGE along with negative controls containing no PNGase F and positive controls, in which the glycoproteins were subjected to conventional multiple step deglycosylation with SDS based denaturation and PNGase F incubation for 30 minutes at 37 °C. Figure 7 shows the results of this study, where it can be seen that for each of the tested proteins there is a significant decrease in protein apparent molecular weight after they are subjected to the rapid deglycosylation procedure. Moreover, the apparent molecular weight decreases are visually comparable to those observed for proteins deglycosylated by the control method. These results demonstrate that the fast deglycosylation approach facilitated by a unique formulation of Rapid PNGase F and *Rapi*Gest SF Surfactant produces deglycosylation comparable to a conventional approach but in only a fraction of the time required.



(-) Neg Control / Conventional / 2 Steps without PNGase F: SDS + DTT, 95°C, 2 min / NP-40 + Reaction Buffer, 37°C, 30 min
(+) Pos Control / Conventional / 2 Steps with PNGase F: SDS + DTT, 95°C, 2 min / NP-40 + Reaction Buffer + PNGase F, 37°C, 30 min
(R) 2 Step Rapid Deglycosylation / Reaction Buffer + RapiGest ≥80°C, 2 min / + PNGase F, 50°C, 5 min

PNGase F inactivated after the deglycosylation step via heat denaturation at 70°C for 20 min

Figure 7. Gel Electrophoresis Assay for Deglycosylation of Glycoproteins. A negative control (-) shows the migration distance and apparent molecular weight of the native glycoproteins, and a positive control (+) shows the migration distance and decreased apparent molecular weight of deglycosylated proteins as obtained by conventional two step deglycosylation using SDS denaturation and a subsequent 30 minute incubation with PNGase F at 37 °C. Results demonstrating the complete deglycosylation of these glycoproteins with a fast procedure involving a two step approach with RapiGest-assisted heat denaturation and a subsequent 5 minute incubation with Glycoworks Rapid PNGase F at 50°C are also shown (R). Coomassie blue staining was used for band visualization.

Robust, quantitative HILIC SPE

As mentioned earlier, the final step in an *N*-glycan sample preparation aims to extract the labeled glycans in preparation for their analysis. An effective approach for extraction of labeled glycans from reaction byproducts has been devised using solid phase extraction (SPE). In particular, this SPE is designed to selectively extract *Rapi*Fluor-MS labeled *N*-glycans from a mixture comprised of deglycosylated protein, PNGase F, buffer/formulation components, *Rapi*Gest Surfactant, and labeling reaction byproducts, which otherwise interfere with analysis of the labeled glycans by HILIC column chromatography (Figure 8). For the *Rapi*Fluor-MS *N*-Glycans kit, a GlycoWorks µElution plate is provided that contains a silica based aminopropyl sorbent specifically selected for this application.[19-22] Due to its highly polar nature, the GlycoWorks SPE sorbent readily and selectively retains polar compounds such as glycans. In addition, this sorbent possesses a weakly basic surface that provides further selectivity advantages based on ion exchange and electrostatic repulsion. It is also worth noting that the GlycoWorks µElution Plate is designed for minimal elution volumes such that samples can be immediately analyzed without a dry down step. Moreover, the GlycoWorks µElution Plate is constructed as a 96 well format, meaning it can be used to perform high throughput experiments or used serially (with appropriate storage, see the Care and Use Manual) for low throughput needs.

In this HILIC SPE process, the sorbent is first conditioned with water and then equilibrated to high acetonitrile loading conditions. Thereafter, glycan samples that have been diluted with acetonitrile are loaded and washed free of the sample matrix using an acidic wash solvent comprised of 1% formic acid in 90% acetonitrile. This washing condition achieves optimal SPE selectivity by introducing electrostatic repulsion between the aminopropyl HILIC sorbent and reaction byproducts and by enhancing the solubility of the matrix components. After washing, the labeled, released glycans are next eluted from the HILIC sorbent. Since the GlycoWorks SPE sorbent has a weakly basic surface, and the capacity for anion exchange, just as it has the capacity for cation repulsion, it is necessary to elute the labeled glycans with an eluent of significant ionic strength. We have, as a result, developed an elution buffer comprised of a pH 7 solution of 200 mM ammonium acetate in 5% acetonitrile (p/n 186007991). Upon their elution, the *Rapi*Fluor-MS labeled glycans can be diluted with a mixture of organic solvents (acetonitrile and dimethylformamide) and directly analyzed by UPLC or HPLC HILIC column chromatography using fluorescence and/or ESI-MS detection.

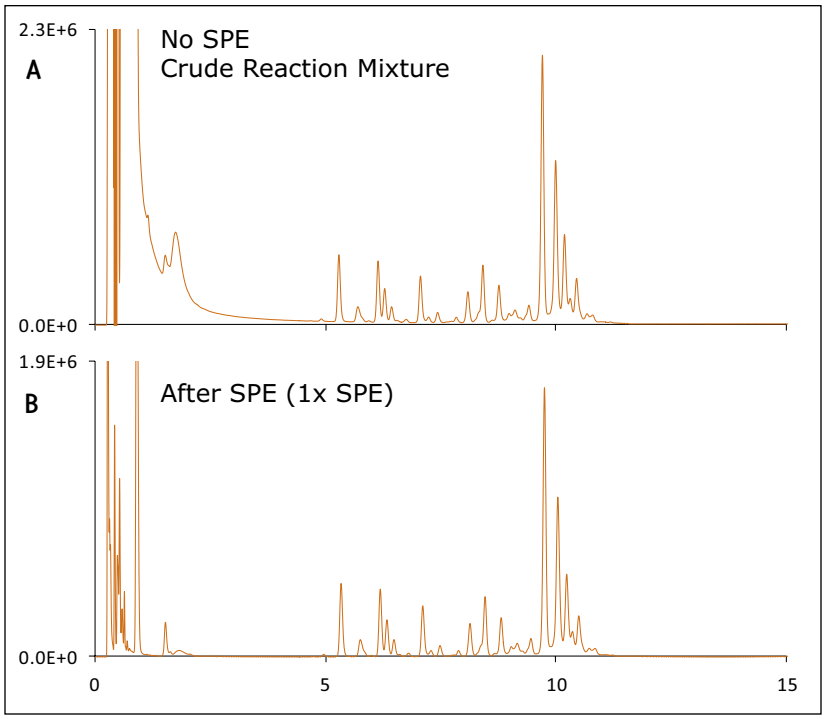


Figure 8. HILIC SPE to Remove Chromatographic Interference. (A) A test mixture comprised of RapiFluor-MS labeled glycans from pooled human IgG and bovine fetuin separated on an ACQUITY UPLC BEH Amide 130Å, 1.7 µm, 2.1 x 50 mm Column and detected via fluorescence (labeled N-glycans from 0.4 µg glycoprotein, 1 µL injection crude reaction mixture). (B) The test mixture after extraction by HILIC SPE (labeled N-glycans from 0.4 µg glycoprotein, 10 µL injection of ACN/DMF diluted SPE eluate).

As with other aspects of the *Rapi*Fluor-MS *N*-Glycan Kit, this GlycoWorks SPE step has been extensively evaluated. A test mixture was created to assess the recovery of a diverse set of *Rapi*Fluor-MS labeled *N*-glycans, ranging from small neutral glycans to high molecular weight, tetrasialylated glycans. Such a mixture was prepared by releasing and labeling *N*-glycans from both pooled human IgG and bovine fetuin. An example analysis of this test mixture by HILIC column chromatography and fluorescence detection is shown in Figure 9A. Species representing extremes in glycan properties are labeled, including an asialo FA2 glycan and a glycan with a tetrasialylated, triantennary structure (A3S1G3S3). To evaluate the effects of the SPE process, this mixture was subjected to a second pass of GlycoWorks SPE and again analyzed by HILIC chromatography and fluorescence detection, as shown in Figure 9B. It can be observed that the sample processed twice by SPE presents a labeled glycan profile comparable to the profile observed for the sample processed only once by SPE. Indeed, this SPE step has been found to exhibit an absolute recovery of approximately 70-80% for all purified glycans and more importantly highly accurate relative yields. Figure 9C shows the relative abundances for four glycans (FA2, FA2G2S1, A3G3S3, and A3S1G3S3) as determined for samples subjected to one pass versus two passes of SPE. The largest deviation in relative abundance was observed for the tetrasialylated A3S1G3S3, in which case relative abundances of 5.7% and 6.1% were determined for samples processed by one and two passes of SPE, respectively. With these results, it is demonstrated that GlycoWorks SPE provides a mechanism to immediately analyze a sample of extracted, labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of *N*-glycans.

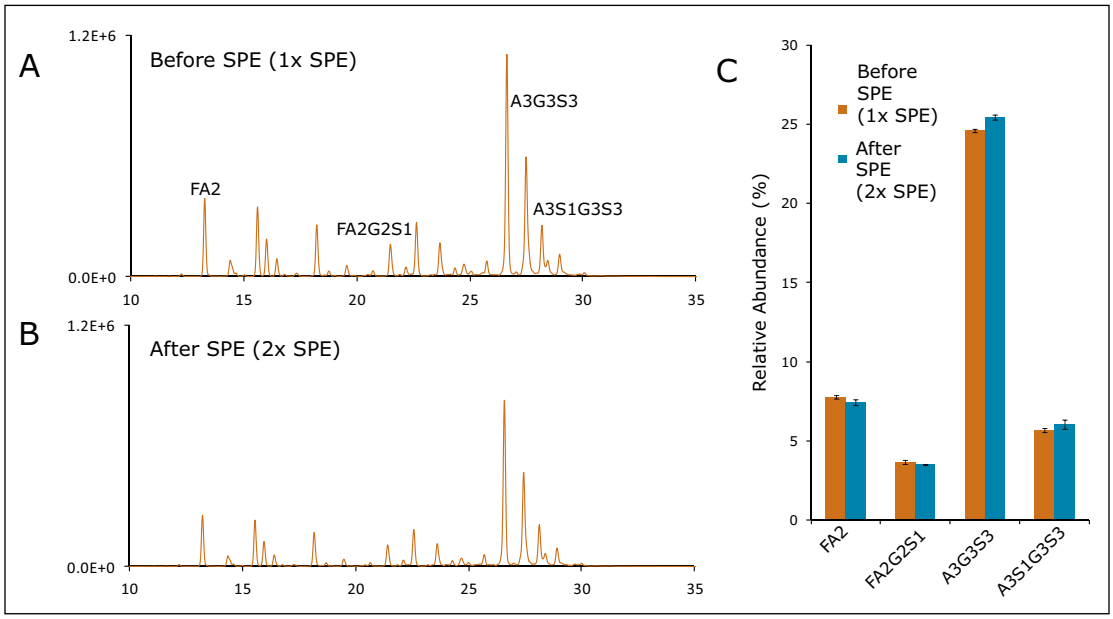


Figure 9. Extraction of RapiFluor-MS Labeled N-glycans by SPE with a GlycoWorks HILIC µElution Plate. (A) A test mixture comprised of RapiFluor-MS labeled glycans from pooled human IgG and bovine fetuin separated on an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column and detected via fluorescence (labeled N-glycans from 0.4 µg glycoprotein, 10 µL injection of ACN/DMF diluted sample). (B) The test mixture after extraction by HILIC SPE. (C) Relative abundances determined for a set of RapiFluor-MS labeled glycans before and after GlycoWorks HILIC SPE.

CONCLUSIONS

Conventional approaches to the preparation of *N*-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity. With the development of the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit, we address these shortcomings by enabling unprecedented sensitivity for glycan detection while also improving the throughput of *N*-glycan sample preparation. With this approach, glycoproteins are deglycosylated in approximately 10 minutes to produce *N*-glycosylamines. These glycans are then rapidly reacted with the novel *Rapi*Fluor-MS reagent within a 5 minute reaction and are thereby labeled with a tag comprised of an efficient fluorophore and a highly basic tertiary amine that yields enhanced sensitivity for both fluorescence and MS detection. In a final step requiring no more than 15 minutes, the resulting *Rapi*Fluor-MS labeled glycans are extracted from reaction byproducts by means of µElution HILIC SPE that has been rigorously developed to provide quantitative recovery of glycans (from neutral to tetrasialylated species) and to facilitate immediate analysis of samples. Accordingly, an analyst can complete an *N*-glycan sample preparation, from glycoprotein to ready-to-analyze sample, in just 30 minutes when using the sensitivity enhancing *Rapi*Fluor-MS labeling reagent.

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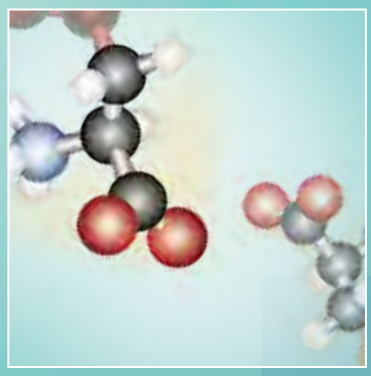
HIC for Hydrophobic Moieties and Drug Antibody Ratio (DAR)

Hydrophobic interaction chromatography (HIC) is a powerful analytical method used for the separation of molecular variants of therapeutic proteins, including monoclonal antibodies. The method has been employed for monitoring various post-translational modifications, including proteolytic fragments and domain misfolding, tryptophan oxidation, aspartic acid isomerization, and carboxy terminal heterogeneity.

Hydrophobic interaction chromatography (HIC) is also a powerful analytical technique for the analysis of antibody-drug conjugates. It is the method of choice for determination of the drug-to-antibody ratio (DAR) and drug load distribution for cysteine (Cys)-linked antibody-drug conjugates (ADCs).

Solution highlights

Protein-Pak Hi Res Hydrophobic Interaction Columns (HIC) and HIC Protein Standard Mix can help in the characterization of recombinant proteins, monoclonal antibodies, and/or antibody drug conjugates (ADCs). The use of non-porous particles containing a low density of butyl ligands on Waters Protein-Pak Hi Res HIC Column results in fewer protein-ligand interactions and yields outstanding resolution in less time compared to use of many traditional porous HIC offerings. In addition, quality control testing with defined protein standards helps ensure consistent batch-to-batch performance of these columns.



Automating the Determination of Drug-to-Antibody Ratio (DAR) of Antibody Drug Conjugates (ADCs) Based on Separation by Hydrophobic Interaction Chromatography (HIC)

Robert Birdsall, Eoin Cosgrave, Henry Shion, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increase productivity through the automation of DAR calculations
- Streamline data processing with custom calculations for efficient data review
- Integrate functionality to improve reporting of results

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC TUV Detector

Protein-Pak™ Hi Res HIC Column

Empower® 3 Software

KEY WORDS

Antibody drug conjugate, ADC, cytotoxic agent, drug payload, linker, cysteine conjugate, drug antibody ratio, DAR, hydrophobic, HIC, therapeutic antibody, protein

INTRODUCTION

Antibody drug conjugates (ADCs) represent a rapidly growing class of biotherapeutic drugs for the treatment of cancer.[1] ADCs offer the selectivity of an antibody with the potency of a cytotoxic agent such as a synthetic drug.[2] ADC design, in part, relies on predictable conjugation chemistry that preserves antibody binding activity (Figure 1A) while facilitating reproducible characteristics that can be used as metrics for assessing critical quality attributes (CQAs) to ensure a safe and effective ADC product.[3]

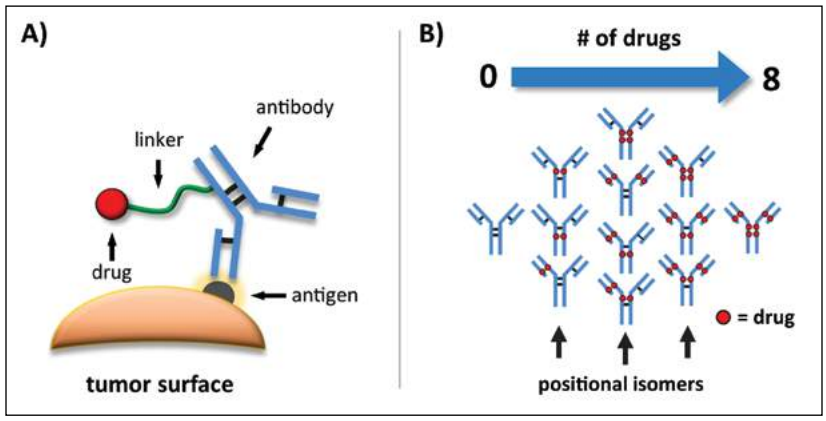


Figure 1. Biotherapeutic ADC Modality from Effective Drug Design. A) An ADC facilitates a targeted treatment of cancerous tumors, in which a drug payload is brought within close proximity of a tumor through the specificity of a therapeutic antibody. B) Example of cysteine-conjugated ADCs, illustrating the conjugation of drugs to the antibody via inter-chain disulfide bonds. Expected drug loads occur in intervals of 2, 4, 6, and 8.

Cysteine-conjugated ADCs are a sub-class of biotherapeutic drugs that are manufactured with well-known conjugation chemistry. Drug payloads are attached through a linker to monoclonal antibodies (mAbs) via the thiol groups. Thiols are generated by the reduction of inter-chain disulfide bonds, and expected drug load occurs in intervals of 2, 4, 6, and 8 as shown in Figure 1B due to the formation of two thiols from each disulfide.

EXPERIMENTAL

The Protein-Pak Hi Res HIC column (4.6 x 100 mm, 2.5 µm, p/n 186007583) was conditioned prior to use according to its care and use manual. Chemical reagents were purchased from Sigma-Aldrich and used as received. A cysteine-conjugated ADC was prepared by a collaborator at a concentration of 10 mg/mL in formulation buffer. Samples were prepared for analysis at a concentration of 2 mg/mL in 1M ammonium sulfate ($(NH_4)_2SO_4$).

Conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption Wavelength:	280 nm
Vials:	Total Recovery vial: 12x32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	Protein-Pak Hi Res HIC, 4.6 x 100 mm, 2.5 µm
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase A:	125 mM Phosphate buffer, pH 6.7 with 2.5 M $(NH_4)_2SO_4$
Mobile phase B:	125 mM phosphate buffer, pH 6.7
Mobile phase C:	Isopropyl alcohol
Mobile phase D:	Water

Gradient

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.700	50	0	5	45
10.00	0.700	0	50	5	45
15.00	0.700	0	50	5	45
15.01	0.700	50	0	5	45
30.00	0.700	50	0	5	45

Informatics for data collection & processing

Empower 3 Software, SR1, FR2

The conjugation process, which is dependent on the reactant concentrations, can result in variable drug-to-antibody-ratio (DAR, Figure 2) and subsequently impact the efficacy and safety of the ADC. Therefore monitoring the drug distribution and drug load of ADCs during the manufacturing process is of critical importance for pharmaceutical companies.

In the absence of a dedicated workflow, analysts will often perform manual calculations based on hydrophobic interaction chromatography (HIC) peak area for the determination of DAR based on drug load distribution. Waters' chromatographic data system (CDS), Empower 3 Software, can be used to streamline the analysis of ADCs through an integrated approach to data acquisition, processing, and reporting of experimental results. This process, which can be fully automated, makes the Empower CDS ideal for increasing productivity through efficient method deployment in the characterization of ADCs.

The objective of this application note is to demonstrate the ability to automatically determine DAR values corresponding to the drug load distribution of cysteine-conjugated ADCs using Empower 3 Chromatography Data Software. A cysteine-conjugated ADC was used as a model conjugate to test the application.

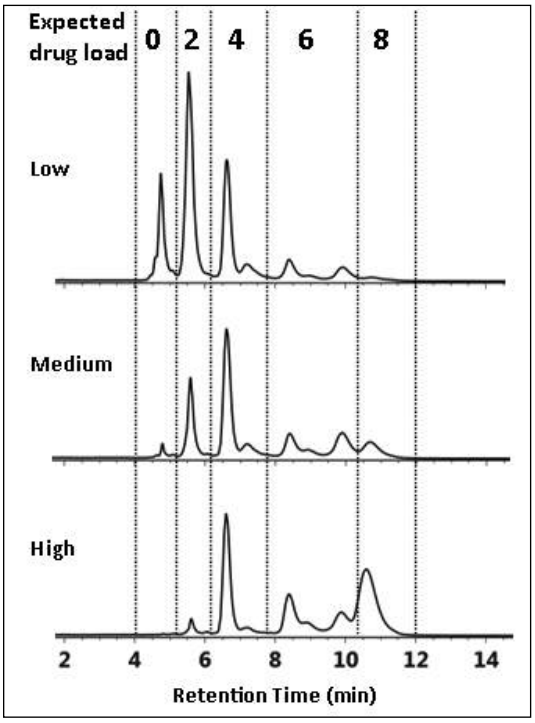


Figure 2. Monitoring drug load variability. Three batches of cysteine-linked ADCs were synthesized, each with a different level of drug conjugation (low, medium, high) and separated using hydrophobic interaction chromatography (see experimental). The drug load distribution shifted from low to high corresponding to an increase in the load of the hydrophobic drug.

RESULTS AND DISCUSSION

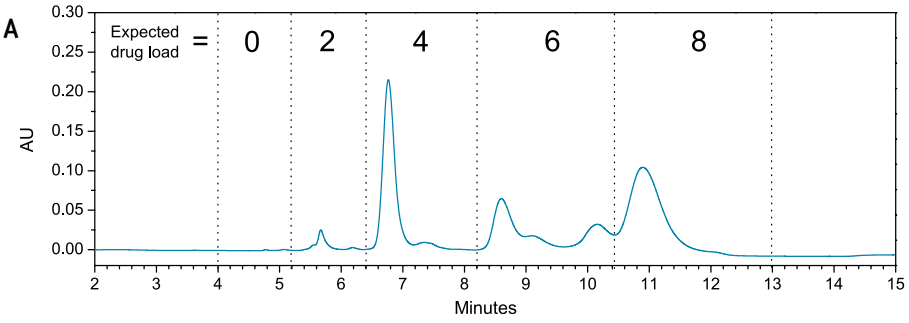
Efficient component management for challenging biotherapeutic samples

Chromatograms of separations of cysteine-conjugated ADCs using HIC are often comprised of multiple peaks where groups of peaks represent positional isomers of a conjugated antibody (Figure 3A).[3]

With its high-fidelity separations, the ACQUITY UPLC H-Class System delivers reproducible analyses; the researcher can then take advantage of informatics tools in Empower 3 Software to accurately group peaks by retention time for calculation of the DAR value based on drug distribution. This results in a highly efficient approach to method development during characterization.

As shown in Figure 3A, the multiple components associated with individual drug loads (DAR 0 through DAR 8) are entered into the Timed Groups tab (Figure 3B) of the processing method based on expected retention times. The ability to manage identification of components in separations like this makes Empower 3 Software ideal for characterizing challenging biotherapeutics such as ADCs.

A



B

Integration | Smoothing/Offset | Components | Impurity | Peak Ratios (MS Ion Ratios) | Default Amounts/Purity | Named Groups | Timed Groups | Suitability | Limits | Noise

	Name	Start (min)	Stop (min)	Source of Calibration X Value
1	DAR 0	4.000	5.200	Not Calibrated, Sum Peaks for Quantitation
2	DAR 2	5.200	6.400	Not Calibrated, Sum Peaks for Quantitation
3	DAR 4	6.400	8.200	Not Calibrated, Sum Peaks for Quantitation
4	DAR 6	8.200	10.400	Not Calibrated, Sum Peaks for Quantitation
5	DAR 8	10.400	13.000	Not Calibrated, Sum Peaks for Quantitation

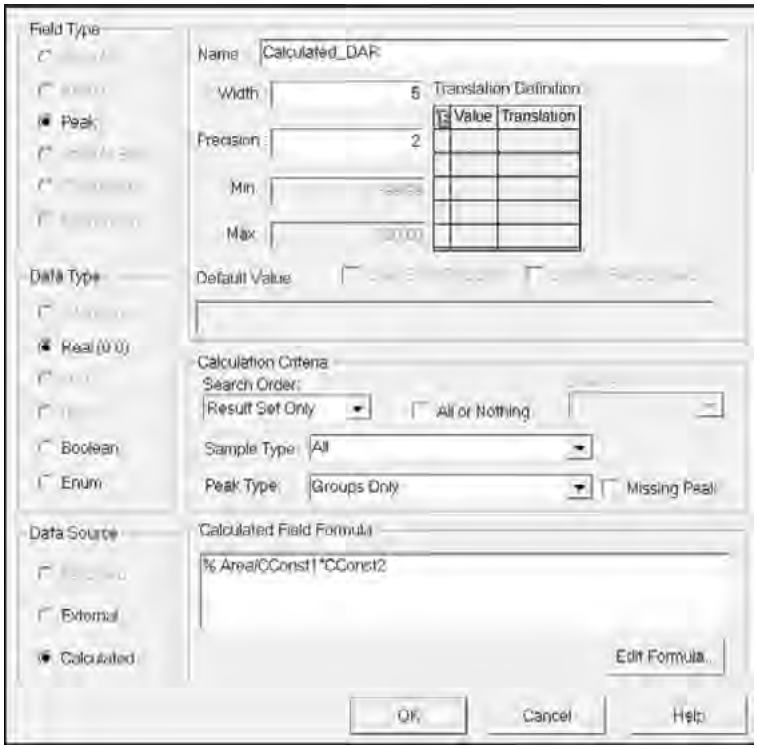
Figure 3. Managing components through method design. A) Empower offers the ability to manage groups of separation components (peaks) associated with individual drug loads. B) Using the Timed Groups feature of the processing method, retention time windows can be used for the grouping of multiple components for efficient peak identification and management in analysis results.

Improving productivity through automated calculation of DAR values

Empower 3 Software provides a myriad of informatics tools – which are designed to increase productivity in data analysis – that can be deployed for the automation of calculations associated with CQAs of cysteine-conjugated ADCs such as DAR values. This is achieved through the use of the Custom Fields, which are generated in the project properties from the system configuration manager as shown in Figure 4A.

Efficient processing of results using Empower is, in part, made possible through the ability to set criteria for custom field calculations. As shown in Figure 4A, by selecting Groups Only in the Peak Type window, the custom calculation shown in the formula window will only be applied to the grouped data as defined in Figure 3B. Furthermore, Empower's flexibility to use numerical constants from the Component Manager (Figure 4B) facilitates the use of custom calculations such as DAR values for cysteine-conjugated ADCs.

A



B

Integration | Smoothing/Offset | Components | Impurity | Peak Ratios (MS Ion Ratios) | Default Amounts/Purity | Named Groups | Timed Groups | Suitability | Limits

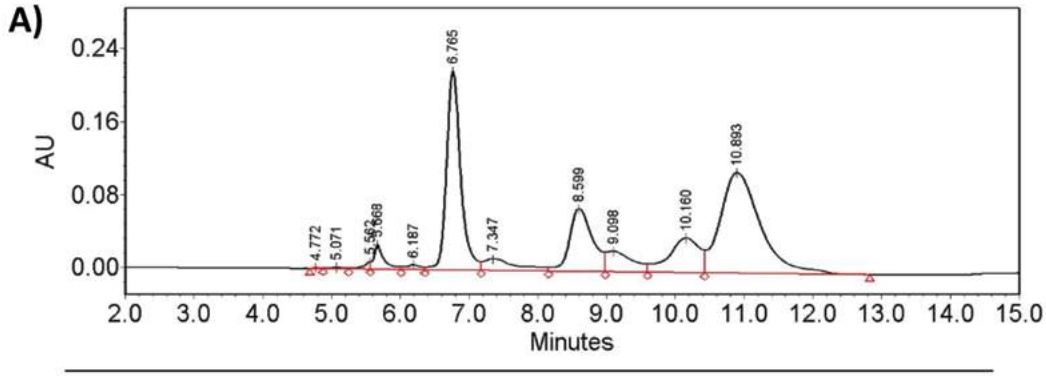
	Name	Component Type	Retention Time (min)	CConst1	CConst2
1	DAR 0	Main Component	4.000	100.00	0.00
2	DAR 2	Main Component	5.200	100.00	2.00
3	DAR 4	Main Component	6.400	100.00	4.00
4	DAR 6	Main Component	8.200	100.00	6.00
5	DAR 8	Main Component	10.400	100.00	8.00

Figure 4. Automating calculations using flexible custom fields. A) Empower 3 Software's Custom Fields feature is flexible and can accommodate custom calculations that use B) numerical constants in the calculation formula. In this example, CConst2 (Expected DAR) associated with the HIC separation of the cysteine-conjugated ADCs is used in the calculated field formula (DAR = % Area / 100 x Expected DAR).

The custom fields, upon initial set-up, can be incorporated into the analysis workflow. To demonstrate this, 20 µg of a cysteine-conjugated ADC sample at a concentration of 2 mg/mL in 1M $(NH_4)_2SO_4$ was injected on a Protein-Pak Hi Res HIC column (4.6 x 100 mm, 2.5 µm) and separated using a 10-minute gradient as shown in Figure 5A. As shown in Figure 5B, Empower automatically reports the retention time and associated peak area for the individual peaks as well as the grouped peaks as defined in Figure 3B.

As part of the processing method, Empower automatically calculates and displays the individual DAR values based on drug distribution (DAR 0 = 0.00, DAR 2 = 0.08, DAR 4 = 1.32, DAR 6 = 1.70, DAR 8 = 2.75) for the cysteine-conjugated ADC sample using the custom field calculation defined in Figure 4A. This process, which automates the calculation of CQAs, demonstrates that the Empower 3 Software is well-suited for increasing productivity in the characterization of antibody drug conjugates.

A)



B)

	Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	Int Type	Calculated_DAR
8		6.765	3089401	27.42	217325	VV	
9		7.347	321935	2.86	11808	VV	
10		7.931	39516	0.35	3207	VV	
11		8.598	1523740	13.53	67630	VV	
12		9.094	523160	4.64	20682	VV	
13		10.160	1060635	9.42	35666	VV	
14		10.897	4279225	37.99	108315	VB	
15	DAR 0	4.051	40995	0.36	4049	Group	0.00
16	DAR 2	5.562	386488	3.43	39551	Group	0.07
17	DAR 4	6.765	3450852	30.63	232340	Group	1.23
18	DAR 6	8.598	3107534	27.59	123978	Group	1.66
19	DAR 8	10.897	4279225	37.99	108315	Group	3.04

Individual peaks

Grouped peaks

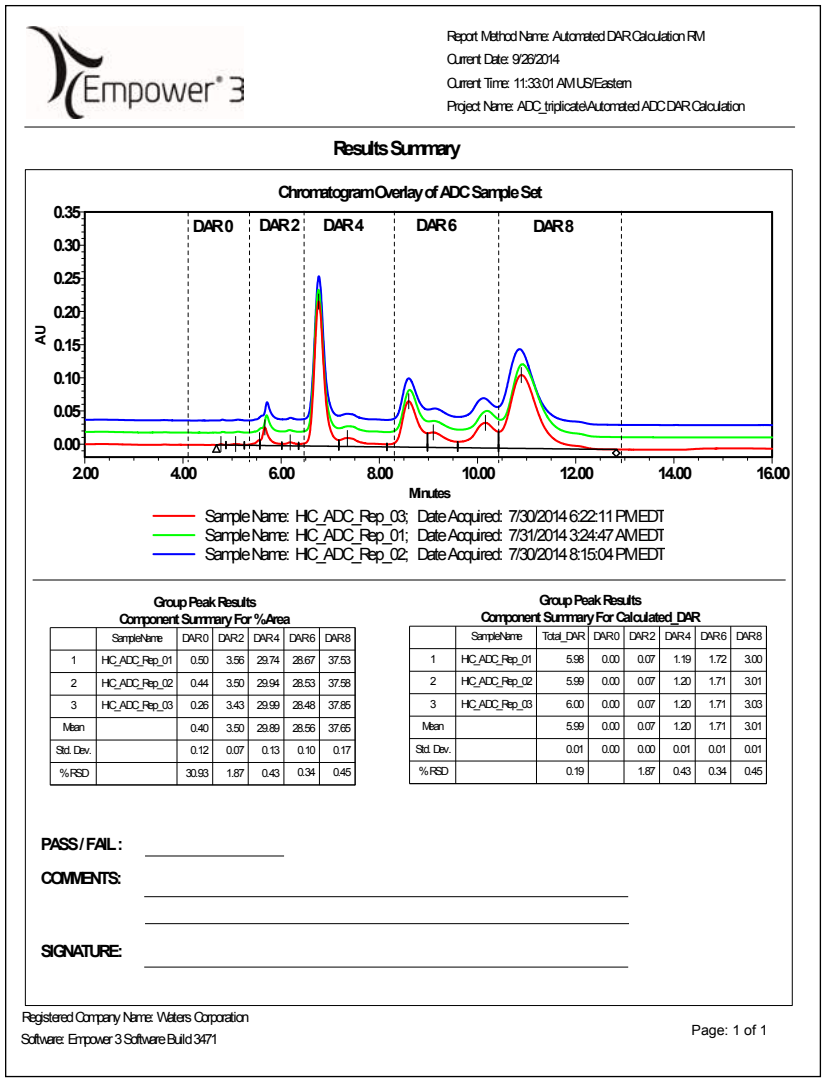
Figure 5A. Incorporation of Custom Fields in data analysis. A) Integration of a cysteine-conjugated ADC separation using a Protein-Pak Hi Res HIC Column with custom calculations. B) As a part of the processing method, Empower 3 Software seamlessly incorporates identification of individual and groups of chromatographic peaks, while custom field calculations for the individual DAR values are displayed in the right column for the grouped peaks.

Informatics tools that automate reporting of CQAs

Empower 3 Software additionally features powerful reporting functionality that is designed to provide researchers with meaningful analytical data and summaries for management review. Report templates can be readily constructed and customized for assessment of results.

An example of a report template designed for reporting the DAR and drug distribution for cysteine-conjugated ADC characterization is shown in Figure 6. Using the results from Figure 5B, a summary report of the relative area and corresponding individual and total DAR values based on drug distribution along with the corresponding statistical results (*e.g.*, mean and % RSD) is generated after data acquisition and processing.

Offering the flexibility of custom reporting templates that can be designed to meet a laboratory's specific analytical and communication needs, Empower 3 Software is a powerful partner to an ACQUITY UPLC H-Class System. Together they provide an integrated method development approach for the acquisition, processing, and reporting of results in the characterization of cysteine-conjugated ADCs.



	SampleName	DAR0	DAR2	DAR4	DAR6	DAR8
1	HIC_ADC_Rep_01	0.50	3.56	29.74	28.67	37.53
2	HIC_ADC_Rep_02	0.44	3.50	29.94	28.53	37.58
3	HIC_ADC_Rep_03	0.26	3.43	29.99	28.48	37.85
Mean		0.40	3.50	29.89	28.56	37.65
Std. Dev.		0.12	0.07	0.13	0.10	0.17
%RSD		30.93	1.87	0.43	0.34	0.45

	SampleName	Total_DAR	DAR0	DAR2	DAR4	DAR6	DAR8
1	HIC_ADC_Rep_01	5.98	0.00	0.07	1.19	1.72	3.00
2	HIC_ADC_Rep_02	5.99	0.00	0.07	1.20	1.71	3.01
3	HIC_ADC_Rep_03	6.00	0.00	0.07	1.20	1.71	3.03
Mean		5.99	0.00	0.07	1.20	1.71	3.01
Std. Dev.		0.01	0.00	0.00	0.01	0.01	0.01
%RSD		0.19		1.87	0.43	0.34	0.45

Figure 6. Report Template example. Custom calculations for the individual and total DAR values based on drug distribution are shown in the report for the cysteine-conjugated ADC sample.

CONCLUSIONS

With a modality that has the potential to redefine cancer treatment, interest from pharmaceutical companies to deliver ADC biotherapeutics to market is intensifying. To support this, characterization methods that are efficient and adaptable to evaluate novel CQAs associated with this new class of biotherapeutic drug are needed.

Empower 3 Software represents one of Waters' solutions to these challenging problems. This chromatographic data system can be used to streamline the analytical workflow through an integrated approach to data acquisition, processing, and reporting of experimental results. As one part of its integrated informatics toolset, custom field calculations are seamlessly incorporated into the ADC data analysis workflow to address processing challenges facing today's analysts.

This end-to-end automatable workflow illustrates that Waters' integrated analytical technology solutions are ideal for increasing productivity through efficient method deployment in the characterization of antibody drug conjugates.

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Multidimensional Liquid Chromatography (2D-LC)

Bringing well-characterized, high quality drug to patients quickly is a major task in pharmaceutical analysis. Two-dimensional liquid chromatography (2D-LC) has been used to solve the separation challenges that cannot be achieved by one-dimensional LC (1D-LC). Chromatographers can exploit orthogonal mechanisms of interaction between proteins and the columns of each dimension to realize higher effective peak capacities up to the product of the resolving power of each individual dimension.

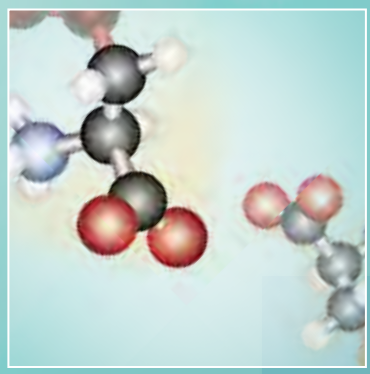
In two-dimensional liquid chromatography (2D-LC), two independent liquid phase separation systems are applied to the sample. 2D-LC can be done by transferring a portion of the first dimension effluent onto the second dimension, in what is known as "heart cutting" chromatography, or by sequentially transferring the entirety of the first dimension effluent, in many small aliquots, onto the second dimension; in what is referred to as "comprehensive" 2D chromatography.

Solution highlights

Characterization methods of biotherapeutic proteins that provide increased information without compromising productivity require efficient solutions that are adaptable to the high-throughput environment of industry. **The ACQUITY UPLC H-Class Bio System with 2D Technology** is Waters' solution to these challenges.

The ACQUITY UPLC H-Class Bio System with 2D Technology offers an efficient method for the characterization of biotherapeutics with the ability to combine orthogonal detection techniques on-line for improved productivity. Moreover, the heart-cut feature offered with the system makes it straightforward to fractionate and desalt challenging biological samples.

Compatibility with multiple column configurations and the ability to automate the process offers analysts the flexibility needed to maximize information in the characterization of biotherapeutics without compromising productivity.



Two-Dimensional Liquid Chromatography for Quantification and MS Analysis of Monoclonal Antibodies in Complex Samples

Sean M. McCarthy, Thomas E. Wheat, Ying Qing Yu, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Waters® ACQUITY UPLC® H-Class Bio System with 2D Technology provides a single system approach for rapid protein titer measurement and mass profiling of proteins. This method can be used during clone selection and process scale-up to determine yield and monitor the mass profile.

WATERS SOLUTIONS

ACQUITY UPLC H-Class Bio System with 2D Technology

Xevo® G2 Q-Tof™

BiopharmaLynx™ for MassLynx® Software

MassPREP™ Micro Desalting Column

KEY WORDS

Affinity chromatography, two-dimensional (2D) chromatography, monoclonal antibodies, Protein A immobilization, affinity purification and quantification, mass profile

INTRODUCTION

Identification, characterization, and quantification of monoclonal antibodies (mAbs) are required at many stages of biopharmaceutical research and development. The primary analytical tools for these assays are liquid chromatography coupled with UV or mass spectrometry (MS). Both techniques can be compromised by interferences in the sample matrix, including high salt concentrations, other proteins, or the components of cell culture media.

A high throughput analytical technique should combine sample preparation and chromatographic techniques to ensure accurate and robust quantification. Affinity chromatography on immobilized Protein A can be used to isolate the antibody from a complex matrix, while reverse phase (RP) LC is useful for introducing a salt-free, concentrated sample into an MS ion source.

To accomplish both affinity purification and MS analysis in a high throughput manner, we utilized the ACQUITY UPLC H-Class Bio System with 2D Technology. The 2D system allows for simultaneous purification and quantification of monoclonal antibodies by Protein A affinity chromatography, and determination of mass profile by MS analysis after desalting on a short RP column. This online 2D UPLC® method requires little to no sample preparation, and an analysis is quickly completed with an instrument duty cycle time of seven minutes.

EXPERIMENTAL

Sample preparation

Samples of trastuzumab were prepared by diluting the sample to a concentration of 1 mg/mL in DMEM cell culture media containing 1 mg/mL of bovine serum albumin (BSA).

UPLC conditions

LC system: ACQUITY UPLC H-Class Bio System with 2D Technology:

- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (1D)
- ACQUITY UPLC H-Class Bio Sample Manager (FTN)
- ACQUITY UPLC Column Manager A
- ACQUITY UPLC Tunable UV Detector
- ACQUITY UPLC Binary Solvent Manager (2D)

First dimension

Column:	Poros A 20, 2.1 x 30 mm (Applied Biosystems)
Column temp.:	20 °C
Flow rate:	1.0 mL/min
Mobile phase A:	50 mM Phosphate Buffer, pH 7.0, 150 mM NaCl
Mobile phase B:	12 mM HCl, pH 1.9, 150 mM NaCl
Detection:	UV 280 nm

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	1.0	100	0	0	0	Initial
1.00	1.0	100	0	0	0	1
1.10	1.0	0	100	0	0	1
4.00	1.0	0	100	0	0	1
4.10	1.0	100	0	0	0	1
7.00	1.0	100	0	0	0	1

Second dimension

Column:	MassPREP Micro Desalting Column (P/N 186004032)
Column temp.:	80 °C
Flow rate:	0.5 mL/min (desalting and regeneration), 0.2 mL/min (analysis)
Mobile phase A:	0.1% Formic acid
Mobile phase B:	0.1% Formic acid in ACN

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	100	0	Initial
1.80	0.5	100	0	6
3.50	0.5	100	0	6
3.51	0.2	100	0	6
5.00	0.2	5	95	6
5.10	0.5	100	0	6
5.70	0.5	5	95	6
5.80	0.5	100	0	6
6.40	0.5	5	95	6
6.50	0.5	100	0	6

Column manager

Initial:	Left Valve Position 1
1.5 min:	Left Valve Position 2
1.8 min:	Left Valve Position 1

MS conditions

MS system:	Xevo G2 Q-Tof
Capillary:	3 kV
Sampling cone:	45 V
Extraction cone:	2 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	600.0 L/h

RESULTS AND DISCUSSION

The ACQUITY UPLC H-Class Bio System with 2D Technology can be configured to collect a heart cut (time slice) from a first-dimension separation and divert the fraction for subsequent analysis. Determination of the correct time segment is performed experimentally by measuring the elution time of the peak of interest. The analyst can then enter timed events to control valve positions.

The plumbing diagram shows the system configured for affinity purification followed by RP analysis for intact mass. The sample is introduced into the affinity column in position 1. Following removal of unwanted components (such as BSA and media components), the valve is switched to position 2. The antibody is eluted as a concentrated peak directly onto the head of the desalting column. After collection, the valve is returned to position 1 then the second dimension desalting and gradient are performed.

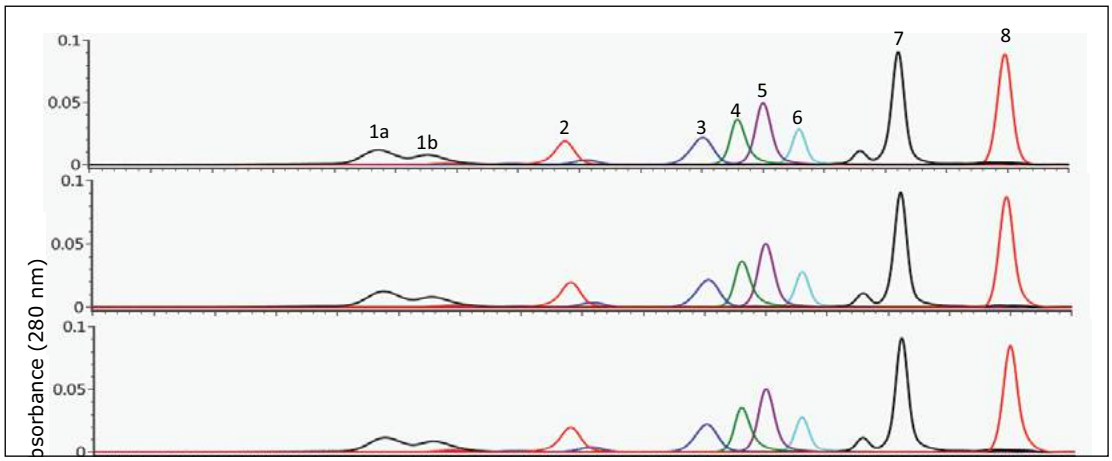


Figure 1. Instrument plumbing configuration. The sample is introduced to the 1D column in position 1. At a specified time, the left valve is switched to position 2 to divert flow to the 2D column. After collection, the valve is returned to position 1 and the second dimension gradient is initiated. A splitter is used between the 1D and 2D to compensate for the differences in mass load needed for each analysis. The splitter can be bypassed by shunting the split flow to waste when the 1D mass load is compatible with the 2D.

In heart cut applications such as this high throughput analysis, it is important that the retention time be reproducible and and carryover be minimal to ensure accurate collection and quantification. As shown in Figure 2, the ACQUITY UPLC H-Class Bio System with 2D Technology has both of these attributes. Our data shows reproducible retention time and peak area, allowing for accurate collection and quantification, with no evidence of carryover in blank injections. We also found very good linearity over a range of mass loads, as shown in Figure 3.

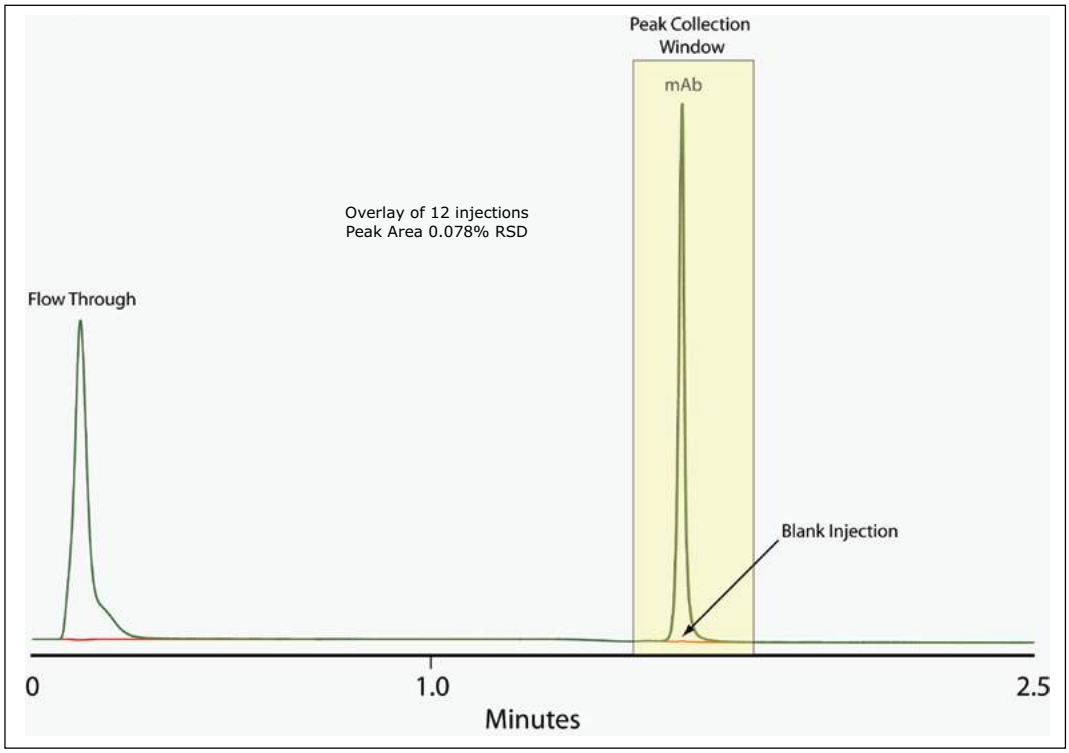


Figure 2. Overlay of 12 1D Protein A affinity chromatograms of trastuzumab, 1 mg/mL in cell culture media (DMEM) with 1 mg/mL BSA. Excellent reproducibility of retention time, peak shape, and peak area were found. Blank injections do not show any carryover.

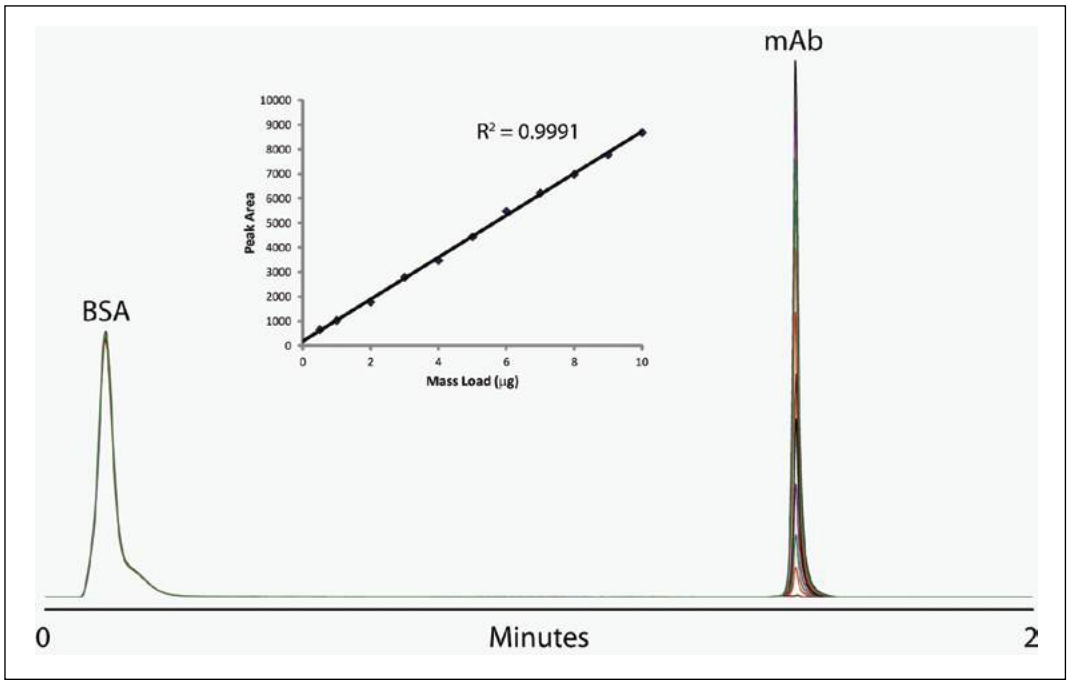


Figure 3. Overlay of serially diluted trastuzumab, from 0.5 µg to 10 µg in cell culture media with 1 mg/mL BSA. Response for mAb is linear over the entire mass load range. Samples run in triplicate with RSD for each mass load <0.1%.

Important aspects of this application include the reproducibility of collection from the first dimension and reproducibility of the MS response. As demonstrated by the overlaid total ion chromatogram (TIC) traces in Figure 4, replicate injections at each mass load overlay very well, and signal intensity increases with mass load. To prevent overloading the second dimension column, we utilized a PEEK tee, shown in Figure 1, as a flow splitter to divert approximately 70% of the first dimension eluent to waste. The split flow going to waste can be shunted; thereby, diverting the entire first dimension flow to the second dimension, if necessary.

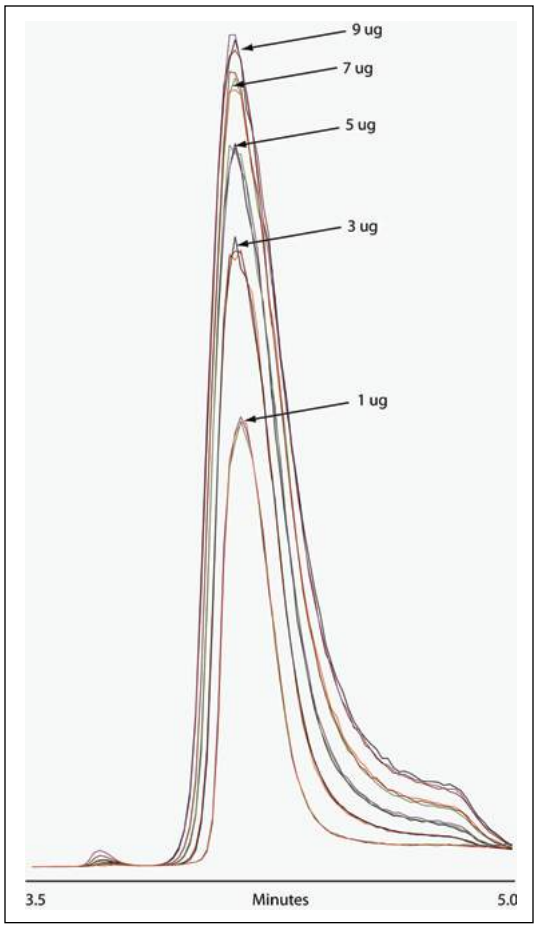


Figure 4. Overlay of total ion chromatograms from desalting column for various mass loads collected from first dimension separation.

For this application, we utilized BiopharmaLynx Software to process the data. The processed spectra for 10-µg and 0.5-µg injections on the affinity column are shown in the top and bottom panels of Figure 5. Since flow was split for each injection, the effective mass loads for each sample were approximately 3 µg and 0.15 µg, respectively. We found excellent agreement between the highest and lowest mass loads used in this application.

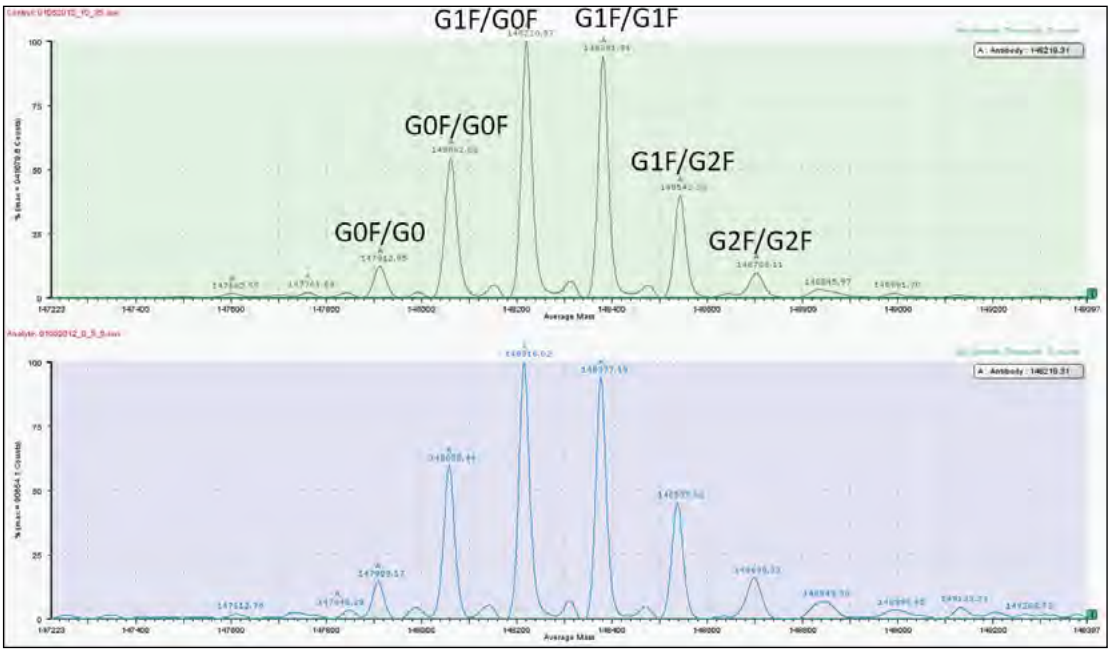


Figure 5. Automated intact protein mass deconvolution using BiopharmaLynx. Data can be batch-processed and quickly screened for mass and glycoprofile. Additionally, data can be compared to a control sample to identify and quickly assess similarities and differences between samples.

CONCLUSION

Using the ACQUITY UPLC H-Class Bio System with 2D Technology, a method was developed to determine monoclonal antibody concentration and mass profile from a single injection. The 2D chromatographic methods were easily defined using an inlet method editor. The system delivered the necessary reproducibility in terms of retention time, recovery, and response for samples from complex matrices using both UV and MS detection. By streamlining sample preparation and enabling high-throughput MS analysis, this online 2D UPLC system can improve a biopharmaceutical organization's productivity in monitoring mass profiles and determining yield during clone selection and production scale-up for monoclonal antibody products.

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BRIEF REPORT

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A rapid on-line method for mass spectrometric confirmation of a cysteine-conjugated antibody-drug-conjugate structure using multidimensional chromatography

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Keywords: 2DLC, ADC, antibody-drug-conjugate, cysteine-drug-conjugate, drug-antibody-ratio, LC/LC/MS, multidimensional chromatography, positional isomers

Abbreviations: ADC, antibody-drug conjugate; 1D, 1st dimension; 2D, 2nd dimension; BSM, binary solvent manager; CQA, critical quality attribute; DAR, drug-antibody-ratio; DTT, dithiothreitol; HIC, hydrophobic interaction chromatography; IgG, immunoglobulin G; LC-UV, liquid chromatography ultra-violet; mAb, monoclonal antibody; MP, mobile phase; MS, mass spectrometry; QSM, quaternary solvent manager; QTOF, quadrupole time-of-flight; RP, reversed phase; SDS-CE, sodium dodecyl sulfate-capillary electrophoresis; TCEP, (tris(2-carboxyethyl)phosphine); TUV, tunable ultra-violet; UV, ultra-violet.

Cysteine-conjugated antibody-drug conjugates (ADCs) are manufactured using controlled partial reduction and conjugation chemistry with drug payloads that typically occur in intervals of 0, 2, 4, 6, and 8. Control of heterogeneity is of particular importance to the quality of ADC product because drug loading and distribution can affect the safety and efficacy of the ADC. Liquid chromatography ultra-violet (LC-UV)-based methods can be used to acquire the drug distribution profiles of cysteine-conjugated ADCs when analyzed using hydrophobic interaction chromatography (HIC). However, alternative analysis techniques are often required for structural identification when conjugated drugs do not possess discrete ultra-violet absorbance properties for precise assessment of the drug-to-antibody ratio (DAR). In this study, multidimensional chromatography was used as an efficient method for combining non-compatible techniques, such as HIC, with analysis by mass spectrometry (LC/LC/QTOF-MS) for rapid on-line structural elucidation of species observed in HIC distribution profiles of cysteine-conjugated ADCs. The methodology was tested using an IgG1 mAb modified by cysteine conjugation with a non-toxic drug mimic. Structural elucidation of peaks observed in the HIC analysis (1st dimension) were successfully identified based on their unique sub-unit masses via mass spectrometry techniques once dissociation occurred under denaturing reversed phase conditions (2nd dimension). Upon identification, the DAR values were determined to be 2.83, 4.44, and 5.97 for 3 drug load levels (low-, medium-, and high-loaded ADC batches), respectively, based on relative abundance from the LC-UV data. This work demonstrates that multidimensional chromatography coupled with MS, provides an efficient approach for on-line biotherapeutic characterization to ensure ADC product quality.

Introduction

Antibody-drug conjugates (ADCs) represent a growing class of biotherapeutics currently being investigated for the treatment of cancer.[1-5] The efficacy of ADCs, in part, is attributed to the underlying architecture of the conjugate, wherein a monoclonal antibody (mAb) is combined with a cytotoxin. The selectivity of the mAb toward over-expressed cell surface antigens associated with cancerous tumors facilitates the targeted delivery of a covalently linked cytotoxic agent, or "drug," adjacent to the tumor surface. This therapeutic approach offers the selectivity of an antibody[6] for targeted treatment of tumor cells while minimizing systemic toxicity effects from the highly potent drug.[2,7,8] Successful launches of brentuximab vedotin (Adcetris®)[9] and ado-trastuzumab emtansine (Kadcyla®)[3] for the treatment of Hodgkin's lymphoma and breast cancer, respectively, illustrate the potential impact of these

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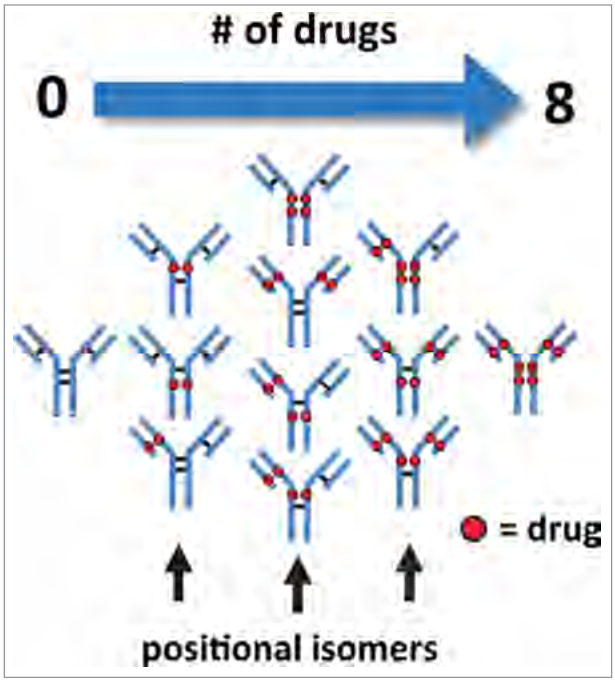


Figure 1. Illustration of cysteine-conjugated ADCs with various drug load distributions. Reduction of inter-chain disulfide bonds allows the conjugation of drugs through a maleimide-containing linker via the newly generated sulfhydryl groups. Conjugation of drugs via reduced inter-chain disulfide bonds generate ADCs with expected drug loads occurring in intervals of 2, 4, 6, and 8 with associated possible positional isomers.

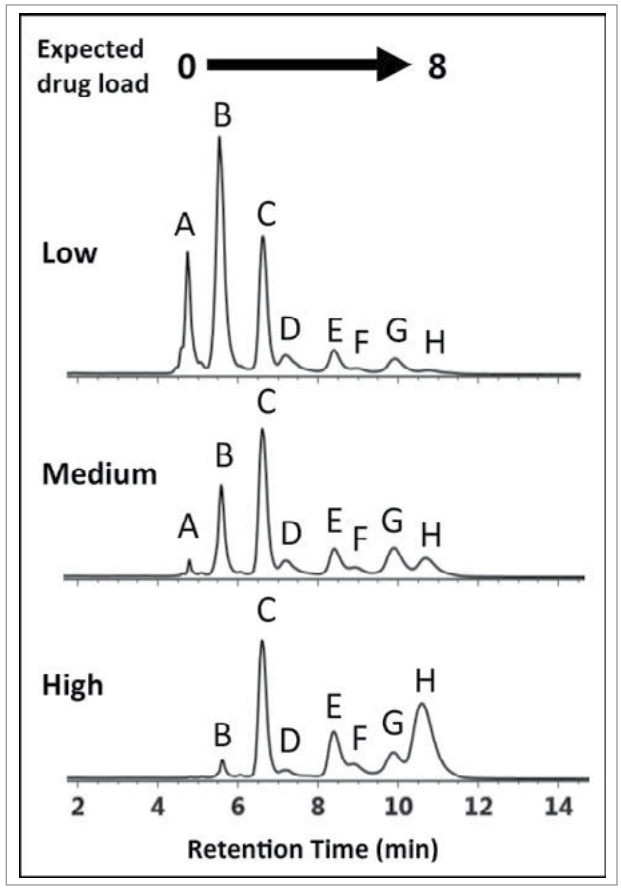


Figure 2. HIC chromatograms showing distribution profiles of cysteine-conjugated ADCs. Three batches of ADCs were synthesized, each with a different level of drug load (Low-, Medium-, and High-loaded) and analyzed using a 10 min gradient with a HIC column, 4.6 × 100 mm, 2.5 µm (see experimental). The distribution profiles exhibited multiple peaks that differed from expected profiles preventing unambiguous correlation of DAR 2, 4, 6, and 8 species with the peaks (B)–(H).

emerging biotherapeutic agents in cancer treatment. Engineering ADCs with preserved antibody binding activity and reproducible physicochemical properties that can be used as metrics during development and process control is highly desirable.[10,11]

Cysteine-conjugated ADCs are a sub-class of biotherapeutics that are manufactured with well-known conjugation chemistry. This type of ADC is typically less heterogeneous with respect to the quantity and distribution of drugs conjugated to the mAb[12] in comparison to ADCs created via lysine-based conjugation methods.[13] Control of heterogeneity is of particular importance as drug load and drug distribution can affect the efficacy, toxicology, and half-life/clearance properties of the ADC.[10,14,15] In theory, the conjugation process produces a mixture of isoforms with the number of drugs conjugated in intervals of 0, 2, 4, 6, and 8 from the controlled reduction of the inter-chain disulfide bonds, each of which generates 2 sulfhydryl groups (**Fig. 1**).[12] As a result, the reduced complexity of cysteine-conjugated ADCs allows for the use of liquid chromatography (LC)-based analytical methods such as hydrophobic interaction chromatography (HIC) to assess the heterogeneity of conjugated antibody.[15,6]

The conjugation process, when not entirely optimized, can present diagnostically different HIC distribution profiles (**Fig. 2**) that deviate from profiles containing the expected 5 peaks representing DAR values of 0, 2, 4, 6, and 8.[11,12,17] In the absence of a reference standard or for conjugated drugs that do not possess discrete ultra-violet (UV) absorbance properties, shoulder peaks distributed throughout the chromatogram cannot be unambiguously assigned based on LC-UV methods alone.[12,15,16] These peaks, which may represent ADC isoforms,[12,18] incomplete drug conjugation,[17] or post-translational modifications on the mAb,[19,20] can affect the efficacy and safety[10,15] of the ADC. Additional analysis is required for structural identification if precise assessment of drug loading and distribution are to be determined. For identification purposes, current LC-UV-based methods require the inclusion of an additional purification step followed by analysis using orthogonal techniques such as reversed phase (RP) LC or denaturing capillary electrophoresis (SDS-CE).[12,14,15,17]

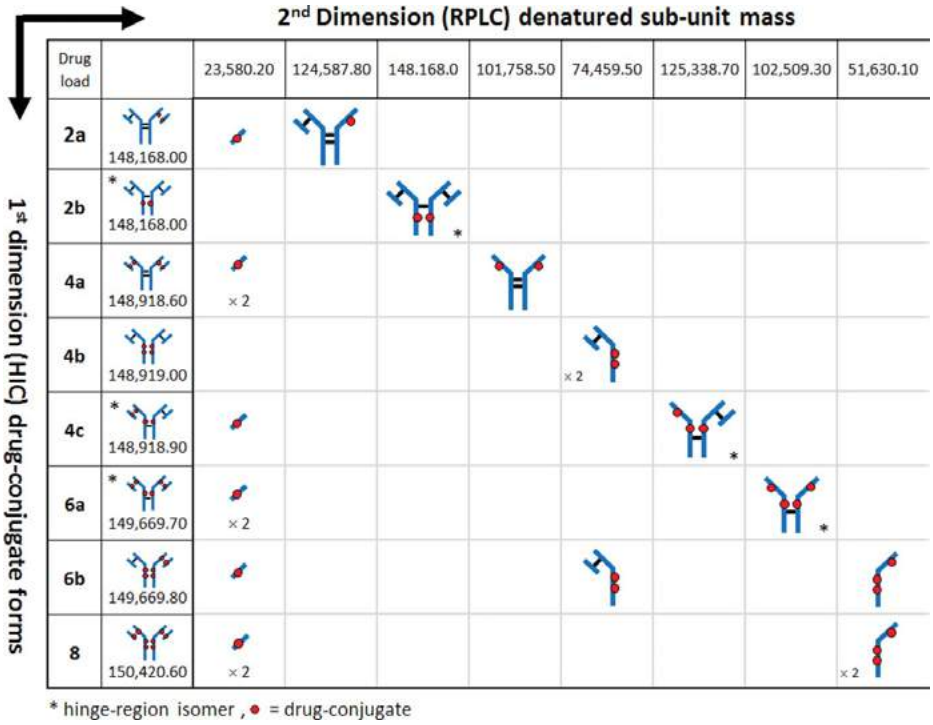
In comparison, non-denaturing mass spectrometry (MS)-based methods have been adopted successfully in the intact analysis of ADCs, including the elucidation of sub-unit conjugated forms and site distribution of conjugated drugs.[21-25] Recently, Valliere-Douglass et al. described a non-denaturing MS-based method for the determination of relative drug distribution in ADCs based on ion abundance and mass from the deconvoluted spectrum of cysteine-conjugated ADCs.[23] A high degree of correlation in relative

abundance for DAR species was observed when compared to traditional LC(HIC)-UV-based methods, despite the question raised on the possible ionization efficiency discrepancy of higher drug-conjugated species. Using a similar approach, Chen et al. improved ionization efficiency by enzymatically removing conjugated drug species while also observing a high degree of correlation with off-line LC(HIC)-UV methods.[22] Innovative techniques that incorporate ion mobility (IM)-MS have also demonstrated their utility in the characterization of IgGs and ADCs.[25-29] Debane and colleagues demonstrated the utility of native MS methods as well as IM-MS in the assessment of drug distribution in cysteine-conjugated ADCs.[25] Similar to Valliere-Douglass et al. and Chen et al., their work found a high degree of correlation between semi-quantitative MS-based approaches and traditional HIC-UV-based methods. Interestingly, Debane et al. found that an offline HIC/native MS approach was able to detect the presence of unexpected odd-numbered conjugated species. The utility of MS-based techniques in the characterization of ADCs is firmly established in the literature. However, similar to LC-UV-based methods, additional steps involving enzymatic treatment are necessary for the elucidation of positional isoforms of cysteine-conjugated ADCs.[24,30] Because this modality has the potential to redefine cancer treatment, rapid methods to identify ADC isoforms during biotherapeutic characterization are highly desirable.

Recent work has demonstrated multidimensional chromatography approaches to be effective in the characterization of impurities,[31] glycopeptides,[32] production excipients,[33] and formulation degradants[34] associated with therapeutic drugs. The straightforward experimental design associated with this methodology provides an elegant solution for the structural identification of peaks observed in LC-UV-based separations of ADCs, bypassing the need for sample preparation procedures such as enzymatic digests and manual fraction collection. As shown in **Table 1**, the coupling of 2 orthogonal separation dimensions increases the separation capacity in cysteine-conjugated ADC analyses and facilitates a method for structural elucidation of peaks observed in ADC HIC distribution profiles. In the 1st dimension (y-axis), ADCs are separated by their hydrophobicity using established HIC techniques. Positional isoforms, which may be represented as additional peaks in the 1st dimension separations (peaks (C)-(H), **Fig. 2**), can be identified by unique sub-unit masses (x-axis) via MS techniques once dissociation occurs under denaturing conditions. In this fashion, multidimensional chromatography renders an efficient method for combining otherwise non-compatible techniques, such as HIC and MS analysis (LC/LC/QTOF-MS) for rapid structural elucidation of species observed in HIC distribution profiles of cysteine-conjugated ADCs.[35,36]

Experimental results from an analysis of 3 batches of an ADC at 3 different DARs by the 2D-LC/QTOF method are presented here. The method preserves intact ADC characterization information while facilitating the online fractionation and identification of peaks under investigation from cysteine-conjugated ADC HIC distribution profiles. The unambiguous identification of peaks associated with distribution profiles in HIC separations of

Table 1. Multidimensional analysis. HIC separation in the 1st dimension (y-axis) separates cysteine-conjugated ADCs based on their hydrophobicity associated with increasing DAR species. When subjected to an orthogonal 2nd dimension (x-axis) separation such as RPLC-MS, cysteine-conjugated ADCs dissociate into their respective sub-units due to denaturation by the mobile phases and temperature employed. The discrete masses generated from the unique sub-units for each conjugated species facilitate structural identification of positional isoforms associated with cysteine-conjugated ADCs



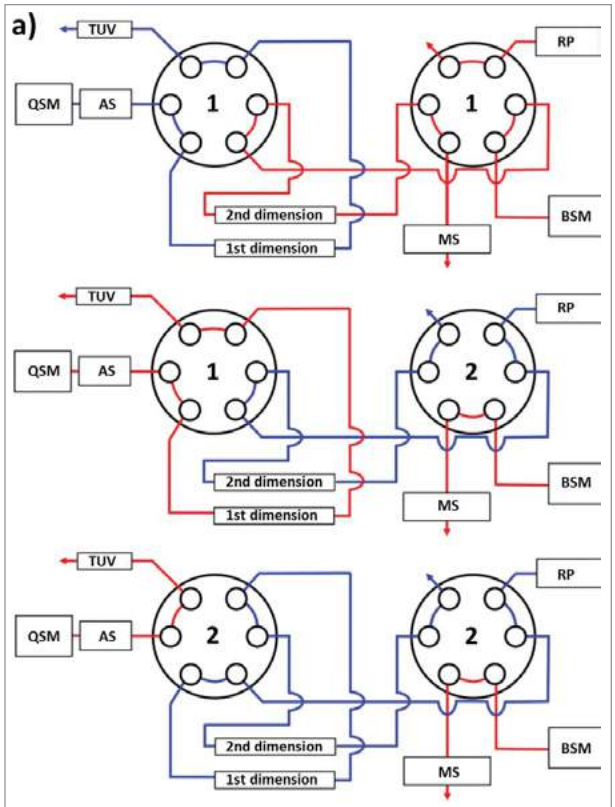
cysteine-conjugated ADCs increase the confidence for accurate determination of DAR and ADC profiles. The results demonstrate that the 2D LC/MS method is well suited for rapid analysis of ADC isoforms for the development and characterization of ADCs.

Results

An IgG1 mAb was modified by cysteine conjugation to a non-toxic drug that mimics the hydrophobicity of a cytotoxic drug to give ADC batches at 3 DARs and analyzed by HIC chromatography to assess drug distribution profiles. As seen in **Figure 2**, multiple peaks were observed in all 3 ADC batches presumably representing individual DARs of 0, 2, 4, 6, and 8. Based on the underlying separation mechanism of HIC, as well as knowledge of the possible isoforms that could be formed by cysteine conjugation using a partial reduction/conjugation process, it was assumed that the elution order was associated with increasing DAR species. The identity of peak (A) was readily confirmed experimentally using the retention time alignment of the unconjugated mAb in a separate HIC separation (supplemental material). The trending reduction in intensity of peaks (A–C) combined with the increasing intensity of peaks (E) and (H) across the low- to high-loaded ADC batches suggests that these peaks represent individual ADC DAR species of 0, 2, 4, 6, and 8, respectively.[15,16] However, assignment of peaks (D)-(H) based on distribution profiles and intensity trends from LC-UV data is challenging considering that conjugation conditions, mAb identity, and the presence of isoforms can affect ADC distribution profiles. To determine if unambiguous peak identification and improved confidence in determining critical quality attributes[11] (CQAs) such as individual and total DAR values could be achieved, an MS-based approach was evaluated.

Mass confirmation of peaks observed in ADC HIC distribution profiles would provide a method for determining the individual DAR values for ambiguous peaks, e.g., (F)-(G) in **Figure 2**. However, the high concentration and low volatility of the salts used in HIC separations are not compatible with direct MS analysis.[35,36] Coupling LC (HIC)-UV to RPLC-MS with selective sampling of LC (HIC)-UV profiles would render an efficient online method for the assessment of CQAs associated with unassigned peaks. In contrast to non-denaturing MS methods, the denaturing conditions used in traditional RPLC, when coupled to HIC, provides a means to remove non-volatile buffer components while simultaneously dissociating ADCs into their respective non-covalently-linked sub-units for identification of isoforms by mass. Such information is currently not directly reflected with non-denaturing MS methods. To this end, an on-line multidimensional chromatography approach was used for elucidating the peak identities.

Coupling of orthogonal analytical techniques (HIC with RPLC/QTOF-MS) was achieved through the use of two 6-port, 2-position valves housed in a column manager (**Fig. 3**). Fractions (or "heart-cuts") from peaks of interest observed in the first dimension HIC separation were selected by coordinated timed switches between the 2 valves so that protein species contained in the HIC fraction could be transferred from the 1st dimension column onto the 2nd dimension column. Subsequent separation on



b) **Heart-cut valve timing example**

Time (min)	Left valve position	Right valve position
Initial	1	1
4.95	1	2
5.00	2	2
5.20	1	2
5.25	1	1

Figure 3. Instrument configuration schematic. (**A**) A column manager housing two 6-port 2-position valves was configured as illustrated by the schematic to perform heart-cuts of the 1st dimension separation. Valve position is denoted numerically as position 1 and 2. Abbreviations are defined as QSM: quaternary solvent manager, AS: auto sampler, TUV: tunable ultraviolet detector, BSM: binary solvent manager, RP: regenerative pump, MS: mass spectrometer. (**B**) Heart-cuts were performed in 0.2 minute intervals at or near the apex of the peak under investigation. An example of the heart-cut being performed on peak (**C**) from **Figure 5c** is illustrated in the example. The heart-cut was bracketed with a 3 second interval to purge residual ammonium sulfate in the fluidic path post 1st dimension column.

the 2nd dimension column (and MS detection) allows the protein species to be identified.

To test the validity of this approach, a separation of the latest-eluting species observed in the HIC distribution profile, which represents the highest DAR species, was targeted for experimentation. To maximize the amount of protein transferred from higher-loaded species (peaks (C)-(H)) to the 2nd dimension column in a relatively small volume, the high loaded ADC batch (**Fig. 2**) was selected for this and subsequent analyses. To ensure column pressure tolerances were not exceeded when both columns were engaged in the fluidic path, a relatively short HIC column (4.6 × 35 mm, 2.5 μm) was employed. Peak profiles were assessed and deemed sufficiently comparable to the profiles observed with the 100 mm version (**Fig. 2**) without the need to scale the method for the shorter column. As shown in **Figure 4a**, a 0.20 min heart-cut was programmed to transfer a 0.100 mL fraction of the late-eluting species (peak (H)) from the HIC separation (visualized by the gray box) to the 2nd dimension RP (C_4) column. The heart-cut was performed post-peak apex to ensure that relatively more homogeneous species were selected and transferred to the 2nd dimension analysis. Desalting of the sample on the RP column, which facilitated coupling of a method that is normally not compatible with MS instrumentation, was performed using a high percentage of aqueous component of the 2nd dimension RPLC mobile phases (100% water containing 0.1% formic acid) with the MS-valve position set to waste. As shown in **Figure 4b**, the subsequent RPLC separation of the fraction of interest resulted in 2 peaks, which represent the dissociated sub-units of the fractionated species. Deconvolution of the raw mass spectrum for each peak is shown in **Figure 4c**. From the deconvoluted spectrum, a mass for the early eluting peak was found to match the predicted mass for a light-chain conjugated to one drug (23,580.0 Da, mass error −8.48 ppm) and masses for the late eluting peak were found to be consistent with the heavy-chain (G0F/G1F glycoforms) with 3 drugs (51,630.6 Da, mass error +9.97 ppm). As shown in **Table 1**, these 2 masses can only be observed for the DAR 8 ADC species, since the 2 dissociated sub-unit peaks in the 2nd dimension separation represent a corresponding sub-unit pair (**Fig. 4b** inset). With mass confirmation of peak (H) as the DAR 8 ADC species, and no higher-loaded species observed across the low-, medium-, and high-loaded ADC samples, the remaining peaks must have DAR values < 8. A complete list of MaxEnt1 parameters and mass shifts can be found in the supplemental material.

Using the same experimental approach, peaks ((C), (E), and (F)) were investigated (**Fig. 5**) using 0.20 min heart-cuts centered at the apex of the target peak. Fractionation of peak (F), as shown in **Figure 5a**, resulted in 3 peaks in the 2nd dimension separation. The deconvoluted masses associated with each peak could only arise from the positional isoform represented by the 6-loaded ADC species (i.e., 6b in **Table 1**). Peak (E), as shown in **Figure 5b**, was determined to be the DAR 6 isomer 6a (see **Table 1**). Meanwhile, analysis of peak (C) confirmed the identity to be the DAR 4 isomer 4a (**Fig. 5c**, **4a**, **Table 1**). A mass difference of ~ 17 Da was observed for 2nd dimension peak 2 from HIC peak (C). This mass loss is likely the result of dehydration of the fragment (i.e., loss of one water molecule), which can occur in the mass spectrometer, HIC, or RP-HPLC environments, or originate in the native conjugate; localization of which requires further studies. However, isoform assignment was not impeded due to the contrast in mass shifts associated with the sub-unit forms shown in **Table 1**, affirming the robustness of such an approach for peak assignment of cysteine-conjugated ADCs.

Interestingly, peak (C) appears to be the major DAR 4 species across all 3 ADC batches based on peak intensities in **Figure 2**, suggesting a kinetically more favorable reaction product compared to the other DAR 4 ADC isomers.[12,37] In addition, for the DAR 6 ADC species, the intensities of peak (F) and peak (G) are lower than that from peak (E) across all 3 samples. Closer inspection of relative peak area indicated peak (F) at 14.1% and peak

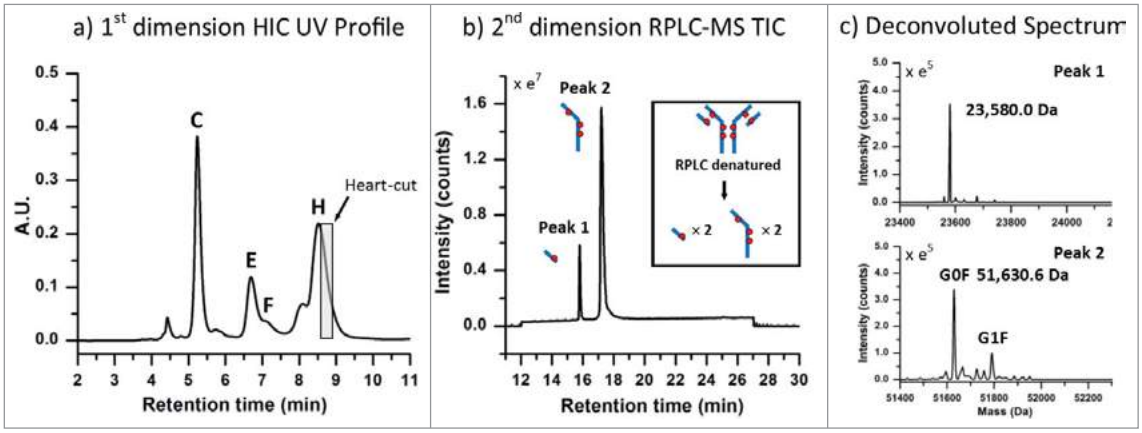


Figure 4. ADC analysis using an LC(HIC)/LC(RP)/QTOF-MS approach. (**A**) A 0.20 min heart-cut (100 μL) was initiated post-apex on the latest eluting peak from the HIC separation (peak (H)) of the high cysteine-conjugated ADC batch. (**B**) The transferred fraction was desalted and separated using a 15 min gradient by RPLC with expected dissociated sub-units shown in the inset. (**C**) MS spectra were deconvoluted and determined to be indicative of the light-chain (23,580.0 Da) and heavy-chain (51,630.6 Da) containing 1 and 3 drugs, respectively.

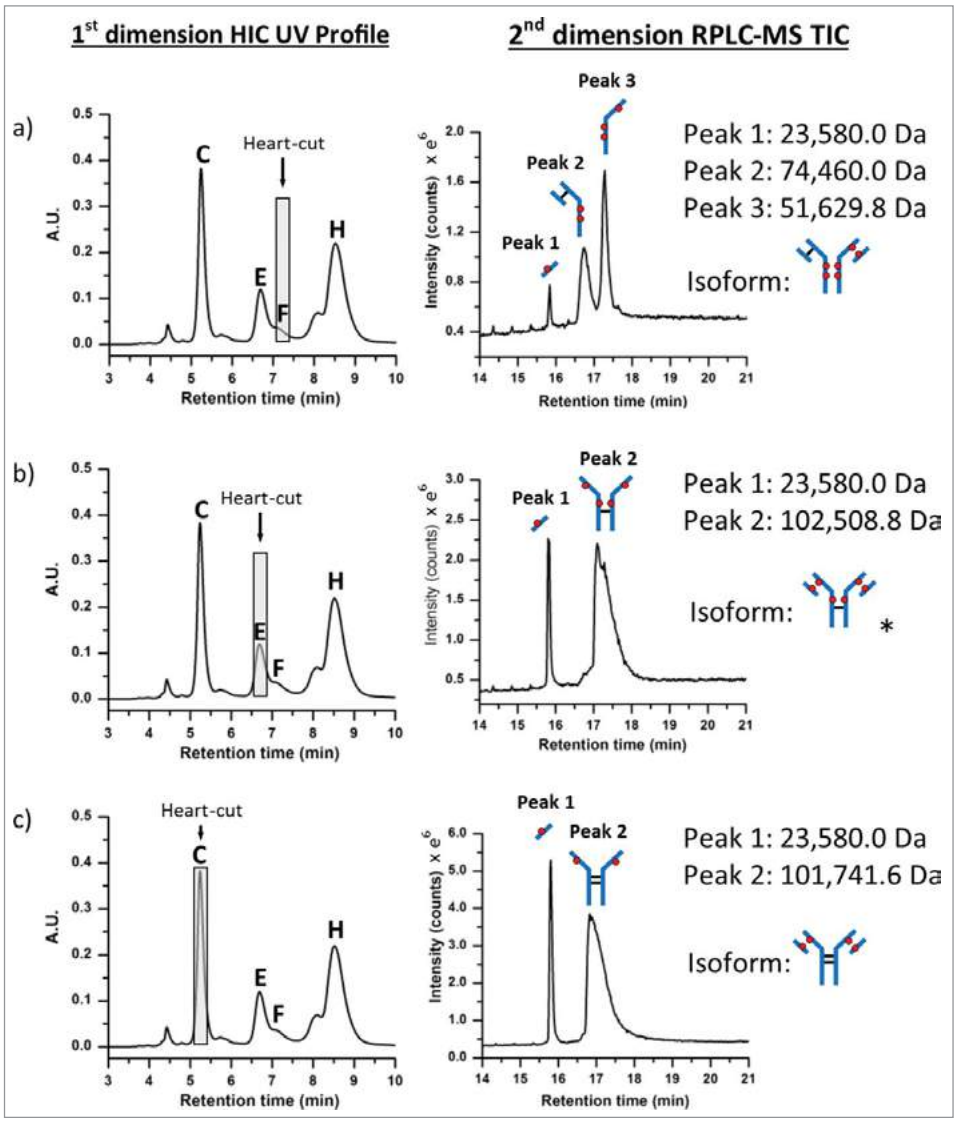


Figure 5. Positional isoform identification using HIC/RP/QTOF-MS. (**A**) A 0.20 min heart-cut fraction of peak (**F**) dissociated into 3 unique sub-unit masses under RPLC conditions which corresponded to an ADC with 6 drugs (DAR 6) as illustrated by the isoform structure provided. (**B**) A 0.20 min heart-cut fraction of peak (**E**) was also determined to be an isomer of DAR 6 with the corresponding structure shown in the illustration. (**C**) A 0.20 min heart-cut of peak (**C**) was confirmed to be an ADC bearing 4 drugs with both light-chain and their corresponding heavy-chain sulfhydryl sites occupied with drugs as shown in the illustration.

(G) at 42.2% maintained similar peak area with respect to peak (E) at 43.7% across the 3 samples. The results suggest that the accessibility of the hinge-region cysteine residues plays a critical role in reaction kinetics of cysteine drug-conjugation chemistry, and conjugation of thiol groups from the cysteine residues that form the light chain–heavy chain disulfide bond is a preferred pathway for this IgG1 mAb under the reduction/conjugation conditions employed. The existence of 2 inter-chain disulfide bonds in the hinge region of all IgG1 molecules suggests that peak (E) may represent either one of the isomers or both isomers. The confirmation of which individual isomer exist (or both) would require additional experiments involving purification and enrichment of the peak, followed by enzymatic treatment for analysis,[30] which is beyond the scope of the current work. Nonetheless, unambiguous identification of the major HIC peaks of cysteine-conjugated ADCs was achieved using the multidimensional chromatography approach. With the additional peaks being identified as ADC isoforms, drug distribution profiles could be readily assigned across all 3 cysteine-conjugated ADC batches as shown in **Figure 6**. With this knowledge, DAR values based on relative abundance from the LC-UV data were determined to be 2.83, 4.44, and 5.97 for the low-, medium-, and high-loaded ADC batches, respectively (**Table 2**).

Discussion

Targeted immunoconjugate-based therapies such as ADCs offer the potential to redefine our understanding and approach to the treatment of cancer. Cysteine-conjugated ADCs using conventional reduction/alkylation.[12] represent a sub-class of ADCs that offer reduced complexity that are readily characterized using LC-UV-based techniques. Accurate assessment of CQAs such as ADC drug distribution and associated DAR values, which can affect efficacy and safety, is critical for the research and development of ADCs. Development of immunoconjugate therapies with diagnostically different chromatographic profiles that deviate from expected profiles requires the use of orthogonal techniques that augment existing characterization methods, which allows accurate assessment of CQAs. The utility of MS-based techniques to gain additional insight in the characterization of biotherapeutics has been well established in literature.[21-29] However, techniques that are incompatible with MS analysis are often encountered in the characterization of ADCs such as in the case of HIC-UV, which is frequently used to assess drug distribution and drug load attributes.[11] Efficient methods that complement existing characterization techniques and can be readily adapted into the biopharmaceutical production environment are highly desirable.

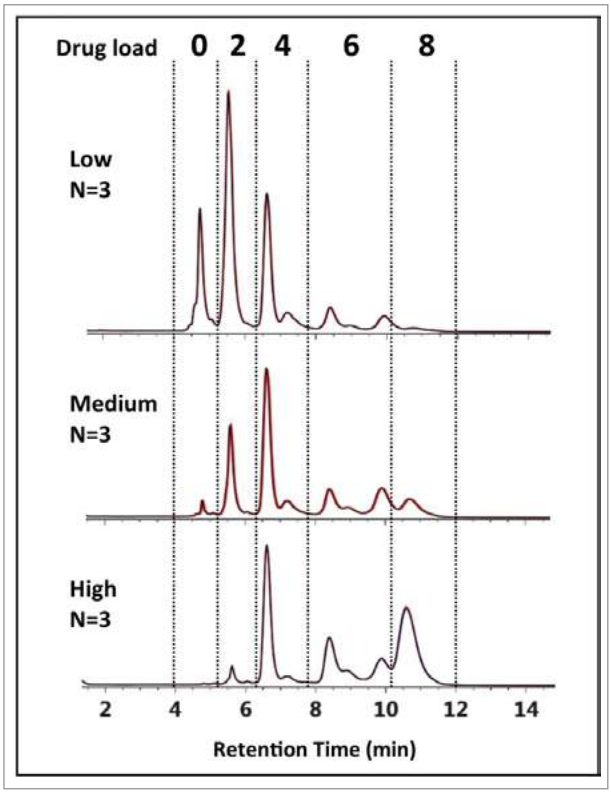


Figure 6. HIC-UV assessment of drug load distribution. Using peak identification determined from the multidimensional chromatography-MS method, drug load distribution was assessed in triplicate for the 3 batches of cysteine-conjugated ADCs. Overlays of the chromatograms (black, red, blue) are shown to demonstrate the reproducibility and robustness of the methodology.

This work has demonstrated that multidimensional chromatography renders a viable interface for the hyphenation of normally non-compatible methods with MS instrumentation. The 2D methodology presented here is particularly useful in providing rapid characterization of positional isoforms associated with conventional cysteine ADCs based on their unique sub-unit structures as well as DAR determinations. It is also applicable in general to characterization of HIC peaks for ADCs with other conjugation chemistries, including site-specific cysteine conjugation,[10,38,39] although information obtained may be tailored to the specific chemistry used for conjugation.

Material and Methods

Chemicals and reagents were purchased from Sigma Aldrich unless otherwise stated. MS-grade solvents were used for mobile phase preparation. Aqueous buffers were prepared with water purified using a lab water filtration system (Millipore).

ADC Stock Solutions

ADCs were prepared according to conventional reduction protocols.[12] To show the proof of concept and for ease of handling, a noncytotoxic drug-mimic was selected as the drug for this study. Tris(2carboxyethyl)phosphine hydrochloride (TCEP) was added to a solution of the IgG1 mAb in phosphate-buffered saline (pH 7.2) to reduce the inter-chain disulfide bonds. The maleimide-containing drug-mimic ($C_{23}H_{24}N_2O_3$, MW = 376.46 Da) was then added and allowed to react. TCEP and drug-mimic concentrations were controlled to produce 3 stock samples with increasing DAR (low-, medium-, and high-loaded samples). Following the conjugation reaction, the ADC mixtures were buffer-exchanged into 20 mM histidine buffer (pH 6.0) and adjusted to a concentration of ~ 10.0 mg/mL. An unconjugated mAb stock was also prepared at ~ 10.0 mg/mL. The stock solutions were stored at −80°C prior to use.

Sample Preparation

HIC samples were prepared at a concentration of 2.0 mg/mL in 1M $(NH_4)_2SO_4$ using a 62.5mM sodium phosphate buffer, pH 6.7. Analyses were performed on a 10.0 µL injection volume. A reversed phase chromatography control sample was prepared through partial reduction of the unconjugated mAb. Briefly, a 1.0 mg/mL solution of unconjugated mAb prepared in 25mM phosphate buffer, pH 7.8, was reduced in 1 mM dithiothreitol (DTT) at 37°C for 20 min. An equal amount of sample was mixed with 0.1% formic acid (v/v) in purified water to give a final concentration of 0.5 mg/mL.

Table 2. Drug-to-antibody ratio determination. Individual DAR contributions for each drug loaded species (e.g., DAR 0, 2, 4, 6, 8) was calculated based on the sum relative peak area for the distribution species denoted by the dashed lines in **Figure 6** The average DARs were determined to be 2.83, 4.44, and 5.97 for the low-, medium-, and high-loaded ADC batches, respectively

	Drug load																
N=3	0			2			4			6			8				
Batch	Area (%)	RSD (%)	DAR contribution	Area (%)	RSD (%)	DAR contribution	Area (%)	RSD (%)	DAR contribution	Area (%)	RSD (%)	DAR contribution	Area (%)	RSD (%)	DAR contribution	Total DAR	RSD (%)
Low	16.73	0.28	0	40.71	0.23	0.81	28.56	0.24	1.14	12.54	0.50	0.75	1.50	10.11	0.12	2.83	0.38
Med	2.80	0.34	0	18.85	0.46	0.38	41.69	0.22	1.67	26.87	0.13	1.61	9.79	1.69	0.78	4.44	0.20
High	0.37	1.45	0	3.42	0.14	0.07	30.67	0.18	1.23	28.60	0.32	1.72	36.90	0.37	2.95	5.97	0.06

Chromatography

An ACQUITY H-Class Bio equipped with a commercially available 2D technology configuration (Waters Corp.) was used for the experiments. **Figure 3A** is a schematic of the instrument setup denoting column, pump, and plumbing configuration. Mobile phase (MP) reservoirs for the 1st dimension quaternary solvent manager (QSM) were prepared as follows: MP A: 2.5 M $(NH_4)_2SO_4$ in 125 mM sodium phosphate buffer, pH 6.7, MP B: 125 mM sodium phosphate buffer, pH 6.7, MP C: n-propanol, MP D: 18 MΩ purified water. A 4.6 × 35 mm, 2.5 µm n-butyl HIC column (Waters Corp.) was conditioned with the unconjugated mAb until the integrated area and retention time stabilized using a 10 min gradient from 1.25 M $(NH_4)_2SO_4$, in 65 mM sodium phosphate buffer, pH 6.7, 5% n-propanol (50% MP A : 0% MP B : 5% MP C : 45% MP D) to 65 mM sodium phosphate buffer, pH 6.7, 5% n-propanol (0% MP A : 50% MP B : 5% MP C : 45% MP D). Mobile phase conditions were held constant for 5 min at the end of each gradient followed by 15 min of column reconditioning at initial conditions. Column temperature and flow rate were set at 25°C and 0.500 mL/min, respectively.

Mobile phase reservoirs for the 2nd dimension binary solvent manager (BSM) were prepared as follows: MP A: aqueous solution containing 0.1% formic acid v/v, MP B: acetonitrile containing 0.1% formic acid v/v. A 2.1.mm × 50.mm, 1.7 µm reversed-phase C_4 column (BEH C_4, Waters Corp.) was conditioned with the reduced unconjugated mAb in a 1D configuration until the integrated area and retention time stabilized. For RP separations, fractions of interest were transferred from the 1st dimension HIC column to the 2nd dimension C_4 column using a regenerative pump with the left and right valves set in position 2 (**Fig. 3a**). Once transferred, a 12 min gradient from 100% A to 80% A was performed to stack and de-salt the fraction of interest using the binary solvent manager. After the 12-min desalting period, a 15 min separation gradient was performed from 80% to 50% MP A. At the end of the gradient, a 3 min column reconditioning step was performed at initial conditions. Column temperature and flow rate were set at 80°C and 0.500 mL/min, respectively.

Transfer of fractions of interest or "heart-cuts" from the 1st dimension column to the 2nd dimension column were programmed through the events tab using the column manager control interface. Valve position timing is illustrated in **Figure 3b** and were adjusted for each peak apex under investigation. A regenerative pump delivering an isocratic gradient of MP B from the 1st dimension separation at 0.500 mL/min was used to perform the heart-cut and to reduce the amount of residual ammonium sulfate in the fluidic path post 1st dimension column. A 0.05 min delay was programmed at the beginning and end of each heart-cut to purge the fluidic path of residual ammonium sulfate using 50% MP B : 5% MP C : 45% MP D from the 1st dimension reservoirs. A tunable ultra-violet (TUV) detector (Waters Corp.) equipped with a 5-mm titanium flow cell was incorporated post the 1st dimension separation to monitor the heart-cut procedure. Single wavelength detection was performed at an A_{max} of 220 nm with a sampling rate of 20 Hz.

Ms settings

A quadrupole time-of-flight mass spectrometer (Xevo G2 QTof, Waters Corp.) was used for MS analysis post 2nd dimension column (**Fig. 3a**). Continuum data were acquired in sensitivity mode with positive polarity. A mass range from 500 to 4,000 m/z was used for data collection, and the MS data was only collected from 12 to 27 min as defined in the chromatography section. The flow was directed to waste via the MS valve event manager when the 2D system was operated outside the acquisition time window. Additional instrument settings were set as follows: capillary voltage 3.00 kV, sample cone, 80.0 V, extraction cone, 4.0 V, source temperature 100°C, desolvation temperature 350.°C, and desolvation gas flow 600 L/Hr. Data from the MS analysis for the ADC subunits and the light chain and heavy chain of native mAbs were processed by MaxEnt 1 algorithm within MassLynx. Typically twenty iterations of MaxEnt 1 deconvolution were performed on the raw spectrum, and a mass accuracy error ≤10 ppm was achieved for the light-chain and non-deglycosylated heavy-chain. Individual parameter settings can be found in the supplemental material.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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Characterization of Biotherapeutics: ACQUITY UPLC H-Class Bio with 2D Part 1 of 3: On-line Desalting of Biotherapeutic Samples for Increased Productivity

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APPLICATION BENEFITS

- Increase productivity through the automation of the fractionation processes using liquid chromatography
- Increase efficiency through the on-line desalting of complex biotherapeutic samples

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System with 2D Technology

ACQUITY UPLC Photodiode Array (PDA) Detector

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP, 7 µm, 4.6 x 100 mm, SCX Column

ACQUITY UPLC Protein BEH Technology™ C_4, 300Å, 1.7 µm, 2.1 x 50 mm Column

KEY WORDS

Multidimensional chromatography, 2D-LC, enrichment, desalting, cation exchange, antibody, IEX, bioseparation, therapeutic, protein, 2D technology

INTRODUCTION

Characterization of charge variants in biotherapeutic proteins often relies on charge-based separation techniques such as ion exchange chromatography (IEX).[1,2] While IEX separations are powerful in gathering information such as charge variant composition, the use of salts and buffer components prevents direct coupling to mass spectrometry (MS) to elucidate peak identity.[3] Chromatographic fractions of interest are often manually collected and desalted offline prior to MS analysis, which negatively impacts overall analysis time and productivity.

Waters' solution to this challenge is the ACQUITY UPLC H-Class Bio System with 2D Technology featuring a heart-cut technique. The fidelity of this 2D UPLC® system makes it well suited for the automated fractionation and desalting of peaks of interest for increased productivity. Tandem column configurations (e.g., IEX/RPLC), when used with synchronized valve switching, can be used to heart-cut peaks of interest from the 1st dimension column to the 2nd dimension column. The 2nd dimension column acts as a trapping/desalting column where samples are desalted and eluted in solvents compatible with down-stream analyses, such as MS analysis or enzymatic digestion.

The objective of this three-part application series is to collectively demonstrate that the ACQUITY UPLC H-Class Bio System with 2D Technology offers advantages for the on-line fractionation and desalting (part 1) of challenging biological samples and is a viable interface for IEX with MS analysis (part 2). The system's ability to increase productivity across platforms will also be demonstrated with the on-line enrichment of low abundance species for peptide analysis (part 3). A therapeutic monoclonal antibody, infliximab, was used to demonstrate this.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class Bio with 2D Technology
	1st dimension pump: ACQUITY UPLC Quaternary Solvent Manager, ACQUITY UPLC Column manager
	2nd dimension pump: ACQUITY UPLC Binary Solvent Manager, ACQUITY UPLC Autosampler with FTN
Detectors:	(1st dimension) ACQUITY UPLC TUV (2nd dimension) ACQUITY UPLC PDA
Absorption wavelength:	280 nm
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm (p/n 186004930)
	ACQUITY UPLC BEH C_4, 300Å, 1.7 µm, 2.1 x 50 mm, (p/n 186004495)
Column temp.:	25 °C (IEX); 80 °C (C4)
Sample temp.:	4 °C
Injection vol.:	2 µL unless otherwise stated

IEX/RPLC Pump configuration

Quaternary solvent manager:

Flow rate:	0.500 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Auto•Blend Plus™ setting:	20 mM MES buffer, pH 6.5, 25–65 mM NaCl in 15 minutes

Binary solvent manager:

Flow rate:	0.250 mL/min for heart-cut, otherwise 0.500 mL/min
Mobile phase A:	18MΩ H_2O, 0.1% FA
Mobile phase B:	Acetonitrile, 0.1% FA
Gradient:	5–85% B in 10 minutes

Data management

MassLynx® Software v4.1 (SCN 8.62)

The Waters Protein-Pak Hi Res SP, 7-µm, 4.6 x 100 mm, strong cation exchange column (p/n 186004930) and ACQUITY UPLC Protein BEH C_4 Column, 300Å, 1.7 µm, 2.1 x 50 mm, (p/n 186004495) were conditioned prior to use. Chemical reagents were purchased from Sigma Aldrich and used as received. The monoclonal antibody infliximab was received at a concentration of 20 mg/mL in formulation buffer.

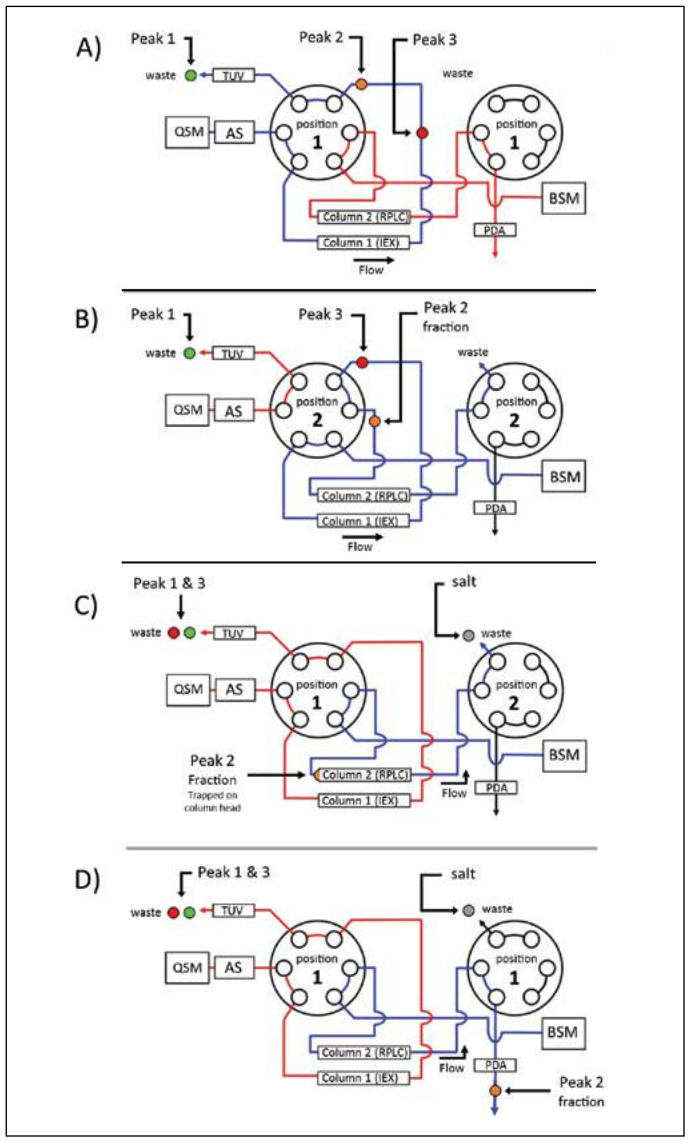

Figure 1. A plumbing diagram of the ACQUITY UPLC H-Class Bio System with 2D Technology and corresponding valve positions to perform a heart-cut are illustrated in A–D. A) Three charge variants (peak 1–3) are separated in the 1st dimension (IEX) using the ACQUITY UPLC Quaternary Solvent Manager to deliver a salt gradient. B) Synchronized valve switching diverts the flow path to the 2nd dimension where a fraction (heart-cut) of peak 2 is directed to the 2nd dimension column (RPLC). C) Independent valve control allows the left valve to re-engage flow to the 1st dimension column while the right valve remains in position 2 where the heart-cut of peak 2 has unbound salt removed under aqueous conditions from the ACQUITY UPLC Binary Solvent Manager. D) The right valve is returned to position 1 after desalting the heart-cut fraction and a standard reversed phase gradient is used to elute the fraction for down-stream analyses.

RESULTS AND DISCUSSION

ACQUITY UPLC H-Class Bio System with 2D Technology featuring heart-cut technology

The ACQUITY UPLC H-Class Bio System with 2D Technology featuring the heart-cut process is readily deployed with a two-column configuration as shown in Figure 1. With both valves in position 1 (Figure 1A), the flow from both the quaternary solvent manager and binary solvent manger are independent of each other, allowing for independent gradients to be performed on column 1 and 2.

The heart cut is performed when the valve positions are temporarily switched to position 2 (Figure 1B), combining the flow paths (Figure 1B blue trace) where effluent from column 1 is redirected to column 2. The ACQUITY UPLC Column Manager supports independent valve control as shown in Figure 1C. With the left valve in position 1 and the right valve in position 2 the flow paths of each column are isolated again, with the 2nd dimension column being eluted to waste. This allows for unbound salts to be washed from the heart-cut fraction, which is trapped at the column head of the 2nd dimension column, using the aqueous phase of the 2nd dimension.

Once desalted, the heart-cut fraction can be readily eluted in a mobile phase amenable to down-stream analyses using the ACQUITY UPLC Binary Solvent Manager.

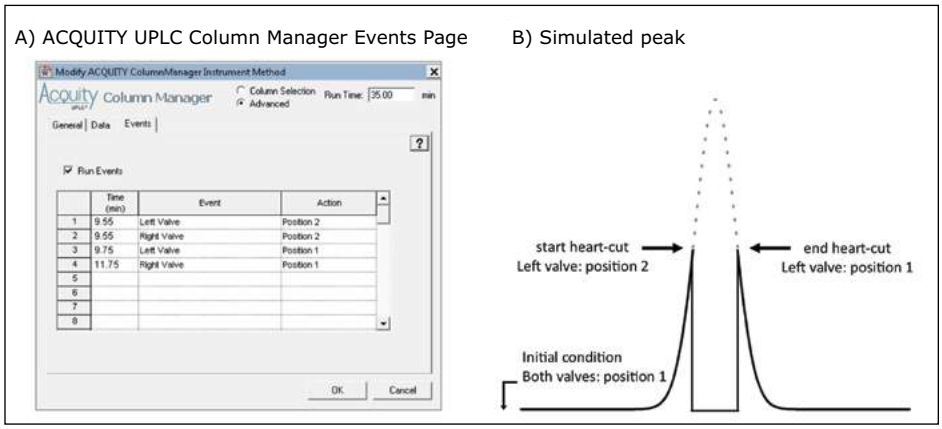

Figure 2. Programming the heart-cut.
A) The heart-cut process is readily programmed from the integrated event table of the ACQUITY UPLC Column Manager.
B) The simulated peak trace illustrates the expected chromatographic profile from the 1st dimension when performing a heart-cut.

The heart-cut process is readily engaged using the integrated event table of the ACQUITY UPLC Column Manager as shown in Figure 2A. When enabled the event table allows for independent programming of the left and right valve and their respective position. An illustration of the expected effect of the valve switches on the chromatographic profile are shown in Figure 2B where the "heart" of the peak is re-directed to the 2nd dimension column, bypassing the ACQUITY UPLC TUV Detector as illustrated in Figure 1B, resulting in the characteristic profile. Adaptable column configurations, combined with an easy-to-use integrated interface, demonstrates the ACQUITY UPLC H-Class Bio System with 2D Technology is well suited for fractionation, desalting of challenging biological samples.

On-line desalting of lysine variants using 2D UPLC

Biotherapeutics are often processed in non-denaturing buffers containing salts, surfactants, and sugars. Additives such as these often need to be removed via desalting/buffer exchange columns or diluted to facilitate down-stream analyses that incorporate mass spectrometry or enzymatic digests. The heart-cut technology featured in the ACQUITY UPLC H-Class Bio System with 2D Technology is readily deployed with a two column configuration (IEX/RPLC) as shown in Figure 1 for on-line desalting of biotherapeutics.

The Waters Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm, SCX Column was used as the 1st dimension column with the Auto•Blend Plus Technology delivering a 15-minute salt gradient from 25–65 mM NaCl in 20 mM MES buffer, pH 6.8. Figure 3 shows a deglycosylated sample of infliximab where a heart-cut is being performed on the +0 lysine truncation variant from 9.55 min to 9.75 min using the ACQUITY UPLC Binary Solvent Manager Pump at a flow rate of 0.250 mL/min to elute the fraction to the 2nd dimension column.

As seen in the inset of Figure 3A the optical trace when performing the heart-cut exhibited the characteristic profile as illustrated in Figure 2B. The eluent flow containing the fraction of interest was redirected to an ACQUITY UPLC BEH300 C_4 Column (2nd dimension) where it was retained at the head of the column. The heart-cut fraction was desalted for 5 minutes at a flow rate of 0.500 mL/min using 100% of the aqueous component of the 2nd dimension column (H_2O, 0.1%FA v/v) as shown in Figure 3B. The desalted fraction of interest was eluted in the 2nd dimension using a 10 minute gradient from 0%–95% acetonitrile 0.1% FA, starting at the 15 minute mark using the ACQUITY UPLC Binary Solvent Manager.

The successful fractionation and desalting of a heart-cut fraction demonstrates the utility of using an ACQUITY UPLC H-Class Bio System with 2D Technology to increase efficiency in sample preparation of complex biotherapeutics.

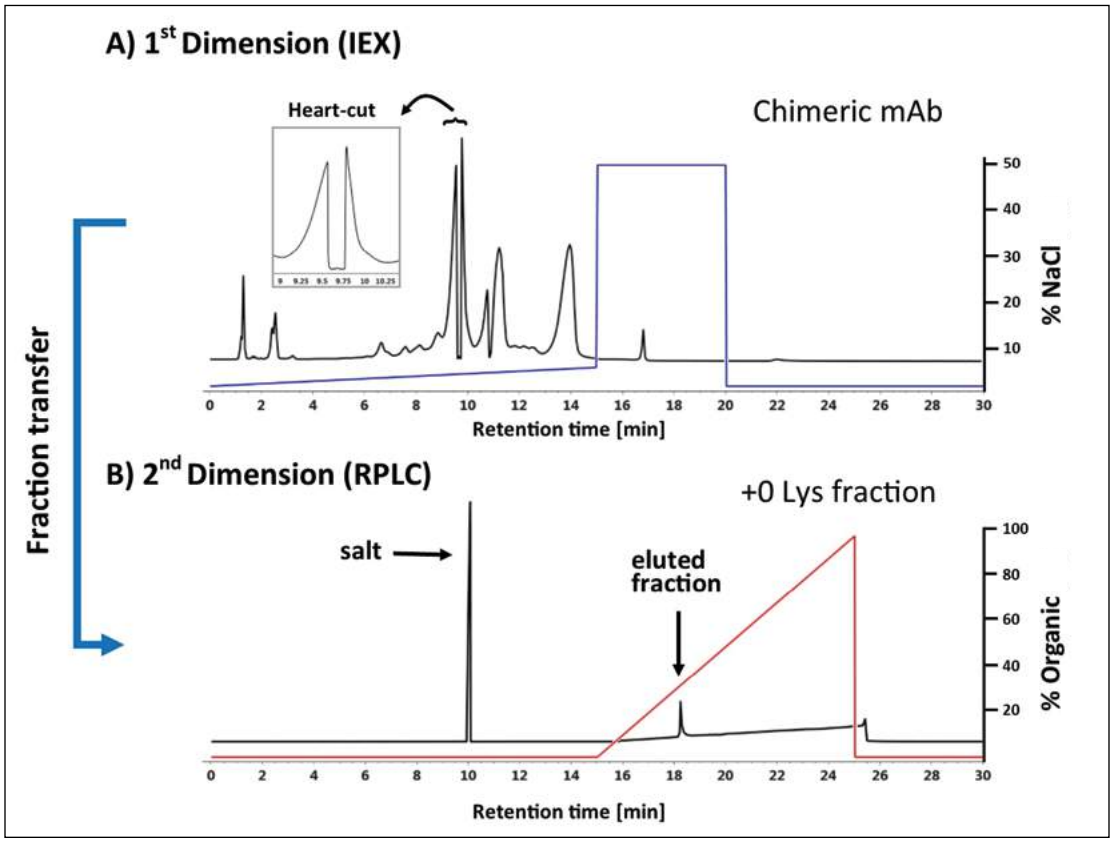

Figure 3. On-line Desalting of a heart-cut fraction.

A) The +0 Lys charge variant of infliximab was fractionated using the heart-cut technology and transferred to the

B) 2nd dimension column where the salt was removed from the sample prior to elution with an organic solvent.

**The sharp drop in absorbance at 11 minutes proceeding the heart-cut was due to the change in mobile phase composition passing through the 1st dimension column from the ACQUITY UPLC Binary Solvent Manager Pump.*

CONCLUSION

Increasing demand for informational content in the characterization of biotherapeutics, combined with a fast-paced work environment, require efficient solutions that offer flexibility. The ACQUITY UPLC H-Class Bio System with 2D Technology is Waters' solution to these challenges. The heart-cut feature offered with the system is well suited for fractionation and desalting of challenging biological samples. Compatibility with multiple column configurations and the ability to automate the process offers today's analyst the flexibility needed to maximize information in the characterization of biotherapeutics without compromising productivity.

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Characterization of Biotherapeutics: ACQUITY UPLC H-Class Bio with 2D Part 2 of 3: Rendering a Viable Interface for IEX with ESI-MS Analysis

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APPLICATION BENEFITS

- Increased productivity through the automation of the fractionation processes using an ACQUITY UPLC H-Class Bio System with 2D Technology
- Improved efficiency through on-line desalting of complex biological therapeutic samples
- 2D UPLC® is a viable option for interfacing IEX with ESI-MS

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System with 2D Technology

ACQUITY UPLC Photodiode Array (PDA) Detector

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP, 7 µm, 4.6 x 100 mm, SCX Column

ACQUITY UPLC Protein BEH Technology C_4, 300Å, 1.7 µm, 2.1 x 50 mm Column

Xevo® G2 QTof MS

KEY WORDS

Multidimensional chromatography, 2D-LC, enrichment, desalting, cation exchange, antibody, IEX, bioseparation, therapeutic protein

INTRODUCTION

Charge variants, such as C-terminal lysine variants in monoclonal antibodies, are known to be sensitive to manufacturing processes.[1-3] Developing efficient methods for characterization and identification of charge variants in biotherapeutic proteins for monitoring and control of production processes is an area of continued interest in the pharmaceutical industry.

Orthogonal techniques such as ion exchange chromatography (IEX) and mass spectrometry (MS) are often employed to maximize the information gained in the characterization of biotherapeutics.[4] While IEX separations are powerful in gathering information such as charge variant composition, the use of salts and buffer components prevents direct coupling to mass spectrometry (MS) to elucidate peak identity.[5] Chromatographic fractions of interest are often manually collected, and desalted offline prior to MS analysis, which negatively impact overall analysis time and productivity.

Part 1 of this three-part application series demonstrated that the Waters ACQUITY UPLC H-Class Bio System with 2D Technology offers an efficient solution for increasing productivity in the fractionation and desalting of charge variants in complex biotherapeutic samples. When coupled to a mass spectrometer, the ACQUITY UPLC H-Class Bio System with 2D Technology renders an efficient method for combining complementary orthogonal techniques employed in the monitoring of C-terminal lysine variants in therapeutic monoclonal antibodies without compromising productivity.

The objective of this application note is to demonstrate that the ACQUITY UPLC H-Class Bio System with 2D Technology is a viable option for interfacing IEX to electrospray ionization (ESI)-MS. A therapeutic monoclonal antibody, infliximab, was used as a model protein to evaluate the functionality.

EXPERIMENTAL

The Waters Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm, strong cation exchange column (p/n 186004930) and ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm, 2.1 x 50 mm Column (p/n 186004495) were conditioned prior to use. Chemical reagents were purchased from Sigma Aldrich and used as received. The monoclonal antibody infliximab was received at a concentration of 20 mg/mL.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio with 2D Technology 1st dimension pump: ACQUITY UPLC Quaternary Solvent Manager, ACQUITY UPLC Column manager 2nd dimension pump: ACQUITY UPLC Binary Solvent Manager, ACQUITY UPLC Autosampler with FTN, ACQUITY UPLC Column Manager
Detectors:	(1st dimension) ACQUITY UPLC TUV (2nd dimension) ACQUITY UPLC PDA
Absorption wavelength:	280 nm
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm (p/n 186004930) ACQUITY UPLC BEH C_4, 300Å, 1.7 µm, 2.1 x 50 mm (p/n 186004495)
Column temp.:	25 °C (IEX); 80 °C (C4)
Sample temp.:	4 °C
Injection vol.:	2 µL unless otherwise stated

IEX/RPLC Pump Configuration

Quaternary solvent manager:

Flow rate: 0.500 mL/min

Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Auto•Blend Plus™ setting:	20 mM MES buffer, pH 6.5, 25–65 mM NaCl in 15 minutes

Binary solvent manager:

Flow rate:	0.250 mL/min for heart-cut, otherwise 0.500 mL/min
Mobile phase A:	18MΩ H_2O, 0.1% FA
Mobile phase B:	Acetonitrile, 0.1% FA
Gradient:	5–85% B in 10 minutes

MS conditions

Capillary:	3kV
Sample cone:	45 V
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas:	800 L/h

Data Management

MassLynx Software v4.1 (SCN 8.62)

RESULTS AND DISCUSSION

ACQUITY UPLCH-Class Bio System with 2D Technology featuring heart-cut technology

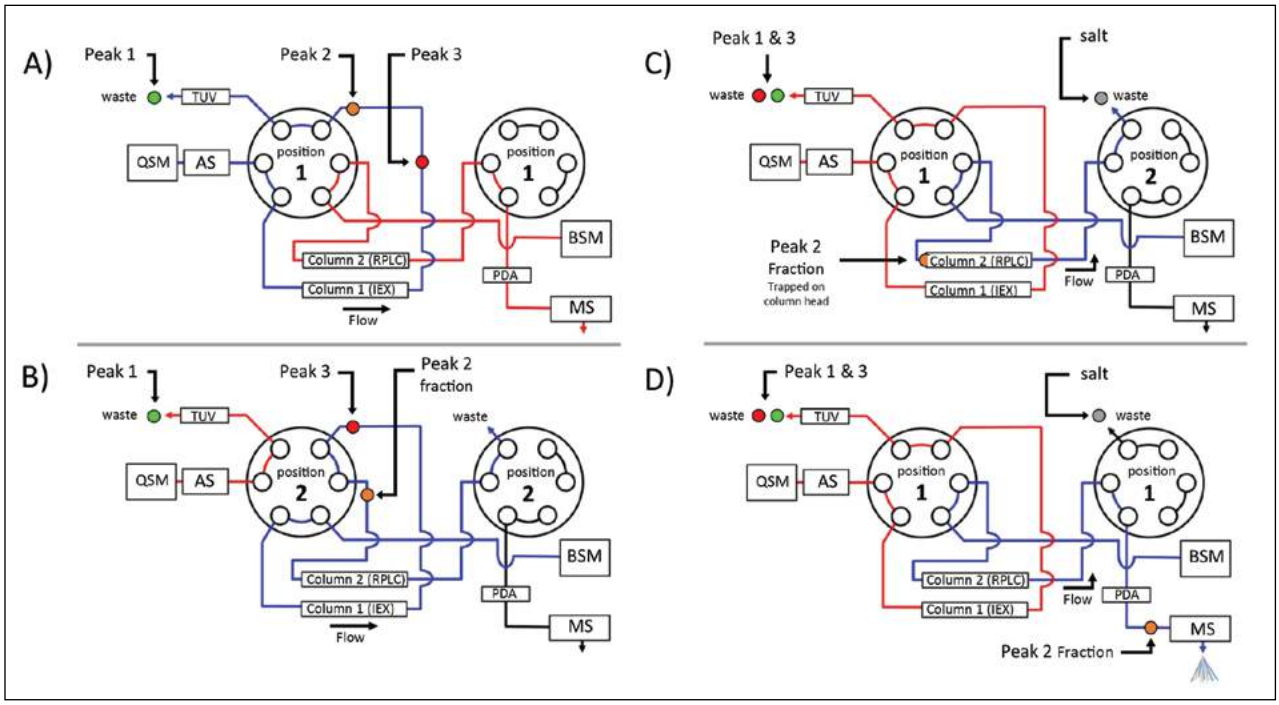

Figure 1. A plumbing diagram of the ACQUITY UPLC H-Class Bio System with 2D Technology and corresponding valve positions to perform a heart-cut are illustrated in A–D. A) Three charge variants (peak 1–3) are separated in the 1st dimension (IEX) using the ACQUITY UPLC Quaternary Solvent Manager to deliver a salt gradient. B) Synchronized valve switching diverts the flow path to the 2nd dimension where a fraction (heart-cut) of peak 2 is directed to the 2nd dimension column (RPLC). C) Independent valve control allows the left valve to re-engage flow to the 1st dimension column while the right valve remains in position 2 where the heart-cut of peak 2 has unbound salt removed under aqueous conditions from the ACQUITY UPLC Binary Solvent Manager. D) The right valve is returned to position 1 after desalting the heart-cut fraction and a standard reversed phase gradient is used to elute the fraction for MS analysis.

As described in part 1 of this three part series, the ACQUITY UPLC H-Class Bio System with 2D Technology featuring the heart-cut process is readily deployed with a two-column configuration as shown in Figure 1. With both valves in position 1 (Figure 1A), the flow from both the quaternary solvent manager and binary solvent manger are independent of each other, allowing for independent gradients to be performed on column 1 and 2.

The heart cut is performed when the valve positions are temporarily switched to position 2 (Figure 1B), combining the flow paths (Figure 1B blue trace) where eluent from column 1 is redirected to column 2. The ACQUITY UPLC Column Manager supports independent valve control as shown in Figure 2C. With the left valve in position 1 and the right valve in position 2 the flow paths of each column are isolated again, with the 2nd dimension column being eluted to waste. This allows for unbound salts to be washed from the heart-cut fraction, which is trapped at the column head of the 2nd dimension column, using the aqueous phase of the 2nd dimension.

Once desalted, the heart-cut fraction can be readily eluted in a mobile phase amendable to MS analysis using the ACQUITY UPLC Binary Solvent Manager, demonstrating the ACQUITY UPLC H-Class Bio System with 2D Technology facilitates a viable option for interfacing IEX to MS analysis.

Auto•Blend Plus for reproducible 1st dimension separations

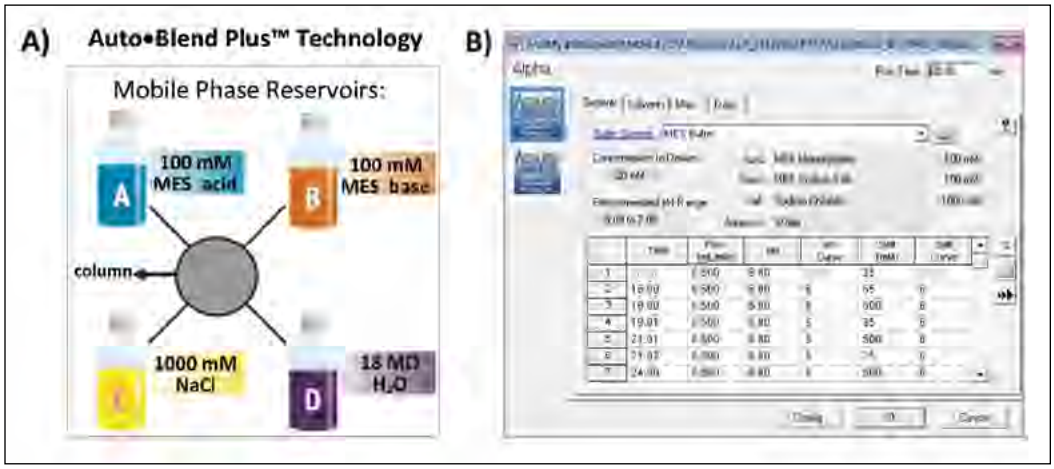

Figure 2. Auto•Blend Plus Technology. A) Auto•Blend Plus Technology automatically blends the desired gradients from concentrated stocks using pure solutions in combination with a quaternary solvent management system. B) The intuitive interface can be programmed to generate gradient conditions with either constant salt or pH.

One aspect of successful multidimensional chromatography relies on reproducible 1st dimension separations. Aqueous separations such as IEX can involve preparing multiple sets of buffers over the course of a project. This iterative process is time consuming and has an increased potential for error in buffer preparation resulting in irreproducible separations.

Auto•Blend Plus Technology is an integrated software solution designed to increase productivity and reproducibility of chromatographic separations. Auto•Blend Plus incorporates a solvent management system using pure solutions and concentrated stocks (Figure 2). The end user is presented with an easy-to-use gradient table interface, where the gradient is expressed directly in terms of pH and ionic strength. The software then automatically calculates the percentage of acid and base required for the specified pH using the known pK_a value of the chosen buffer system or a small empirical calibration table. Auto•Blend Plus allows for multiple buffer compositions to be tested from a single set of pure components and can be easily automated to increase productivity.

Biotherapeutic characterization employing orthogonal techniques on-line with 2D UPLC/MS

Biotherapeutics undergo routine analysis throughout the manufacturing process to ensure regulatory guidelines are met with regards to product quality. Orthogonal techniques that can be employed to characterize the homogeneity (or lack thereof) in biotherapeutics drugs without compromising productivity are highly desirable.

The ACQUITY UPLC H-Class Bio System with 2D Technology is capable of combining complementary orthogonal detection techniques on-line for improved productivity. The heart-cut technology featured in the system is readily deployed with a two column configuration (IEX/RPLC) as shown in Figure 1 for on-line desalting of biotherapeutic samples, which was demonstrated in part I of this application series.

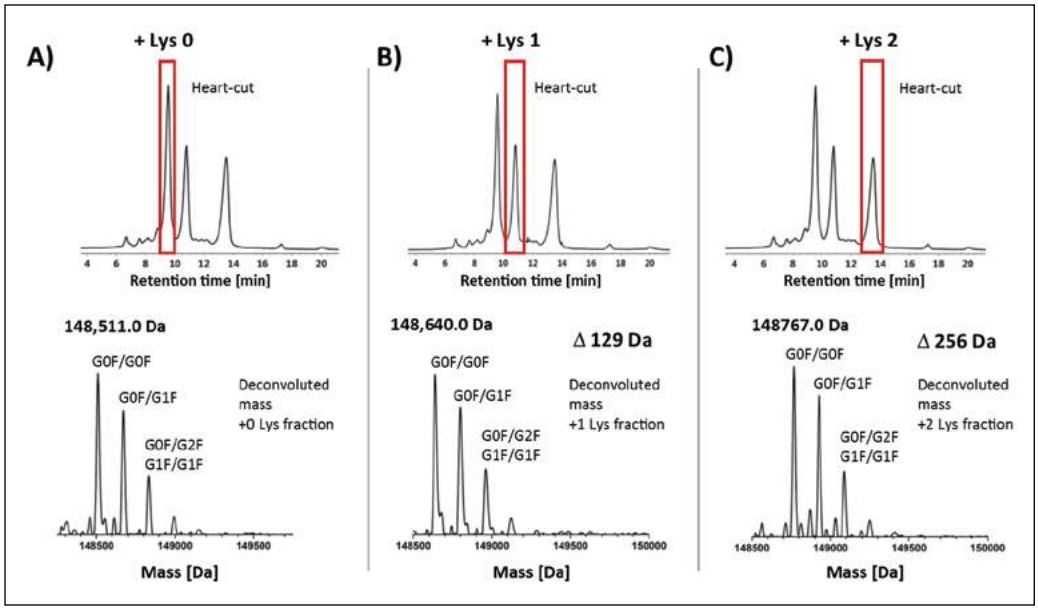

Figure 3. Lysine variant characterization using 2D UPLC/MS. Individual heart-cuts of the A) Lys +0, B) Lys +1, and C) Lys +2 were performed on the bio therapeutic infliximab. Heart-cut fractions were desalted on-line and eluted to a Xevo Mass Spectrometer. Deconvoluted mass are shown below the corresponding heart-cut.

To demonstrate the MS amenability of this technology in comparability analyses, a Xevo G2 QTof Mass Spectrometer was interfaced with the 2D UPLC system after the ACQUITY UPLC Photodiode Array Detector as shown in Figure 1. Heart-cuts were performed on the individual lysine variants as outlined by the red box in Figure 3A–C. Using the same method and gradients as described in part 1, heart-cut fractions were desalted and eluted with the BSM pump to the source of the Xevo Mass Spectrometer (settings in MS conditions).

From the deconvoluted mass spectrums shown in Figure 3, the infliximab +0 Lys, +1 Lys, and +2 Lys peak mass were determined to be 148,511.0 Da, 148,640.0 Da, and 148,767.0 Da, respectively. The +1 Lys and +2 Lys peaks were determined to have a mass difference of Δ129 Da, and Δ256 Da, respectively, which correlates to the addition of one and two lysine residues (average mass 128 Da) while the glycosylation profile was confirmed to be identical between the major isoforms.

This application demonstrates that the ACQUITY UPLC H-Class Bio System with 2D Technology is well-suited for desalting of biological samples and renders a viable option to interface IEX with ESI-MS analysis as an orthogonal detection technique.

CONCLUSION

Characterization methods of biotherapeutic proteins that provide increased informational content without compromising productivity require efficient solutions that are adaptable to the high-throughput environment of industry. The ACQUITY UPLC H-Class Bio System with 2D Technology offers an efficient method for the characterization of biotherapeutics with the ability to combine orthogonal detection techniques on-line for improved productivity.

The heart-cut feature offered with the system is well suited for fractionation and desalting of challenging biological samples. Compatibility with multiple column configurations and the ability to automate the process offers today's analyst a flexible and efficient means to maximize the information obtained during characterization of biotherapeutics.

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Characterization of Biotherapeutics: ACQUITY UPLC H-Class Bio with 2D Part 3 of 3: On-line Enrichment of Low Abundance Species

Robert Birdsall, Vera Ivleva, Sean M. McCarthy, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increased productivity through the automation of the fractionation processes using liquid chromatography
- Improved efficiency through simultaneous quantitation, desalting, and sample enrichment of a biotherapeutic sample
- Increased sample recovery by minimizing sample preparation and testing aliquots for protein content

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System with 2D Technology

ACQUITY UPLC Photodiode Array (PDA) Detector

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Protein-Pak™ Hi Res SP, 7 µm, 4.6 x 100 mm, SCX Column

ACQUITY UPLC Protein BEH Technology C_4, 300Å, 1.7 µm, 2.1 x 50 mm Column

ACQUITY UPLC Peptide BEH Technology C_{18}, 130Å, 1.7 µm, 2.1 x 100 mm Column

KEY WORDS

Multidimensional chromatography, 2DLC, enrichment, desalting, cation exchange, antibody, IEX, bioseparation, therapeutic, protein, ACQUITY, H-Class Bio, 2D technology

INTRODUCTION

Characterization of biotherapeutic proteins often employ separation techniques that incorporate non-denaturing aqueous buffers such as ion exchange chromatography (IEX).[1-3] As a result, the low volatility of the mobile phase, and presence of additives such as salt, prevents direct coupling of the separation to mass spectrometry (MS) to elucidate peak identities.[4]

Parts 1 and Part 2 of this three-part series demonstrated that the ACQUITY UPLC H-Class Bio System with 2D Technology enables the fractionation and desalting of biotherapeutic samples, while facilitating a direct interface for IEX to MS analysis. However, downstream analyses such as peptide mapping still require peaks of interest to be manually collected for enzymatic treatment.

Furthermore, peaks of interest may be present in low abundance, requiring an enrichment process where several fractions are pooled to ensure adequate protein is available for optimal enzymatic digest efficiency and a subsequent assay.[5] Factors such as these can require manned instrument operation and negatively impact productivity in the fast-paced pharmaceutical environment.

The ACQUITY UPLC H-Class Bio System with 2D Technology is Waters' solution for the on-line enrichment of peaks of interest. The 2nd dimension can be used to enrich low abundance fractions where the 2nd dimension column acts as a trapping/desalting column to retain and enrich the protein variant from multiple first dimension heart-cuts. Furthermore, quantitation of desalted/enriched samples can be calculated directly from the peak area rather than testing an aliquot of the enriched fraction, thus allowing for maximum sample recovery with minimal sample preparation.

The objective of this application note is to demonstrate that the ACQUITY UPLC H-Class Bio System with 2D Technology is well suited for fractionation and enrichment of low abundant species in biological samples. A therapeutic monoclonal antibody, infliximab, was used to demonstrate this.

EXPERIMENTAL

LC conditions (enrichment study)

LC system:	ACQUITY UPLC H-Class Bio with 2D Technology 1st dimension pump: ACQUITY UPLC Quaternary Solvent Manager, ACQUITY UPLC Column manager 2nd dimension pump: ACQUITY UPLC Binary Solvent Manager, ACQUITY UPLC Autosampler with FTN
Detectors:	(1st dimension) ACQUITY UPLC TUV (2nd dimension) ACQUITY UPLC PDA
Absorption Wavelength:	280 nm
Vials:	Total recovery vial: 12x32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm (p/n 186004930) ACQUITY UPLC BEH C_4, 300Å, 1.7 µm, 2.1 x 50 mm (p/n 186004495)
Column temp.:	25 °C (IEX); C_4: 40 °C
Sample temp.:	4 °C
Injection vol.:	2 µL unless otherwise stated

IEX/RPLC Pump Configuration

Quaternary solvent manager:	
Flow rate:	0.500 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O
Auto•Blend Plus™ setting:	20 mM MES buffer, pH 6.5, 25–65 mM NaCl in 15 minutes
Binary solvent manager:	
Flow rate:	0.250 mL/min for heart-cut, otherwise 0.500 mL/min
Mobile phase A:	18MΩ H_2O, 0.1% Formic acid
Mobile phase B:	Acetonitrile, 0.1% Formic acid
Gradient:	5–85% B in 10 minutes

LC conditions (peptide analysis)

LC system:	ACQUITY UPLC H-Class Bio
Vials:	Total recovery vial: 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	ACQUITY UPLC Peptide BEH C_{18}, 130Å, 1.7 µm, 2.1 x 100 mm (p/n 186003544)
Column temp.:	65 °C
Sample temp.:	4 °C
Mobile phase A:	18 MΩ water
Mobile phase B:	Acetonitrile
Mobile phase C:	Acetonitrile, 1 % formic acid, v/v

Gradient table:

Time (min)	Flow rate (mL/min)	A	B	C	D
0.00	0.200	87.0	3.0	10.0	0.0
60.00	0.200	48.0	42.0	10.0	0.0
61.00	0.200	10.0	80.0	10.0	0.0
64.00	0.200	10.0	80.0	10.0	0.0
65.00	0.200	87.0	3.0	10.0	0.0
70.00	0.200	87.0	3.0	10.0	0.0

MS conditions

Instrument:	Xevo® G2-S QTof
Capillary:	3kV
Sample cone:	80V
Source temp:	120 °C
Desolvation temp.:	300 °C
Desolvation gas:	800 L/h

Data management

Enrichment study:	MassLynx® Software v4.1 (SCN 8.62)
Peptide mapping:	UNIFI® Scientific Information System v1.7

The Waters Protein-Pak Hi Res SP, 7 µm, 4.6 x 100 mm, strong cation exchange column (p/n 186004930) and ACQUITY UPLC Protein BEH C_4, 300Å, 1.7 µm, 2.1 x 50 mm Column (p/n 186004495) were conditioned prior to use. Chemical reagents were purchased from Sigma Aldrich and used as received. Carboxypeptidase B (p/n LS005305) was purchased from Worthington Labs and used as received. PNGaseF (p/n P0705S) was purchased from New England Biolabs Inc. and used as received. Modified trypsin was purchased from Promega and used as received. The monoclonal antibody infliximab was received at a concentration of 20 mg/mL. Deglycosylation of infliximab was performed overnight (12 hours) at 37 °C using PNGaseF as per the manufacturer's protocol. After deglycosylation, the sample was divided into two equal aliquots, with one aliquot being treated with Carboxypeptidase B and incubated at 37 °C for 2 hours as per the manufacturer's protocol. Trypsin digests of enriched fractions were performed overnight at 37 °C as per the manufacturer's protocol.

RESULTS AND DISCUSSION

ACQUITY UPLC H-Class Bio System with 2D Technology featuring heart-cut technology

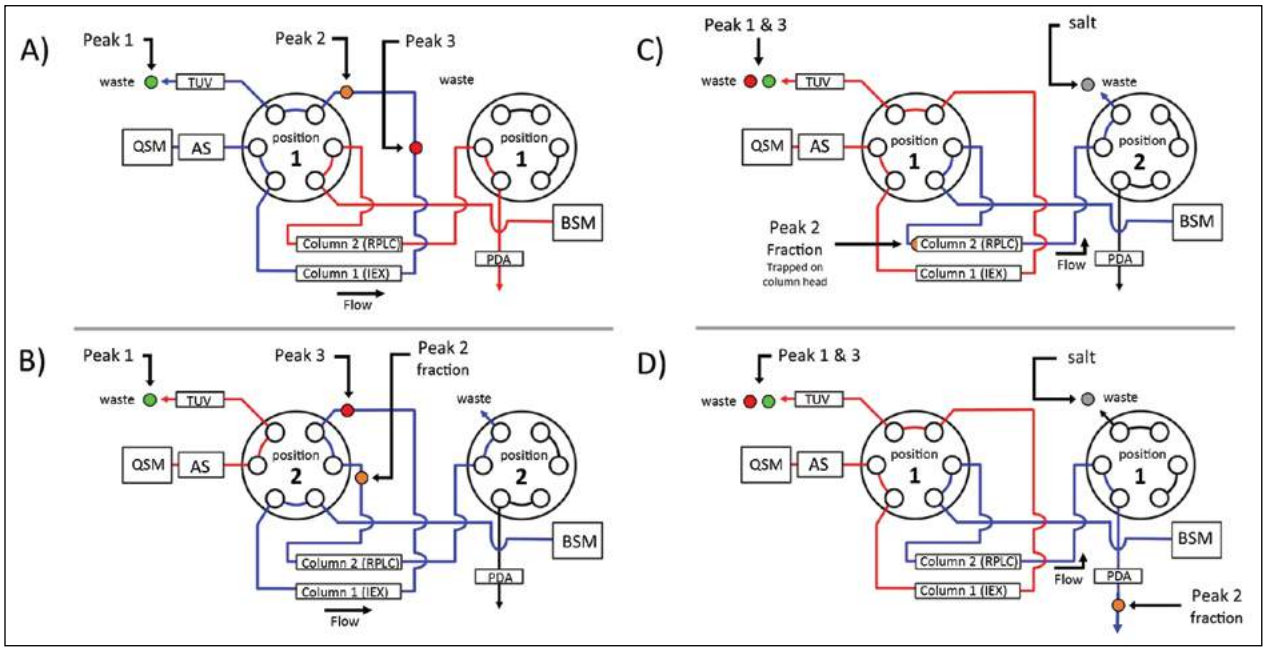

Figure 1. A plumbing diagram of the Waters ACQUITY UPLC H-Class Bio System with 2D Technology and corresponding valve positions to perform a heart-cut are illustrated in A–D. A) Three charge variants (peak 1-3) are separated in the 1st dimension (IEX) using the ACQUITY UPLC Quaternary Solvent Manager to deliver a salt gradient. B) Synchronized valve switching diverts the flow path to the 2nd dimension where a fraction (heart-cut) of peak 2 is directed to the 2nd dimension column (RPLC). C) Independent valve control allows the left valve to re-engage flow to the 1st dimension column while the right valve remains in position 2 where the heart-cut of peak 2 has unbound salt removed under aqueous conditions from the ACQUITY UPLC Binary Solvent Manager. D) The right valve is returned to position 1 after desalting the heart-cut fraction and a standard reversed phase gradient is used to elute the fraction for collection.

As described in parts 1 and 2 of this three-part series, the ACQUITY UPLC H-Class Bio System with 2D Technology featuring the heart-cut process is readily deployed with a two-column configuration as shown in Figure 1. With both valves in position 1 (Figure 1A), the flow from both the quaternary solvent manager and binary solvent manger are independent of each other, allowing for independent gradients to be performed on column 1 and 2.

The heart-cut is performed when the valve positions are temporarily switched to position 2 (Figure 1B), combining the flow paths (Figure 1B blue trace) where eluent from column 1 is redirected to column 2. The ACQUITY UPLC Column Manager supports independent valve control as shown in Figure 1C. With the left valve in position 1 and the right valve in position 2 the flow paths of each column are isolated again, with the 2nd dimension column being eluted to waste. This allows for unbound salts to be washed from the heart-cut fraction, which is trapped at the column head of the 2nd dimension column, using the aqueous phase of the 2nd dimension.

Once desalted, the heart-cut fraction can be readily eluted in a mobile phase amendable to down-stream analyses using the ACQUITY UPLC Binary Solvent Manager. With fraction collection occurring post-optical detection, as shown in Figure 1D, peak area can be used for relative quantification of the eluted fraction.

On-line enrichment of low abundance species for peptide analysis

Peptide analysis of species such as the acidic variants in the infliximab sample (Figure 2A) can be challenging to characterize due to their low abundance. As an example, the acidic variant eluting at ~6.6 minutes would require approximately 1.0 minute to collect individually for characterization. A 10-µg injection at a flow rate of 0.500 mL/min would result in a fraction concentration of 0.6 ng/µL with the fractionated sample still requiring a buffer exchange, which could lead to additional protein loss.

Low abundance peaks such as these are often manually collected over several runs where the final samples are pooled to ensure adequate protein is available for multiple enzymatic digests. This process requires manned instrument hours and can negatively impact productivity.

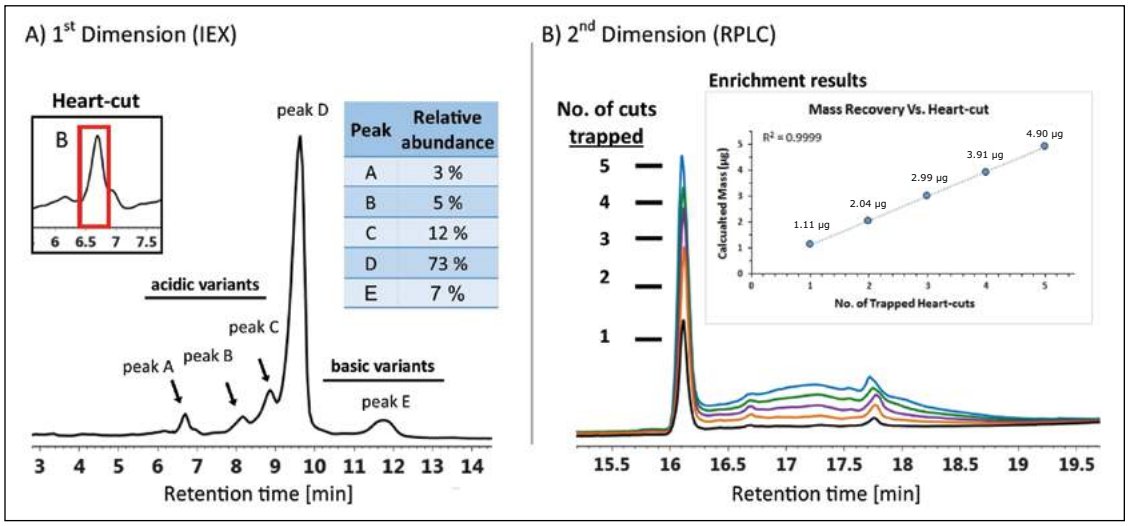

Figure 2. On-line enrichment of low-abundance species. A) A 3-µL injection (60 µg) of carboxypeptidase B treated infliximab sample separated on a Waters Protein-Pak Hi Res SP strong cation exchange column using Auto•Blend Plus to deliver a 15-minute salt gradient, from 25 mM to 65 mM NaCl, in 20 mM MES buffer at pH 6.8. A heart-cut was performed from 6.40 minutes to 6.90 minutes as shown in the inset figure. B) Heart-cuts were eluted off the 2nd dimension column using a 10-minute gradient from 5% to 85% organic mobile phase. Overlays of chromatographic traces with enrichment of 1 to 5 heart-cuts are shown with their corresponding recovered mass plotted in the inset graph.

The ACQUITY UPLC H-Class Bio System with 2D Technology offers the ideal solution for fractionation and enrichment of biotherapeutics with high protein recovery. Using the column configuration as before, Auto•Blend Plus was used to deliver a salt gradient from 25 mM to 65 mM NaCl in 20 mM MES buffer at pH 6.5, to separate a 3-µL injection of carboxypeptidase B treated sample of infliximab. The acidic variant (peak A) eluting at ~6.6 minutes as shown in the inset of Figure 2A was arbitrarily selected for fractionation and enrichment. To evaluate fraction enrichment of a biotherapeutic using the ACQUITY UPLC H-Class Bio System with 2D Technology, 1 to 5 heart-cuts from sequential 1st dimension separations were retained on the 2nd dimension column and eluted for quantification. The enriched sample was collected post PDA detector to allow for quantification.

For the 1st dimension, a heart-cut was performed from 6.40 minutes to 6.90 minutes as shown in the inset figure of Figure 2A. The heart-cut fractions were retained on the 2nd dimension column using a mobile phase composition of 95 % water, 0.05 % TFA: 5 % acetonitrile, 0.05 % TFA. Enriched fractions were eluted from the 2nd dimension column using a 10 minute gradient from 5% to 85% acetonitrile with 0.05% TFA with manual collection of the eluent occurring from 16–19.5 minutes (Figure 2B).

For quantification, a six-point calibration plot was constructed (not shown) using the same CPB treated sample of infliximab with mass loads ranging from 1.3 µg to 7.5 µg. As shown in the inset of Figure 7B, the calculated recovered mass is proportional to the number of heart-cuts used in the enrichment process and demonstrates the fidelity of the instrument to deliver a reproducible method for the enrichment of low abundance species.

To demonstrate the applicability of on-line enrichment using the ACQUITY UPLC H-Class Bio System with 2D Technology, the same enrichment process was applied to the main peak (peak D) as shown in Figure 2A and both enriched fractions were subjected to peptide analysis comparison to elucidate difference, if any, contributing to the charge variant.

Enriched fractions were directly transferred to a vacuum centrifuge and dried at 30 °C followed by suspension in 0.100 M Tris buffer, pH 7.6 at a concentration of 1.25 mg/mL and 1.43 mg/mL for peak A (acidic peak) and peak D (main peak), respectively. Samples were enzymatically digested with modified trypsin from Promega as per the manufacturer's protocol. An ACQUITY UPLC Peptide BEH C_{18}, 130Å, 1.7 µm, 2.1 x 100 mm Column was used for the analysis.

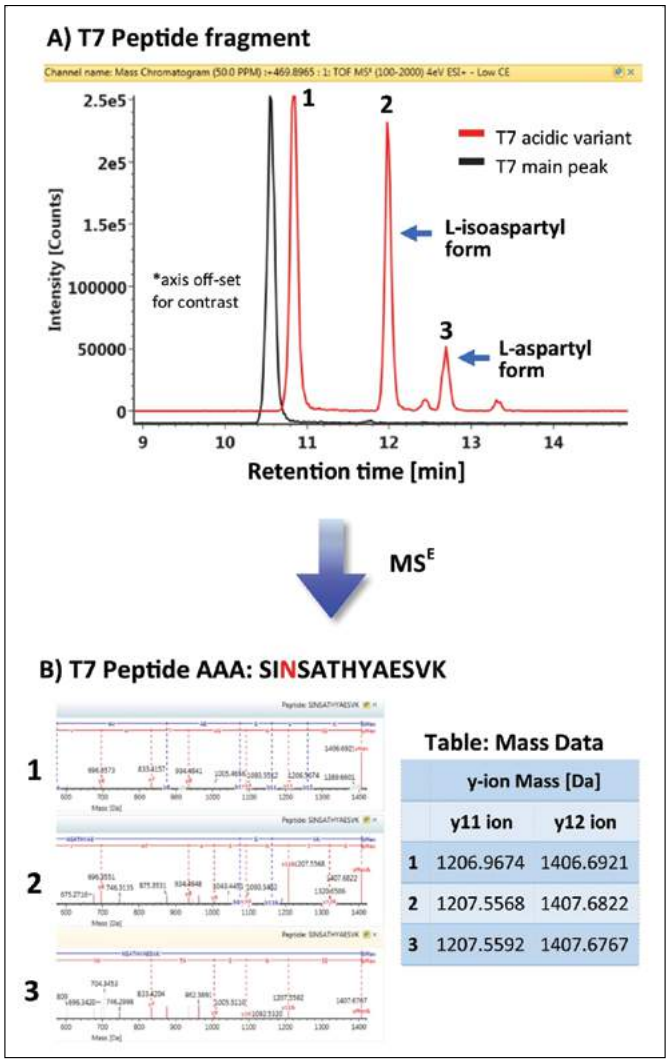

Figure 3. Peptide analysis. A) Peptide fragment T7 was identified as containing a deamidated residue (peak 2 and 3) in the acidic variant when compared to the main peak of infliximab. The deamidation of asparagine undergoes modification from asparagine to a succinimide intermediate that degrades into isoaspartic acid and aspartic acid (~ 3:1 ratio).[6] B) MS^E analysis of the peptides identified a 1 Da shift in the isoapartic and aspartic acid containing deamidated peaks (peak 2 and 3), evident in the y-ions associated with the acidic variant T7 peptide. The characteristic 3:1 ratio of L-isoapartic acid to L-aspartic acid was observed in the BPI profile. A 1 Da shift associated with the deamidation event was observed in y-ions of the acidic fractions amino acid analysis table.

Three replicate samples were separated with a 13% to 52% gradient using acetonitrile, 0.1% FA v/v, in 60 minutes. The Biopharmaceutical Platform Solution with UNIFI was used for data analysis of the peptide mapping experiments.

Using a workflow targeted for identification of deamidation events, UNIFI Software was able to identify peptide fragment T7 as shown in Figure 3A as containing a deamidated residue responsible in part for the charge variant of the acidic fraction. The deamidation of asparagine (residue N) undergoes modification from asparagine to a succinimide intermediate that degrades into isoaspartic acid and aspartic acid in a 3:1 ratio.[6] These byproducts of the deamidation are observed in the peptide map, following the elution of the unmodified T7 peptide, as +1 Da isoforms.

MS^E fragmentation analysis of these species (shown in Figure 3B) revealed that the observed 1 Da mass shifts could indeed be localized to the third residue (N) in the peptide, confirming asparagine deamidation.

This experiment demonstrates that the ACQUITY UPLC H-Class Bio System with 2D Technology is well-suited for fractionation and enrichment of challenging separations encountered during the characterization of biotherapeutics.

CONCLUSION

This comprehensive three-part series demonstrates that the ACQUITY H-Class Bio System with 2D Technology is well suited for the automated fractionation, desalting, and enrichment of biologically complex samples. The ACQUITY UPLC H-Class Bio System with 2D Technology offers an efficient solution with the flexibility needed for increasing informational content in the characterization of biotherapeutics without compromising productivity.

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