Chapter 2

Characterization of Biopharmaceuticals Focusing on Antibody Therapeutics

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Additional information is available at the end of the chapter

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Abstract

Biopharmaceuticals are highly complex molecules and also require high quality for safety and efficacy in human uses. For well-characterized products, the desired level of quality should be monitored and controlled during the manufacturing processes. A series of workflow for analytical characterization should be applied for product quality throughout those processes. In this chapter, several analytical techniques are introduced for assessing characteristics of biopharmaceuticals focusing on monoclonal antibodies (mAbs). Analytical characterization for primary structure was performed by mass spectrometry (MS), and assessment of post-translational modifications (PTMs) was done by conventional approaches. The analytical assessments were also done by multi-attribute method (MAM) approach using mass spectrometer (MS), and the performance of MAM was compared to conventional approaches.

Keywords: biopharmaceutical, analytical characterization, primary structure, mass spectrometry, post-translational modification (PTM), multi-attribute method (MAM)

1. Introduction

Biopharmaceuticals can be defined as protein drugs that are produced by recombinant DNA technology, such as hormones, enzymes, monoclonal antibodies, and fusion proteins used for therapeutic or diagnostic purposes [1]. The first biopharmaceutical, insulin, was introduced in 1982 [2], and since then, over 250 biopharmaceutical products are authorized for marketing in the two major regions, United States of America (USA) and Europe (EU). Those products can be classified into monoclonal antibodies (mAb), hormones, growth factors, vaccines, cytokines, blood factors, and others [3]. This trend with increasing number of biopharmaceuticals

on the market results in gaining interest for drug development industry, and biopharmaceuticals are considered as fast growing and promising area for drug development [3–6].

The approval of mAb-related products is dramatically increased in the recent years [6, 7]. Over 90 mAb-related products are approved by European Medicines Agency (EMA) and US Food and Drug Administration (FDA). Those can be classified into mAb, Fc-fusion, Fab, antibody-drug conjugate (ADC), bispecific mAb (bsAb), and bispecific T cell engager (BiTE). Among them, mAbs are the major product, consisting of 77% of total. Others represent rest 23% of total, Fc-fusion (12%), ADC (5%), Fab (3%), bsAb (2%), and BiTE (1%), respectively. After the first approval of full-length mAb in 1998, mAbs are major product in the biopharmaceutical industry. This increasing number gives high revenue for pharmaceutical companies, and seven mAb-related products are positioned in top 10 drugs in the world, 2017, including Humira, Enbrel, Rituxan, Remicade, AVASTIN, Herceptin, and Lantus [8].

Mylotarg is the first approved ADC in 2000, which combined a mAb targeting leukemic blast cells with a bacterial toxin (calicheamicin) [7, 9]. ADC is a complex generated between a mAb and small molecule or a peptide, and mAb gives the selective delivery for targeting of cytotoxic drugs [1, 9–11]. Since the first approval, four additional ADC products are approved in Europe and USA. bsAb has two different antigen binding sites recognizing two different epitopes in a single mAb, and this dual specificity gives more specific targeting and higher efficacy [12–14]. Currently, three bsAbs are approved by EMA or US FDA. The first bsAb, Removab, was approved in 2009 but voluntarily withdrawn in 2013. Fc-fusion proteins are fusions of the IgG Fc domain with a desired linked protein, enhancing pharmacokinetic properties (serum half-life) and pharmacodynamics properties (ADCC and CDC) [6, 15]. Following the first approval of Fc-fusion protein, Enbrel in 1998, eight Fc-fusion proteins are authorized for the marketing in the region of Europe and USA.

Biosimilars, known as follow-on biologics, which follow termination of patent protection of original biopharmaceutical products, are developed and approved since 2006. Following the first approval from EMA, over 35 biosimilars are authorized for the European market and over 20 biosimilars are approved from FDA since 2015 [16–18]. A biosimilar is a biological product that is highly similar to and has no clinically meaningful differences from an existing FDA-approved reference product in terms of safety, purity, and potency (safety and effectiveness) [19]. Structural and clinical similarities are to be proven for the biosimilar authorization.

Biopharmaceuticals are highly complex molecules compared to small molecule drugs and should be monitored and controlled during the manufacturing processes for well-characterized products [20–22]. The characterization of biopharmaceuticals is challenging, which utilize the state-of-the-art technology to meet the international harmonized guidelines, Q5E and Q6B [23, 24]. For proper characterization, critical quality attributes (CQAs) have to be defined and evaluated that may impact on safety, purity, and potency. CQA is defined by ICHQ8(R2) as a physical, chemical, biological, or microbiological property of characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality [25].

Within this chapter, methodologies for in-depth physicochemical characterization are introduced for recombinant biopharmaceuticals mainly focused on mAbs. Analytical characterization for primary structure was performed by mass spectrometry, and assessment of post-translational modifications (PTMs) was done with conventional and multi-attribute method (MAM) approaches. The performance of MAM was compared to conventional approach.

2. Structural characterization for identity

2.1. Intact mass determination of mAb

The molecular weight of a protein is an important parameter in the physicochemical properties of the protein. MS with high resolution and accuracy, such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or electrospray ionization quadrupole time-of-flight (ESI-QTOF), has become a reliable and sensitive technique for proteins and peptides to determine the molecular weight [26, 27]. Other high resolution mass spectrometers, Fourier transform ion cyclotron resonance (FTICR) and orbitrap MS, have been recently used for the intact mass analysis [28–30]. The workflow of ESI was further extended to native MS, having the capability to investigate intact mAb for structural analysis and heterogeneities from PTM [31, 32]. Applying intact mass analysis, it not only gives molecular mass of the intact protein for comparison with the theoretical mass but also provides quick information about primary structure of protein or sequence variants, such as isoforms, truncation, mutations, addition of signal sequences, or PTMs including glycosylations [31–33].

ESI-QTOF is widely used for measuring intact mass because of high resolution, mass accuracy, and easy connection to high performance liquid chromatography (HPLC) for online analysis [34]. ESI produces multiply charged ions, and those ions can be deconvoluted for molecular mass of proteins [34]. Typical results of mass determination for mAbs, bevacizumab, rituximab, and trastuzumab using ESI-QTOF are shown in **Figure 1**. MS spectra in inset of **Figure 1** show multiply charged ions characterized by ESI-spectrum, a number of peaks corresponding a statistical distribution of different charge states. The accuracy was less than 50 ppm, providing tools for the identification of PTMs, glycosylation, C-terminal Lysine deletion (ΔK), or Gln/Glu cyclization (pE) (**Figure 1**).

2.2. Subunit mass determination of mAb

Subunits or fragments of mAbs can be obtained by reduction of disulfide bonds or proteolysis to reduce complexities for large size mAbs [36, 37]. Chemical reduction of mAb's disulfide bond generates free heavy chains and light chains, having ~50 kDa and ~25 kDa molecular mass, respectively. Recently, a new protease IdeS (Immunoglobulin G-degrading enzyme of *Streptococcus pyogenes*), specifically cleaving between the two consecutive glycine residues of the hinge region, has been described for mAb fragmentations [29, 37–39]. IdeS treatment of

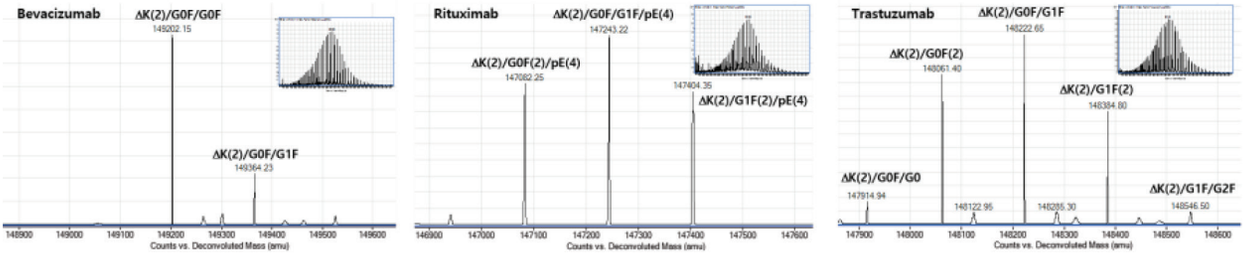

Figure 1. Intact mass of mAbs determined by deconvolution using ESI-QTOF. Inserts show charge envelope having multiply charged ions. Glycan structures (G0F, G1F, and G2F) were adapted from [35]. ΔK: C-terminal Lys deletion and pE: pyro-glutamate.

mAbs with subsequent chemical reduction generates three subunits, Fd, LC, and Fc/2, having ~25 kDa molecular mass. Those subunits can be separated by reverse phase (RP)-HPLC analysis and be analyzed using online MS analysis. A typical example of IdeS treatment of mAb is shown in **Figure 2**. The subunits of rituximab were generated by IdeS digestion with subsequent chemical reduction and separated by RP-HPLC. The molecular masses of the subunits were measured by online ESI-QTOF. Three subunits from IdeS-digested rituximab, Fc/2, LC, and Fd, were clearly separated on the chromatogram of RP-HPLC (**Figure 2**). The deconvolution of ESI-QTOF spectra for subunits gives molecular mass information (**Figure 3**, inset). The molecular masses for Fc/2, LC, or Fd subunits were 25.4, 23.0, or 25.3 kDa, respectively. This not only provides tools for N-glycan profiling but also allows identification of PTMs such as C-terminal Lys deletion and cyclization of N-terminal glutamine (**Figure 2**).

2.3. Peptide mapping of mAb

The peptide mapping is a gold standard for biopharmaceutical characterization not only as an identity test but also to demonstrate the integrity of disulfide bonds [40–43]. This analytical method provides detailed information of primary structure for a given protein and enables the control of the protein sequence down to the level of single amino acids by coupling with mass spectrometry [44–46]. Based on the analysis of peptide mapping, it is possible to confirm genetic stability (correct translation), identify post-translation modification, and demonstrate the integrity of disulfide bonds [47–50].

Peptide mapping was carried out by digesting protein samples with endoprotease, such as trypsin, and subsequent separation of peptide fragments by RP-HPLC. The peptide fragments are then monitored by UV absorption and identified by MS. Prior to protease digestion, denaturation of the test protein with known concentration is needed to ensure complete digestion. The measured absorbance of a protein sample solution is used to calculate the concentration from its absorptivity at 280 nm (A280) either experimentally determined or empirically calculated [22, 51]. Denaturation can be done using chaotropic reagent, urea, SDS, guanidine, or

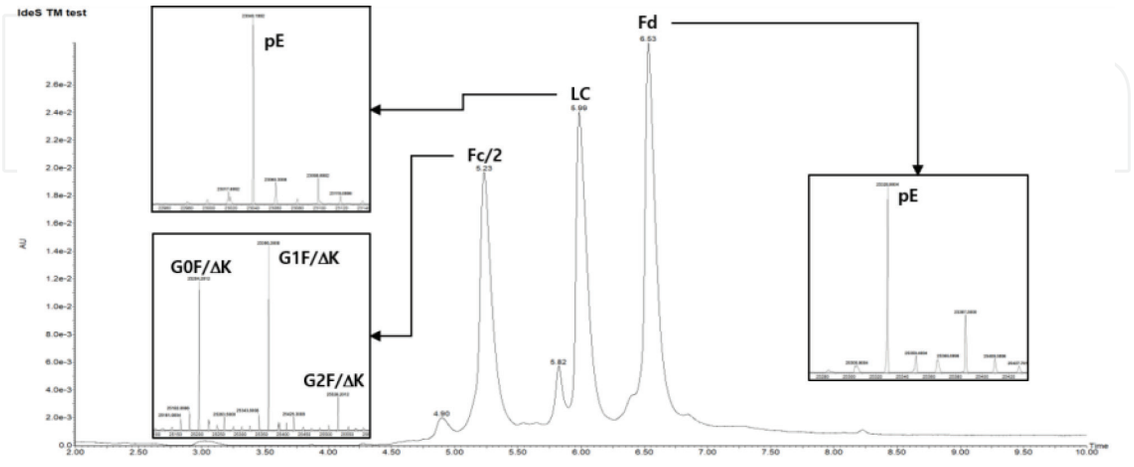

Figure 2. UV chromatogram of RP-HPLC for IdeS-digested Rituximab. Three subunits (Fc/2, LC, and Fd) are resolved on the chromatogram, and deconvoluted monoisotopic masses for each subunit are shown in insets. Glycan structures (G0F, G1F, and G2F) were adapted from [35]. ΔK: C-terminal Lys deletion and pE: pyro-glutamate.

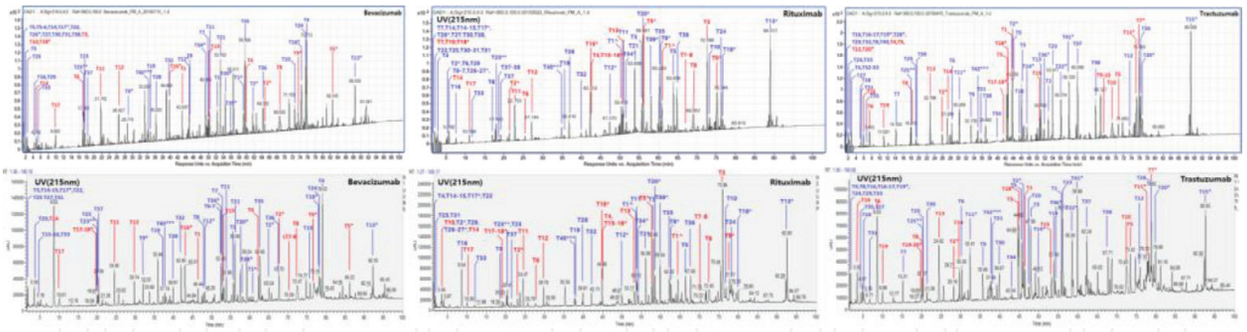

Figure 3. Identified peptides of three mAbs, bevacizumab, rituximab, and trastuzumab, by peptide mapping analysis using QTOF (Top) or Orbitrap (Bottom). Blue: peptides from heavy chain, Red: peptides from light chain. *Alkylated peptides, **N-glycopeptide, ***C-terminal Lys deleted peptide, and ^pyroglutamic acid Q N-term.

acid-labile surfactant, RapiGest [50, 51]. Denatured proteins are further reduced and alkylated. Reduction of disulfide bonds can be done with dithiothreitol (DTT), 2-Mercapto-ethanol, or tris(2-carboxyethyl)phosphine (TCEP) and alkylation with iodoacetamide or iodoacetic acid to prevent free cysteine groups after reduction [52, 53].

Many proteases are available for protein digestion, each having their own characteristics in terms of specificity, efficiency, and optimum digestion conditions [54, 55]. Trypsin is the most commonly used protease for peptide mapping analysis because it has a well-defined specificity. It hydrolyzes only the peptide bonds in which the carbonyl group is followed either by an arginine (Arg) or lysine (Lys) residue and the cleavage will not occur if proline is positioned on the carboxyl side of Lys or Arg [56, 57].

After digestion, resulting peptides are separated by RP-HPLC, detected by UV-absorption, and identified by mass spectrometry. The identity of the samples was confirmed by comparing the peak profile taking into account the number of detected peaks, retention times, and peak areas of the individual peaks. Furthermore, the measured masses of the found tryptic peptides were compared to the theoretical masses calculated from the amino acid sequence to verify the correct identification of the single peptides. Recently, the development of ultra-high performance chromatography (UHPLC) and sub-2 μm solid core particle columns leads to improve peak resolution and width, resulting in enhancing efficiency, sensitivity, and reproducibility for peptide identification [58–63]. To identify peptides with MS, peptides need to be transferred into gas phase by ionization such as ESI or MALDI [64, 65]. ESI is commonly used for peptide mapping in combination with HPLC separations. The hybrid mass instruments, Q-Orbitrap and QTOF with ESI ionization, are routinely used for peptide mapping of mAbs [66, 67].

Typical results of peptide mapping of bevacizumab, rituximab, and trastuzumab are shown in **Figure 3**. For the peptide mapping analysis, protein samples were digested with trypsin, and the resulting peptides were separated using RP-UHPLC, detected by UV-absorption, and identified by online ESI-QTOF or Orbitrap (Fusion). Peptide map analysis covers the full sequence of mAbs, with the exception of the small polar peptides, giving over 98% coverage for overall sequences. Along with the intact peptides, modified peptides were also identified, such as glycopeptides, C-terminal Lys deleted peptides, and peptides with Gln cyclization.

2.4. Disulfide bond identification for mAb

Protein disulfide bonds are produced by the covalent bonding of two thiol groups between cysteine amino acids. They are essential for structural and functional roles of proteins and give stability of proteins [68, 69]. The number of disulfide bonds and their positions are a critical attribute for biopharmaceuticals to ensure safety and efficacy [70, 71]. Peptide mapping coupled with LC-MS offers fast and accurate workflow for characterizing disulfide bonds of proteins. The typical workflow for identifying disulfide bonds in proteins is done by peptide mapping with the conditions of reduced and non-reduced digestion, and these two conditions are compared using LC-MS and LC-MS/MS [72, 73].

The typical result of disulfide bond identification for trastuzumab is shown in **Figure 4**. It is known that trastuzumab (IgG1) has 32 cysteine residues, which are cross-linked by four interchain disulfide bonds (two set of a disulfide bond between heavy and light chains, two between heavy chains) and two set of six intrachain disulfide bonds [74]. When trastuzumab is compared for tryptic map between reduced and non-reduced condition, 16 tryptic peptides (T) having cysteine residues (for heavy chain (H)— H:T2 (Cys22), H:T11 (Cys96), H:T14 (Cys147), H:T15 (Cys203), H:T19 (Cys223), H:T20 (Cys229, Cys232), H:C27(Cys229, Cys232), H:T22 (Cys264), H:T28 (Cys324), H:T36 (Cys370), and H:T41 (Cys428); for light chain (L)—L:T2 (Cys23), L:T7 (Cys88), L:T11 (Cys134), L:T18 (Cys194), and L:T20 (Cys214)) are expected under reduced condition as shown in **Figure 4**. Under non-reduced condition, six peptides linked by an intra-chain disulfide bond (H:T2 = H:T11, H:T14 = H:T15, H:T22 = H:T28, H:T36 = H:T41, L:T2 = L:T7, and L:T11 = L:T18), a peptide linked by a disulfide bond between heavy and light chains (L:T19-20 = H:T19), and a peptide linked by two disulfide bonds between heavy chains (H:T20 = H:T20) are generated as shown in **Figure 4**.

Collision-induced dissociation (CID) for disulfide-bonded peptide usually generates b and y ions from two individual peptides as shown in **Figure 5**, requiring side-by-side comparison between reduced and non-reduced peptide map [75]. Electron-transfer dissociation (ETD) usually generates disulfide-bonded fragment ions from both peptides, simultaneously detecting disulfide-bonded fragments and disulfide-bonded precursor peptide and simplifying the workflow for CID fragmentation without parallel side-by-side comparison [76]. The data-independent MSE approach collects mass data of precursors and fragments of eluting peptide from protein digests in an unbiased manner for peptide mapping and disulfide bond analyses [77, 78]. An example of disulfide bond analysis using MSE is shown in **Figure 5**. The MSE spectrum not only contains b and y ions from the two individual peptides (H:T2 and H:T11)

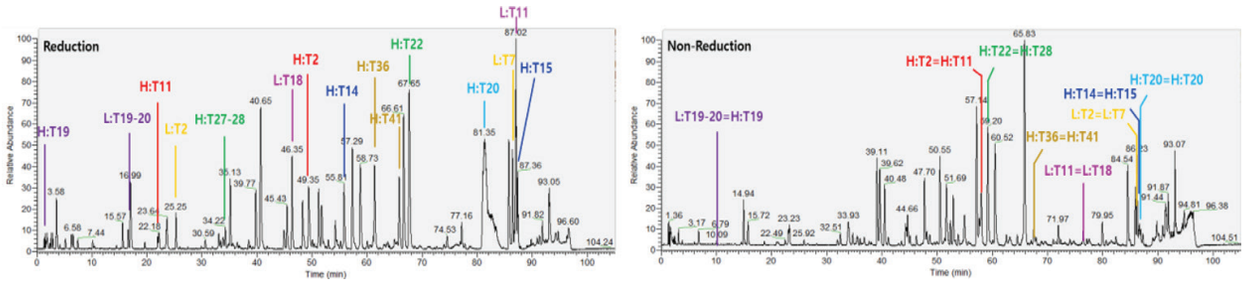

Figure 4. Identification of disulfide bonds for trastuzumab by peptide mapping under reduced (left) and non-reduced (right) tryptic digestions.

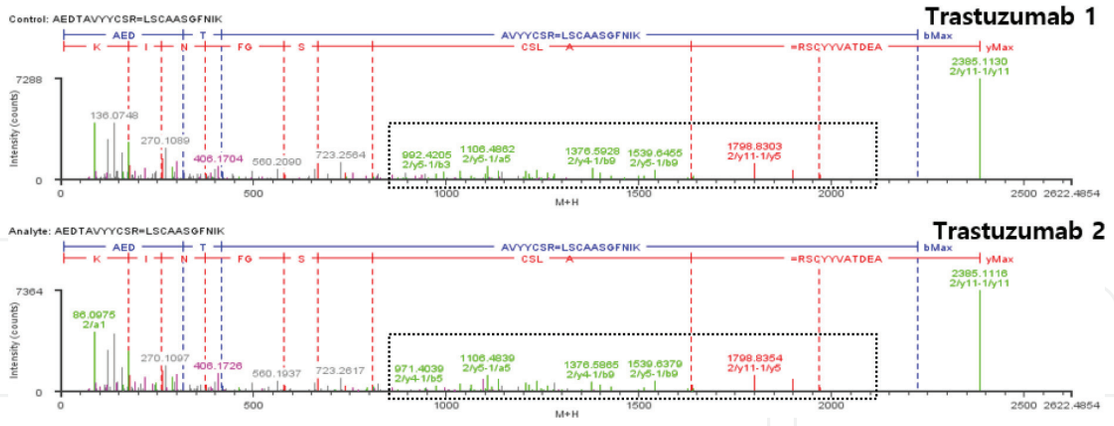

Figure 5. Fragmentation of disulfide-bonded peptide (H:T2 = H:T11) from trastuzumab by MSE approach using CID.

but also has ions corresponding to disulfide-bonded fragments from both peptides shown (H:T2 = H:T11) in dotted box in **Figure 5**.

3. Post-translational modification (PTM)

PTMs can be classified into two major classes, enzymatic and chemical modifications. Enzymatic modifications are defined as enzyme-catalyzed processing of proteins after translation by kinases, phosphatases, proteases, transferases, ligases, etc. [79]. Most common PTMs in this class are glycosylation, disulfide bond formation, and proteolytic cleavage of the protein. Chemical modifications are generated during upstream and downstream processing, formulation, and storage, including oxidation, deamidation, isomerization, glycation, and Gln/Glu cyclization [80]. Those PTMs can affect activity, stability, and immunogenicity and thus must be well-characterized, controlled, and monitored during development processes [20, 21, 81]. Physicochemical and biological analyses are required for monitoring those PTMs [82–86]. Peptide mapping approach is one of those method capable for the site-specific identification and quantitation of various PTMs. Recently multi-attribute method (MAM) has been developed as MS-based method that is able to identify and quantify several attribute at once [48, 87, 88]. The conventional methods such as hydrophilic interaction chromatography (HILIC) for oligosaccharide analysis, cation-exchange (CEX) chromatography, and capillary electrophoresis sodium dodecyl sulfate (CE-SDS) can be replaced by MAM approach (**Table 1**).

3.1. Glycosylation of mAb

Glycosylation is a major PTM, influencing protein folding and conformation. Thus, heterogeneity due to glycosylation may affect the bioactivity [89, 90]. Most mAbs have a single N-glycosylation site in Fc subunit (e.g., heavy chain N300), although some mAbs may have an additional glycosylation site in Fab subunit. Glycosylation on mAbs is known to affect their effector functions such as complement activation, antibody-dependent cell cytotoxicity (ADCC), cytotoxic endocytosis of immune complexes leading to antigen presentation, and inhibition of B lymphocytes, monocytes, and basophils [90–93].

Attribute	Description	Current workflow	Future development
N-term sequences	Identity	Edman Degradation	Multi-attribute method (MAM)
N-term variants	Gln/Glu cyclization	CEX-HPLC	
C-term variants	C-term Lys deletion	CEX-HPLC	
Charge variants	Deamidation/Isomerization	CEX-HPLC	
Clips	Fragment	CE-SDS	
Glycans	Glycosylation	HILIC	

HILIC: hydrophilic interaction chromatography, CEX-HPLC: cation-exchange high performance liquid chromatography, and CE-SDS: capillary electrophoresis-sodium dodecyl sulfate.

Table 1. MS-based MAM for attribute control compared to current workflow.

Glycan profiling can be done by releasing glycan moieties from mAbs using PNGase F and cleaving the linkage between GlcNAc and Asn. The released N-glycans are done by fluorescent labeling and subsequently analyzed using HPLC with fluorescence detector (FLD) coupled to MS instrument [94]. The recent development of HILIC and ultra-high pressure liquid chromatography with sub-2 μm amide-bonded stationary phases enables to separate labeled N-glycans with high resolution [95–97]. The typical result of N-glycan profiling is shown in **Figure 6**. Based on the detected mass by MS analysis, potential structures are all assigned for each peak, and the relative contents are calculated for the assigned structures from FLD detection (**Table 2**). G0F and G1F are major glycans on trastuzumab. The contents of afucosylated N-glycans are around 9.9% and that of high mannose type N-glycan around 1.7% of total. Sialylated N-glycans are not detected on this analysis.

For MAM approach for glycan profile, glycopeptides were identified by peptide mapping of mAb, trastuzumab. Different N-glycans were identified on the N300 of the H:T25 peptide (EEQYNSTYR), such as G0F-GlcNac, G0, G0F, Man5, G1, G1F, G2F, and G2F + 1SA. Intact peptide without N-glycan was also detected. **Figure 7** shows the identified glycopeptide from peptide mapping analysis.

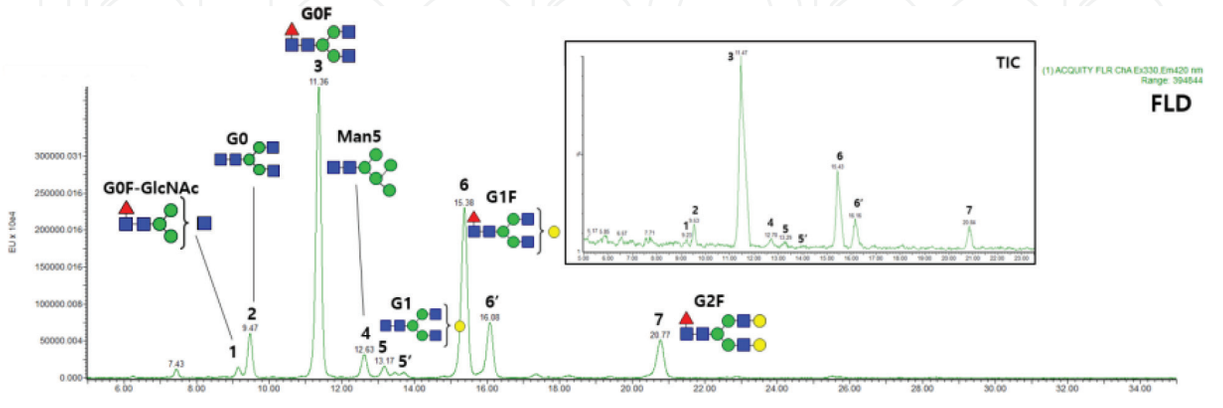

Figure 6. N-glycan profiling of mAb, trastuzumab, using HILIC-FLD-MS. Total ion chromatogram (TIC) and FLD chromatogram of N-glycans are shown. Inset represents TIC. Glycan structures (G0F, G1F, G2F, or etc.) were adapted from [35].

Peak	1	2	3	4	5 and 5′	6 and 6′	7
Glycan	G0F-GlcNAc	G0	G0F	Man5	G1	G1F	G2F
Relative content (%)	0.48 ± 0.09	6.48 ± 0.96	43.58 ± 1.81	1.66 ± 0.22	1.71 ± 0.11	39.10 ± 1.73	6.98 ± 0.49

Glycan structures (G0F, G1F, G2F, or etc.) were adapted from [35].

Table 2. N-glycan profiling of mAb, trastuzumab, determined by HILIC-FLD-MS.

Based on MS/MS analysis, potential structures are assigned for glycopeptide peak. The relative contents are calculated from XIC for the assigned structures (**Table 3**). The mass accuracy was determined by Eq. (1) and less than ±5 ppm. G0F and G1F are major glycans on trastuzumab. The contents of afucosylated N-glycans are around 12.2% and that of high mannose type N-glycan around 6.42% of total. A sialylated N-glycan was detected less than 1%.

Calculation of error for determined peptide by MS is shown in Eq. (1)

$$\text{Error (ppm)} = [(\text{Determined Mass-Calculated Mass})/\text{Determined Mass}] \times 10^6 \quad (1)$$

To compare N-glycan profiling results between HILIC and MAM approaches, the relative contents for each N-glycan between two approaches are visualized and a linear regression analysis was performed. The results are shown in **Figure 8**. The relative amounts of G0, G0F, and G1F are slightly higher in HILIC than MAM but those of Man5 and G2F are higher in MAM than HILIC (**Figure 9**). From the regression analysis, the slope was 0.816, intercept was 2.307, and correlation coefficient was 0.958, which indicates a correlation between HILIC and MAM approaches.

3.2. Deamidation and isomerization of mAb

The major cause of charge variants in mAbs is deamidation and isomerization [98–100]. Asparagine (Asn) and glutamine (Gln) are susceptible to deamidation, but glutamine is deamidated at a much lower rate than that of asparagine [101–103]. Deamidation of Asn is

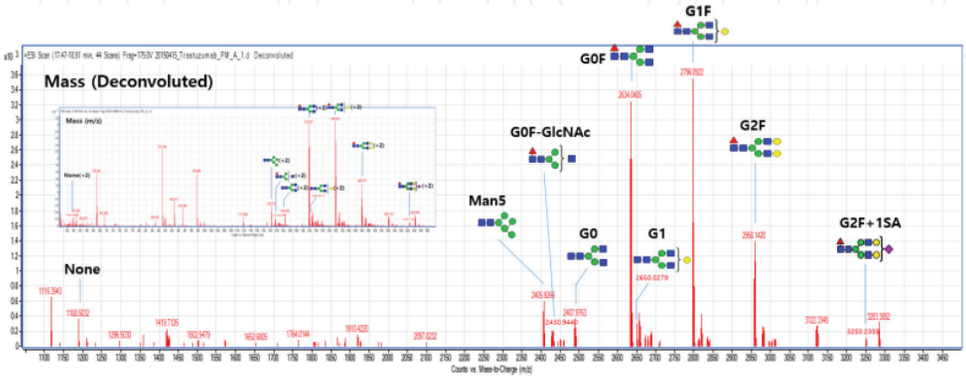

Figure 7. Glycopeptide having different N-glycans identified by peptide mapping analysis of trastuzumab. Inset represents MS1 spectrum before deconvolution. Glycan structures (G0F, G1F, G2F, or etc.) were adapted from [35].

Peptide	Number	Glycan attached	Calculated mass (Da)	Determined mass (Da)	Error (ppm)	Relative content (%)
EEQYNSTYR	H:T25		1188.5047	1188.502	−2.27	2.18 ± 0.06
EEQYNSTYR	H:T25**	Man5	2404.9276	2404.9249	−1.12	6.42 ± 0.27
EEQYNSTYR	H:T25**	G0F-GlcNAc	2429.9592	2429.9531	−2.51	1.23 ± 0.04
EEQYNSTYR	H:T25**	G0	2486.9807	2486.9744	−2.53	3.21 ± 0.19
EEQYNSTYR	H:T25**	G0F	2633.0386	2633.0395	0.34	36.25 ± 0.68
EEQYNSTYR	H:T25**	G1	2649.0335	2649.031	−0.94	2.56 ± 0.18
EEQYNSTYR	H:T25**	G1F	2795.0914	2795.093	0.57	34.05 ± 0.80
EEQYNSTYR	H:T25**	G2F	2957.1443	2957.1398	−1.52	13.25 ± 0.85
EEQYNSTYR	H:T25**	G2F + 1SA	3248.2397	3248.2295	−3.14	0.86 ± 0.00

Glycan structures (G0F, G1F, G2F, or etc.) were adapted from Ref. [35]. **: N-glycosylated Peptide.

Table 3. N-glycan profiling of mAb, trastuzumab, determined by MAM approach.

a non-enzymatic process, converting Asn to a 5-ringed cyclic succinimide intermediate that is hydrolyzed to form a mixture of isoaspartic acid (isoAsp or isoD) and aspartic acid (Asp) [104]. Isomerization follows the same mechanism of deamidation but occurs at aspartic acid (Asp) residues to form iso-Asp through the succinimide intermediate [105, 106]. This results in product heterogeneity and complicates manufacturing consistency [107]. Deamidation and isomerization are also known to have significant impact on in vitro potency, product heterogeneity, shelf-life stability, manufacturing consistency, and yield [108–110].

CEX-HPLC and isoelectric focusing (IEF) have been successfully developed to investigate the charge variants due to deamidation and isomerization [111, 112]. Peptide mapping analysis

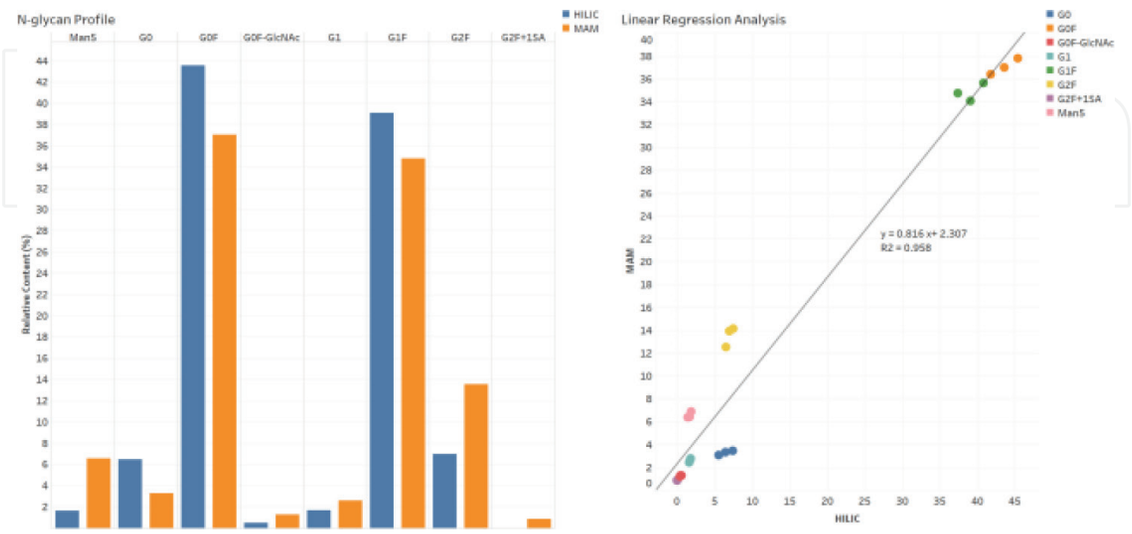

Figure 8. Glycan profiles determined by HILIC and MAM approaches.

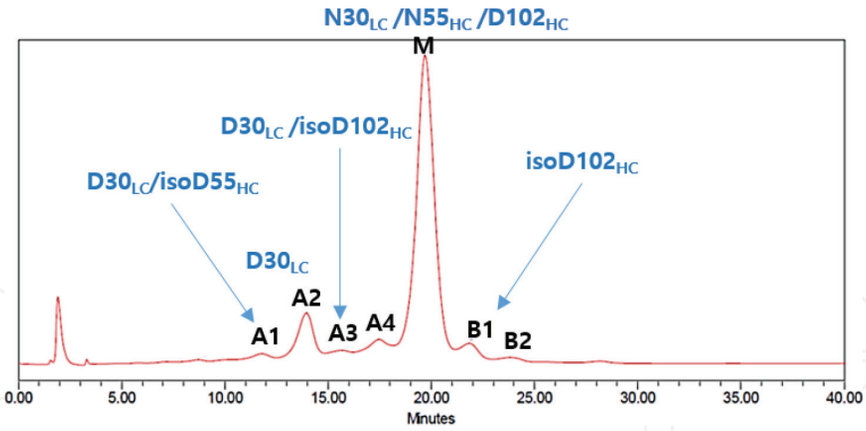

Figure 9. Profiling of charge variants from mAb, trastuzumab, by CEX-HPLC analysis. N: asparagine, D: aspartate, isoD: iso-aspartate, HC: heavy chain, and LC: light chain.

based on LC-MS/MS is commonly applied for identification of site and quantification of deamidation and isomerization, including the succinimide intermediated [113, 114].

CEX-HPLC can detect the occurrence of deamidation or C-terminal Lys deletion as well as glycosylation variants. The result of charge variant profiling for mAb, trastuzumab, is shown in **Figure 9**. Four acidic variants and two basic variants were detected on the CEX chromatogram. Each fraction for charge variants was collected, and the pooled fractions were further analyzed to characterize modification site by peptide mapping analysis. This analysis gives the structural information for each variant, and most of the charge variants are produced by deamidation and isomerization, localized on the CDR regions of mAb, trastuzumab (**Figure 9**).

UV detection of CEX chromatography allows to quantify charge variant peaks and the relative contents are calculated (**Table 4**). The content of major form without deamidation or isomerization on CDR regions is around 71.2%, that of acidic charge variants (A1-A4) are around 22.4%, and that of basic variants (B1-B2) is around 6.4% of total (**Table 4**).

Peak	N30(LC)	N55(HC)	D102(HC)	Relative content (%)
A1	D/N	isoD/N	D/D	0.24 ± 0.05
A2	D/N	N/N	D/D	10.60 ± 0.30
A3	D/N	N/N	isoD/D	3.89 ± 0.18
A4	N/N	N/N	D/D	7.68 ± 0.10
M	N/N	N/N	D/D	71.14 ± 0.58
B1	N/N	N/N	isoD/D	5.06 ± 0.26
B2	N/N	N/N	isoD/D	1.38 ± 0.05

N: asparagine, D: glutamate, isoD: iso-glutamate.

Table 4. The relative contents of charge variants identified by CEX analysis.

For MAM approach for charge variant profile including deamidation and isomerization, modified peptides were identified from peptide mapping analysis of mAb, trastuzumab. Deamidation on Asn30 of light chain was detected and isomerization on Asp102 of heavy chain was also detected by peptide mapping analysis using LC-MS/MS. However, deamination and isomerization on N55 of heavy chain were not detected. **Figure 10** shows the results of deamidation and isomerization on Asn30 of light chain and Asp102 of heavy chain, respectively.

Based on MS/MS analysis, peptide sequences are confirmed for intact and modified peptides. The relative contents are calculated from XIC for the intact and modified peptides (**Table 5**). The mass accuracy was determined by Eq. (1) and less than ±5 ppm. Isomerization on Asp102 of heavy chain was detected and its relative content was around 4.6% of total (**Table 5**). Intact and isomerized peptide has same molecular mass, and thus, it is not possible to distinguish from each other only by mass, but those peptides have different retention time on the chromatogram (**Figure 10**). Deamidation on Asn30 of light chain was detected and its relative content was around 7.7% of total (**Table 5**). Deamination and isomerization on N55 of heavy chain were not detected.

To compare profiling results of deamidation and isomerization between CEX and MAM approaches, the relative contents for each modified peptide between two approaches are visualized and a linear regression analysis was performed. The result from CEX contains information not from peptide levels but from full mAb, and thus CEX data were recalculated for the level of each amino acid comparable for MAM data. The results are shown in **Figure 11**. The relative amounts of deamidation and isomerization are very similar between CEX and MAM approaches (**Figure 11**). From the regression analysis, the slope was 1.038, intercept was 0.267, and correlation coefficient was 0.988, which indicates a very good correlation between HILIC and MAM approaches.

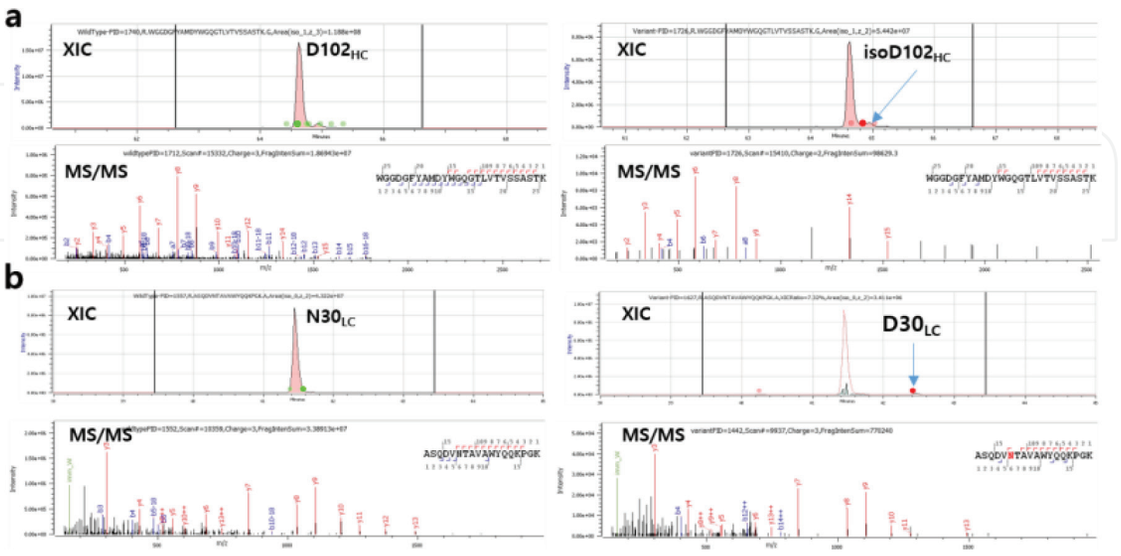

Figure 10. Isomerization on Asp102 of heavy chain (a) and deamidation on Asn30 of heavy chain (b). N: asparagine, D: aspartate, isoD: iso-aspartate, HC: heavy chain, LC: light chain.

Peptide	Number	Change	Calculated mass (Da)	Mass (Da)	Error (ppm)	Relative content (%)
WGGDGFYAM DYWGQGTLV TVSSASTK	H:T12	D102	2783.2537	2783.2551	0.50	95.36 ± 0.28
WGG$_{iso}$DGFYAM DYWGQGTLV TVSSASTK	H:T12*	D102 to $_{iso}$D102	2783.2537	2783.2595	2.08	4.63 ± 0.28
IYPTNGYTR	H:T6	N55	1083.5349	1083.5345	−0.37	100
IYPT$_{iso}$DGYTR	H:T6*	N55 to $_{iso}$D55	1083.5349	—	—	0
ASQDVNTAV AWYQQKPGK	L:T3	N30	1989.9908	1989.9938	1.51	92.35 ± 0.24
ASQDVDTAV AWYQQKPGK	L:T3*	N30 to D30	1990.9749	1990.9774	1.26	7.70 ± 0.24

N: asparagine, D: aspartate, isoD: iso-aspartate, H: heavy chain, L: light chain, and T: tryptic peptide.
*: Deamidated or isomerized peptide.

Table 5. The relative contents of deamidation and isomerization identified by MAM analysis.

3.3. C-terminal Lys deletion of mAb

C-terminal Lys variants are clipped modification found at heavy chain C-terminus of mAbs produced in mammalian cell cultures, usually produced by proteolysis of endogenous carboxypeptidases during the manufacturing process [115, 116]. C-terminal Lys deletion has been known for no impact on antibody function, such as biologic activity, structural stability, pharmacokinetics, or bioavailability in rats [117]. However, there have been debates about impacts of C-terminal Lys deletion on Fc effector functions [117, 118].

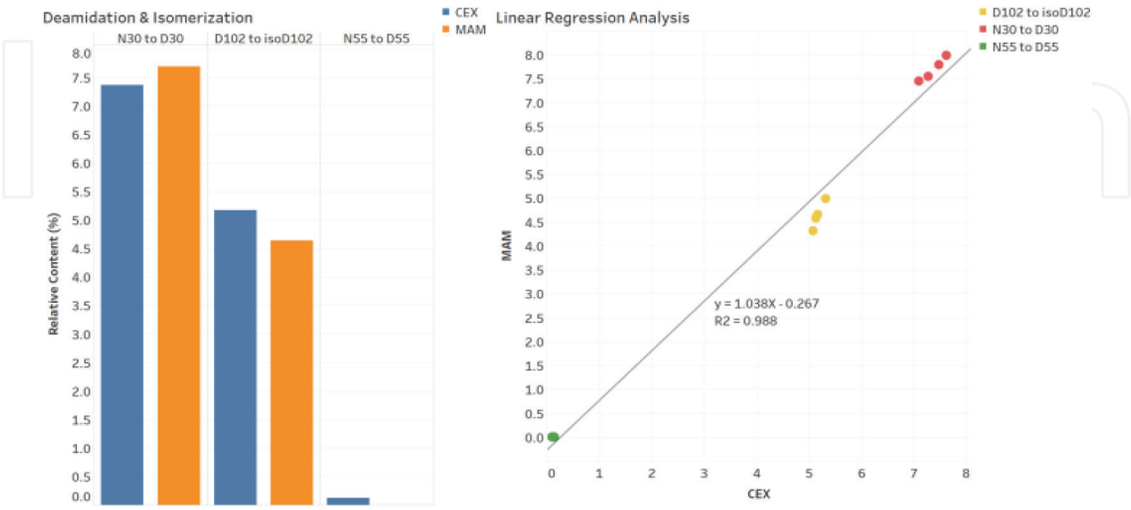

Figure 11. Profiles of deamidation and isomerization determined by CEX and MAM approaches. N: asparagine, D: aspartate, isoD: iso-aspartate, HC: heavy chain, and LC: light chain.

Because Lys residue is positively charged, leading to charge heterogeneity of mAb products. Thus, it is still a quality parameter for characterization. Due to charge variation, the modified and unmodified structures can be separated by CEX, IEF, and cIEF [117, 119]. Loss of the terminal Lys residue gives mass shift, which can be also detected and quantified by mass spectrometry [115, 118]. The modified structures can be separated and also identified from the unmodified structures by comparing the results from carboxypeptidase treatment [115, 120]. Carboxypeptidase treatment removes C-terminal Lys of mAbs and the disappeared peaks on the chromatogram can be identified as the unmodified variants [115, 120–122].

The typical results of CEX analysis are shown in **Figure 12**, which identify and quantify C-terminal Lys variants of mAb, adalimumab. Five acidic variants and three basic variants are detected (**Figure 12**). With the treatment of carboxypeptidase, all of the basic peaks were disappeared on the chromatogram (**Figure 12**, inset). Thus, those peaks are the unmodified peaks having C-terminal Lys.

UV detection of CEX chromatography allows to quantify C-terminal Lys variant peaks and the relative contents are calculated (**Table 6**). The content of major form is around 64.7%, that of C-terminal Lys variants on both heavy chains (A1-A4 and M) are around 77.5%, that of C-terminal Lys variants on one heavy chain (B1 and B2) are around 18.3%, and that of intact C-terminal Lys (B3) is around 4.1% of total (**Table 6**).

For MAM approach for C-terminal Lys variant profile, modified peptides were identified from peptide mapping analysis of mAb, adalimumab. **Figure 13** shows the results of C-terminal Lys variant of adalimumab. Based on MS/MS analysis, peptide sequences are confirmed for intact and modified peptides. XICs of confirmed peptides were determined and quantified. The mass accuracy was determined by Eq. (1) and less than ±5 ppm. C-terminal Lys variant was detected and its relative content was around 13% of total (**Table 7**).

To compare profiling results of C-terminal Lys variants between CEX and MAM approaches, the relative contents for each modified peptide between two approaches are visualized and a linear regression analysis was performed. The result from IEX contains information not from peptide levels but from full mAb, and thus, CEX data were recalculated for the level of each

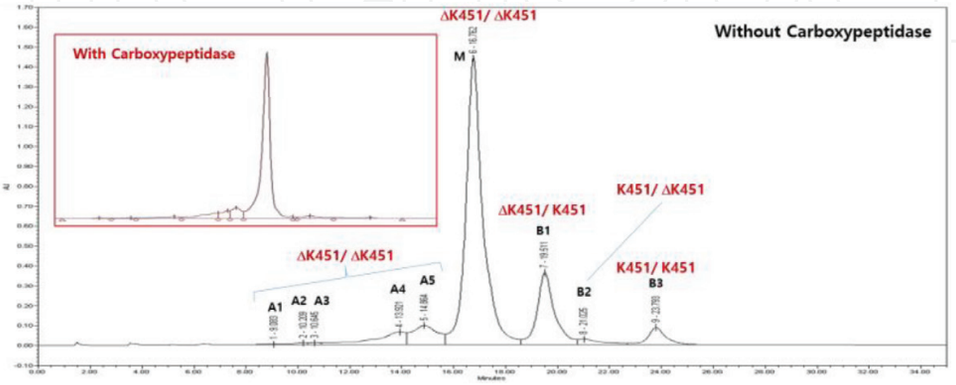

Figure 12. Profiling of C-terminal Lys variants from mAb, adalimumab, by CEX analysis. Inset represents the CEX chromatogram after carboxypeptidase treatment. K: Lys and ΔK: Lys deletion.

Peak	C-terminal structure of heavy chains	Relative content (%)
A1	Lys Deleted/Lys Deleted	0.10 ± 0.05
A2	Lys Deleted/Lys Deleted	0.54 ± 0.14
A3	Lys Deleted/Lys Deleted	0.29 ± 0.04
A4	Lys Deleted/Lys Deleted	5.52 ± 0.20
A5	Lys Deleted/Lys Deleted	6.422 ± 0.21
M	Lys Deleted/Lys Deleted	64.65 ± 0.34
B1	Lys Deleted/Intact Lys	16.55 ± 0.21
B2	Intact Lys/Lys Deleted	1.78 ± 0.12
B3	Intact Lys/Intact Lys	4.15 ± 0.09

A: acidic, M: major, B: basic.

Table 6. The relative contents of C-terminal Lys variants from adalimumab identified by IEX analysis.

amino acid comparable for MAM data. Along with adalimumab results, those of trastuzumab were also visualized. The results are shown in **Figure 14**. The relative amounts of C-terminal Lys variants from adalimumab and trastuzumab are very similar between CEX and MAM approaches (**Figure 14**). From the regression analysis for adalimumab and trastuzumab, the slope was 0.970, intercept was 2.935, and correlation coefficient was 0.998, which indicates a very good correlation between CEX and MAM approaches.

3.4. N-terminal cyclization of mAb

N-terminal cyclization (pyroGlu or pE) variants are generated by the rearrangement of Gln or Glu at the N-terminus of mAbs, which can be done by spontaneous or enzymatic reactions [123, 124]. The conversion rate from Gln to pyroGlus is much faster than that from Glu and nearly completed over 95% in mAbs having N-terminal Gln, which is known that this conversion occurs primarily in bioreactors [123]. The N-terminal cyclizations of mAbs converting Gln/Glu to pyroGlu do not impact on their structure, activity, in vivo clearance, and other pharmacokinetic properties [124].

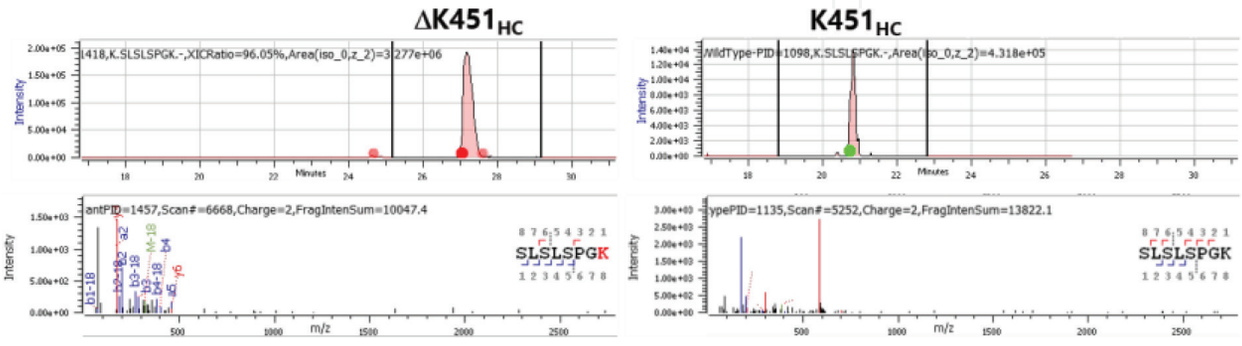

Figure 13. Identification of C-terminal Lys variant from adalimumab by peptide mapping analysis. K: Lys, ΔK: Lys deletion, and HC: heavy chain.

Peptide	Number	Modification	Calculated mass (Da)	Mass (Da)	Error (ppm)	Relative content (%)
SLSLSPGK	H:T40		787.4440	787.4434	−0.76	13.01 ± 0.38
SLSLSPG	H:T40*	K → ΔK	659.3489	659.3489	0.00	86.99 ± 0.38

K: Lys and Δ K: C-terminal Lys deletion. *: Deamidated or isomerized peptide.

Table 7. The relative contents of C-terminal Lys variants from adalimumab identified by MAM analysis.

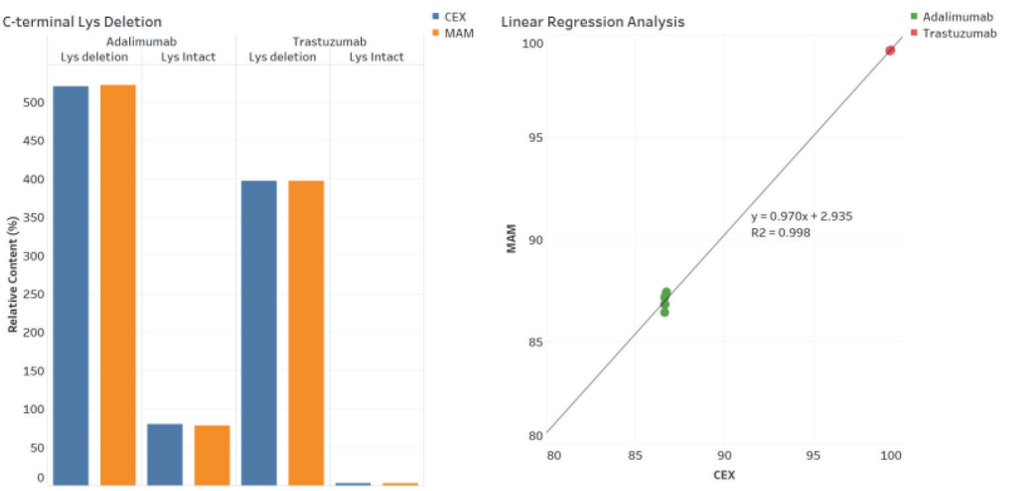

Figure 14. Profiles of C-terminal Lys variants for adalimumab and trastuzumab determined by CEX and MAM approaches.

The conversion from Gln to pyroGlu renders mAbs more acidic and the conversion from Glu to pyroGlu gives a basic shift. Thus, the N-terminal cyclization increases charge heterogeneity of mAb products, which can be detected by charge-based methods such as CEX, IEF, and cIEF. The conversion of Gln or Glu to pyroGlu gives a mass shift −17 or − 18 Da, respectively, compared to the unmodified peptide, and this can be assessed using peptide mapping and intact mass analysis by MS.

The typical results of CEX analysis are shown in **Figure 15**, which identify and quantify N-terminal cyclization variants of mAb, rituximab. On the chromatogram of rituximab, several acidic variants and two major basic variants are detected (**Figure 15**). Each fraction for basic charge variants was collected and the pooled fractions were further analyzed to characterize modification site by peptide mapping analysis. This analysis gives the structural information for each variant, and the basic variant (B2) are produced by N-terminal cyclization of light chain, rituximab (**Figure 16**).

UV detection of IEX chromatography allows to quantify this N-terminal cyclization variant and the relative content of it is calculated (**Table 8**). The content of major form is around 89.4% and that of N-terminal cyclization variant on a light chain is around 1.9% (**Table 8**).

For MAM approach for detecting and quantifying N-terminal cyclization of mAb, rituximab, the conversion from N-terminal Gln to pyroGlu was identified by peptide mapping analysis.

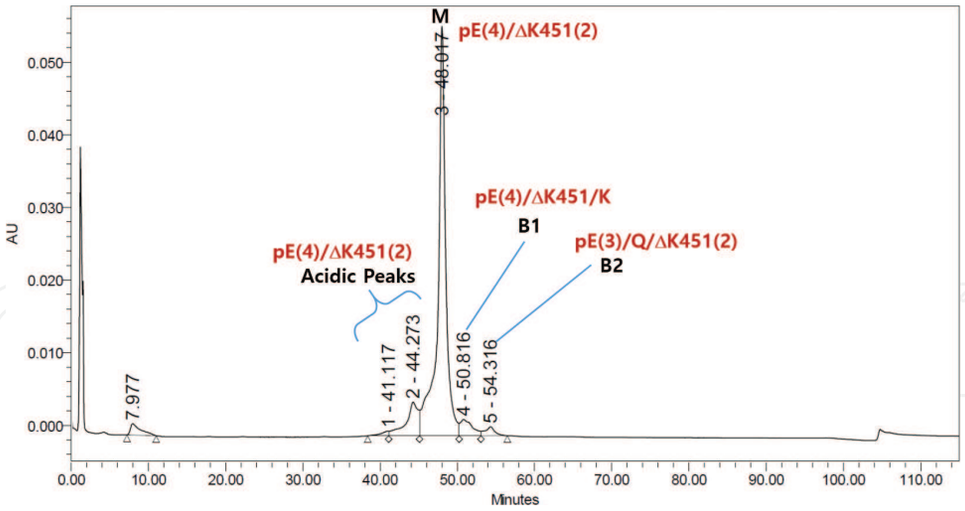

Figure 15. Profiling of N-terminal cyclization variant from mAb, rituximab, by IEX analysis. K: Lys, ΔK: Lys deletion, and pE: pyro-glutamate.

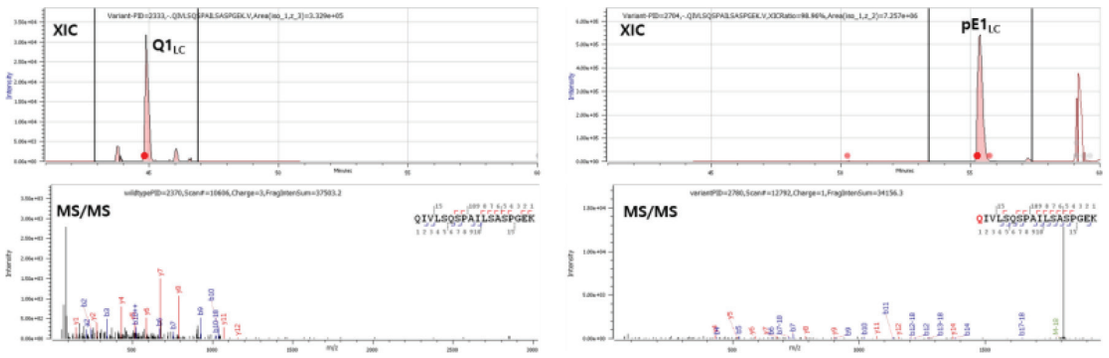

Figure 16. Identification of N-terminal cyclization variant from rituximab by peptide mapping analysis. Q: glutamine, pE: pyro-glutamate, and LC: light chain.

N-terminal cyclization on Gln1 of light chain was detected but N-terminal cyclization on Gln1 of heavy chain was not detected by peptide mapping analysis. **Figure 16** shows the results of N-terminal cyclization on Gln1 of light chain of mAb, rituximab.

Based on MS/MS analysis, peptide sequences are confirmed for intact and modified peptides. The relative contents are calculated from XIC for the intact and modified peptides. The mass accuracy was determined by Eq. (1) and less than ±5 ppm. N-terminal cyclization variant of light chain was detected and its relative content was around 99% of total (**Table 9**).

To compare profiling results of N-terminal cyclization variant between CEX and MAM approaches, the relative contents for each modified peptide between two approaches are compared and visualized. The relative amounts of N-terminal cyclization variant from rituximab are very similar between CEX and MAM approaches (**Figure 17**).

3.5. Other PTMs of mAb

Many other PTMs can be identified and quantified using MAM approach, including oxidations of Met and Trp, glycation, cysteine variants, truncation, mutations, etc. Those PTMs

Peak	N-terminal structure of light chains	Relative content (%)
Acidic	pyroGlu/pyroGlu	5.26 ± 0.01
M	pyroGlu/pyroGlu	89.40 ± 0.12
B1	pyroGlu/pyroGlu	3.42 ± 0.08
B2	Gln/pyroGlu	1.92 ± 0.05

M: major and B: basic.

Table 8. The relative contents of N-terminal cyclization variant from rituximab identified by CEX analysis.

Peptide	Number	Change	Calculated mass (Da)	Mass (Da)	Error (ppm)	Relative content (%)
QIVLSQSPAI LSASPGEK	L:T1		1823.9993	1823.9949	−2.41	0.93 ± 0.01
pEIVLSQSPAI LSASPGEK	L:T1	pyroGlu	1806.9727	1806.9731	0.22	99.08 ± 0.01

L: light chain, T: tryptic peptide, and pE: pyro-glutamate.

Table 9. The relative contents of N-terminal cyclization variant from rituximab identified by MAM analysis.

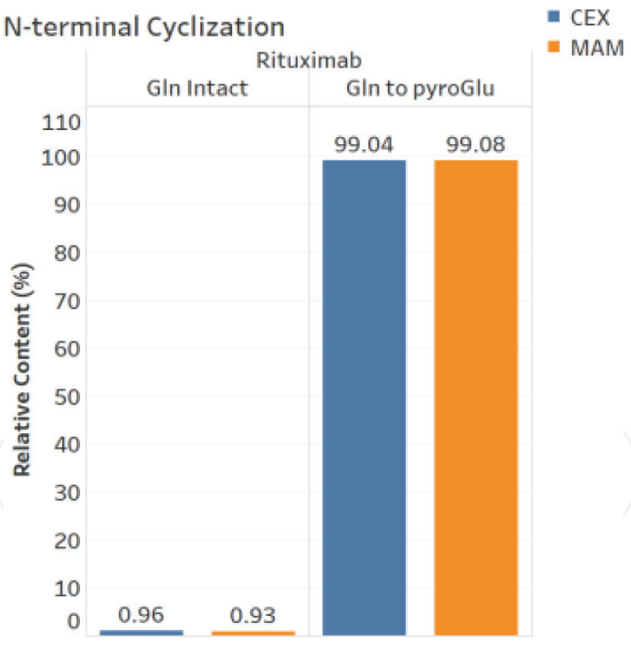

Figure 17. Profiles of N-terminal cyclization variants for rituximab.

result in mass shifts compared to those intact peptides, and this gives clues for detecting PTMs by considering the mass differences. Most of those PTMs may not be separated from their unmodified form by conventional approaches. For those cases, MAM approach is a possible alternative for quantifying those PTMs.

4. Conclusions

The analytical characterization of biopharmaceutical is still challenging for biotech industry to meet the requirements. Conventional methods, such as chromatography and electrophoresis, are routinely used because they are easy to use, robust, and, cost effective. Current trends for characterization are in-depth and well characterized. Current advances in instrumentation can help to follow those trends and characterize very complex heterogeneity from various PTMs. MS is the most powerful instrument among them, which provides high resolution, accurate, and confident data with rich information from primary structure (intact mass and peptide mapping) to high order structures (PTMs and HDX).

In this chapter, several workflows are summarized for intact mass determination, primary structure analysis, and determination and quantitation of various PTMs using chromatography with online detection by MS. Those conventional approaches were assessed by the current MAM approaches primarily by peptide mapping analysis using MS.

MAM approach has been introduced, which is able to identify and quantify several attributes at once. In this chapter, glycosylation, deamidation/isomerization, C-terminal Lys variants, and N-terminal cyclization are investigated by using MAM approach, and the performance was compared to the conventional methods such as HILIC oligosaccharide analysis and CEX charge variant analysis. The results confirmed that MAM approach is quite comparable for those from conventional independent approaches.

In this chapter, we showed that MAM approach for biopharmaceutical characterization is quite comparable for typical conventional approaches using HILIC and CEX. This result conveys that MAM workflow can be extended to other related area of biopharmaceutical development as follows. MAM approach may help to select best cell lines for producing biopharmaceuticals, to support process control for upstream and downstream, and monitor critical attributes for production. MAM approach will also gain attention for the development of biosimilar requiring in-depth structural analysis for similarity.

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Conflict of interest

The authors have nothing to disclose.

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References

[1] Parr MK, Montacir O, Montacir H. Physicochemical characterization of biopharmaceuticals. Journal of Pharmaceutical and Biomedical Analysis. 2016;**130**:366-389. DOI: 10.1016/j.jpba.2016.05.028

[2] The MJ. Human insulin: DNA technology's first drug. American Journal of Hospital Pharmacy. 1989;**46**(11 Suppl 2):S9-S11

[3] Walsh G. Biopharmaceutical benchmarks 2014. Nature Biotechnology. 2014;**32**:992-100. DOI: 10.1038/nbt.3040

[4] Sato AK, Viswanathan M, Kent RB, Wood CR. Therapeutic peptides: Technological advances driving peptides into development. Current Opinion in Biotechnology. 2006;**17**:638-642. DOI: 10.1016/j.copbio.2006.10.002

[5] Uhlig T, Kyprianou T, Martinelli FG, Oppici CA, Heiligers D, Hills D, Calvo XR, Verhaert P. The emergence of peptides in the pharmaceuticalbusiness: From exploration to exploitation. EuPA Open Proteomics. 2014;**4**:58-69. DOI: 10.1016/j.euprot.2014.05.003

[6] Jafari R, Zolbanin NM, Rafatpanah H, Majidi J, Kazemi T. Fc-fusion proteins in therapy: An updated view. Current Medicinal Chemistry. 2017;**24**(12):1228-1237. DOI: 10.2174/0929867324666170113112759

[7] Rogstad S, Faustino A, Ruth A, Keire D, Boyne M, Park J. A retrospective evaluation of the use of mass spectrometry in FDA biologics license applications. Journal of the American Society for Mass Spectrometry. 2017;**28**:786-794. DOI: 10.1007/s13361-016-1531-9

[8] Top 20 Drugs in the World 2017 [Internet]. Available from: https://igeahub.com/2017/08/08/top-20-drugs-in-the-world-2017/ [Accessed: 01-04-2018]

[9] Zolot RS, Basu S, Million RP. Antibody-drug conjugates. Nature Reviews. Drug Discovery. 2013;**12**(4):259-260. DOI: 10.1038/nrd3980

[10] Carter PJ, Lazar GA. Next generation antibody drugs: Pursuit of the 'high-hanging fruit. Nature Reviews. Drug Discovery. 2018;**17**(3):197-223. DOI: 10.1038/nrd.2017.227

[11] Wakankar A, Chen Y, Gokarn Y, Jacobson FS. Analytical methods for physicochemical characterization of antibody drug conjugates. MAbs. 2011;**3**(2):161-172. DOI: 10.4161/mabs.3.2.14960

[12] Fan G, Wang Z, Hao M, Li J. Bispecific antibodies and their applications. Journal of Hematology & Oncology. 2015;**8**:130. DOI: 10.1186/s13045-015-0227-0

[13] De Nardis C, Hendriks LJA, Poirier E, Arvinte T, Gros P, Bakker ABH, de Kruif J. A new approach for generating bispecific antibodies based on a common light chain format and the stable architecture of human immunoglobulin G(1). The Journal of Biological Chemistry. 2017;**292**(35):14706-14717. DOI: 10.1074/jbc.M117.793497

[14] Godar M, de Haard H, Blanchetot C, Rasser J. Therapeutic bispecific antibody formats: A patent applications review (1994-2017). Expert Opinion on Therapeutic Patents. 2018;**28**(3):251-276. DOI: 10.1080/13543776.2018.1428307

[15] Strohl WR. Current progress in innovative engineered antibodies. Protein & Cell. 2018;**9**(1):86-120. DOI: 10.1007/s13238-017-0457-8

[16] Johnson JA. Biologics and Biosimilars: Background and Key Issues. CRS Report. 2017;R44620. Available from: https://fas.org/sgp/crs/misc/R44620.pdf

[17] Crespi-Lofton J, Skelton JB. The growing role of biologics and biosimilars in the United States: Perspectives from the APhA Biologics and Biosimilars Stakeholder Conference. Journal of the American Pharmaceutical Association. 2017;**57**(5):e15-e27. DOI: 10.1016/j.japh.2017.05.014

[18] Peterson J, Budlong H, Affeldt T, Skiermont K, Kyllo G, Heaton A. Biosimilar products in the modern U.S. health care and regulatory landscape. Journal of Managed Care & Specialty Pharmacy. 2017;**23**(12):1255-1259. DOI: 10.18553/jmcp.2017.23.12.1255

[19] U.S. Food and Drug Administration. Biosimilar and Interchangeable Products [Internet]. Available from: https://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/ucm580419.htm [Accessed: 15-04-2018]

[20] Song KE, Byeon J, Moon DB, Kim HH, Choi Y-J, Suh J-K. Structural identification of modified amino acids on the interface between EPO and its receptor from EPO BRP, human recombinant erythropoietin by LC/MS analysis. Molecules and Cells. 2014;**37**(11):819-826. DOI: 10.14348/molcells.2014.0214

[21] Byeon J, Choi Y-J, Suh J-K. Structural characterization of modification on the interface between a ligand and its receptor for biopharmaceuticals. Receptors & Clinical Investigation. 2015;**2**(2):1-5. DOI: 10.14800/rci.536

[22] Byeon J, Kang KH, Jung H-K, Suh J-K. Assessment for quantification of biopharmaceutical protein using a microvolume spectrometer on microfluidic slides. BioChip Journal. 2017;**11**(1):21-29. DOI: 10.1007/s13206-016-1104-9

[23] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q5E guideline, Comparability of biotechnological/biological products subject to changes in their manufacturing process. 2004

[24] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q6B guideline, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. 1999

[25] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q8 R2 guideline, Pharmaceutical development. 2009. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/ Q8_R1/Step4/Q8_R2_Guideline.pdf

[26] Li Y, Champion MM, Sun L, Champion PA, Wojcik R, Dovichi NJ. Capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry as an alternative proteomics platform to ultraperformance liquid chromatography-electrospray ionization-tandem mass spectrometry for samples of intermediate complexity. Analytical Chemistry. 2012;**84**(3):1617-1622. DOI: 10.1021/ac202899p

[27] Srebalus Barnes CA, Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass Spectrometry Reviews. 2007;**26**(3):370-388. DOI: 10.1002/mas.20129

[28] Bondarenko PV, Second TP, Zabrouskov V, Makarov AA, Zhang Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. Journal of the American Society for Mass Spectrometry. 2009;**20**(8):1415-1424. DOI: 10.1016/j.jasms.2009.03.020

[29] Tran BQ, Barton C, Feng J, Sandjong A, Yoon SH, Awasthi S, Liang T, Khan MM, Kilgour DPA, Goodlett DR, Goo YA. Comprehensive glycosylation profiling of IgG and IgG-fusion proteins by top-down MS with multiple fragmentation techniques. Journal of Proteomics. 2016;**134**:93-101. DOI: 10.1016/j.jprot.2015.10.021

[30] He L, Anderson LC, Barnidge DR, Murray DL, Hendrickson CL, Marshall AG. Analysis of monoclonal antibodies in human serum as a model for clinical monoclonal Gammopathy by use of 21 tesla FT-ICR top-down and middle-down MS/MS. Journal of the American Society for Mass Spectrometry. 2017;**28**(5):827-838. DOI: 10.1007/s13361-017-1602-6

[31] Thompson NJ, Rosati S, Heck AJ. Performing native mass spectrometry analysis on therapeutic antibodies. Methods. 2014;**65**(1):11-17. DOI: 10.1016/j.ymeth.2013.05.003

[32] Beck A, Debaene F, Diemer H, Wagner-Rousset E, Colas O, Van Dorsselaer A, Cianférani S. Cutting-edge mass spectrometry characterization of originator, biosimilar and biobetter antibodies. Journal of Mass Spectrometry. 2015;**50**(2):285-297. DOI: 10.1002/jms.3554

[33] Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. Nature Methods. 2007;**4**(10):817-821. DOI: 10.1038/nmeth1097

[34] Macht M. Top-down characterization of biopharmaceuticals. Trends in Analytical Chemistry. 2013;**48**:62-71. DOI: 10.1016/j.trac.2013.01.016

[35] Liu L. Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins. Journal of Pharmaceutical Sciences. 2015;**104**(6):1866-1884. DOI: 10.1002/jps.24444

[36] Zhang Z, Pan H, Chen X. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrometry Reviews. 2009;**28**(1):147-176. DOI: 10.1002/mas.20190

[37] Ayoub D, Jabs W, Resemann A, Evers W, Evans C, Main L, Baessmann C, Wagner-Rousset E, Suckau D, Beck A. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs. 2013;**5**(5):699-710. DOI: 10.4161/mabs.25423

[38] Wang B, Gucinski AC, Keire DA, Buhse LF, Boyne MT 2nd. Structural comparison of two anti-CD20 monoclonal antibody drug products using middle-down mass spectrometry. The Analyst. 2013;**138**(10):3058-3065. DOI: 10.1039/c3an36524g

[39] Fornelli L, Ayoub D, Aizikov K, Beck A, Tsybin YO. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Analytical Chemistry. 2014;**86**(6):3005-3012. DOI: 10.1021/ac4036857

[40] Hartman PA, Stodola JD, Harbour GC, Hoogerheide JG. Reversed-phase high-performance liquid chromatography peptide mapping of bovine somatotropin. Journal of Chromatography. 1986;**360**(2):385-395. DOI: 10.1016/S0021-9673(00)91687-X

[41] Recny MA, Scoble HA, Kim Y. Structural characterization of natural human urinary and recombinant DNA-derived erythropoietin. Identification of des-arginine 166 erythropoietin. The Journal of Biological Chemistry. 1987;**262**(35):17156-17163. Available from: http://www.jbc.org/content/262/35/17156.full.pdf

[42] Dougherty JJ Jr, Snyder LM, Sinclair RL, Robins RH. High-performance tryptic mapping of recombinant bovine somatotropin. Analytical Biochemistry. 1990;**190**(1):7-20. DOI: 10.1016/0003-2697(90)90126-T

[43] Schär M, Börnsen KO, Gassmann E. Fast protein sequence determination with matrix-assisted laser desorption and ionization mass spectrometry. Rapid Communications in Mass Spectrometry. 1991;**5**(7):319-326. DOI: 10.1002/rcm.1290050705

[44] Bongers J, Cummings JJ, Ebert MB, Federici MM, Gledhill L, Gulati D, Hilliard GM, Jones BH, Lee KR, Mozdzanowski J, Naimoli M, Burman S. Validation of a peptide mapping method for a therapeutic monoclonal antibody: What could we possibly learn about a method we have run 100 times? Journal of Pharmaceutical and Biomedical Analysis. 2000;**21**(6):1099-1128. DOI: 10.1016/S0731-7085(99)00181-8

[45] Hernandez P, Müller M, Appel RD. Automated protein identification by tandem mass spectrometry: Issues and strategies. Mass Spectrometry Reviews. 2006;**25**(2):235-254. DOI: 10.1002/mas.20068

[46] Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR 3rd. Protein analysis by shotgun/bottom-up proteomics. Chemical Reviews. 2013;**113**(4):2343-2494. DOI: 10.1021/cr3003533

[47] Byeon J, Yim Y-R, Kim H-H, Suh J-K. Structural identification of a non-glycosylated variant at Ser126 for O-glycosylation site from EPO BRP, human recombinant erythropoietin by LC/MS analysis. Molecules and Cells. 2015;**38**(6):496-505. DOI: 10.14348/molcells.2015.2256

[48] Wang Y, Li X, Liu YH, Richardson D, Li H, Shameem M, Yang X. Simultaneous monitoring of oxidation, deamidation, isomerization, and glycosylation of monoclonal antibodies by liquid chromatography-mass spectrometry method with ultrafast tryptic digestion. MAbs. 2016;**8**(8):1477-1486. DOI: 10.1080/19420862.2016.1226715

[49] Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. Protein Science. 1995;**4**(11):2411-2423. DOI: 10.1002/pro.5560041120

[50] Proc JL, Kuzyk MA, Hardie DB, Yang J, Smith DS, Jackson AM, Parker CE, Borchers CH. A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. Journal of Proteome Research. 2010;**9**(10):5422-5437. DOI: 10.1021/pr100656u

[51] Yu YQ, Gilar M, Lee PJ, Bouvier ES, Gebler JC. Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. Analytical Chemistry. 2003;**75**(21):6023-6028. DOI: 10.1021/ac0346196

[52] Han JC, Han GY. A procedure for quantitative determination of tris(2-carboxyethyl) phosphine, an odorless reducing agent more stable and effective than dithiothreitol. Analytical Biochemistry. 1994;**220**(1):5-10. DOI: 10.1006/abio.1994.1290

[53] Svoboda M, Meister W, Vetter W. A method for counting disulfide bridges in small proteins by reduction with mercaptoethanol and electrospray mass spectrometry. Journal of Mass Spectrometry. 1995;**30**:1562-1566. DOI: 10.1002/jms.1190301107

[54] Switzar L, Giera M, Niessen WM. Protein digestion: An overview of the available techniques and recent developments. Journal of Proteome Research. 2013;**12**(3):1067-1077. DOI: 10.1021/pr301201x

[55] Tsiatsiani L, Heck AJ. Proteomics beyond trypsin. The FEBS Journal. 2015;**282**(14):2612-2626. DOI: 10.1111/febs.13287

[56] Bunkenborg J, Espadas G, Molina H. Cutting edge proteomics: Benchmarking of six commercial trypsins. Journal of Proteome Research. 2013;**12**(8):3631-3641. DOI: 10.1021/pr4001465

[57] Giansanti P, Tsiatsiani L, Low TY, Heck AJ. Six alternative proteases for mass spectrometry-based proteomics beyond trypsin. Nature Protocols. 2016;**11**(5):993-1006. DOI: 10.1038/nprot.2016.057

[58] Plumb R, Castro-Perez J, Granger J, Beattie I, Joncour K, Wright A. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry. 2004;**18**(19):2331-2337. DOI: 10.1002/rcm.1627

[59] Motoyama A, Venable JD, Ruse CI, Yates JR 3rd. Automated ultra-high-pressure multidimensional protein identification technology (UHP-MudPIT) for improved peptide identification of proteomic samples. Analytical Chemistry. 2006;**78**(14):5109-5118. DOI: 10.1021/ac060354u

[60] Omamogho JO, Glennon JD. Comparison between the efficiencies of sub-2 μm C18 particles packed in narrow bore columns. Analytical Chemistry. 2011;**83**(5):1547-1556. DOI: 10.1021/ac102139a

[61] Water TH. Recent innovations in UHPLC columns and instrumentation. Trends in Analytical Chemistry. 2014;**63**:14-20. DOI: 10.1016/j.trac.2014.07.016

[62] González-Ruiz V, Olives AI, Martín MA. Core-shell particles lead the way to renewing high-performance liquid chromatography. Trends in Analytical Chemistry. 2015;**64**:17-28. DOI: 10.1016/j.trac.2014.08.008

[63] Kochling J, Wu W, Hua Y, Guan Q, Castaneda-Merced J. A platform analytical quality by design (AQbD) approach for multiple UHPLC-UV and UHPLC-MS methods development for protein analysis. Journal of Pharmaceutical and Biomedical Analysis. 2016;**125**:130-139. DOI: 10.1016/j.jpba.2016.03.031

[64] Ghosh PK. Introduction to Protein Mass Spectrometry. 1st ed. London: Academic Press; 2015. Available from: https://www.sciencedirect.com/science/book/9780128021026

[65] Domon B, Aebersold R. Mass spectrometry and protein analysis. Science. 2006;**312**(5771):212-217. DOI: 10.1126/science.1124619

[66] Yu L, Remmele RL Jr, He B. Identification of N-terminal modification for recombinant monoclonal antibody light chain using partial reduction and quadrupole time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry. 2006;**20**(24):3674-3680. DOI: 10.1002/rcm.2790

[67] Wang D, Wynne C, Gu F, Becker C, Zhao J, Mueller HM, Li H, Shameem M, Liu YH. Characterization of drug-product-related impurities and variants of a therapeutic monoclonal antibody by higher energy C-trap dissociation mass spectrometry. Analytical Chemistry. 2015;**87**(2):914-921. DOI: 10.1021/ac503158g

[68] Colgrave ML, Craik DJ. Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: The importance of the cyclic cystine knot. Biochemistry. 2004;**43**(20):5965-5975. DOI: 10.1021/bi049711q

[69] Fass D. Disulfide bonding in protein biophysics. Annual Review of Biophysics. 2012;**41**:63-79. DOI: 10.1146/annurev-biophys-050511-102321

[70] Goyder MS, Rebeaud F, Pfeifer ME, Kálmán F. Strategies in mass spectrometry for the assignment of Cys-Cys disulfide connectivities in proteins. Expert Review of Proteomics. 2013;**10**(5):489-501. DOI: 10.1586/14789450.2013.837663

[71] Wiesner J, Resemann A, Evans C, Suckau D, Jabs W. Advanced mass spectrometry workflows for analyzing disulfide bonds in biologics. Expert Review of Proteomics. 2015;**12**(2):115-123. DOI: 10.1586/14789450.2015.1018896

[72] Gorman JJ, Wallis TP, Pitt JJ. Protein disulfide bond determination by mass spectrometry. Mass Spectrometry Reviews. 2002;**21**(3):183-216. DOI: 10.1002/mas.10025

[73] Lakbub JC, Shipman JT, Desaire H. Recent mass spectrometry-based techniques and considerations for disulfide bond characterization in proteins. Analytical and Bioanalytical Chemistry. 2018;**410**(10):2467-2484. DOI: 10.1007/s00216-017-0772-1

[74] Liu H, May K. Disulfide bond structures of IgG molecules: Structural variations, chemical modifications and possible impacts to stability and biological function. MAbs. 2012;**4**(1):17-23. DOI: 10.4161/mabs.4.1.18347

[75] Wu SL, Jiang H, Hancock WS, Karger BL. Identification of the unpaired cysteine status and complete mapping of the 17 disulfides of recombinant tissue plasminogen activator using LC-MS with electron transfer dissociation/collision induced dissociation. Analytical Chemistry. 2010;**82**(12):5296-5303. DOI: 10.1021/ac100766r

[76] Wu SL, Jiang H, Lu Q, Dai S, Hancock WS, Karger BL. Mass spectrometric determination of disulfide linkages in recombinant therapeutic proteins using online LC-MS with electron-transfer dissociation. Analytical Chemistry. 2009;**81**(1):112-122. DOI: 10.1021/ac801560k

[77] Xie H, Gilar M, Gebler JC. Characterization of protein impurities and site-specific modifications using peptide mapping with liquid chromatography and data independent acquisition mass spectrometry. Analytical Chemistry. 2009;**81**(14):5699-5708. DOI: 10.1021/ac900468j

[78] Xie H, Chakraborty AB, Chen W. Towards fast mapping of protein disulfide bonds: An integrated workflow for automatic assignment of disulfide pairing. In: Proceedings of Practical Applications of Mass Spectrometry in the Biotechnology Industry; 8-10 September 2010; Marina Del Ray. CA: Mass Spec; 2010. p. 107. Available from: http://www.waters.com/webassets/cms/ library/docs/720003751en.pdf

[79] Walsh CT, Garneau-Tsodikova S, Gatto GJ Jr. Protein posttranslational modifications: The chemistry of proteome diversifications. Angewandte Chemie (International Ed. in English). 2005;**44**(45):7342-7372. DOI: 10.1002/anie.200501023

[80] Li W, Kerwin JL, Schiel J, Formolo T, Davis D, Mahan A, Benchaar SA. Structural elucidation of post-translational modifications in monoclonal antibodies. In: Schiel JE, Davis DL, Borisov OV, editors. State-of-the-art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NIST mAb Case Study. Washington, DC: American Chemical Society; 2015. pp. 119-183. DOI: 10.1021/bk-2015-1201.ch003

[81] Walsh G. Post-translational modifications of protein biopharmaceuticals. Drug Discovery Today. 2010;**15**:773-780. DOI: 10.1016/j.drudis.2010.06.009

[82] Brinks V, Hawe A, Basmeleh AH, Joachin-Rodriguez L, Haselberg R, Somsen GW, Jis5oot W, Schellekens H. Quality of original and biosimilar epoetin products. Pharmaceutical Research. 2011;**28**(2):386-393. DOI: 10.1007/s11095-010-0288-2

[83] Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nature Reviews. Drug Discovery. 2012;**11**:527-540. DOI: 10.1038/nrd3746

[84] Diepold K, Bomans K, Wiedmann M, Zimmermann B, Petzold A, Schlothauer T, Mueller R, Moritz B, Stracke JO, Mølhøj M, Reusch D, Bulau P. Simultaneous assessment of Asp isomerization and Asn deamidation in recombinant antibodies by LC-MS following incubation at elevated temperatures. PLoS One. 2012;**7**(1):e30295. DOI: 10.1371/ journal.pone.0030295

[85] Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianferani S. Characterization of therapeutic antibodies and related products. Analytical Chemistry. 2013;**85**:715-736. DOI: 10.1021/ac3032355

[86] Fekete S, Guillarme D, Sandra P, Sandra K. Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals. Analytical Chemistry. 2016;**88**:480-507. DOI: 10.1021/acs.analchem.5b04561

[87] Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Balland A. Development of a quantitative mass spectrometry multiattribute method for characterization, quality control testing and disposition of biologics. MAbs. 2015;**7**:881-890. DOI: 10.1080/19420862

[88] Wang T, Chu L, Li W, Lawson K, Apostol I, Eris T. Application of a quantitative LC–MS multiattribute method for monitoring site-specific glycan heterogeneity on a monoclonal antibody containing two N-linked glycosylation sites. Analytical Chemistry. 2017;**89**:3562-3567. DOI: 10.1021/acs.analchem.6b04856

[89] Huhn C, Selman MH, Ruhaak LR, Deelder AM, Wuhrer M. IgG glycosylation analysis. Proteomics. 2009;**9**(4):882-913. DOI: 10.1002/pmic.200800715

[90] Abès R, Teillaud JL. Impact of glycosylation on effector functions of therapeutic IgG. Pharmaceuticals (Basel). 2010;**3**(1):146-157. DOI: 10.3390/ph3010146

[91] Sibéril S, Dutertre CA, Fridman WH, Teillaud JL, Fcgamma R. The key to optimize therapeutic antibodies? Critical Reviews in Oncology/Hematology. 2007;**62**(1):26-33. DOI: 10.1016/ j.critrevonc.2006.12.003

[92] Takahashi M, Kuroki Y, Ohtsubo K, Taniguchi N. Core fucose and bisecting GlcNAc, the direct modifiers of the N-glycan core: Their functions and target proteins. Carbohydrate Research. 2009;**344**(12):1387-1390. DOI: 10.1016/j.carres.2009.04.031

[93] Abès R, Dutertre CA, Agnelli L, Teillaud JL. Activating and inhibitory Fcgamma receptors in immunotherapy: Being the actor or being the target. Expert Review of Clinical Immunology. 2009;**5**(6):735-747. DOI: 10.1586/eci.09.57

[94] Zhang Q, Li Z, Wang Y, Zheng Q, Li J. Mass spectrometry for protein sialoglycosylation. Mass Spectrometry Reviews. 2017. DOI: 10.1002/mas.21555

[95] Tharmalingam T, Adamczyk B, Doherty MA, Royle L, Rudd PM. Strategies for the profiling, characterisation and detailed structural analysis of N-linked oligosaccharides. Glycoconjugate Journal. 2013;**30**(2):137-146. DOI: 10.1007/s10719-012-9443-9

[96] Lauber MA, Yu YQ, Brousmiche DW, Hua Z, Koza SM, Magnelli P, Guthrie E, Taron CH, Fountain KJ. Rapid preparation of released N-glycans for HILIC analysis using a labeling reagent that facilitates sensitive fluorescence and ESI-MS detection. Analytical Chemistry. 2015;**87**(10):5401-5409. DOI: 10.1021/acs.analchem.5b00758

[97] Reusch D, Haberger M, Falck D, Peter B, Maier B, Gassner J, Hook M, Wagner K, Bonnington L, Bulau P, Wuhrer M. Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-part 2: Mass spectrometric methods. MAbs. 2015;**7**(4):732-742. DOI: 10.1080/19420862.2015.1045173

[98] Terashima I, Koga A, Nagai H. Identification of deamidation and isomerization sites on pharmaceutical recombinant antibody using H(2)(18)O. Analytical Biochemistry. 2007;**368**(1):49-60. DOI: 10.1016/j.ab.2007.05.012

[99] Liu H, Gaza-Bulseco G, Chumsae C. Glutamine deamidation of a recombinant monoclonal antibody. Rapid Communications in Mass Spectrometry. 2008;**22**(24):4081-4088. DOI: 10.1002/rcm.3831

[100] Yang H, Zubarev RA. Mass spectrometric analysis of asparagine deamidation and aspartate isomerization in polypeptides. Electrophoresis. 2010;**31**(11):1764-1772. DOI: 10.1002/elps.201000027

[101] Robinson AB, Scotchler JW, McKerrow JH. Rates of nonenzymatic deamidation of glutaminyl and asparaginyl residues in pentapeptides. Journal of the American Chemical Society. 1973;**95**(24):8156-8159

[102] Scotchler JW, Robinson AB. Deamidation of glutaminyl residues: Dependence on pH, temperature, and ionic strength. Analytical Biochemistry. 1974;**59**(1):319-322. DOI: 10.1016/0003-2697(74)90040-2

[103] Tyler-Cross R, Schirch V. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. The Journal of Biological Chemistry. 1991 Nov 25;**266**(33):22549-22556. Available from: http://www.jbc.org/content/266/33/22549.full.pdf

[104] Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. The Journal of Biological Chemistry. 1987;**262**(2):785-794. Available from: http://www.jbc.org/content/262/2/785.full.pdf

[105] Wakankar AA, Borchardt RT, Eigenbrot C, Shia S, Wang YJ, Shire SJ, Liu JL. Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. Biochemistry. 2007;**46**(6):1534-1544. DOI: 10.1021/bi061500t

[106] Sreedhara A, Cordoba A, Zhu Q, Kwong J, Liu J. Characterization of the isomerization products of aspartate residues at two different sites in a monoclonal antibody. Pharmaceutical Research. 2012;**29**(1):187-197. DOI: 10.1007/s11095-011-0534-2

[107] Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J. Heterogeneity of monoclonal antibodies. Journal of Pharmaceutical Sciences. 2008;**97**(7):2426-2447. DOI: 10.1002/jps.21180

[108] Wakankar AA, Liu J, Vandervelde D, Wang YJ, Shire SJ, Borchardt RT. The effect of cosolutes on the isomerization of aspartic acid residues and conformational stability in a monoclonal antibody. Journal of Pharmaceutical Sciences. 2007;**96**(7):1708-1718. DOI: 10.1002/jps.20823

[109] Rehder DS, Chelius D, McAuley A, Dillon TM, Xiao G, Crouse-Zeineddini J, Vardanyan L, Perico N, Mukku V, Brems DN, Matsumura M, Bondarenko PV. Isomerization of a single aspartyl residue of anti-epidermal growth factor receptor immunoglobulin gamma2 antibody highlights the role avidity plays in antibody activity. Biochemistry. 2008;**47**(8):2518-2530. DOI: 10.1021/bi7018223

[110] Yan Y, Wei H, Fu Y, Jusuf S, Zeng M, Ludwig R, Krystek SR Jr, Chen G, Tao L, Das TK. Isomerization and oxidation in the complementarity-determining regions of a monoclonal antibody: A study of the modification-structure-function correlations by hydrogen-deuterium exchange mass spectrometry. Analytical Chemistry. 2016;**88**(4):2041-2050. DOI: 10.1021/acs.analchem.5b02800

[111] Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, Shire SJ, Bjork N, Totpal K, Chen AB. Identification of multiple sources of charge heterogeneity in a recombinant antibody. Journal of Chromatography. B, Biomedical Sciences and Applications. 2001;**752**(2):233-245. DOI: 10.1016/S0378-4347(00)00548-X

[112] Chu GC, Chelius D, Xiao G, Khor HK, Coulibaly S, Bondarenko PV. Accumulation of succinimide in a recombinant monoclonal antibody in mildly acidic buffers under elevated temperatures. Pharmaceutical Research. 2007;**24**(6):1145-1156. DOI: 10.1007/s11095-007-9241-4

[113] Kroon DJ, Baldwin-Ferro A, Lalan P. Identification of sites of degradation in a therapeutic monoclonal antibody by peptide mapping. Pharmaceutical Research. 1992;**9**(11):1386-1393

[114] Chelius D, Rehder DS, Bondarenko PV. Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies. Analytical Chemistry. 2005;**77**(18):6004-6011. DOI: 10.1021/ac050672d

[115] Dick LW Jr, Qiu D, Mahon D, Adamo M, Cheng KC. C-terminal lysine variants in fully human monoclonal antibodies: Investigation of test methods and possible causes. Biotechnology and Bioengineering. 2008;**100**(6):1132-1143. DOI: 10.1002/bit.21855

[116] Luo J, Zhang J, Ren D, Tsai WL, Li F, Amanullah A, Hudson T. Probing of C-terminal lysine variation in a recombinant monoclonal antibody production using Chinese hamster ovary cells with chemically defined media. Biotechnology and Bioengineering. 2012;**109**(9):2306-2315. DOI: 10.1002/bit.24510

[117] Jiang G, Yu C, Yadav DB, Hu Z, Amurao A, Duenas E, Wong M, Iverson M, Zheng K, Lam X, Chen J, Vega R, Ulufatu S, Leddy C, Davis H, Shen A, Wong PY, Harris R, Wang YJ, Li D. Evaluation of heavy-chain C-terminal deletion on product quality and pharmacokinetics of monoclonal antibodies. Journal of Pharmaceutical Sciences. 2016;**105**(7):2066-2072. DOI: 10.1016/j.xphs.2016.04.027

[118] Antes B, Amon S, Rizzi A, Wiederkum S, Kainer M, Szolar O, Fido M, Kircheis R, Nechansky A. Analysis of lysine clipping of a humanized Lewis-Y specific IgG antibody and its relation to Fc-mediated effector function. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 2007;**852**(1-2):250-256. DOI: 10.1016/j.jchromb.2007.01.024

[119] van den Bremer ET, Beurskens FJ, Voorhorst M, Engelberts PJ, de Jong RN, van der Boom BG, Cook EM, Lindorfer MA, Taylor RP, van Berkel PH, Parren PW. Human IgG is produced in a pro-form that requires clipping of C-terminal lysines for maximal complement activation. MAbs. 2015;**7**(4):672-680. DOI: 10.1080/19420862.2015.1046665

[120] Kim DG, Kim HJ, Kim HJ. Effects of carboxypeptidase B treatment and elevated temperature on recombinant monoclonal antibody charge variants in cation-exchange chromatography analysis. Archives of Pharmacal Research. 2016;**39**(10):1472-1481. DOI: 10.1007/s12272-016-0818-5

[121] Liu J, Eris T, Li C, Cao S, Kuhns S. Assessing analytical similarity of proposed amgen biosimilar ABP 501 to adalimumab. BioDrugs. 2016;**30**(4):321-338. DOI: 10.1007/s40259-016-0184-3

[122] Miao S, Fan L, Zhao L, Ding D, Liu X, Wang H, Tan WS. Physicochemical and biological characterization of the proposed biosimilar tocilizumab. BioMed Research International. 2017;**2017**. DOI: 4926168. DOI: 10.1155/2017/4926168

[123] Dick LW Jr, Kim C, Qiu D, Cheng KC. Determination of the origin of the N-terminal pyroglutamate variation in monoclonal antibodies using model peptides. Biotechnology and Bioengineering. 2007;**97**(3):544-553. DOI: 10.1002/bit.21260

[124] Liu YD, Goetze AM, Bass RB, Flynn GC. N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies. The Journal of Biological Chemistry. 2011;**286**(13):11211-11217. DOI: 10.1074/jbc.M110.185041