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# Characterization of Biopharmaceuticals Focusing on Antibody Therapeutics

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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)

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## 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

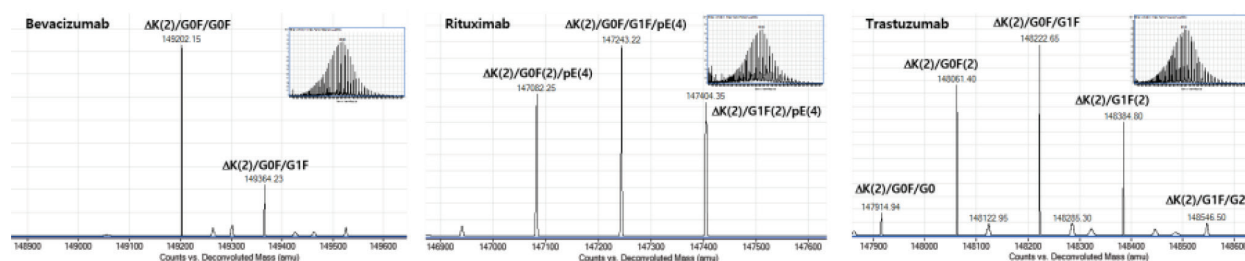
### 2.1. Intact mass determination of mAb

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 ( $\Delta K$ ), 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



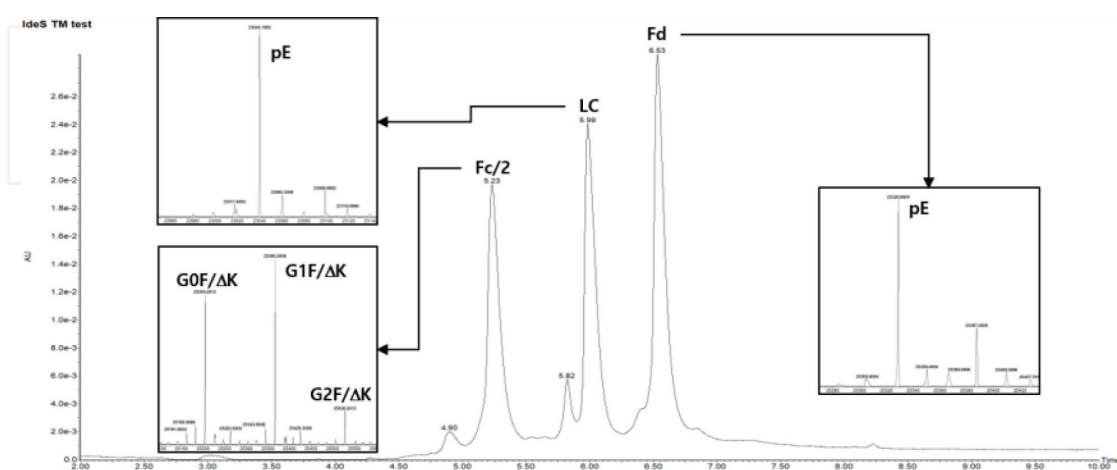
**Figure 1.** Intact mass of mAbs determined by deconvolution using ESI-QTOF. Inserts show charge envelope having multiply charged ions. Glycan structures (G0F, G1F, and G2F) were adapted from [35].  $\Delta K$ : C-terminal Lys deletion and pE: pyro-glutamate.

mAbs with subsequent chemical reduction generates three subunits, Fc/2, LC, and Fd, 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**).

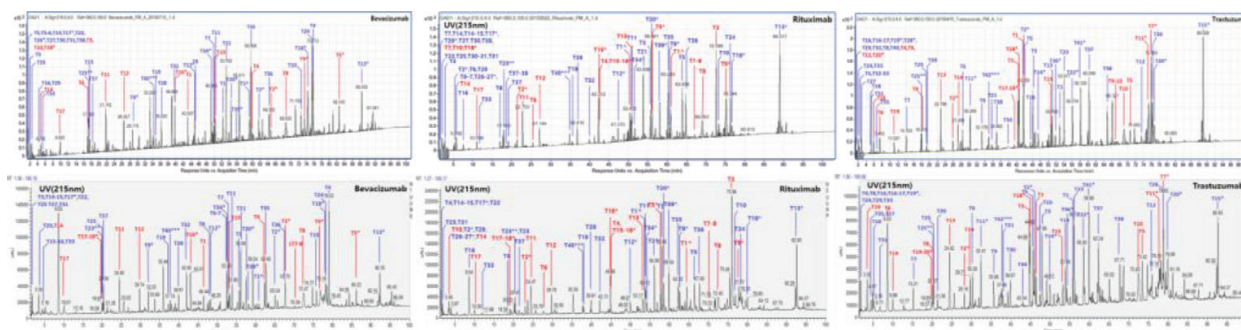
### 2.3. Peptide mapping of mAb

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 ( $A_{280}$ ) 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].  $\Delta$ K: 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  $\mu\text{m}$  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].

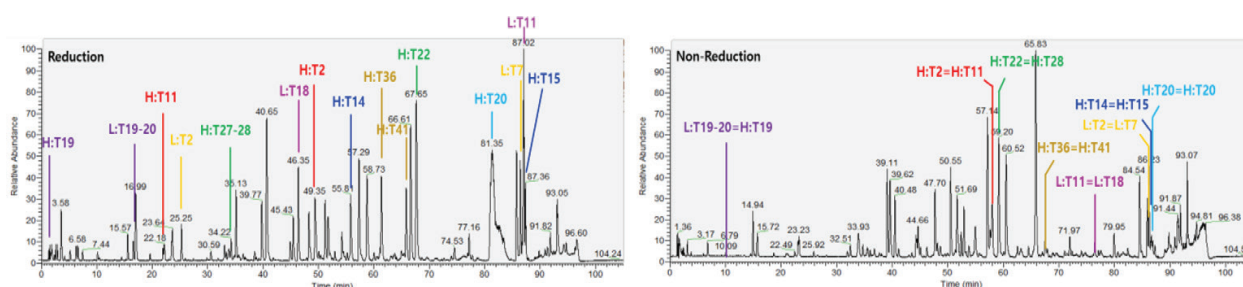
Typical results of peptide mapping of bevacizumab, rituximab, and trastuzumab are shown in **Figure 3**. For the peptide mapping analysis, protein samples were digested with trypsin, and the resulting peptides were separated using RP-UHPLC, detected by UV-absorption, and identified by online ESI-QTOF 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

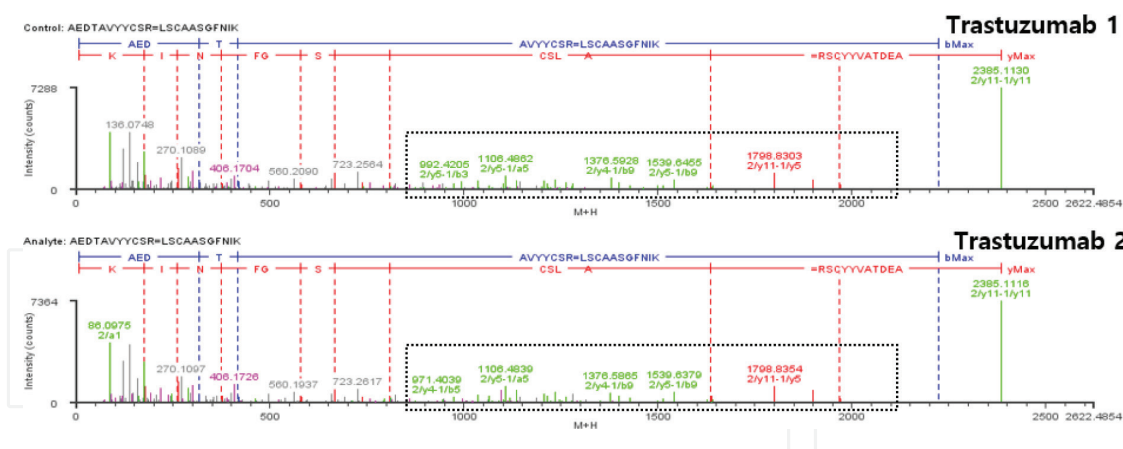
Protein disulfide bonds are produced by the covalent bonding of two thiol groups between cysteine amino acids. They are essential for structural and functional roles of proteins and give stability of proteins [68, 69]. The number of disulfide bonds and their positions are a critical attribute for biopharmaceuticals 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].



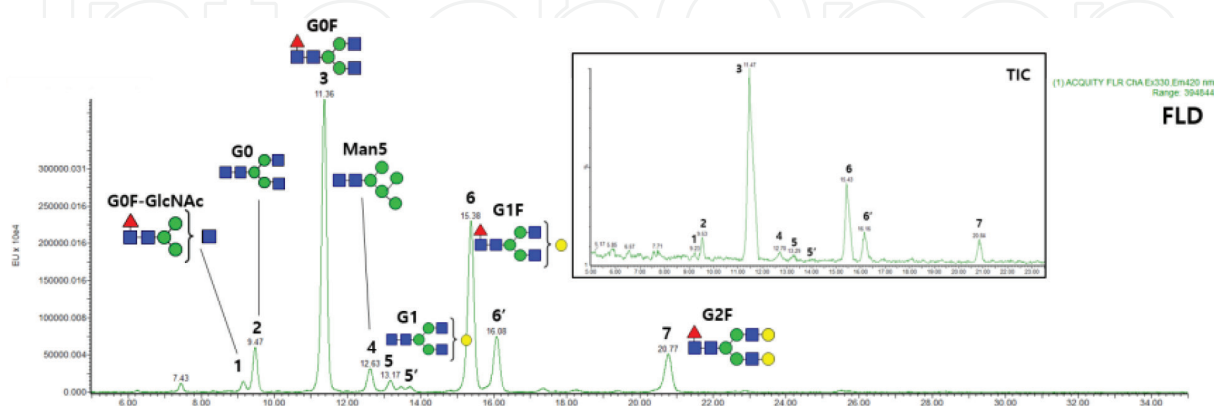
Attribute	Description	Current workflow	Future development
N-term sequences	Identity	Edman Degradation	Multi-attribute method (MAM)
N-term variants	Gln/Glu cyclization	CEX-HPLC	
C-term variants	C-term Lys deletion	CEX-HPLC	
Charge variants	Deamidation/Isomerization	CEX-HPLC	
Clips	Fragment	CE-SDS	
Glycans	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  $\mu\text{m}$  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%.

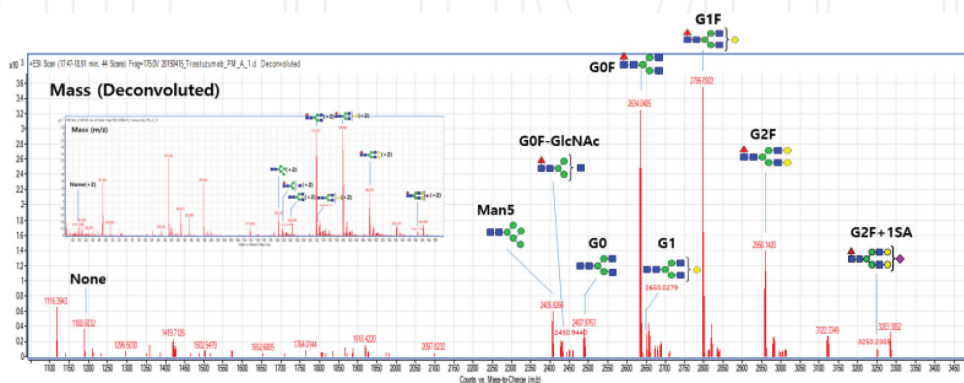
Calculation of error for determined peptide by MS is shown in Eq. (1)

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

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].

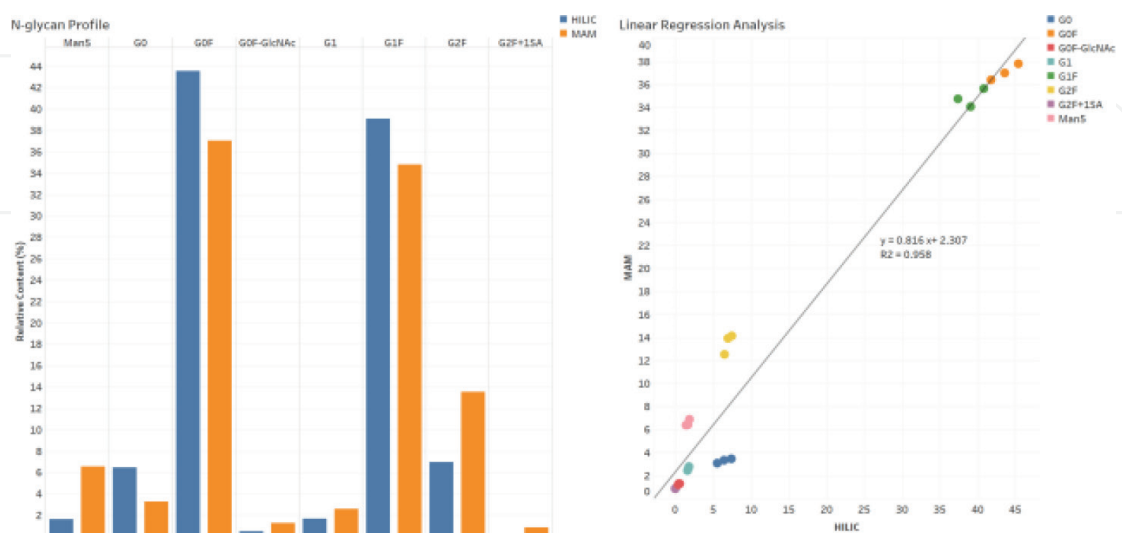
Peptide	Number	Glycan attached	Calculated mass (Da)	Determined mass (Da)	Error (ppm)	Relative content (%)
EEQYNSTYR	H:T25		1188.5047	1188.502	-2.27	2.18 ± 0.06
EEQYNSTYR	H:T25**	Man5	2404.9276	2404.9249	-1.12	6.42 ± 0.27
EEQYNSTYR	H:T25**	G0F-GlcNAc	2429.9592	2429.9531	-2.51	1.23 ± 0.04
EEQYNSTYR	H:T25**	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.

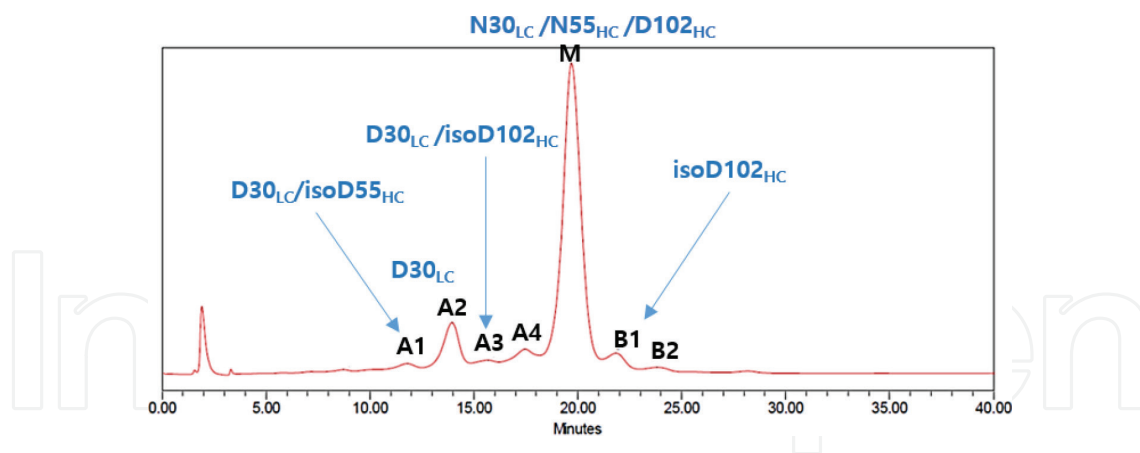
**Table 3.** N-glycan profiling of mAb, trastuzumab, determined by MAM approach.

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

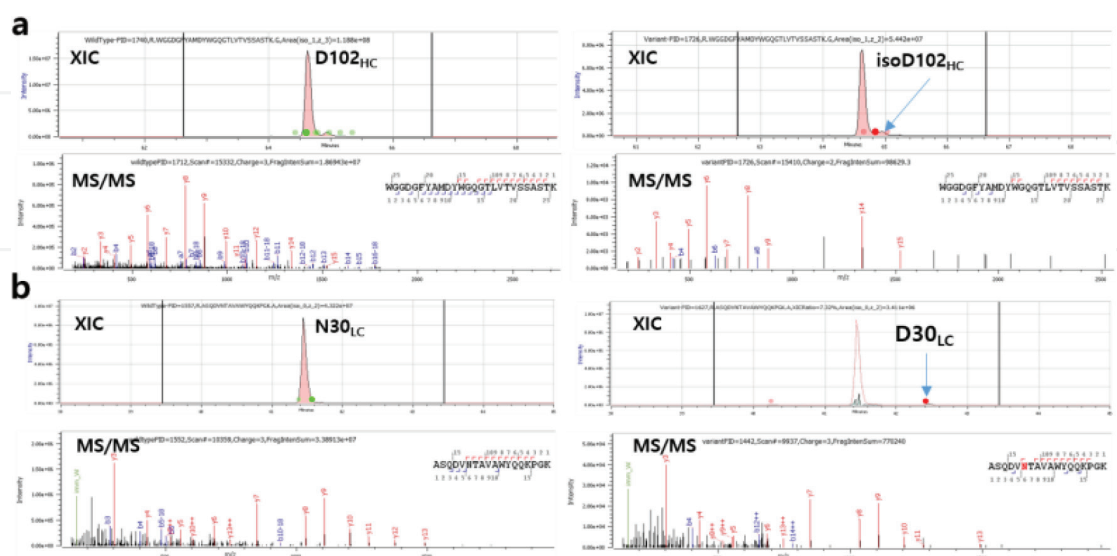
N: asparagine, D: glutamate, isoD: iso-glutamate.

**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, deamidation 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  $\pm 5$  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**). Deamidation 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 DYWGQGLV TVSSASTK	H:T12	D102	2783.2537	2783.2551	0.50	95.36 ± 0.28
WGG <sub>iso</sub> DGFYAM DYWGQGLV TVSSASTK	H:T12*	D102 to isoD102	2783.2537	2783.2595	2.08	4.63 ± 0.28
IYPTNGYTR	H:T6	N55	1083.5349	1083.5345	-0.37	100
IYPT <sub>iso</sub> DGYTR	H:T6*	N55 to isoD55	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

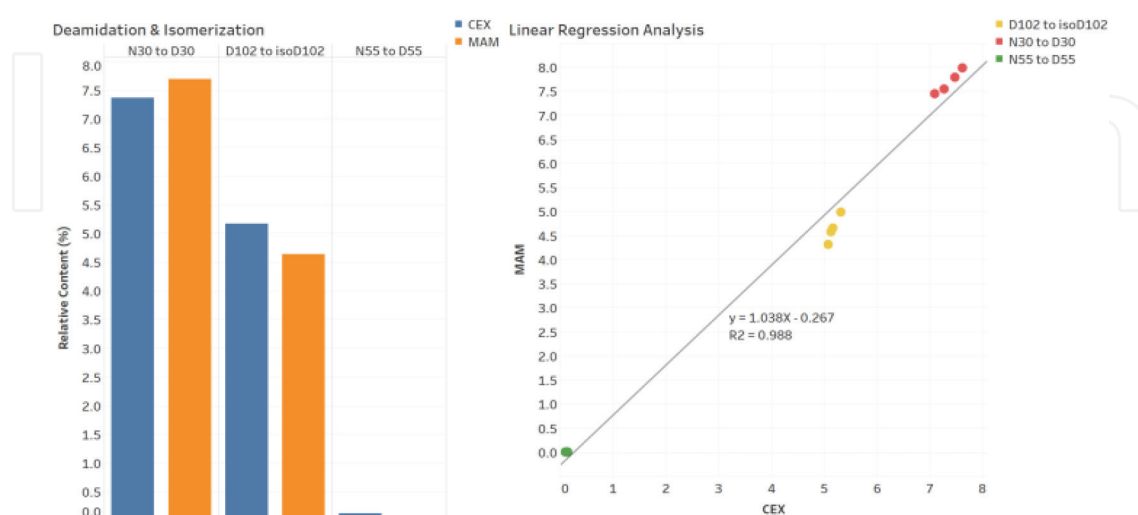
N: asparagine, D: aspartate, isoD: iso-aspartate, H: heavy chain, L: light chain, and T: tryptic peptide.

\*: 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.

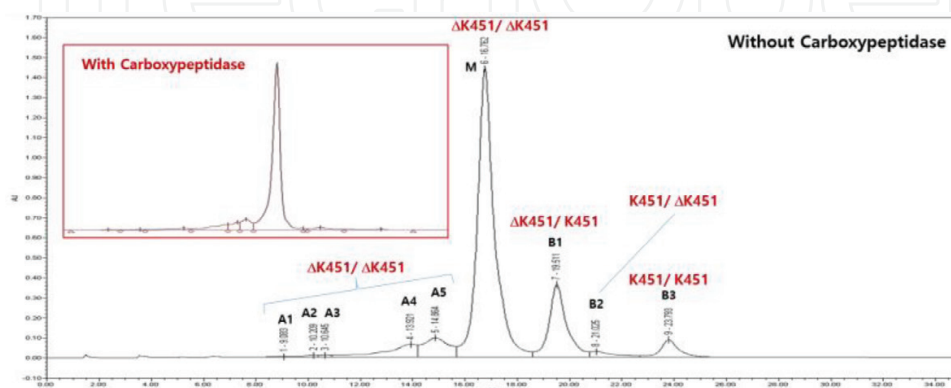
Because Lys residue is positively charged, leading to charge heterogeneity of mAb products. Thus, it is still a quality parameter for characterization. Due to charge variation, the modified and unmodified structures can be separated by CEX, IEF, and cIEF [117, 119]. Loss of the terminal Lys residue gives 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  $\pm 5$  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  $\Delta$ K: 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

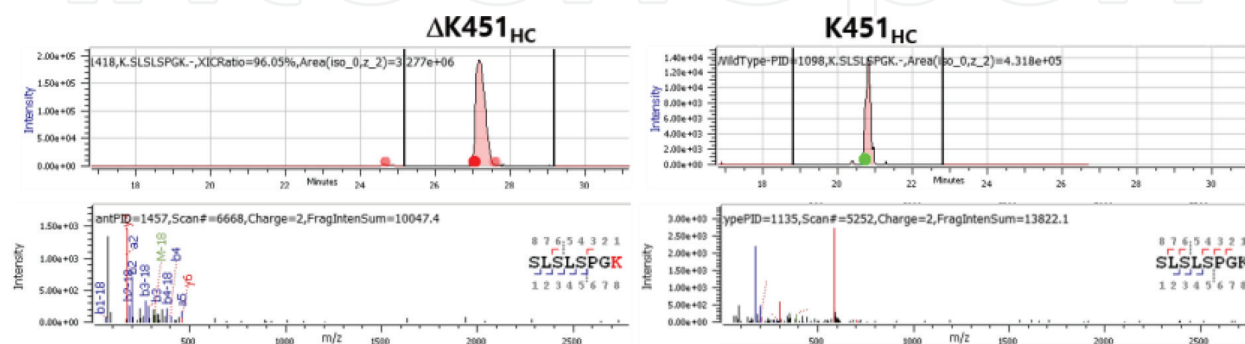
A: acidic, M: major, B: basic.

**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 pyroGlu 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].



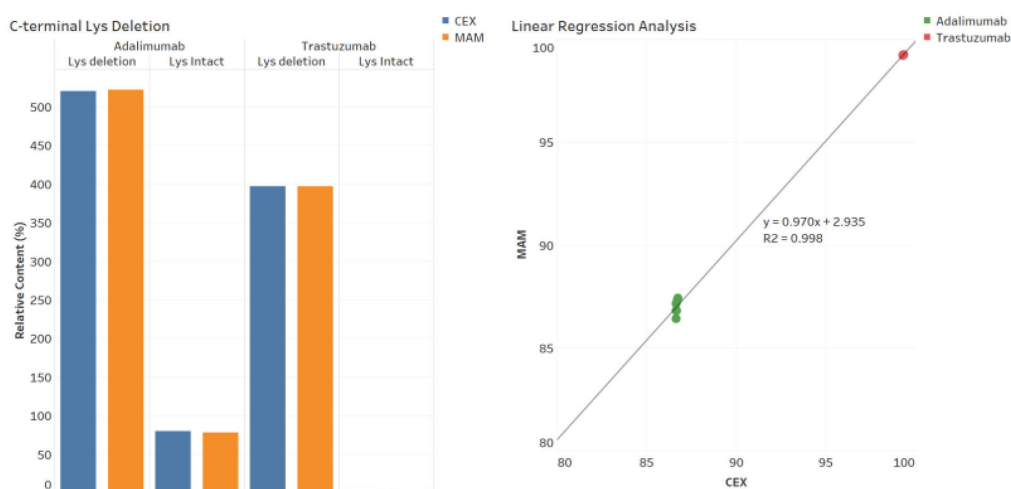
**Figure 13.** Identification of C-terminal Lys variant from adalimumab by peptide mapping analysis. K: Lys, ΔK: Lys deletion, and HC: heavy chain.



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.



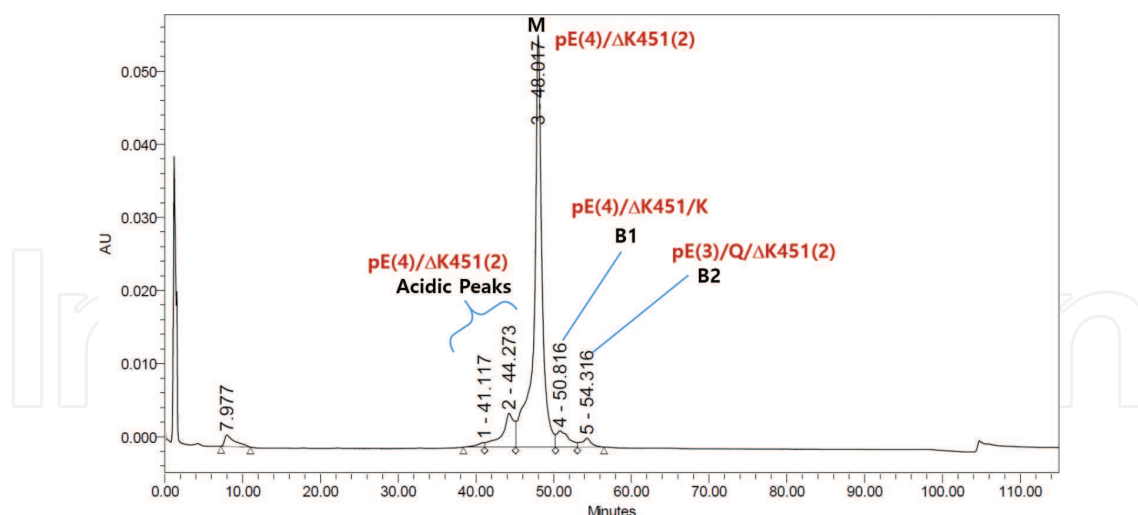
**Figure 14.** Profiles of C-terminal Lys variants for adalimumab and trastuzumab determined by CEX and MAM approaches.

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.

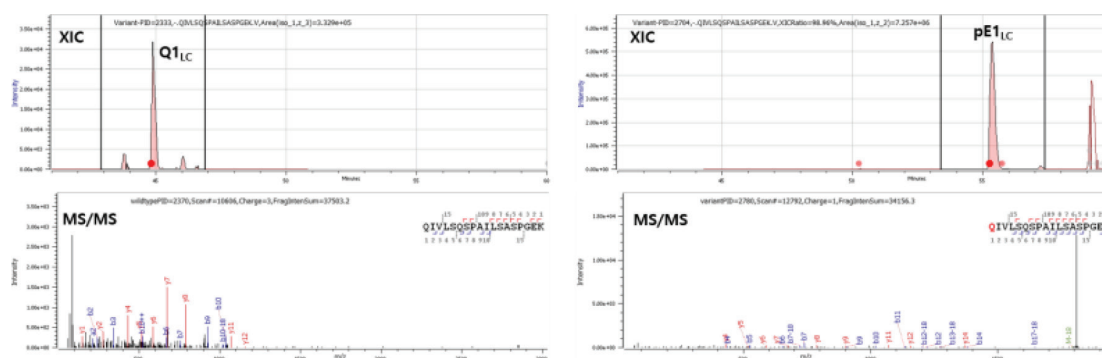
The typical results of CEX analysis are shown in **Figure 15**, which identify and quantify N-terminal cyclization variants of mAb, rituximab. On the chromatogram of rituximab, several acidic variants and two major basic variants are detected (**Figure 15**). Each fraction for basic charge variants was 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  $\pm 5$  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

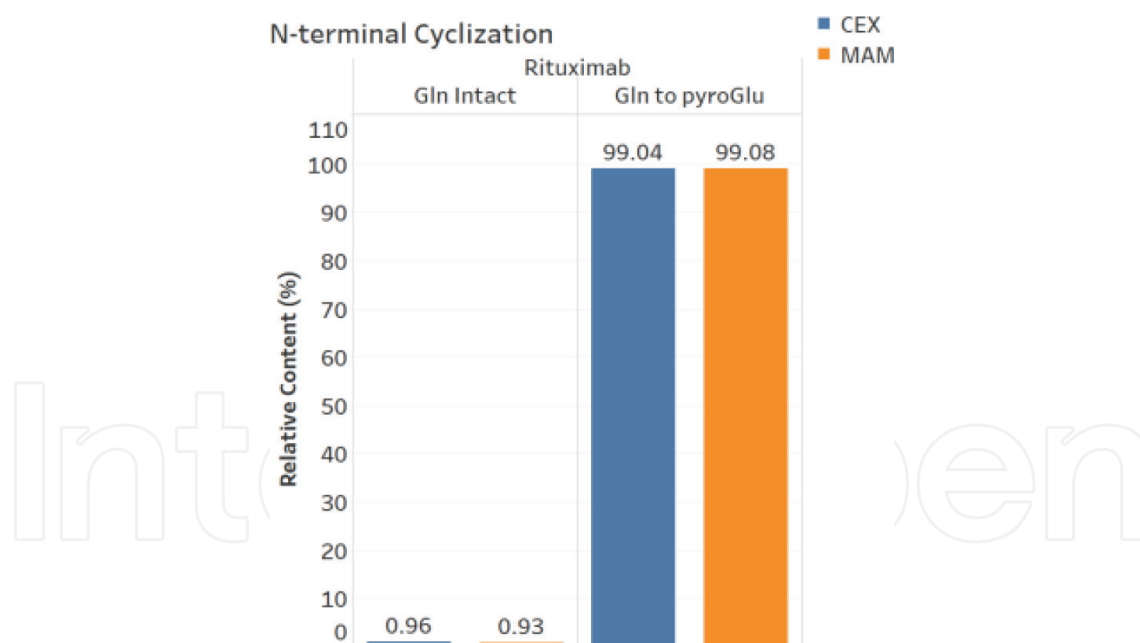
M: major and B: basic.

**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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