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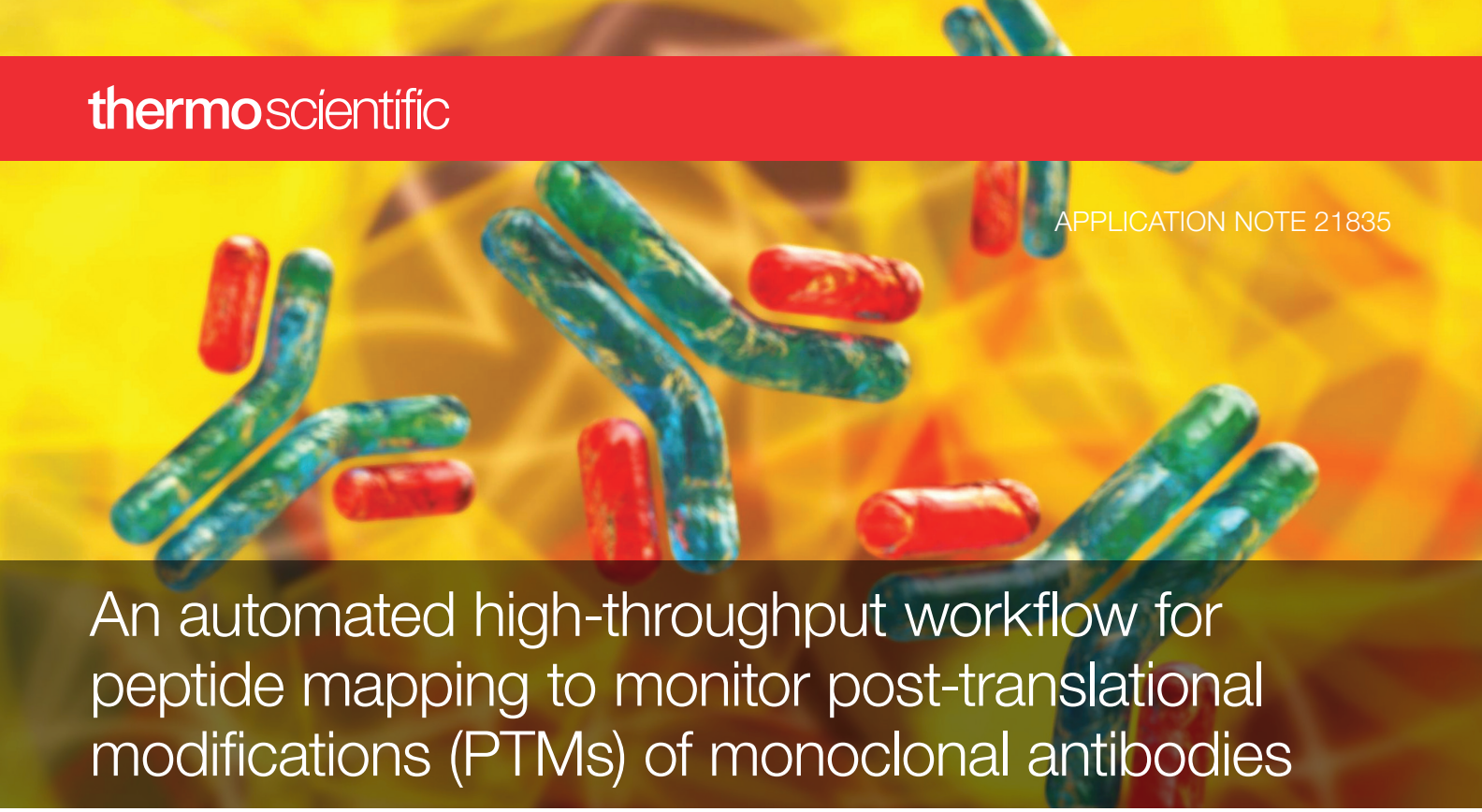

An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies

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Keywords

NIBRT, biopharmaceuticals, biotherapeutics, CQA, monoclonal antibodies, mAb, IgG, post-translational modifications, PTM, peptide mapping, sequence coverage, bevacizumab, cetuximab, adalimumab, rituximab, trastuzumab, high throughput, Magnetic SMART Digest kit, KingFisher Duo Prime purification system, Vanquish Flex Binary UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Application benefits

- Speed and simplicity of data preparation in addition to automation for highly reproducible peptide mapping results
- Simple and rapid protein digestion for a peptide mapping workflow of different monoclonal antibodies (chimeric, humanized, and fully human) in less than two hours
- Automated magnetic bead technology allows excellent recovery of samples with great reproducibility and efficiency with less hands-on time
- High confidence in results with excellent quality data; excellent coverage and low levels of sample preparation-induced post-translational modifications (PTMs)

Goal

Report on the benefits of automated high-throughput trypsin digestion for highly reproducible peptide mapping of five top-selling monoclonal antibodies (chimeric, humanized and fully human) using the magnetic Thermo Scientific™ SMART Digest™ Trypsin Kit as a bulk resin option on the Thermo Scientific™ KingFisher™ Duo Prime purification system. The study focused on reproducibility, protein sequence coverage, and identification of post-translational modifications (PTMs), including deamidation, oxidation,

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N-terminal pyroglutamination, C-terminal lysine loss, glycation, and glycosylation.

Introduction

Complex glycoproteins, specifically monoclonal antibodies, are currently the most prevalent type of biotherapeutics in development. Monoclonal antibodies (mAbs), which offer high specificity and low side effects, are used to treat many types of cancer, autoimmune and inflammatory diseases, infections, and metabolic disorders, yielding their impressive success as human medicines.[1] These large proteins have molecular weights near 150 kDa and are composed of two identical ~50 kDa heavy chains (HC) and two identical ~25 kDa light chains (LC). They also contain at least 16 disulfide bonds that maintain three-dimensional structure and biological activity. Although they share similar secondary protein structures, different mAbs vary greatly in the sequence of variable regions.

Since the commercialization of the first therapeutic mAb product in 1986, this class of biopharmaceutical products has grown significantly so that, as of December 1st, 2017, seventy-six mAb products have been approved in the US or Europe for the treatment of a variety of diseases, and many of these products have also been approved for other global markets.[2,3] Rituximab (Rituxan®), cetuximab (Erbitux®), bevacizumab (Avastin®), trastuzumab (Herceptin®), and adalimumab (Humira®) were five of the top-ten selling mAbs in 2012.[4]

Post-translational modification (PTM) refers to the covalent and generally enzymatic modification of proteins during or after protein biosynthesis. Proteins are synthesized by ribosomes translating mRNA into polypeptide chains, which may then undergo PTM to form the mature protein product. PTMs are important components in cell signaling and can occur on the amino acid side chains or at the protein's C- or N- termini. They can extend the chemical repertoire of the 20 standard amino acids by modifying an existing functional group or introducing a new one. PTMs affect structural and functional aspects of therapeutic proteins, and the effects can be detrimental, that is, heterogeneity and immunogenicity, even though the modification may originally be required for functional activity of the polypeptide.[5]

Most therapeutic proteins approved or in development bear at least one or more PTMs. Variants of proteins produced for medicinal purposes can occur during manufacturing, handling, and storage, and can impact the activity and stability of the biotherapeutic. Their characterization is challenging due to their size and inherent heterogeneity caused by PTMs, among which glycosylation is probably the most prominent.[6,7]

Peptide mapping is used routinely to study PTMs and is capable of pinpointing the amino acid residue within the sequence at which the modification has occurred. Peptide mapping is commonly used in the biopharmaceutical industry to establish product identity by confirming the primary structure of a product.[8,9] For recombinant protein pharmaceuticals, peptide mapping is used for the initial "proof of structure" characterization. It confirms expression of the desired amino acid sequence and characterizes any PTM, for subsequent lot-to-lot identity testing, in support of bioprocess development and clinical trials. It is also used as the current method of choice for monitoring the "genetic stability" of recombinant cell lines.

This application note describes a full workflow solution for peptide mapping of five different monoclonal antibodies using the Magnetic SMART Digest resin option on a KingFisher Duo Prime purification system, in combination with the high-resolution, accurate-mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ Plus mass spectrometer, and high-resolution chromatographic separation with the Thermo Scientific™ Vanquish™ Flex UHPLC system. Thermo Scientific™ BioPharma Finder™ software was used to interrogate the high-quality data sets.

Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical™) (P/N 10505904)
- Acetonitrile, Optima LC/MS grade (Fisher Chemical) (P/N 10001334)
- Water with 0.1% formic acid (v/v), Optima LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima LC/MS grade (Fisher Chemical) (P/N 10118464)
- Trifluoroacetic acid (TFA) (Fisher Chemical) (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Dithiothreitol (DTT) (Fisher Bioreagents™) (P/N 10386833)

- Iodoacetic acid, sodium salt 99% (IA) (Acros Organics™) (P/N 10235940)
- Thermo Scientific™ KingFisher™ Deepwell plates, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime purification system (P/N 5400110)

Vanquish Flex Binary UHPLC system including:

- Binary Pump F (P/N VF-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A)
- System Base Vanquish Horizon (P/N VH-S01-A)

Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)

Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Samples (mAbs)

Five commercially available monoclonal antibodies were supplied at different concentrations (Table 1).

Table 1. Monoclonal antibodies used in the study

Drug	Brand name	Concentration	Type
Bevacizumab	Avastin®	25 mg/mL	Recombinant IgG1 humanized mAb
Cetuximab	Erbitux®	25 mg/mL	Recombinant IgG1 kappa, chimeric murine/human mAb
Adalimumab	Humira®	5 mg/mL	Recombinant fully human mAb
Rituximab	MabThera®	10 mg/mL	Recombinant IgG1 kappa, chimeric murine/human mAb
Trastuzumab	Herceptin®	15 mg/mL	Recombinant IgG1 kappa, humanized mAb

Sample preparation

Monoclonal antibody samples were prepared in triplicate on different days. For cetuximab biotherapeutic nine replicates were prepared by different analysts on different days.

Protocol for sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and SMART Digest buffer were added to each lane of a KingFisher Deepwell 96-well plate as outlined in Table 2. Bead "wash buffer" was prepared by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using Kingfisher Duo Prime purification system with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 3. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 15.45 µL of 100 mM DTT followed by 15.64 µL 10% TFA (final concentration 11 mM DTT and 1% TFA). Samples were then injected immediately into the LC-MS.

Table 2. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	(SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table 3. Protocol for automated peptide mapping using a KingFisher Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	70 °C heating while mixing 10 °C post temperature	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Mobile phase A: Water with 0.1% formic acid (v/v)
Mobile phase B: Acetonitrile with 0.1% formic acid (v/v)
Flow Rate: 0.3 mL/min
Column Temperature: 25 °C (Still air mode)
Autosampler Temp: 5 °C
Injection Volume: 10 µL
Injection Wash Solvent: Methanol/water, 10:90 (v/v)
Needle Wash: Enabled pre-injection
Gradient: See Table 4 for details

Table 4. Mobile phase gradient for UHPLC separation of peptides

Time (minutes)	Flow (mL/min)	% Mobile Phase B	Curve
0.000	0.300	2.0	5
45.000	0.300	40.0	5
46.000	0.300	80.0	5
50.000	0.300	80.0	5
50.500	0.300	2.0	5
65.000	0.300	2.0	5

Data processing and software

Acquisition software: Thermo Scientific™ Xcalibur™ software version 4.0 MS data analysis software: Thermo Scientific™ BioPharma Finder™ version 3.0

MS Conditions

Detailed MS method parameters are shown in Tables 5 and 6.

Table 5. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Ion Max source with HESI II probe
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	400 °C
Source Voltage	3.8 kV
Capillary Temperature	320 °C
S-lens RF Voltage	50 V

Table 6. MS method parameters utilized for peptide mapping analysis

General	Setting	MS^2 Parameters	Setting
Run Time	0 to 65 min	Resolution Settings	17,500
Polarity	Positive	AGC Target Value	1.0×10^5
Full MS Parameters	**Setting**	Isolation Width	2.0 *m/z*
Full MS Mass Range	200–2,000 *m/z*	Signal Threshold	1.0 × 104
Resolution Settings	70,000	Normalized Collision Energy (HCD)	28
AGC Target Value	3.0×10^6	Top-N MS^2	5
Max Injection Time	100 ms	Max Injection Time	200 ms
Default Charge State	2	Fixed First Mass	–
SID	0 eV	Dynamic Exclusion	7.0 s
Microscans	1	Loop Count	5

MS data processing

Detailed parameter settings are shown in Table 7.

Table 7. Biopharma Finder software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS Signal Threshold	8.0 x 10^4 counts
Typical Chromatographic Peak Width	0.3
Relative MS Signal Threshold (% base peak)	1
Relative Analog Threshold (% of highest peak)	1
Width of Gaussian Filter (represented as 1/n of chromatographic peak width)	3
Minimum Valley to Be Considered as Two Chromatographic Peaks	80.0%
Minimum MS Peak Width (Da)	1.2
Maximum MS Peak Width (Da)	4.2
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift (min)	1.69
Maximum Mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum Peptide Mass	7,000
Mass Accuracy	5 ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	Carboxymethylation
Variable Modifications	**Setting**
N Terminal	Gln→Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation(Q) Glycation (K) Oxidation (MW) Oxidation (C)

Results and discussion

Changes to the mAb structure introduced during the manufacturing process or storage may influence the therapeutic efficacy, bio-availability, clearance, and immunogenic properties, thus altering drug safety and use. Peptide mapping is a widely utilized technique to characterize monoclonal antibodies for the purpose of product identity and as an important stability indicating assay. Peptide mapping by liquid chromatography-mass spectrometry (LC-MS) analysis of enzymatically digested mAb is a powerful method for PTMs characterization to ensure mAb drug function and quality.

Using the magnetic SMART Digest kit in combination with the KingFisher Duo Prime system simplifies the process and reduces the time needed for peptide mapping sample preparation. This methodology provides

significant improvements in reproducibility over existing protocols, resulting in fewer sample failures, higher throughput, and the ability to more easily interrogate data. The following chromatogram (Figure 1) shows nine overlaid traces of cetuximab (Erbitux®) peptides, digested with Magnetic SMART Digest kit. Excellent retention time reproducibility ≤ 0.14 RSD with an average of 0.083% is achieved when using the Vanquish Flex Binary UHPLC system, as indicated in Table 8.

The same peptide mapping workflow was performed using five top-selling mAbs of different categories such as chimeric (rituximab and cetuximab), humanized (trastuzumab and bevacizumab), and fully human (adalimumab) for assessing the sequence coverage for light and heavy chain, as well as for identification and relative quantification of a specific set of modifications: deamidation, oxidation, pyroglutamination, glycation, Lys loss, and glycosylation. Five top mAbs were analyzed after performing an automated SMART digest resulting in peptide mixtures. The obtained base peak chromatograms are similar but show distinct differences (Figure 2). Each protein to be mapped presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity. All antibodies can be identified with 100% sequence coverage when analyzed separately (Table 9).

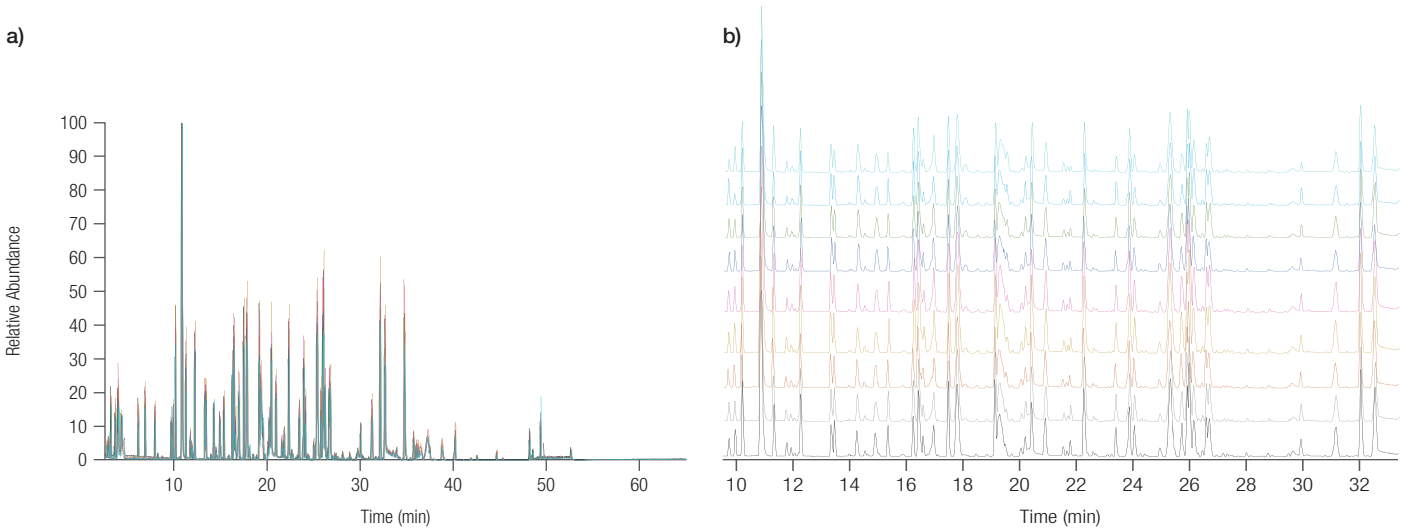

Figure 1. Overlaid base peak chromatograms (BPCs) of nine technical replicates for cetuximab (Erbitux®) Magnetic SMART digested samples (a) and the stacked expanded time region between 9.5 and 33.5 minutes (b)

Table 8. Retention time repeatability (n=9) for fourteen peptides from cetuximab after Magnetic SMART digestion on the King Fisher Duo prime system

Protein	Identification	Peptide Sequence	Average RT (min)	RSD (%)
Cetuximab HC	1:L412-K416	LTVDK	10.138	0.137
Cetuximab HC	1:L67-K71	LSINK	12.216	0.138
Cetuximab HC	1:G33-R38	GVHWVR	14.981	0.139
Cetuximab HC	1:Q1-K5	QVQLK	17.473	0.099
Cetuximab HC	1:A329-K336	ALPAPIEK	17.796	0.109
Cetuximab LC	2:Y50-R61	YASESISGIPSR	19.309	0.108
Cetuximab HC	1:347-R357	EPQVYTLPPSR	20.416	0.078
Cetuximab LC	2:A25-R39	ASQSIGTNIHWYQQR	22.299	0.072
Cetuximab HC	1:S28-R38	SLTNYGVHWVR	23.430	0.064
Cetuximab HC	1:G124-K135	GPSVFPLAPSSK	25.363	0.068
Cetuximab LC	2:D170-K183	DSTYSLSSTLTLSK	26.643	0.046
Cetuximab LC	2:R108-K126	RTVAAPSVFIFPPSDEQLK	29.716	0.065
Cetuximab HC	1:G373-K394	GFYPSDIAVEWESNGQPENNYK	31.242	0.053
Cetuximab HC	1:T395-K411	TTPPVLDSDGSFFLYSK	32.126	0.058

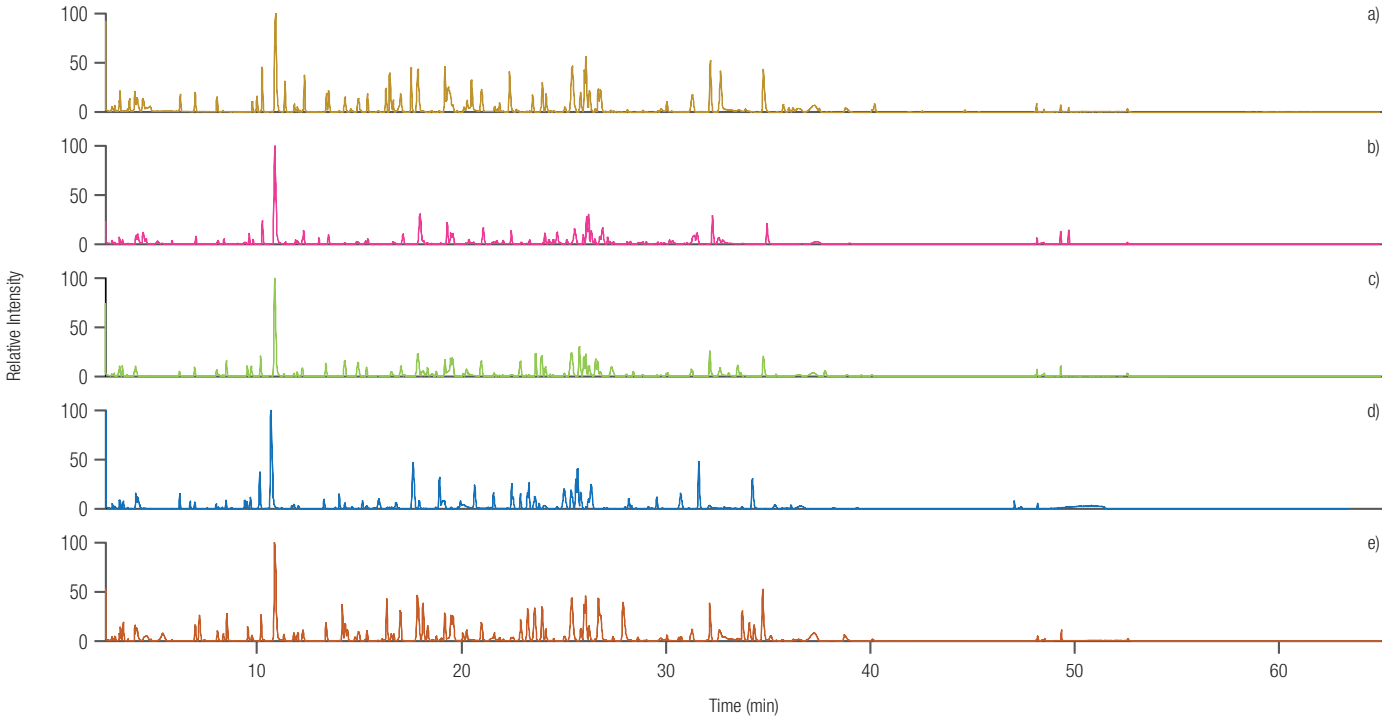

Figure 2. BPCs obtained from peptide mapping experiments of a) cetuximab, b) rituximab, c) bevacizumab, d) trastuzumab, and e) adalimumab after Magnetic SMART digestion with KingFisher Duo Prime system

The number of detected MS peaks in the samples digested with Magnetic SMART Digest kit on the Kingfisher Duo Prime system varied between the different mAbs from 361 to 686 for the light chain and 946 to 1457 for the heavy chain. The chimeric drug product cetuximab showed the largest number of detected MS peaks for heavy chain, while bevacizumab, a humanized IgG1 showed the lowest number of detected MS peaks.

For biotherapeutic protein identity, a peptide map needs high specificity, and complete or nearly complete sequence coverage is important because there maybe not prior knowledge of the alterations to the protein.

The sequence coverage for studied mAbs are shown in Table 9. For all five top mAbs, 100% sequence coverage was achieved for light and heavy chains.

The sequence coverage map (Figure 3) shows the overlap of the different peptides identified with different intensities and in different lengths due to missed cleavages and nonspecific peptides that do not follow protease's rule. As an example, a sequence coverage map is shown for bevacizumab drug product. The colored bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the intensity of the peptide in the MS1 scan.

Table 9. Sequence coverage for the studied recombinant IgG1 mAbs

Proteins	Sample	Number of Peaks	Sequence Coverage (%)
Heavy chain	Cetuximab (Erbitux®)	1457	100.00
	Rituximab (MabThera®)	1187	100.00
	Trastuzumab (Herceptin®)	1169	100.00
	Bevacizumab (Avastin®)	946	100.00
	Adalimumab (Humira®)	1384	100.00
Light chain	Cetuximab (Erbitux®)	533	100.00
	Rituximab (MabThera®)	532	100.00
	Trastuzumab (Herceptin®)	375	100.00
	Bevacizumab (Avastin®)	361	100.00
	Adalimumab (Humira®)	686	100.00

Bevacizumab heavy chain

Bevacizumab light chain

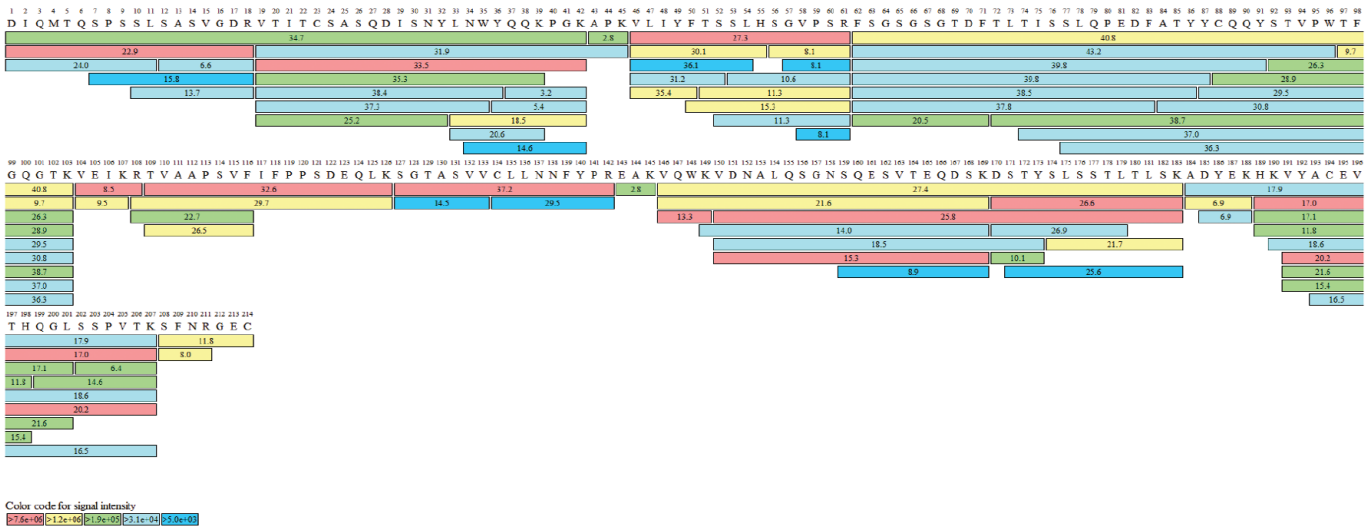

Figure 3. Sequence coverage map of bevacizumab heavy (top) and light chain (bottom), obtained using a 65 min gradient for peptide separation on an Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm column. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > 7.6e+06, yellow > 1.2e+06, green > 1.9e+05, light blue > 3.1e+04, cyan = low abundant > 5.0e+03.

To ensure high confidence in the identified peptides, all peptide sequence matches were required to have ±5 ppm of MS mass error, confidence score ≥ 95, and full MS and MS/MS spectra. Figure 4 shows an example of the selected ion chromatogram (SIC) and corresponding MS/MS spectra for selected peptide ALPAPIEK present on the five top mAb heavy chain, which elutes at 17.80 min. The combination of high-quality MS and MS/MS data ensures a reliable peptide matching.

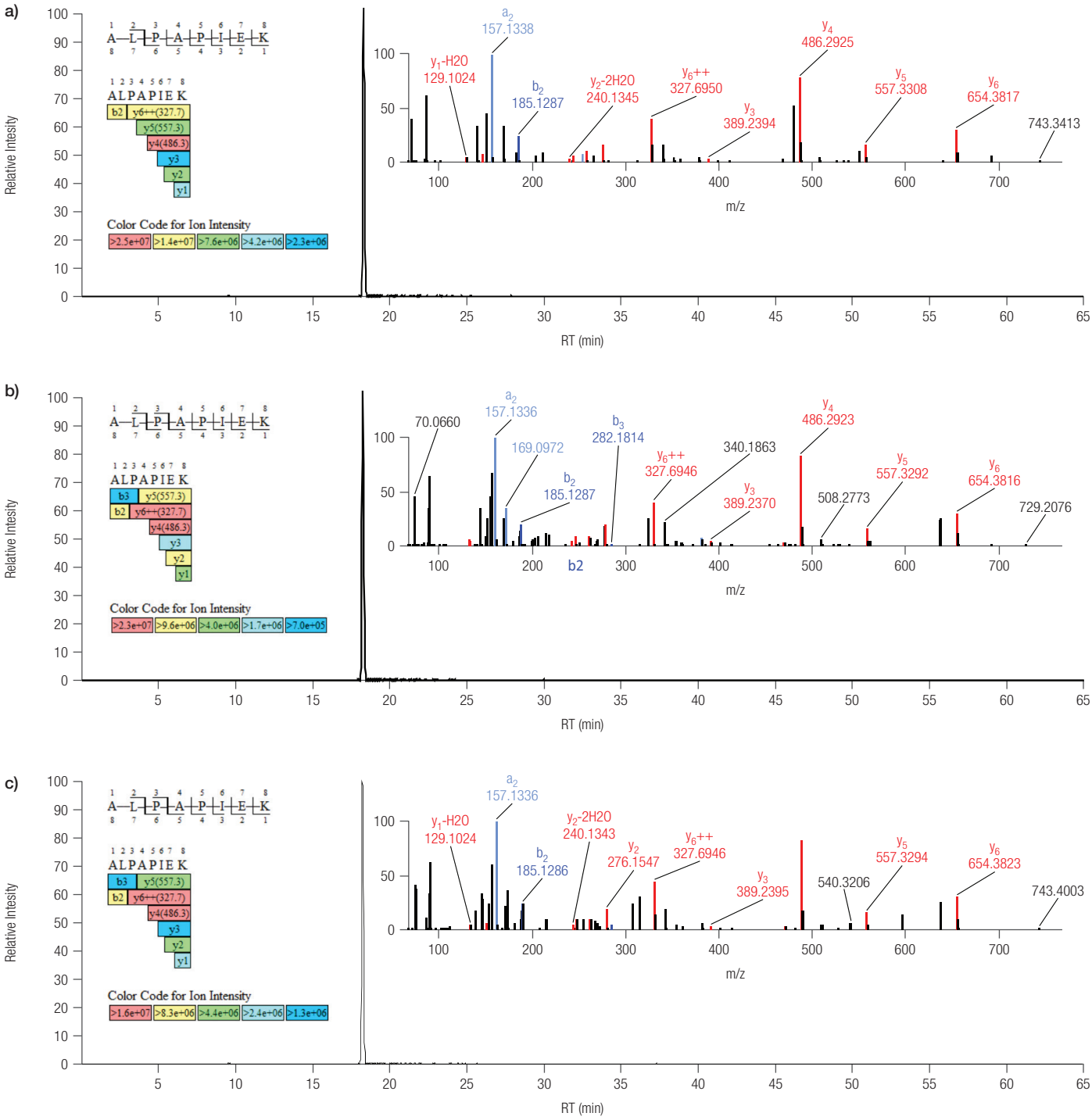

Figure 4. Representative SIC of HC peptide ALPAPIEK, MS/MS spectra, and fragment coverage map from digested (a) cetuximab, (b) rituximab and (c) bevacizumab

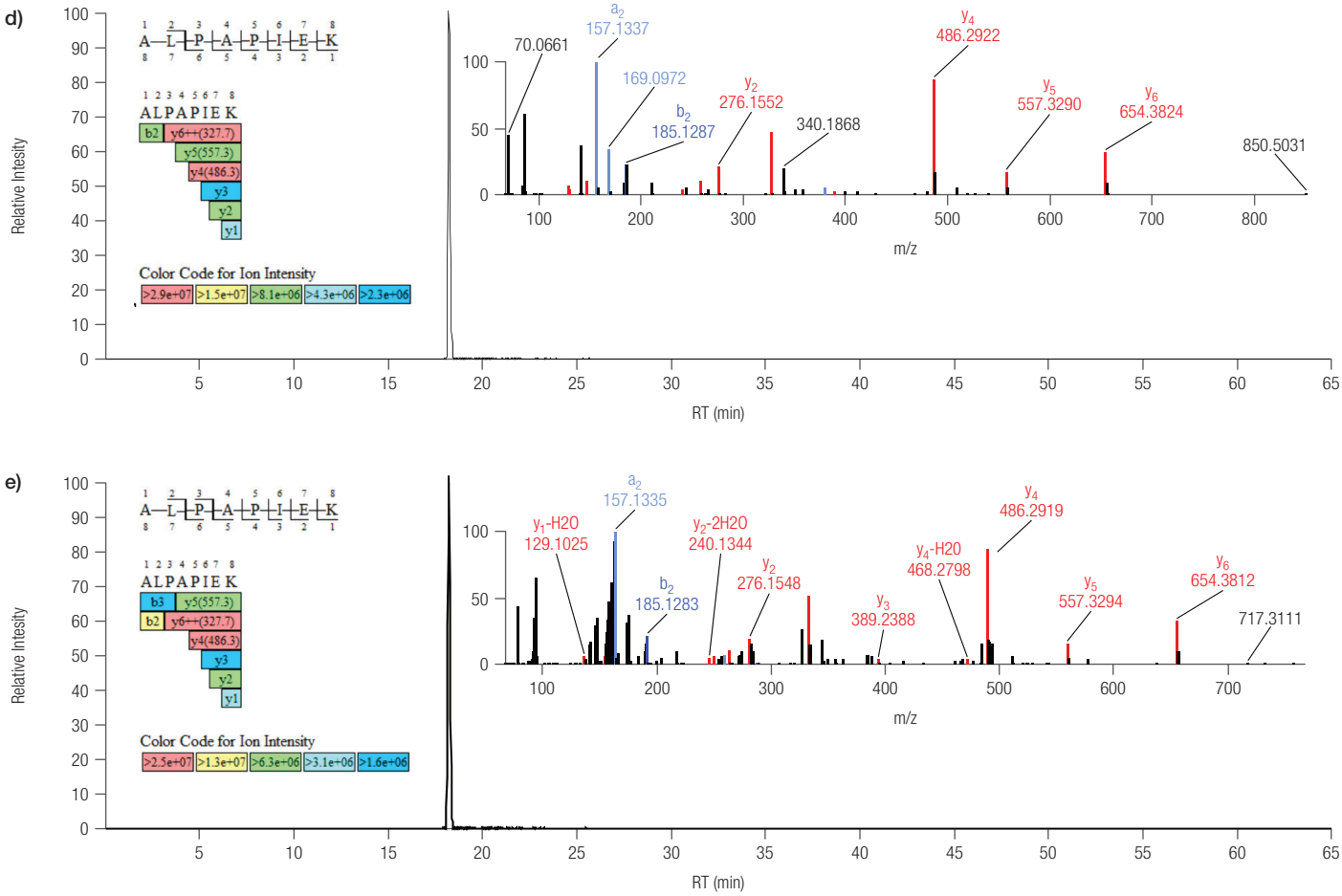

Figure 4. (continued). Representative SIC of HC peptide ALPAPIEK, MS/MS spectra, and fragment coverage map from digested (d) trastuzumab and (e) adalimumab biotherapeutics

Peptide mapping analysis allows the identification and quantification of PTMs. Many common PTMs cause a shift in reversed-phase LC retention relative to the native peptide. In combination with direct MS and MS/MS analysis it can be used to interrogate modifications with relative large mass shifts, such as C-terminal lysine (128 Da), glycation (162 Da), and small mass differences such as deamidation (1 Da), oxidation (16 Da), or pyroglutamination (17 Da), between others.

Tables 10–14 show the identification and comparison of a subset of monitored modifications across the different mAbs studied. PTMs such as N-terminal pyroglutamination, deamidation, oxidation, glycation, C-terminal lysine clipping, and glycosylation are confidently identified in many variations based on MS1 and MS/MS spectra. A tilde (~) before the modification indicates the modification was found on the tryptic peptide but could not be localized on a specific amino acid with MS/MS spectra. The relative abundance of the detected modifications in the five different mAbs usually have relative standard deviations < 10% except for a few modifications above 15% (cetuximab Q37 and N158; and trastuzumab Q199), which were in low abundance. Overall, the method shows that important information regarding PTMs can be obtained equally and accurately at all separation times.

A common structural modification of recombinant proteins is observed through the non-enzymatic deamidation of glutamine (Gln) and asparagine (Asn) residues. The latter occurs in a variety of protein-based pharmaceuticals, including monoclonal antibodies with varying effects on the activity or stability of the therapeutic protein. Thus, determining the deamidation of Asn residues in recombinant proteins is a significant challenge for analytical and protein chemists in the quality control and process departments at biotechnology and pharmaceutical companies.[10] Deamidation of Gln proceeds at a much slower rate than deamidation of Asn residues at peptide level. Liu et al[11] investigated Gln deamidation

of a recombinant monoclonal IgG1 antibody, showing Gln residues at different locations had different susceptibilities to deamidation and it is a highly pH-dependent modification. Figure 5 shows the average relative abundance of four to six of the most abundant deamidation modifications for the five top-studied mAbs. A common deamidation to all the studied mAbs was observed for the residue Q199/Q198 of the LC, with variable levels that go from 0.064% (trastuzumab) to 0.884% (adalimumab). In relation to Asn deamidation, residue N321/N319/N318/N317 (VVSVLTVLHQDWLNGK) seems to be more susceptible to PTMs, being present in the five mAbs at a relative abundance between 1.3% (bevacizumab) and 2.1% (adalimumab). The most abundant deamidation levels were detected for the N55 site from rituximab heavy chain (7.05%) and for the Q27 site from trastuzumab light chain (4.77%).

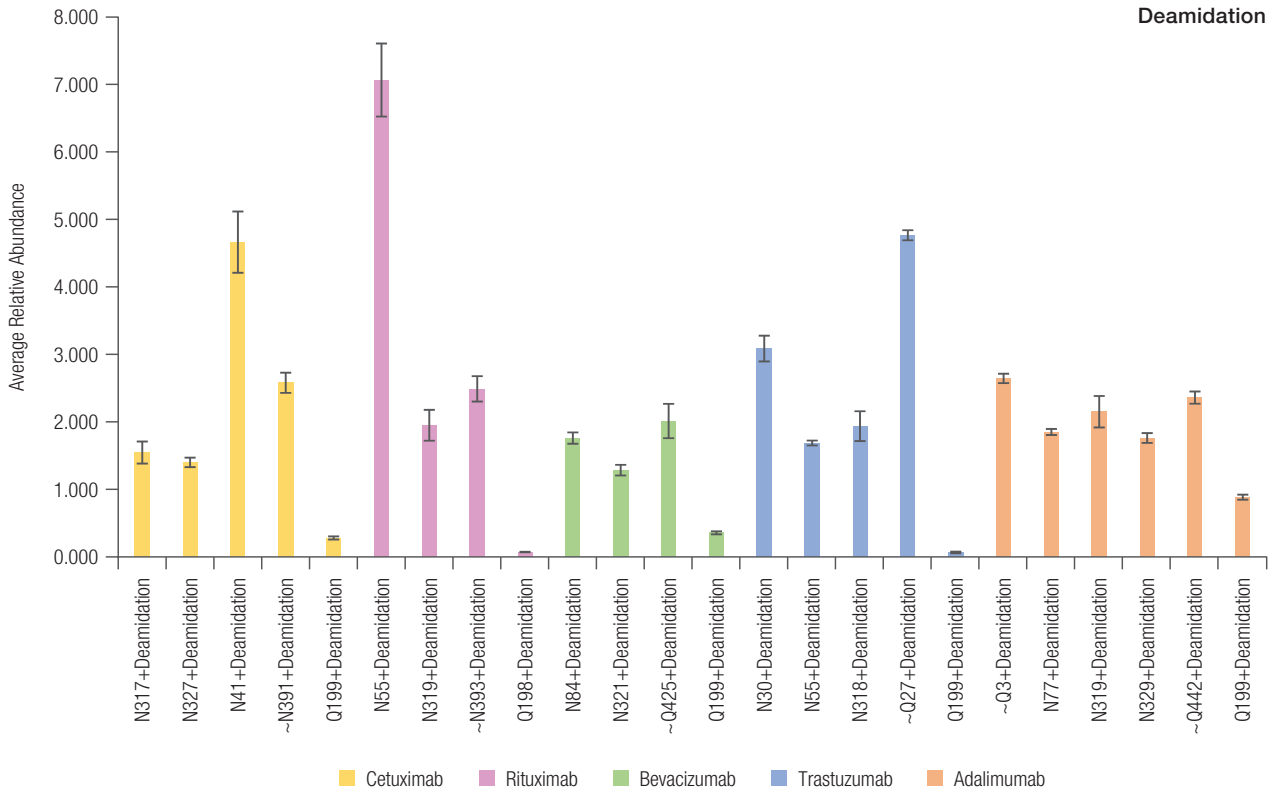

Figure 5. Average relative abundance (n=3) of some identified deamidation modifications for trastuzumab, rituximab, cetuximab, bevacizumab, and adalimumab

On the other hand, protein and peptide microheterogeneity can sometimes be attributed to oxidation of tryptophan (Trp) or methionine (Met) residues. This is also a common PTM observed in proteins and peptides. *In vivo* oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). *In vitro* oxidation can be due to conditions encountered during purification or formulation. Oxidation can also occur during storage and from frequent freeze-thawing cycles. Protein chemists in process development and quality control are concerned with oxidation because it can adversely impact the activity and stability of biotherapeutics.[12] The studied mAbs in the present work show low oxidation levels (<4.0%). Residue M254/M255/M256/M258 (on the peptide sequence DTLMISR) detected in the heavy chain for all the five studied mAbs seems to be more susceptible for oxidation (Figure 6). Adalimumab showed the highest levels for heavy chain M256 (4.0%) and M432 (3.2%), and bevacizumab for M258 (3.3%).

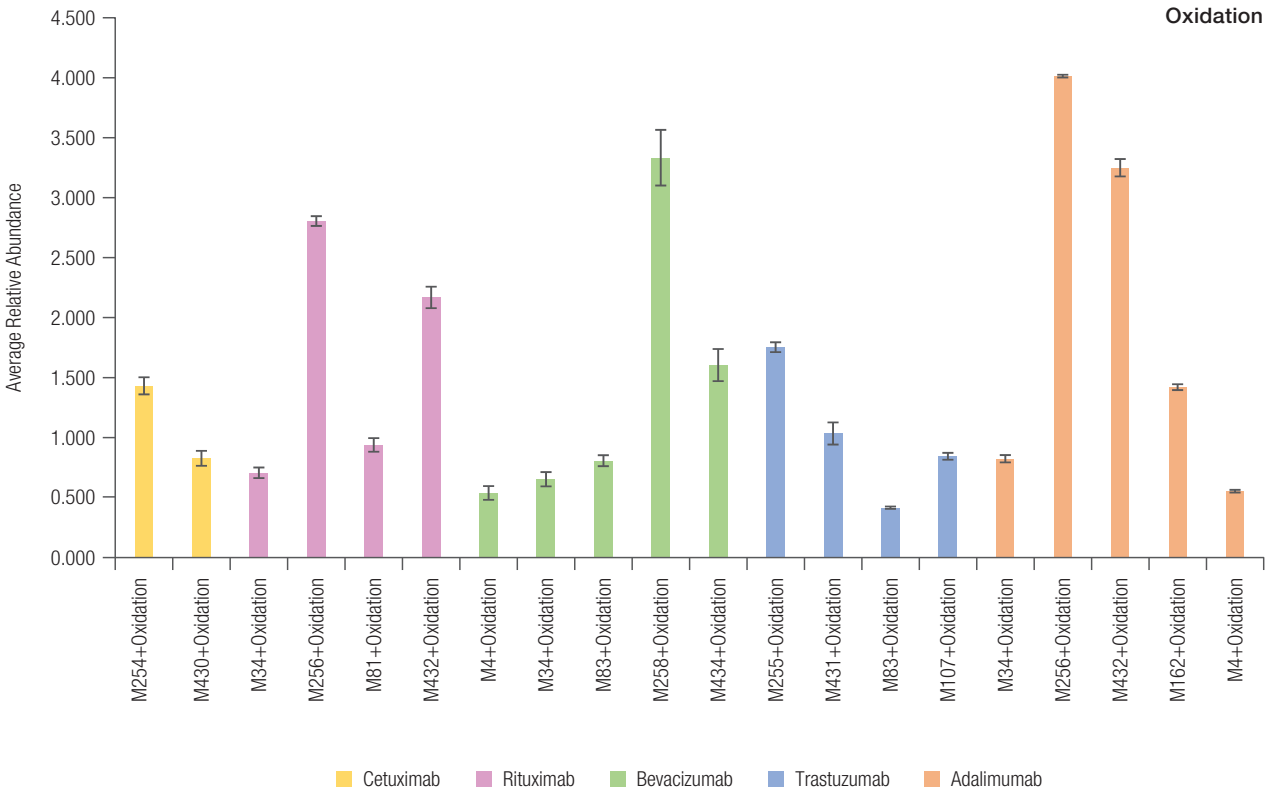

Figure 6. Average relative abundance (n=3) of identified oxidation modifications for cetuximab, rituximab, bevacizumab, trastuzumab, and adalimumab

One of the most important PTMs to characterize and quantify is glycoslyation, which is critical for therapeutic efficacy and safety of the drug. N-glycans have important structural functions as they stabilize the CH2 domain of IgGs. Deglycosylation makes mAbs thermally less stable and more susceptible to unfolding and they are more prone to aggregation. Moreover, functionality of the IgG is influenced by the attached N-glycans and their size.[13] Glycosylation renders a peptide more hydrophilic, meaning it can be easily resolved from its non-glycosylated counterpart. High abundance of glycosylation of the heavy chain is also observed for the five studied mAbs at the Fc part at position N299 (cetuximab), N301 (rituximab and adalimumab), N300 (trastuzumab), or N303 (bevacizumab), where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, A2G2F and A1G0F), afucosylated biantennary (A2G0), and high mannose (M5) structures (Figure 7).

Cetuximab drug product shows another N-glycosylation site at the position N88 on the Fab region (Table 10). The glycan structures observed correspond to several non-human glycan motifs containing α-*1,3-Gal-epitopes* (A2Ga2F, A2Ga1G1F, A3Ga3F, A3Ga2G1F) and the glycan moiety N-glycolylneuraminic acid (A2Sg1G1F, A2Sg1Ga1F, A2Sg2F), which have been reported to cause anaphylaxis in over 30% of patients that received cetuximab.[14] Those findings highlight the importance and need for reliable glycosylation analysis.

The C-terminal lysine (Lys) truncation variant is commonly observed in monoclonal antibodies and recombinant proteins. Although the effect that this variation has on protein activity does not seem to impact the potency or safety profile,[15] the degree of heterogeneity of C-terminal Lys variants reflect the manufacturing consistency and should be monitored for product consistency. Lys loss is detected in all the five studied mAbs with the lowest % of modification for cetuximab (67.13%) and the highest level of modification detected for bevacizumab (97.45%) and trastuzumab (97.73%).

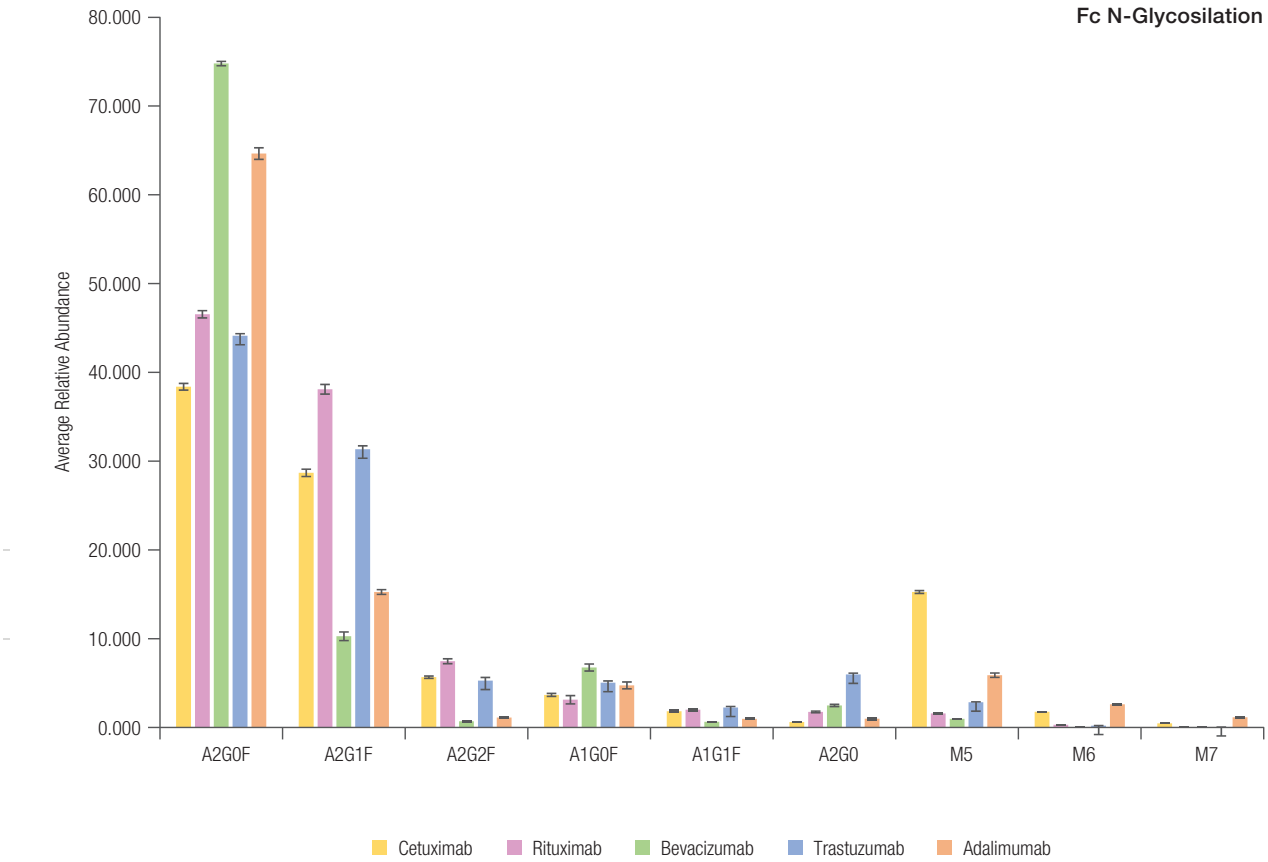

Figure 7. Average relative abundance (n=3) of identified N-glycosylation on the Fc region for cetuximab, rituximab, bevacizumab, trastuzumab, and adalimumab

Another commonly targeted modification is lysine (K) glycations which are listed in Tables 10–14. In total, between eight and twelve lysine glycations could be identified and relatively quantified < 1.58% with an average RSD value ≤ 15%. Overall, similar levels were observed for the different studied IgG1 mAbs.

Almost half the antibodies reported in the literature contain a glutamic acid residue at the N-terminus of the light or the heavy chain. As studied by *Chelius D. et al.*[16] the formation of pyroglutamic acid from N-terminal glutamic acid in the heavy chains and light chains of several antibodies, indicate that it is a non-enzymatic reaction that occurs very commonly. For therapeutic mAbs, pyroGlu can be one of many PTMs or transformations observed during production and storage. N-terminal pyroglutamination was observed for cetuximab and rituximab in high abundance (>85%).

Table 10. Summary of PTMs identified and quantified for cetuximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=9)	RSD (n=9)
CETUXIMAB (chimeric IgG1)				
Heavy chain	Q1+Gln→Pyro-Glu	QVQLK	99.778	0.027
Heavy Chain	N31+Deamidation	SLTNYGVHWVR	0.172	8.421
Heavy Chain	N70+Deamidation	LSINK	0.078	5.746
Heavy Chain	N73+Deamidation	LSINKDNSK	0.939	8.572
Heavy Chain	Q77+Deamidation	SQVFFK	0.173	8.224
Heavy Chain	N288+Deamidation	FNWYVDGVEVHNAK	0.492	5.948
Heavy Chain	N299+Deamidation	EEQYNSTYR	0.130	11.260
Heavy Chain	N317+Deamidation	VVSVLTVLHQDWLNGK	1.546	10.566
Heavy Chain	N327+Deamidation	CKVSNK; VSNK	1.399	5.030
Heavy Chain	N363+Deamidation	NQVSLTCLVK	1.210	6.686
Heavy Chain	Q364+Deamidation	NQVSLTCLVK	0.233	15.143
Heavy Chain	~N391+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.537	6.047
Heavy Chain	~Q421+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	0.459	7.148
Heavy Chain	~N436+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.221	8.461
Light Chain	Q6+Deamidation	DILLTQSPVILSVSPGER	0.296	13.542
Light Chain	Q27+Deamidation	ASQSIGTNIHWYQQR	0.420	13.158
Light Chain	~Q37+Deamidation	ASQSIGTNIHWYQQR	0.157	17.755
Light Chain	N41+Deamidation	TNGSPR	4.664	9.743
Light Chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.691	9.221
Light Chain	Q147+Deamidation	VQWK	0.012	5.418
Light Chain	~Q155+Deamidation	VDNALQSGNSQESVTEQDSK	0.405	10.058
Light Chain	~N158+Deamidation	VQWKVDNALQSGNSQESVTEQDSK;	0.688	18.898
Light Chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.280	8.257
Heavy Chain	M254+Oxidation	DTLMISR	1.431	4.975
Heavy Chain	M430+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK;	0.827	7.532
Heavy Chain	K75+Glycation	DNSKSQVFFK	0.060	14.765
Heavy Chain	K135+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.156	5.155
Heavy Chain	~K290+Glycation	FNWYVDGVEVHNAKTKPR	0.182	3.349
Heavy Chain	K328+Glycation	VSNKALPAPIEK	0.245	3.073
Light Chain	K49+Glycation	LLIKYASESISGIPSR	0.151	7.557
Light Chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.346	3.625
Light Chain	K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.206	9.077
Heavy Chain	K449+Lys Loss	SLSLSPGK	67.128	2.015
Heavy Chain	N299+A1G0	EEQYNSTYR	0.421	5.738
Heavy Chain	N299+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	3.676	9.192
Heavy Chain	N299+A1G0M4	EEQYNSTYR	0.256	5.985
Heavy Chain	N299+A1G0M5	EEQYNSTYR	0.602	2.687
Heavy Chain	N299+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.700	5.358
Heavy Chain	N299+A1G1M4F	EEQYNSTYR	1.710	8.178
Heavy Chain	N299+A1G1M5	EEQYNSTYR	0.832	3.161
Heavy Chain	N299+A1G1M5F	EEQYNSTYR	1.979	2.623
Heavy Chain	N299+A1S1M5	EEQYNSTYR	0.188	4.132
Heavy Chain	N299+A2G0	EEQYNSTYR; TKPREEQYNSTYR	0.616	2.710
Heavy Chain	N299+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	38.399	1.069
Heavy Chain	N299+A2G1	EEQYNSTYR	0.298	2.476

Table 10. (Continued) Summary of PTMs identified and quantified for cetuximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=9)	RSD (n=9)
CETUXIMAB (chimeric IgG1)				
Heavy Chain	N299+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	29.404	1.738
Heavy Chain	N299+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	6.134	1.468
Heavy Chain	N299+A2Ga1G1F	EEQYNSTYR	0.724	2.554
Heavy Chain	N299+A2Ga2F	EEQYNSTYR	0.285	9.907
Heavy Chain	N299+A2S1G1	EEQYNSTYR	0.091	11.504
Heavy Chain	N299+Gn	EEQYNSTYR	0.254	7.589
Heavy Chain	N299+M3	EEQYNSTYR	0.110	27.945
Heavy Chain	N299+M4	EEQYNSTYR	0.718	15.189
Heavy Chain	N299+M5	EEQYNSTYR; TKPREEQYNSTYR	14.502	1.228
Heavy Chain	N299+M6	EEQYNSTYR; TKPREEQYNSTYR	1.760	1.520
Heavy Chain	N299+M7	EEQYNSTYR; TKPREEQYNSTYR	0.426	5.671
Heavy Chain	N299+Unglycosylated	EEQYNSTYR	1.093	8.454
Heavy Chain	N88+A1G0F	MNSLQSNDTAIYYCAR	0.969	5.826
Heavy Chain	N88+A1G1F	MNSLQSNDTAIYYCAR	0.482	3.863
Heavy Chain	N88+A1G1M4F	MNSLQSNDTAIYYCAR	1.305	3.604
Heavy Chain	N88+A1G1M5F	MNSLQSNDTAIYYCAR	0.801	2.973
Heavy Chain	N88+A1S1M5	MNSLQSNDTAIYYCAR	0.192	11.107
Heavy Chain	N88+A2G0F	MNSLQSNDTAIYYCAR	2.491	4.836
Heavy Chain	N88+A2G1F	MNSLQSNDTAIYYCAR	4.433	2.141
Heavy Chain	N88+A2G2F	MNSLQSNDTAIYYCAR	6.832	2.530
Heavy Chain	N88+A2Ga1G1F	MNSLQSNDTAIYYCAR	7.452	1.915
Heavy Chain	N88+A2Ga2F	MNSLQSNDTAIYYCAR	34.807	1.159
Heavy Chain	N88+A2S1Ga1F	MNSLQSNDTAIYYCAR	0.993	1.954
Heavy Chain	N88+A2Sg1G0F	MNSLQSNDTAIYYCAR	0.722	2.152
Heavy Chain	N88+A2Sg1G1F	MNSLQSNDTAIYYCAR	3.991	4.522
Heavy Chain	N88+A2Sg1Ga1F	MNSLQSNDTAIYYCAR	17.037	1.641
Heavy Chain	N88+A2Sg2F	MNSLQSNDTAIYYCAR	2.490	2.532
Heavy Chain	N88+A3G1F	MNSLQSNDTAIYYCAR	0.511	17.567
Heavy Chain	N88+A3G2F	MNSLQSNDTAIYYCAR	1.579	1.789
Heavy Chain	N88+A3G3F	MNSLQSNDTAIYYCAR	0.457	13.301
Heavy Chain	N88+A3Ga1G2F	MNSLQSNDTAIYYCAR	1.159	3.235
Heavy Chain	N88+A3Ga2G1F	MNSLQSNDTAIYYCAR	1.725	1.849
Heavy Chain	N88+A3Ga3F	MNSLQSNDTAIYYCAR	3.784	1.967
Heavy Chain	N88+A3S1Ga2F	MNSLQSNDTAIYYCAR	0.573	2.850
Heavy Chain	N88+A3Sg1G2F	MNSLQSNDTAIYYCAR	0.637	2.161
Heavy Chain	N88+A3Sg1Ga1G1F	MNSLQSNDTAIYYCAR	1.127	1.682
Heavy Chain	N88+A3Sg1Ga2F	MNSLQSNDTAIYYCAR	2.098	2.101
Heavy Chain	N88+A3Sg2G1F	MNSLQSNDTAIYYCAR	0.192	4.822
Heavy Chain	N88+A3Sg2Ga1F	MNSLQSNDTAIYYCAR	0.289	7.464
Heavy Chain	N88+Gn	MNSLQSNDTAIYYCAR	0.112	6.637
Heavy Chain	N88+GnF	MNSLQSNDTAIYYCAR	0.160	4.803
Heavy Chain	N88+M4	MNSLQSNDTAIYYCAR	0.093	13.101
Heavy Chain	N88+M5	MNSLQSNDTAIYYCAR	1.991	3.211

Table 11. Summary of PTMs identified and relatively quantified for rituximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
RITUXIMAB (chimeric IgG1)				
Heavy chain	Q1+Gln→Pyro-Glu	QVQLQQPGAELVKPGASVK	99.919	0.002
Light chain	Q1+Gln→Pyro-Glu	QIVLSQSPAILSASPGEK	85.693	1.158
Heavy chain	N33+Deamidation	ASGYTFTSYNMHWVK; NMHWVK	0.439	5.608
Heavy chain	N55+Deamidation	GLEWIGAIYPGNGDTSYNQK	7.067	7.666
Heavy chain	N163+Deamidation	DYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLY	0.457	4.674
Heavy chain	N290+Deamidation	FNWYVDGVEVHNAK	0.520	1.756
Heavy chain	N301+Deamidation	EEQYNSTYR	0.095	2.323
Heavy chain	N319+Deamidation	VVSVLTVLHQDWLNGK; TVLHQDWLNGK	1.950	11.737
Heavy chain	N365+Deamidation	NQVSLTCLVK	1.211	3.802
Heavy chain	Q366+Deamidation	NQVSLTCLVK	0.273	4.272
Heavy chain	~N393+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.489	7.556
Heavy chain	~Q423+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	0.551	12.770
Heavy chain	~N438+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.300	9.579
Light chain	~Q36+Deamidation	ASSSVSYIHWFQQKPGSSPKPW	0.224	3.650
Light chain	~Q88+Deamidation	VEAEDAATYYCQQWTSNPPTFGGGTK	0.387	12.866
Light chain	~N136+Deamidation	SGTASVVCLLNNFYPR	0.711	4.549
Light chain	Q146+Deamidation	VQWK	0.285	3.753
Light chain	~Q159+Deamidation	VDNALQSGNSQESVTEQDSK	0.319	12.837
Light chain	Q198+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.071	5.828
Heavy chain	M34+Oxidation	ASGYTFTSYNMHWVK; TFTSYNMHWVK; NMHWVK	0.706	6.209
Heavy chain	M81+Oxidation	SSSTAYMQLSSLTSEDSAVYYCAR	0.938	6.038
Heavy chain	M256+Oxidation	DTLMISR	2.805	1.457
Heavy chain	M432+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	2.168	4.135
Heavy chain	M21+Oxidation	VTMTCR	0.203	12.689
Heavy chain	K137+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.302	9.471
Heavy chain	K252+Glycation	PKDTLMISR	0.045	7.924
Heavy chain	~K292+Glycation	FNWYVDGVEVHNAKTKPR	0.431	2.588
Heavy chain	K321+Glycation	VVSVLTVLHQDWLNGKEYK	0.085	2.214
Heavy chain	K330+Glycation	VSNKALPAPIEK	0.536	7.005
Light chain	K148+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.690	12.726
Light chain	K182+Glycation	DSTYSLSSTLTLSKADYEK	0.458	7.301
Light chain	K187+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.585	3.770
Light chain	K189+Glycation	HKVYACEVTHQGLSSPVTK	0.077	10.802
Heavy chain	K451+Lys Loss	SLSLSPGK	96.512	0.037
Heavy chain	N301+A1G0	EEQYNSTYR	0.342	5.911
Heavy chain	N301+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	5.062	2.841
Heavy chain	N301+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.797	11.967
Heavy chain	N301+A1S1F	EEQYNSTYR	0.208	1.153
Heavy chain	N301+A2G0	EEQYNSTYR; TKPREEQYNSTYR	1.611	3.774
Heavy chain	N301+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	40.775	0.845
Heavy chain	N301+A2G1	EEQYNSTYR	0.632	0.804
Heavy chain	N301+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	39.803	2.049

Table 11. (Continued) Summary of PTMs identified and relatively quantified for rituximab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
RITUXIMAB (chimeric IgG1)				
Heavy chain	N301+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	7.511	2.256
Heavy chain	N301+A2S1G0F	EEQYNSTYR	0.483	8.957
Heavy chain	N301+A2S1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.005	11.177
Heavy chain	N301+A2S2F	EEQYNSTYR; TKPREEQYNSTYR	0.478	10.156
Heavy chain	N301+A3G1F	EEQYNSTYR	0.157	3.359
Heavy chain	N301+M5	EEQYNSTYR; TKPREEQYNSTYR	1.454	0.280
Heavy chain	N301+M6	EEQYNSTYR	0.295	7.111
Heavy chain	N301+Unglycosylated	EEQYNSTYR	1.336	17.487

Table 12. Summary of PTMs identified and relatively quantified for trastuzumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
TRASTUZUMAB (humanized IgG1)				
Heavy chain	~Q13+Deamidation	EVQLVESGGGLVQPGGSLR	0.299	14.640
Heavy chain	N28+Deamidation	LSCAASGFNIK	0.051	10.428
Heavy chain	N55+Deamidation	IYPTNGYTR	1.693	2.128
Heavy chain	N77+Deamidation	NTAYLQMNSLR	0.662	1.892
Heavy chain	Q82+Deamidation	NTAYLQMNSLR	0.242	15.735
Heavy chain	N289+Deamidation	FNWYVDGVEVHNAK	0.264	5.494
Heavy chain	N300+Deamidation	EEQYNSTYR	0.073	5.561
Heavy chain	N318+Deamidation	VVSVLTVLHQDWLNGK; TVLHQDWLNGK	1.937	11.390
Heavy chain	N328+Deamidation	CKVSNK	2.545	4.699
Heavy chain	N364+Deamidation	NQVSLTCLVK	1.164	4.098
Heavy chain	Q365+Deamidation	NQVSLTCLVK	0.154	15.557
Heavy chain	~N387+Deamidation	GFYPSDIAVEWESNGQPENNYK	2.376	3.210
Heavy chain	~Q421+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	0.295	13.616
Heavy chain	~N437+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	1.191	6.289
Light chain	~Q27+Deamidation	ASQDVNTAVAWYQQKPGK	4.766	1.565
Light chain	N30+Deamidation	ASQDVNTAVAWYQQKPGK; ASQDVNTAVAWYQQKPGKAPK	3.087	6.181
Light chain	~Q38+Deamidation	ASQDVNTAVAWYQQKPGK	0.222	9.985
Light chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.608	2.685
Light chain	Q199+Deamidation	VYACEVTHQGLSSPVTK	0.064	19.876
Heavy chain	M83+Oxidation	NTAYLQMNSLR	0.415	2.390
Heavy chain	M107+Oxidation	WGGDGFYAMDYWGQGTLVTVSSASTK	0.844	3.392
Heavy chain	M255+Oxidation	DTLMISR	1.753	2.351
Heavy chain	M361+Oxidation	EEMTKNQVSLTCLVK; EEMTK; EPQVYTLPPSREEMTK	0.164	8.929
Heavy chain	M431+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.034	8.911
Light chain	M4+Oxidation	DIQMTQSPSSLSASVGDR	0.381	8.610
Heavy chain	K30+Glycation	LSCAASGFNIKDTYIHWVR	0.820	1.723
Heavy chain	K76+Glycation	FTISADTSKNTAYLQMNSLR	0.179	6.451
Heavy chain	K136+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.497	5.148
Heavy chain	K251+Glycation	PKDTLMISR	0.088	9.273

Table 12. (Continued) Summary of PTMs identified and relatively quantified for trastuzumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
TRASTUZUMAB (humanized IgG1)				
Heavy chain	~K291+Glycation	FNWYVDGVEVHNAKTKPR	0.482	2.329
Heavy chain	K320+Glycation	VVSVLTVLHQDWLNGKEYK	0.116	8.646
Heavy chain	K323+Glycation	EYKCK	0.104	9.495
Heavy chain	K325+Glycation	CKVSNK	1.580	12.067
Heavy chain	K329+Glycation	VSNKALPAPIEK; CKVSNKALPAPIEK	0.335	1.391
Heavy chain	K337+Glycation	ALPAPIEKTISK	0.063	3.309
Heavy chain	K363+Glycation	EEMTKNQVSLTCLVK	0.080	6.908
Light chain	K103+Glycation	SGTDFTLTISSLQPEDFATYYCQQHY TTPPTFGQGTKVEIK	1.152	1.359
Light chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.919	4.916
Light chain	K183+Glycation	DSTYSLSSTLTLSKADYEK	0.369	13.651
Light chain	K188+Glycation	ADYEKHK	0.939	6.465
Light chain	K190+Glycation	HKVYACEVTHQGLSSPVTK	0.113	4.871
Heavy chain	K450+Lys Loss	SLSLSPGK	97.730	0.098
Heavy chain	N300+A1G0	EEQYNSTYR; TKPREEQYNSTYR	2.297	2.306
Heavy chain	N300+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	6.976	9.383
Heavy chain	N300+A1G1	EEQYNSTYR	0.594	1.401
Heavy chain	N300+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	2.180	6.721
Heavy chain	N300+A1S1	EEQYNSTYR	0.091	5.173
Heavy chain	N300+A1S1F	EEQYNSTYR	0.401	2.875
Heavy chain	N300+A2G0	EEQYNSTYR; TKPREEQYNSTYR	5.378	1.718
Heavy chain	N300+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	39.879	0.616
Heavy chain	N300+A2G1	EEQYNSTYR	1.938	0.475
Heavy chain	N300+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	32.283	2.911
Heavy chain	N300+A2G2	EEQYNSTYR	0.165	1.872
Heavy chain	N300+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	5.190	3.141
Heavy chain	N300+A2S1G0F	EEQYNSTYR	0.442	10.083
Heavy chain	N300+A2S1G1F	EEQYNSTYR	0.647	1.787
Heavy chain	N300+A2S2F	EEQYNSTYR	0.234	6.664
Heavy chain	N300+A3G1F	EEQYNSTYR	0.161	4.784
Heavy chain	N300+Gn	EEQYNSTYR	0.069	6.463
Heavy chain	N300+M3	EEQYNSTYR	0.165	17.312
Heavy chain	N300+M4	EEQYNSTYR	0.153	0.493
Heavy chain	N300+M5	EEQYNSTYR; TKPREEQYNSTYR	2.874	2.319
Heavy chain	N300+M6	EEQYNSTYR	0.204	3.781
Heavy chain	N300+Unglycosylated	EEQYNSTYR	1.443	4.976

Table 13. Summary of PTMs identified and relatively quantified for bevacizumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
BEVACIZUMAB (humanized IgG1)				
Heavy chain	~Q3+Deamidation	EVQLVESGGGLVQPGGSLR	0.889	7.378
Heavy chain	N52+Deamidation	GLEWVGWINTYTGEPTYAADFK	0.172	1.118
Heavy chain	Q82+Deamidation	STAYLQMNSLR	0.800	4.216
Heavy chain	N84+Deamidation	STAYLQMNSLR	1.760	4.745
Heavy chain	N292+Deamidation	FNWYVDGVEVHNAK	0.400	4.808
Heavy chain	N303+Deamidation	EEQYNSTYR	0.213	6.696
Heavy chain	N321+Deamidation	VVSVLTVLHQDWLNGK	1.285	6.153
Heavy chain	N367+Deamidation	NQVSLTCLVK	1.165	2.714
Heavy chain	Q368+Deamidation	NQVSLTCLVK	0.493	9.825
Heavy chain	~N395+Deamidation	GFYPSDIAVEWESNGQPENNYK	1.256	13.661
Heavy chain	~Q425+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	2.013	12.658
Light chain	~Q3+Deamidation	DIQMTQSPSSLSASVGDR	0.081	14.929
Light chain	~Q6+Deamidation	DIQMTQSPSSLSASVGDR	0.671	3.767
Light chain	~N34+Deamidation	VTITCSASQDISNYLNWYQQKPGK	0.769	6.008
Light chain	~N137+Deamidation	SGTASVVCLLNNFYPR	0.651	5.199
Light chain	Q147+Deamidation	VQWK	0.412	7.233
Light chain	~Q160+Deamidation	VQWKVDNALQSGNSQESVTEQDSK; VDNALQSGNSQESVTEQDSK	0.877	9.020
Light chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.356	6.204
Heavy chain	M34+Oxidation	LSCAASGYTFTNYGMNWVR; TFTNYGMNWVR	0.653	9.110
Heavy chain	M83+Oxidation	STAYLQMNSLR	0.807	5.659
Heavy chain	M258+Oxidation	DTLMISR	3.333	6.960
	M434+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.604	8.337
Light chain	M4+Oxidation	DIQMTQSPSSLSASVGDR	0.538	10.628
Heavy chain	K65+Glycation	GLEWVGWINTYTGEPTYAADFKR	0.230	9.207
Heavy chain	K98+Glycation	AEDTAVYYCAKYPHYY; AEDTAVYYCAKYPHYYGSSHW	0.415	5.397
Heavy chain	K139+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.425	1.092
Heavy chain	K254+Glycation	PKDTLMISR	0.204	9.839
Heavy chain	~K294+Glycation	FNWYVDGVEVHNAKTKPR	0.505	1.635
Heavy chain	K332+Glycation	VSNKALPAPIEK	0.575	0.299
Light chain	K103+Glycation	TFGQGTKVEIK	1.227	2.171
Light chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.918	3.678
Light chain	K169+Glycation	VDNALQSGNSQESVTEQDSKDST YSLSSTLTLSK	0.239	3.940
Light chain	K183+Glycation	DSTYSLSSTLTLSKADYEK	0.439	13.595
Light chain	K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK; ADYEKHK	0.782	0.929
Light chain	K190+Glycation	HKVYACEVTHQGLSSPVTK	0.135	5.153
Heavy chain	K453+Lys Loss	SLSLSPGK	97.454	0.016

Table 13. (Continued) Summary of PTMs identified and relatively quantified for bevacizumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
BEVACIZUMAB (humanized IgG1)				
Heavy chain	N303+A1G0	EEQYNSTYR	0.837	3.039
Heavy chain	N303+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	6.607	9.799
Heavy chain	N303+A1G1F	EEQYNSTYR	0.761	4.044
Heavy chain	N303+A2G0	EEQYNSTYR; TKPREEQYNSTYR	2.335	3.762
Heavy chain	N303+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	74.864	0.893
Heavy chain	N303+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	10.769	1.695
Heavy chain	N303+A2G2F	EEQYNSTYR	0.735	0.551
Heavy chain	N303+M5	EEQYNSTYR	0.873	3.126
Heavy chain	N303+Unglycosylated	EEQYNSTYR	5.348	1.247

Table 14. Summary of PTMs identified and relatively quantified for adalimumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
ADALIMUMAB (fully human IgG1)				
Heavy Chain	~Q3+Deamidation	EVQLVESGGGLVQPGR	2.644	2.644
Heavy Chain	N77+Deamidation	NSLYLQMNSLR	1.851	2.437
Heavy Chain	Q82+Deamidation	NSLYLQMNSLR	0.952	5.733
Heavy Chain	N84+Deamidation	NSLYLQMNSLR	0.267	2.713
Heavy Chain	Q113+Deamidation	VSYLSTASSLDYWGQGTLVTVSSASTK	1.493	3.818
Heavy Chain	N290+Deamidation	FNWYVDGVEVHNAK	0.691	5.938
Heavy Chain	N301+Deamidation	EEQYNSTYR	0.240	3.262
Heavy Chain	N319+Deamidation	VVSVLTVLHQDWLNGK	2.150	10.848
Heavy Chain	N329+Deamidation	CKVSNK	1.761	4.136
Heavy Chain	N365+Deamidation	NQVSLTCLVK	1.246	1.989
Heavy Chain	Q366+Deamidation	NQVSLTCLVK	0.824	5.030
Heavy Chain	~N388+Deamidation	GFYPSDIAVEWESNGQPENNYK	1.062	6.606
Heavy Chain	~Q423+Deamidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	1.957	9.794
Heavy Chain	~Q442+Deamidation	WQQGNVFSCSVMHEALHNHYTQK	2.360	3.829
Light Chain	~Q3+Deamidation	DIQMTQSPSSLSASVGDR	1.303	1.031
Light Chain	~Q6+Deamidation	DIQMTQSPSSLSASVGDR	1.280	5.373
Light Chain	Q27+Deamidation	ASQGIR	1.078	1.777
Light Chain	N31+Deamidation	NYLAWYQQKPGK	0.975	1.194
Light Chain	~Q37+Deamidation	NYLAWYQQKPGK	2.119	1.391
Light Chain	Q55+Deamidation	LLIYAASTLQSGVPSR	1.165	1.969
Light Chain	Q79+Deamidation	FSGSGSGTDFTLTISSLQPEDVATY	0.821	9.288
Light Chain	Q100+Deamidation	APYTFGQGTK; YNRAPYTFGQGTK; APYTFGQGTKVEIKR	0.911	2.157
Light Chain	~N137+Deamidation	SGTASVVCLLNNFYPR	1.169	2.671
Light Chain	Q147+Deamidation	VQWK	0.883	2.261
Light Chain	~N152+Deamidation	VQWKVDNALQSGNSQESVTEQDSK	0.418	8.678
Light Chain	~Q160+Deamidation	VQWKVDNALQSGNSQESVTEQDSK; VDNALQSGNSQESVTEQDSK	2.309	1.920
Light Chain	Q166+Deamidation	VDNALQSGNSQESVTEQDSK	0.821	8.929
Light Chain	Q199+Deamidation	HKVYACEVTHQGLSSPVTK; VYACEVTHQGLSSPVTK	0.884	4.186

Table 14. (Continued) Summary of PTMs identified and relatively quantified for adalimumab biotherapeutic product after tryptic digestion using the Magnetic Smart Digest kit on the KingFisher Duo Prime system

Protein	Modification	Sequence	Relative Abundance (%) (n=3)	RSD (n=3)
ADALIMUMAB (fully human IgG1)				
Heavy Chain	M34+Oxidation	LSCAASGFTFDDYAMHWVR	0.823	3.711
Heavy Chain	~W53+Oxidation	GLEWVSAITWNSGHIDYADSVEGR	0.083	11.215
Heavy Chain	M83+Oxidation	NSLYLQMNSLR	0.673	5.697
Heavy Chain	M256+Oxidation	DTLMISR	4.014	0.284
Heavy Chain	M432+Oxidation	WQQGNVFSCSVMHEALHNHYTQK; SRWQQGNVFSCSVMHEALHNHYTQK	3.249	2.223
Light Chain	M4+Oxidation	DIQMTQSPSSLSASVGDR; DIQMTQSPSSLSASVGDRVTITCR	0.553	2.021
Heavy Chain	K76+Glycation	DNAKNSLYLQMNSLR	0.090	3.475
Heavy Chain	K137+Glycation	GPSVFPLAPSSKSTSGGTAALGCLVK	0.275	2.992
Heavy Chain	~K292+Glycation	FNWYVDGVEVHNAKTKPR	0.242	1.760
Heavy Chain	K330+Glycation	VSNKALPAPIEK	0.330	2.494
Light Chain	K103+Glycation	APYTFGQGTKVEIK; APYTFGQGTKVEIKR	0.198	1.793
Light Chain	K149+Glycation	VQWKVDNALQSGNSQESVTEQDSK	0.454	1.797
Light Chain	~K188+Glycation	ADYEKHKVYACEVTHQGLSSPVTK	0.444	7.008
Heavy Chain	K451+Lys Loss	SLSLSPGK	78.254	0.559
Heavy Chain	N301+A1G0	EEQYNSTYR	0.511	7.201
Heavy Chain	N301+A1G0F	EEQYNSTYR; TKPREEQYNSTYR	5.562	3.165
Heavy Chain	N301+A1G1F	EEQYNSTYR; TKPREEQYNSTYR	1.115	2.578
Heavy Chain	N301+A2G0	EEQYNSTYR; TKPREEQYNSTYR	0.972	7.113
Heavy Chain	N301+A2G0F	EEQYNSTYR; TKPREEQYNSTYR	64.244	0.718
Heavy Chain	N301+A2G0FB	EEQYNSTYR	0.244	5.530
Heavy Chain	N301+A2G1F	EEQYNSTYR; TKPREEQYNSTYR	15.473	1.181
Heavy Chain	N301+A2G2F	EEQYNSTYR; TKPREEQYNSTYR	1.122	2.580
Heavy Chain	N301+Gn	EEQYNSTYR	0.362	1.247
Heavy Chain	N301+GnF	EEQYNSTYR	0.060	1.931
Heavy Chain	N301+M3	EEQYNSTYR; TKPREEQYNSTYR	0.793	5.631
Heavy Chain	N301+M4	EEQYNSTYR; TKPREEQYNSTYR	0.973	7.936
Heavy Chain	N301+M5	EEQYNSTYR; TKPREEQYNSTYR	5.501	2.622
Heavy Chain	N301+M6	EEQYNSTYR; TKPREEQYNSTYR	2.526	2.694
Heavy Chain	N301+M7	EEQYNSTYR; TKPREEQYNSTYR	1.127	2.949
Heavy Chain	N301+M8	EEQYNSTYR; TKPREEQYNSTYR	0.386	6.322
Heavy Chain	N301+Unglycosylated	EEQYNSTYR	3.464	1.341

Conclusions

- The Magnetic SMART Digest kit provides simple and rapid protein digestion for peptide mapping analysis and PTM investigation of mAbs.

- The analysis of five top monoclonal antibodies using the described global workflow solution results in excellent quality data with high confidence in results. Excellent sequence coverage (100%) and low levels of sample preparation-induced post-translational modifications (PTMs) were observed with the Magnetic SMART Digest kit.

- Easily automated peptide mapping results in less sample handling, increased productivity, and improved reproducibility.

- The combination of the KingFisher Duo Prime system with the Magnetic SMART Digest kit offers a global automated option for biotherapeutic digestions, simplifying the process for reproducible method transfer and reducing the time needed for sample preparation. This not only speeds up the biotherapeutic peptide mapping workflow for high-throughput analysis, but also significantly reduces method development time.

- The Vanquish Flex UHPLC delivers outstanding retention time and peak area precision. The system offers a high-pressure flow path and sample pressurization prior to injection ensuring high peak capacity, retention time stability, and peak area precision, ideal for peptide mapping applications.

- The Q Exactive Hybrid Quadrupole-Orbitrap system has been proven to deliver excellent mass accuracy and highly sensitive MS results for protein identification and detailed peptide mapping. BioPharma Finder 3.0 software can provide automatic data processing, peptide sequence matching, and protein sequence coverage mapping accurately and with high confidence.

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