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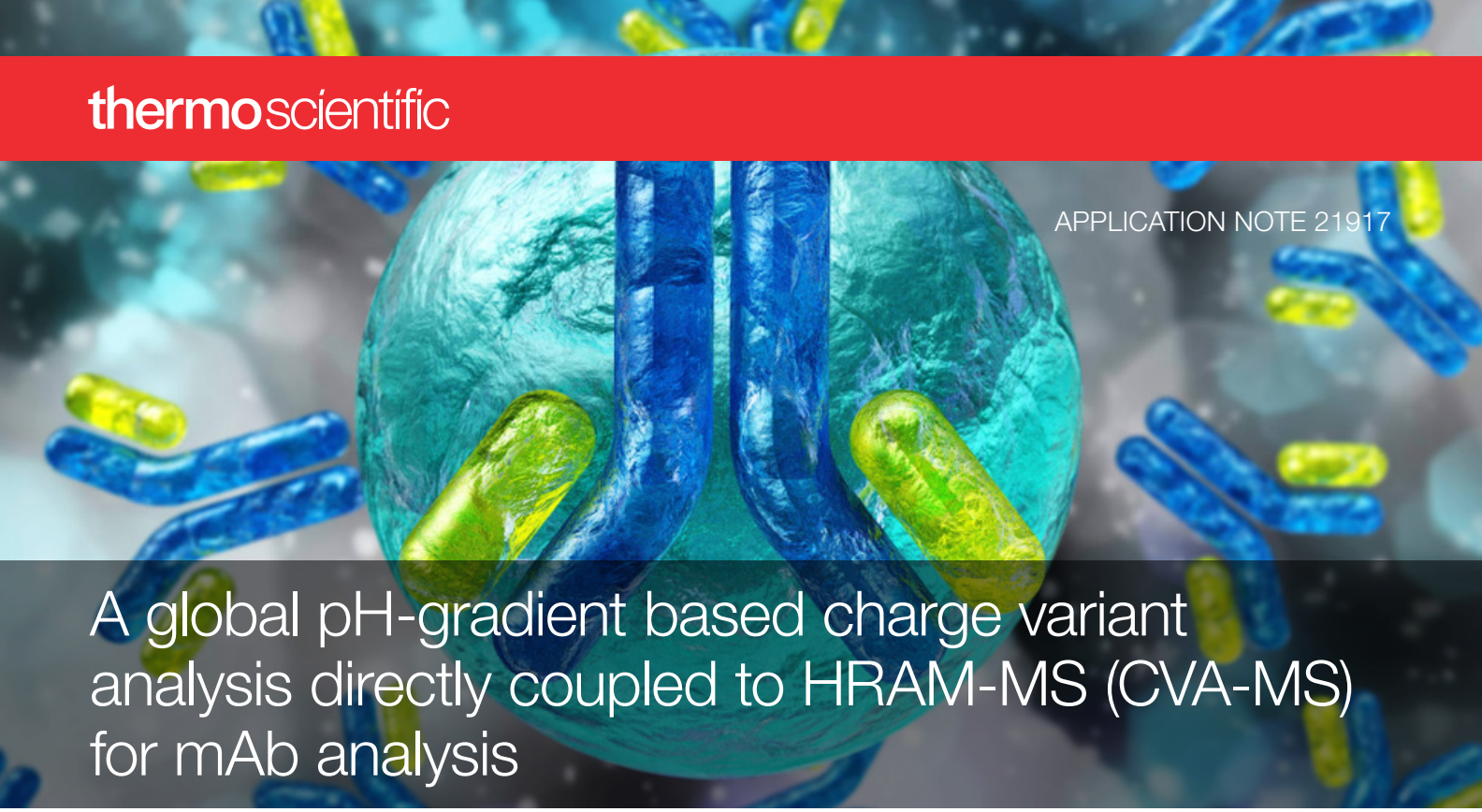

APPLICATION NOTE 21917

A global pH-gradient based charge variant analysis directly coupled to HRAM-MS (CVA-MS) for mAb analysis

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, biosimilar, intact protein analysis, lysine truncation, intact mass, fragmentation, *N*-glycan analysis, fucosylation, multiple critical attributes, MAbPac columns, Vanquish Flex Binary UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, BioPharma Finder

Application benefits

- High information content with no sample preparation using native mass analytical methods for the simultaneous quantification of multiple mAb critical quality attributes that affect efficacy and drug clearance
- Global applicability of the workflow using a Thermo Scientific™ MAbPac™ SCX-10 column for mAb charge variant analysis using volatile buffers with pH-gradients to interface directly with high resolution mass spectrometry
- High data quality obtained using a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap mass spectrometer with BioPharma Option for intact native high resolution accurate mass analysis with on-line charge variant separation of monoclonal antibodies
- Ease of use of Thermo Scientific™ BioPharma Finder™ 3.0 software for intact mass analysis

Goal

To highlight native, intact protein mass analysis techniques for the comprehensive characterization of biotherapeutics. To show the unique coupling of high-resolution accurate mass MS to ion exchange separations of proteins. To characterize several critical quality attributes using a single injection, including relative abundance of mAb N-glycoforms, lysine truncation,

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accurate mass, fragments, deamidation, and charge variants. To demonstrate how the method can be used as a global method for mAb characterization. To show the ease of optimization and utilization of the method while being fast and reproducible.

Introduction

There is a growing interest in the analysis of recombinant monoclonal antibodies (mAbs) and in the study of their biological interaction mechanisms. mAbs offer high specificity and low side effects and are being used to treat many types of cancer, autoimmune, and inflammatory diseases. These attributes have led to their impressive success as human medicines.[1] *N*-glycans present on the Fc region of the monoclonal antibody have an important role in monoclonal antibody stability as they stabilize the CH2 domain of IgGs. Deglycosylation makes a mAb less stable and more prone to aggregation. Moreover, functionality of the IgG is influenced by the attached N-glycans and deglycosylation can change the intended mechanism of action of the drug. The mAb drug clearance from the serum is influenced by the glycan structures as is the affinity of the mAb for natural killer cells, which affects antibody-dependent cellular cytotoxicity (ADCC).[1, 2] As a consequence, the glycan structures on the mAb are closely monitored during production, purification, and storage. C-terminal lysine (Lys) truncation variants are commonly observed in monoclonal antibodies and recombinant proteins. Although these variations do not seem to impact the potency or safety profile, the degree of heterogeneity of C-terminal Lys variants reflect the manufacturing consistency and is monitored for product consistency.[3] Another common structural modification of recombinant proteins is the non-enzymatic deamidation of glutamine (Gln) and asparagine (Asn) residues. Deamidation of monoclonal antibodies has varying effects on function depending on the site of modification.[4] Due to the high similarity in mass, resolving deamidation of Asn residues of intact, recombinant proteins is a significant challenge, however the identification can be assisted by a difference in the elution position in a charge variant separation.[5] All of these modifications can be considered critical attributes that are essential to monitor for drug safety and efficacy and are a requirement for biosimilar comparability.[6] A multitude of analytical techniques need to be used to characterize a biotherapeutic protein. A biosimilar comparison against the reference innovator product also needs to go through a “comparability exercise”.[6] To this end, several analytical testing platforms are needed, which entail several different sample preparation techniques to achieve this in-depth characterization. This adds a certain complexity to the complete characterization of any biopharmaceutical protein and a desire for simple methods that can envelope several of these critical attributes. Information-rich analytical methods, in particular liquid phase separations coupled to mass spectrometry can address these challenges and identify many possible variants.

Several of these modifications on variants can alter the charge distribution on the surface of the mAb and result in charge variants, which is why characterization of these structural variants is a critical requirement. The most common method for charge variant analysis (CVA) utilizes ion exchange chromatography.[7] This technique however is incompatible with mass spectrometry due to the high concentrations of salt used in the eluent system. The current trend to use pH-gradients for protein elution from the ion exchange column reduces the salt requirement of the buffer system.[8] Going one step further to employ volatile buffers in the eluent system for pH-gradient elution has allowed CVA to be directly coupled to high-resolution mass spectrometry (HRMS)[9,10] resulting in a hyphenated method called CVA-MS. This technique provides the chromatographic resolution of ion exchange chromatography coupled to the identification of the separated variants by HRMS. References 9 and 10 are the first examples of publications that utilize this new global method. The biopharmaceutical industry requires fast and robust analytical platforms to fulfil regulatory requirements involved in the Biologics License Applications (BLA) process. A strong new method should be globally applicable to all mAb drugs, easy and reproducible to implement, and give benefits over existing analytical procedures.

In our studies presented here, HRMS was coupled to CVA for the relative quantification of several critical quality attributes on mAb samples from single injections. There was no sample preparation involved in the analysis and the method was shown to be easy to implement and reproducible. How to optimize the method for utilization with different mAb samples and important points to consider are discussed. This application note presents a new global CEX approach for biomolecule CVA using a volatile pH buffer cocktail compatible with HRMS. A MAbPac SCX-10 column was used with a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. This is coupled to a Q Exactive Plus Hybrid Quadrupole-

Orbitrap mass spectrometer equipped with BioPharma Option to allow mass detection up to *m/z* 8000.

The pH buffer platform consists of volatile buffers that give an on-column pH range of 5.3 to 10.2. This pH range should be applicable to most mAb samples. The buffers are volatile but can be prepared as a 5X concentrate, kept in a fridge, and diluted when required to obtain more consistent results. The diluted buffers are recommended to be made up fresh each day. The concentrated stock solution for buffer A are stable in the fridge for at least three months. This makes buffer preparation easier and ensures higher reproducibility. The buffer concentrates are made up by volume and weight without the need for pH titration. This provides easier preparation and increases the reproducibility between users and from day to day. It also prevents contamination of the buffers via the pH electrode and titration process. This platform delivers robust, reproducible gradients applicable to a wide range of mAbs. For method optimization, the separation can be modified by use of different optimized gradients.

The MAbPac SCX-10 LC column works very well in conjunction with this pH buffer platform to allow high-resolution, high-efficiency separation of mAbs and their associated charge variants. The unique nonporous pellicular resin provides the resolving power to separate mAb variants that differ by as little as one charged residue with minor pI differences. A hydrophilic layer surrounds the polymeric beads and eliminates hydrophobic interactions with the resin, resulting in very high peak efficiency. A proprietary grafted cation-exchange surface provides pH selectivity control for high-resolution separations. As the buffering capacity of the volatile buffers is relatively weak, the column dimensions used are 2.1 × 50 mm. This keeps the flow rate low for HRMS data acquisition and allows for fast pH-gradient generation and re-equilibration. The effect of column length with pH-gradient chromatography has been shown to have little impact on the separation.[8,9] A longer column would be undesirable due to the increased buffering capacity causing high response time for the pH-gradients employed and long equilibration times.

The Vanquish Binary Flex UHPLC system is a biocompatible UHPLC system that delivers new benchmarks in accuracy, precision, and sensitivity with low gradient delay for fast gradient generation. Thermo Scientific™ Viper™ Fingertight Fitting connections throughout the system make the setup easy and virtually dead volume free.

All the HRMS data was obtained from a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer equipped with BioPharma Option. The buffer system used in our setup keeps proteins in their native form and preserves the three dimensional structure. This results in a smaller exposed surface to accept charges compared to the analysis under denaturing conditions involving organic solvents and acids. Thus the charge distribution detected by MS under native conditions consists of a smaller number and lower charge states typically detected between *m/z* 4800 and 6800. The benefit of the shift to lower charge states detected at higher *m/z* values is the increased spatial resolution. Detection of species beyond the standard mass range up to *m/z* 6000 is enabled by the BioPharma Option extending mass detection up to *m/z* 8000, which is essential for the intact mAb analysis under native conditions.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Acetic acid (ACS reagent grade, ≥99.7%), (P/N 32209-M)
- Ammonium bicarbonate (BioUltra, reagent grade, ≥99.7%), (P/N 09830)
- Ammonium hydroxide solution (BioUltra, 1 M) (P/N 09859)
- MAbPac SCX-10 RS column, 5 µm, 2.1 × 50 mm (P/N 082675)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample handling equipment

Vanquish Flex Binary UHPLC system including:

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

– VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010)

– Thermo Scientific™ UltiMate™ 3000 PCM-3000 pH and conductivity monitor (P/N 6082.2005)

– Thermo Scientific™ UltiMate™ VWD semi-micro flow cell (P/N 6074.0300)

- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer with BioPharma Option (P/N IQLAAEGAAPFALGMBDK)

Sample preparation

The commercially available monoclonal antibodies trastuzumab, infliximab, bevacizumab, rituximab, and cetuximab were provided by the Hospital Pharmacy Unit of the University Hospital of San Cecilio in Granada, Spain. Adalimumab was provided by St. Vincent's University Hospital in Dublin, Ireland, and the NIST monoclonal antibody reference material (Catalog No RM8671) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Fifty to one hundred micrograms of antibody in formulation buffer were injected per run. Optimized gradient conditions for all mAbs are listed in Table 1.

LC conditions

Mobile phase A:	25 mM ammonium bicarbonate [1.975 g in 200 mL for 5X concentrate] and 30 mM acetic acid in water [1.8 mL in 200 mL for 5X concentrate], final unadjusted pH 5.3
Mobile phase B:	10 mM ammonium hydroxide in water [10 mL of 1 M solution in 200 mL for 5X concentrate], final unadjusted pH 10.9 [results in pH 10.2 from the column due to column buffering effects]
Flow rate:	0.4 mL/min
Column:	MAbPac SCX-10 RS, 5 µm, 2.1 × 50 mm
Column temperature:	30 °C
Pre-column heater:	30 °C
Autosampler temp.:	5 °C
Injection:	50 to 100 µg
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 1 for details

Table 1. Mobile phase gradients for CVA analysis

mAb	Time [min]	%B	Curve
Infliximab	0	30	
	10	55	5
Bevacizumab	0	35	
	10	60	5
Cetuximab	0	30	
	0.5	40	5
	3.5	42	7
	7	55	5
	10	100	5
Adalimumab	0	40	
	10	100	5
Trastuzumab	0	40	
	10	100	5
Rituximab	0	85	
	10	100	5
NIST mAb	0	90	
	10	100	5

Re-equilibration starts with a 100% buffer A flush for 2 minutes followed by equilibration to the starting conditions for a further 8 minutes.

MS conditions

Table 2. Summary of tune and method parameters

Tune Parameters	Spray voltage	3.6 kV
	Capillary temperature	275 °C
	Sheath gas	20 arbitrary units
	Aux gas	5 arbitrary units
	Probe heater temperature	275 °C
	S-Lens RF level	200
	HMR mode	On
	Trapping gas pressure setting	1
Method Parameters	Polarity	Positive
	In-source CID	150 eV
	Microscans	10
	Resolution (@ *m/z* 200)	17,500; 35,000; 70,000
	AGC target	3e6
	Maximum IT	200 ms
	Scan range	2500–8000 *m/z*
	Spectrum data type	Profile

Deconvoluted spectra from the mAb samples were obtained using BioPharma Finder 3.0 software for native intact mass analysis.

Table 3. Biopharma Finder 3.0 software parameter settings for MS data processing

Deconvolution of Manually Selected Area	Parameter	Intact mAb	Fragments
Chromatogram Parameters	*m/z* Range	5000–7000	2500–5000
	Deconvolution Algorithm	ReSpect™	ReSpect
	Model Mass Range	145,000–152,000 Da	40,000–60,000 Da (LMWF) 90,000–110,000 Da (HMWF)
	Mass Tolerance	10 ppm	10 ppm
Deconvolution Algorithm	Charge State Range	20–30	10–20 (LMWF 20–30 (HMWF)
	Minimum Adjacent Charges	3 - 3	3 - 3
Sliding window deconvolution			
Chromatogram parameters	*m/z* range	5000–7000	
Deconvolution algorithm	Deconvolution algorithm	ReSpect	
	Model mass range	145,000 to 152,000 Da	
	Mass tolerance	10 ppm	
	Charge state range	20 to 30	
	Minimum adjacent charges	3 to 3	
Sliding window merging parameters	RT range	3–12 min	
	Target avg. spectrum width	0.3 min	
	Target avg. spectrum offset	28%	
	Merge tolerance	10 ppm	
	Max RT gap	0.166 min	
	Min. number of detected intervals	3	

Results and discussion

The biopharmaceutical industry is committed to a development path that is producing increasing numbers of protein-based biotherapeutics. The importance of quick and reliable analytical methods to characterize monoclonal antibody variants is important to evaluate lot-to-lot consistency, investigate potential problems present along the production pipeline, and biosimilar product characterization to establish comparability with their innovator. As PTMs can be induced during sample preparation, the implementation of methods requiring no sample preparation as described here are highly desirable.

In this study seven biopharmaceuticals (infliximab, cetuximab, adalimumab, rituximab, trastuzumab, bevacizumab, and the NIST mAb standard) with a wide range of isoelectric points [pI] have been tested to show the global applicability of the CVA-MS method. The pI of proteins quoted in the literature often vary depending on the method used to calculate this value. Because of the charge variants present the real value will cover a range rather than a specific value. In the experimental conditions used here the protein is still fully folded in the native state. This means that the only charged sites available to the resin will be on the outside surface of the protein and so the pH of elution reflects the surface

charge available and not the pI calculated from the unfolded or partially unfolded protein.

The principle of CVA through applying a pH-gradient relies on a change in buffer pH over time. As soon as the pH exceeds the value equating to a variant's isoelectric point, the protein is no longer retained and elutes from the column. The use of pH-gradients with relatively weak volatile buffers creates a problem with on-column buffering effects where the column itself buffers against the pH change. The column chosen has a low buffering capacity due to its solid particle format and short length. The strong cation exchange groups also have less buffering effect than weak cation exchange functionality. Even so, buffer B at pH 10.9 has a monitored on-column pH of 10.2. Re-equilibration time from the final high pH value back to the starting pH value is an important aspect for method reproducibility. For this reason, re-equilibration is most efficient with a 100% buffer A flush for 2 minutes, as this is a stronger buffer, then equilibration to the starting conditions of the next run for a further 8 minutes. This was found to be consistent for either gradient used and confirmed with on-line pH-monitoring. pH-gradient protein elution is better described as an isoelectric focusing technique where the proteins are bound to the column, then elution and refocusing is achieved as the pH in the gradient reaches the native pI of the protein. At this point the protein is neutral in charge and no longer binds to the column, eluting in a sharp band at the isoelectric point. This is the reason why column length has little effect on the resolution. The buffers used in this study have a very low salt concentration, so the mechanism of elution is predominantly isoelectric focusing with ion exchange interactions much lower. A stable method that is easily optimized for proteins of different pI values requires the use of a combination of buffers, which, when used together, cover the described range of pH.
A potential problem with volatile MS compatible buffers is the lack of a suitable buffering capacity between pH 7 and 8. Figure 1 shows potential buffers with their buffering capacity zones. This buffering gap results in an uncontrolled, on-column pH jump at these pH values for any linear gradient programmed through these pH values. This constitutes a ballistic pH-gradient between pH 7 and 8 that elutes all proteins and their variants with a pI value in this region at the same retention time (data not shown). To remedy this, gradients required over these pH values have a shallow curved gradient at this point to produce a near linear on-column pH-gradient. This is required for the cetuximab gradient (Table 1, Figure 2). For initial gradient optimization an on-line pH and conductivity monitor (UltiMate 3000 PCM-3000) was used to determine the real on-column pH produced by the gradients programmed into the UHPLC pump.

The optimal resolution setting for the Orbitrap mass spectrometer is a balance between sensitivity and the resolution of species close in *m/z* and very much depends on the composition and complexity of the sample. The variant separation provided by ion exchange simplifies the sample entering the mass spectrometer at a time and can allow higher resolution settings to be used as variants close in mass might be chromatographically resolved. However, even with a charge variant separation, several isoforms can sometimes still be found to co-elute. It must also be remembered that an increase in resolution comes hand in hand with reduced sensitivity, making lower resolution favorable for some low abundant species. We found the resolution setting of 35,000 (at *m/z* 200) to be best suited in the current study to obtain highly accurate masses of even relatively low abundant species (Figure 4). The occurrence of adduction, which is frequently reported in native mass spectrometry, was avoided by the application of a relatively high setting for in-source CID, set to 150 eV in this case.

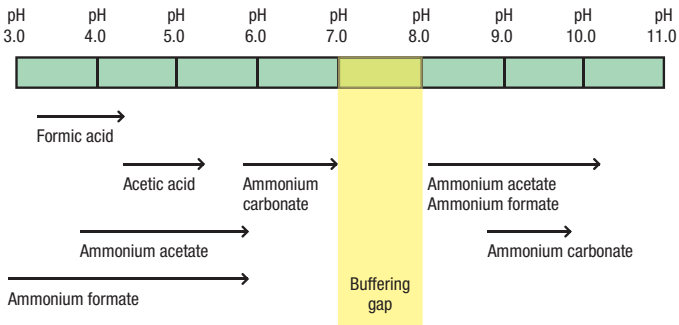


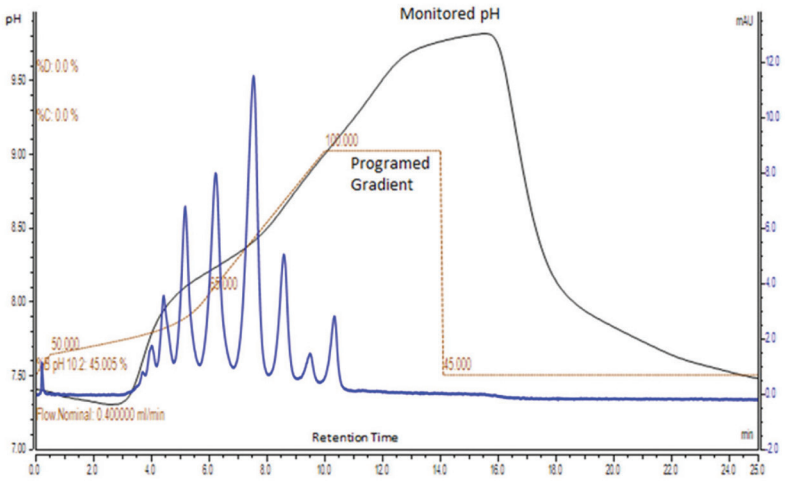
Figure 1. Volatile buffer pH ranges

Figure 2A shows an optimized separation for cetuximab with on-line pH monitoring. This separation is designed to show the effect of a shallow curved gradient over the pH range between 7 and 8. This is the area where it is difficult to keep control of the pH. Eight peaks can be observed in the optimized gradient, all corresponding to charge variants of cetuximab. The shallow curve used in the gradient program successfully prevents a jump in pH to produce a more linear on column pH profile, which can successfully separate the cetuximab charge variants. Without the shallow gradient in this region the pH is uncontrolled and jumps rapidly from pH 7 to 8, co-eluting all the variants in this region (data not shown).

For mAbs with a pI above pH 8, simpler linear gradients can be used as the pH control of this buffer system is quite good in this range and the on-column pH mimics the programmed gradient quite well. As can be seen from Figure 2B, the trastuzumab separation involves binding at pH 8, then a steep initial linear gradient with a shallow optimized gradient for separation.

To demonstrate the global applicability of this workflow, seven different mAbs with varying pI values were tested. Figure 3 shows the gradient optimization of these samples. A linear gradient from 0 to 100% B was used in a scouting method first. Optimization of the gradient for each mAb within a narrower pH range using the same gradient time was then accomplished within two or three following injections. This demonstrates easy and fast method optimization.

A



B

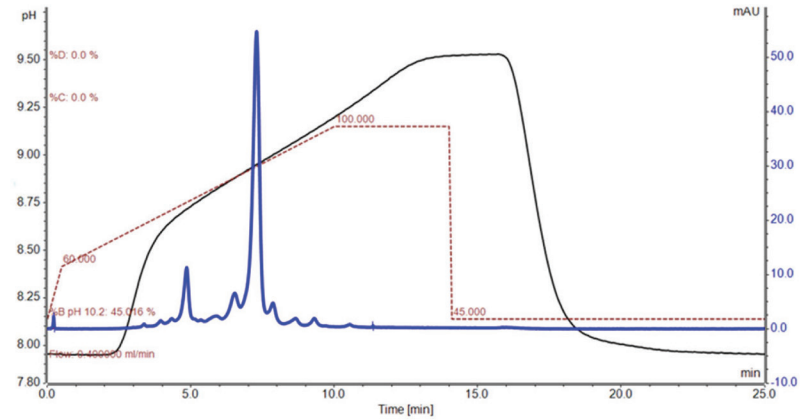


Figure 2. The relationship between the programmed gradient and the actual real time pH-gradient produced on-column with cetuximab analysis (A) and trastuzumab (B). The dotted line represents the programmed gradients used in these example separations. The solid black line represents the actual monitored pH that is produced on the column. The acheived separation produced is reflected by the blue UV-chromatogram detected at 280 nm absorbance.

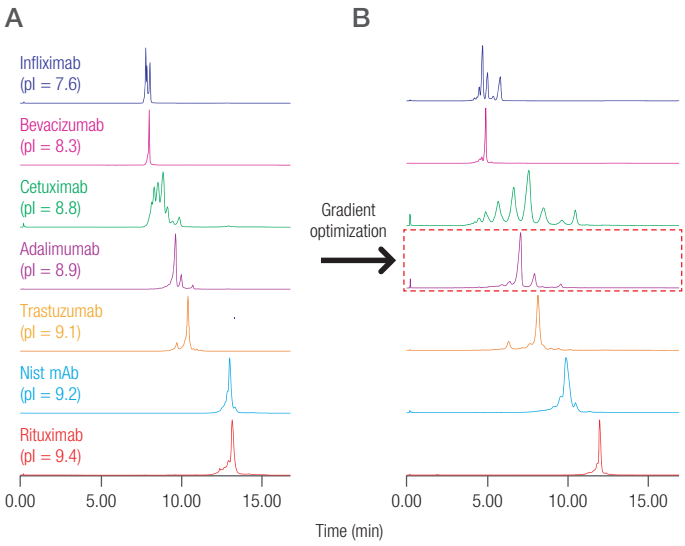


Figure 3. Gradient optimization for CVA-MS analysis of seven different mAbs. (A) Initial gradient from 0 to 100% B in 10 minutes, (B) optimized gradients according to conditions given in Table 1.

Optimization of the MS settings is equally important. The extended mass range accessible with the BioPharma option is required for mAb analysis under native conditions, detected in lower charge states at higher *m/z* values. The resulting increased spatial resolution between charge states can be advantageous for the analysis of highly complex molecules. Two adjacent charge states may overlap in the charge envelope of a denatured spectrum, complicating their analysis. These are usually resolved when acquired under native conditions. A disadvantage of using RP or SEC for MS analysis is that there is no separation of charge variants before MS analysis. As such all the variants will enter the MS at the same time, complicating the analysis and also bearing the risk of not detecting low abundant species. The deconvolution result can therefore be compromised with significantly less species found with reasonable mass accuracy. In contrast, charge variant separation prior to mass detection allows for more sensitive and accurate MS data acquisition. Even low abundant species can provide MS spectra suitable for deconvolution and annotation. Figure 4 shows adalimumab acquired at resolution settings of 17,500, 35,000, and 70,000 (at *m/z* 200). The charge envelope of the chromatographic main peak is displayed in more detail (Figure 4B). An increase in resolution causes a decrease in total signal intensity. Variants close in *m/z* that might co-elute by chance are however more likely to be distinguished which is shown in Figure 4C. Whereas the zoom into the most abundant charge state shows an overlap of two species (5702.89 and 5705.86) at a resolution setting of 17,500.

Higher resolution settings can resolve these variants also resulting in a significant change of the *m/z* value displayed. This in consequence can enable identification of these variants with reasonably high mass accuracy and confidence. Lower abundant species might nevertheless benefit from a lower resolution setting as it can prevent the species from being under the limit of detection.

Glycosylation is important for biological activity and hence needs to be constantly monitored. Using the CVA-MS method, all major glycoforms of all main charge variants can be potently analyzed both qualitatively and quantitatively. The only glycosylations that would cause retention time shifts in CEX themselves are represented by forms containing sialic acids. These would cause a decrease of the protein pI, which in consequence would result in a relatively early elution as for example in the case of cetuximab (data not shown). Another example for an acidic modification would be deamidation, which results in a shift to lower retention time due to the loss of a positive charge.[10] Deamidated and non-deamidated species can be separated chromatographically, which is the only way of obtaining a difference in mass of only 1 Da corresponding to a deamidation event. Figure 6 showcases the separation of lysine truncation variants of adalimumab, each of which are differently glycosylated.

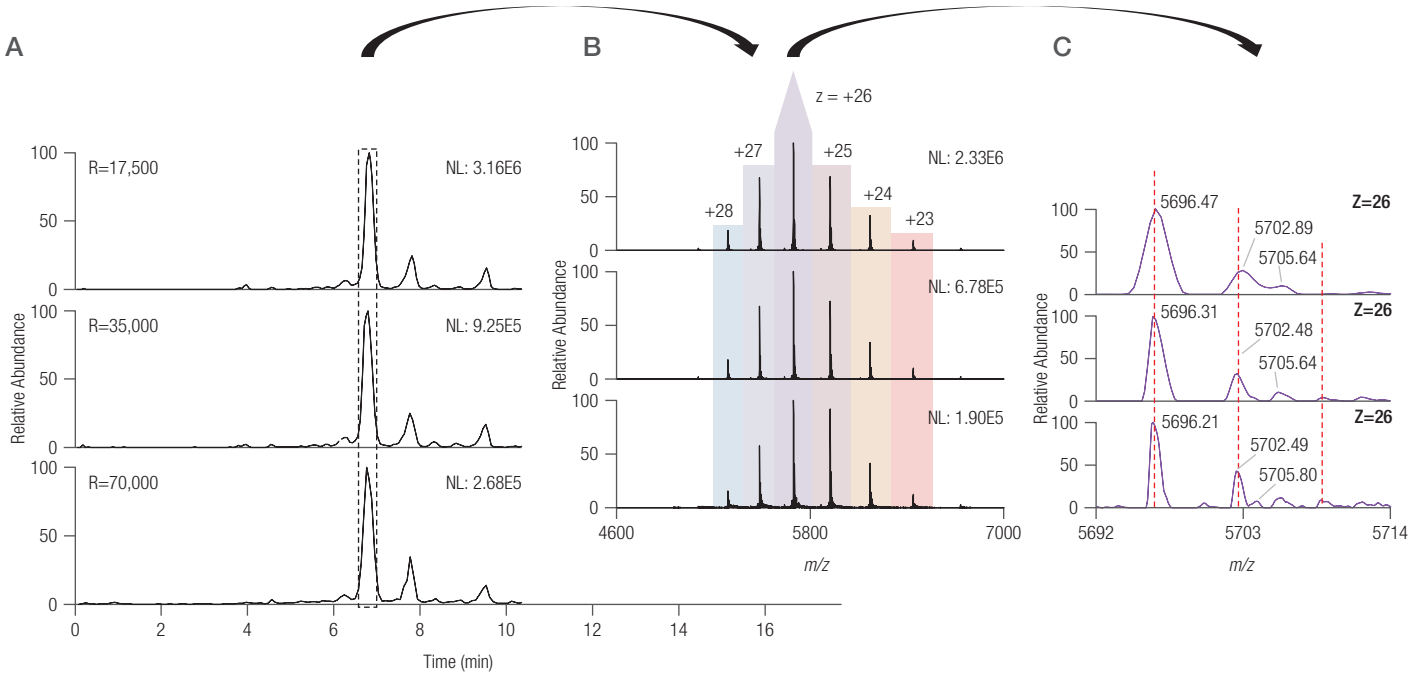
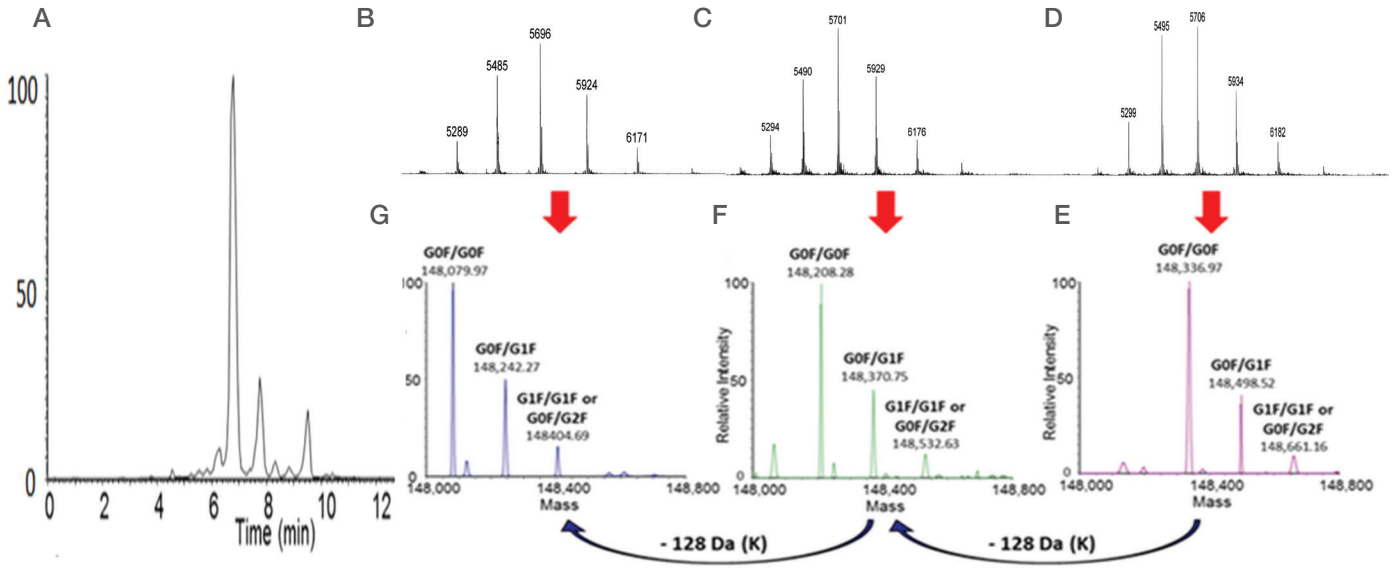


Figure 4. Benefits of higher resolution for the CVA-MS analysis of adalimumab. (A) Base peak chromatogram (BPC) for 100 µg of adalimumab at resolution settings 17,500, 35,000, and 70,000. (B) Charge envelope of the main variant peak. MS signal intensities for BPC and the charge state envelopes are included in the top right of each panel. (C) Magnification of charge state +26 showing the glycoform distribution and a co-eluting species.



H

Lysine Variant	Glycoform	Theoretical Mass (Da)	Experimental Mass (Da)	Δ Mass (ppm)
no K	G0F/G0F	148,080.55	148,079.97	3.9
no K	G0F/G1F	148242.69	148,242.27	2.8
no K	G1F/G1F or G0F/G2F	148,404.83	148,404.69	1.0
1 K	G0F/G0F	148,208.72	148,208.28	3.0
1 K	G0F/G1F	148,370.86	148,370.75	0.7
1 K	G1F/G1F or G0F/G2F	148,533.00	148,532.63	2.5
2 K	G0F/G0F	148,336.89	148,336.97	0.5
2 K	G0F/G1F	148,499.03	148,498.52	3.5
2 K	G1F/G1F or G0F/G2F	148,661.17	148,661.16	0.1

I

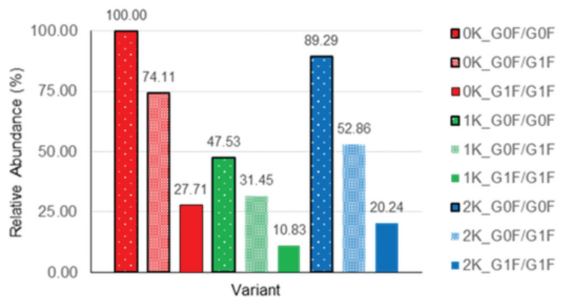


Figure 5. CVA-MS analysis of 100 µg of adalimumab performed with a resolution setting of 35,000. (A) Base peak chromatogram of adalimumab. (B, C, and D) Charge envelopes of species represented in peaks 1, 2, and 3, respectively. (E, F, and G) Glycoform and lysine variant assignment based on the deconvoluted spectrum from spectra represented in each individual chromatographic peak. Theoretical and experimental average molecular masses obtained for the major glycoforms of adalimumab lysine truncation variants are shown (H) with their relative quantitation (I).

The number and relative abundance of different glycans can vary considerably from mAb to mAb. During intact mass analysis of mAbs, annotation of glycoforms can be compromised by co-elution of several closely related proteoforms. The high peak purity following CVA-MS separation of the variants attained here allows high accuracy mass measurements of each peak separately. This enables superior annotation and relative quantitation of glycoforms for each charge variant peak. The superiority of CVA-MS over CVA with conventional detection can be demonstrated with co-eluting variants, which can be distinguished from each other via difference in their intact mass as an additional dimension of measurement.

Figure 6 shows the identification of several attributes achieved in a single injection. Panel A highlights lysine truncation variants of adalimumab, identified in the same manner as the adalimumab variants in Figure 5. In addition, many other variants such as fragments, deamidated variants, and succinimide Asp containing variants, could be identified in the same single LC-MS run.

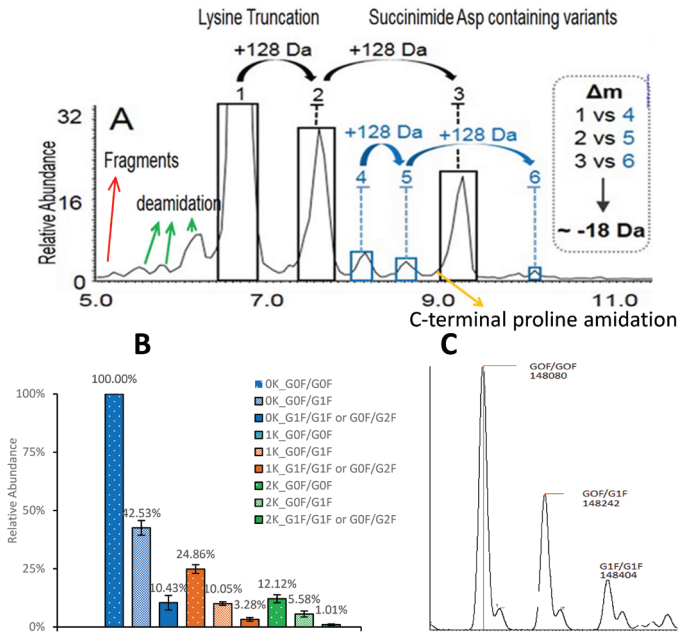


Figure 6. (A) BPC of 100 µg adalimumab sample acquired with resolution setting of 35,000. The peaks labelled from 1 to 3 in black are the main lysine variants. Peaks labelled in blue from 4 to 6 are the succinimide Asp containing versions of these lysine truncated forms. Co-eluting substances present that do not relate to lysine truncation are indicated with a yellow arrow. The green arrows are pointing out differently deamidated forms and the position of fragments is indicated with a red arrow. Panel B shows the glycan ratio for each main lysine variant and panel C the deconvoluted annotated spectrum of the main peak.

Lysine truncation variants (indicated by the black curved arrows in Figure 6A) that also contain a succinimide modification of an aspartic acid residue (blue curved arrow in Figure 6A) can be identified by a shift to a lower retention time and an additional mass shift of 18 Da from the unmodified lysine variants shown in black. The co-eluting species labeled with the yellow arrow could not be unambiguously annotated and was found to be either due to a double C-terminal proline amidation or the occurrence of an N-terminal Asp loss. A digestion with CpB could be done to investigate possible co-eluting peaks under the lysine truncation variants,[10] however this might not be required when using MS detection. The peaks labeled with green arrows are deamidated forms with the first eluting peak carrying two deamidation events and the following two peaks representing singly deamidated species. The identification of deamidation variants which differ by only 1 Da requires high chromatographic resolution and preferably also a higher MS resolution setting to be considered confident. The identification of the site of modification would require fraction collection of these peaks and peptide mapping; however, when this has been performed once the peak can be identified using CVA-MS. Additional early eluting peaks from adalimumab CVA-MS analysis have been confirmed as fragments produced from cleavage at the hinge region. Figure 6B shows the glycan ratios from each of the main lysine truncation variant peaks. All of these show an identical distribution of glycans at high mass accuracy, supporting the confidence in the results. Figure 6C represents the annotated deconvoluted spectrum from the main peak.

Summary

Here, we have presented a method for charge variant analysis of mAbs with on-line MS detection which is markedly superior to anything that has previously been reported in terms of universal applicability and low adduction. Using this method, a comprehensive analysis of a monoclonal antibody can be achieved in a single injection with no sample preparation required. Modifications identified using this method are relative glycoform abundance, various levels of lysine truncation, glycation (not shown, Reference 10) and succinimide formation of the different lysine truncated forms, deamidation, as well as double deamidation, N-terminal Asp loss or C-terminal proline amidation, and fragmentation. The accuracy of the deconvoluted masses using this method are superior to results obtained from reversed-phase methods under denaturing conditions

due to the chromatographic separation of the closely isobaric variants. CVA-MS is therefore a technique that allows the monitoring of multiple attributes at the protein level. The fast analysis without sample preparation will reduce the possibility of any post-translational modifications occurring due to the analytical procedure. This method should be useful for many applications where speed and a more comprehensive characterization may be required.

Conclusions

- Ion exchange chromatography has been successfully interfaced directly to MS for charge variant analysis and direct isoform identification.
- The method requires an optimized volatile buffer cocktail with a carefully selected low buffering capacity, high-resolution ion exchange column.
- The system has global applicability that is demonstrated with several mAb samples, using gradients optimized to the pI of the respective protein.
- Multiple attributes can be determined with a single direct injection of sample including intact mass, charge variant pattern, glycan distribution, lysine truncation, deamidation, succinimide-Asp formation and fragment analysis.
- From a direct injection of drug product without any sample preparation, multiple attributes have been determined that would otherwise require several chromatography methods with different sample preparation protocols. This results in a considerable saving on resources and time.
- BioPharma Finder 3.0 software allowed quick deconvolution of the spectra and confident identification of the mAbs and their variants, providing accurate qualitative and quantitative information.

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