Boosting mass spectrometry-based analytics for biopharma

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Chapter 3

Fast analysis of antibody-derived therapeutics by automated multidimensional liquid chromatography – mass spectrometry

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3.1 Abstract

Characterization of post-translational modifications (PTMs) of therapeutic antibodies is commonly performed by bottom-up approaches, involving sample preparation and peptide analysis by liquid chromatography-mass spectrometry (LC-MS). Conventional sample preparation requires extensive hands-on time and can increase the risk of inducing artificial modifications as many off-line steps - denaturation, disulfide-reduction, alkylation and tryptic digestion - are performed. In this study, we developed an on-line multidimensional (mD)-LC-MS bottom-up approach for fast sample preparation and analysis of (formulated) monoclonal antibodies and antibody-derived therapeutics. This approach allows on-column reduction, tryptic digestion and subsequent peptide analysis by RP-MS. Optimization of the 1D -and 2D flow and temperature improved the trapping of small polar peptides during on-line peptide mapping analysis. These adaptations increased the sequence coverage (95-98% versus 86-94% for off-line approaches) and allowed identification of various PTMs (*i.e*. deamidation of asparagine, methionine oxidation and lysine glycation) within a single analysis. This workflow enables a fast (<2 hours) characterization of antibody heterogeneities within a single run and a low amount of protein (10 µg). Importantly, the new mD-LC-MS bottom-up method was able to detect the polar, fast-eluting peptides: Fc oxidation at Hc-Met-252 and the Fc N-glycosylation at Hc-Asn-297, which can be challenging using mD-LC-MS. Moreover, the method showed good comparability across the different measurements (RSD of retention time in the range of 0.2-1.8% for polar peptides). The LC system was controlled by a standard commercial software package which makes implementation for fast characterization of quality attributes relatively easy.

3.2 Introduction

Therapeutic monoclonal antibodies (mAbs) and new antibody formats are highly complex bio-molecules potentially affected by chemical and enzymatic modifications, also known as post-translational modifications (PTMs) [1, 2]. PTMs - including lysine glycation, deamidation of asparagine, aspartic acid isomerization and methionine oxidation - can occur during manufacturing or storage [3, 4]. As these modifications can affect the pharmacological properties (*i.e.* stability, safety and potency) of the final drug product, their assessment is mandatory to ensure the product quality, efficacy and consistency [5-7]. Structural characterization of mAbs at an amino acid level is commonly performed by bottom-up approaches, involving sample preparation followed by peptide analysis by liquid chromatography–mass spectrometry (LC-MS) [8, 9]. Sample preparation for conventional bottom-up approaches includes many off-line steps such as denaturation, disulfide bridge reduction, alkylation and enzymatic digestion. As a consequence, characterization of mAbs requires extensive hands-on time and can increase the risk of inducing artificial modifications [6, 9]. Furthermore, off-line digestion comes with a few drawbacks, such as long incubation time (several hours), low efficiency and trypsin autodigestion [5]. Hence, there is a high demand for automation of bottom-up approaches which can be used for routine characterization of quality attributes. Bauer et al. [6] proposed an automated sample preparation procedure for peptide mapping using a liquid handling robot, allowing denaturation, disulfide bridge reduction, alkylation and tryptic digestion in four hours. On the downside, these automated robotic platforms are only beneficial when analyzing a large number of samples. Multidimensional LC-MS bridges the automated sample preparation process and the subsequent LC-MS analysis within one set-up, having the opportunity to analyze various samples step-by-step. This technique has been widely adopted for mAb characterization due to the introduction of commercially available 2D-LC systems and immobilized enzyme reactors (IMERs) [7]. For instance, immobilized trypsin reactors have proven to be more efficient than off-line digestion as digestion can be achieved in minutes [5, 10, 11]. In recent years, several studies implemented on-column disulfide bridge reduction and immobilized trypsin reactors between two separation techniques, allowing a full automated bottom-up approach for a peak characterization [5, 7, 8, 11-13]. Gstöttner et al. [5] developed a 4D-LC set-up for the HPLC-peak characterization of mAb charge variants using a commercial 2D-LC system of Agilent which was extended with additional modules. Online fractions of the cation exchange (CEX) separation were taken, followed by reduction, tryptic digestion and peptide analysis by RP-MS. Similarly, Camperi et al. [12] characterized protein modifications of mAbs from cell culture supernatants using protein affinity chromatography (ProA) in the first dimension. Compared to conventional off-line bottom-up approaches, these new automated platforms enable faster identification and characterization of protein modifications (hours versus days) [5, 12, 14]. In addition, upon comparing the off-line and on-line approach, Goyon et al. [11] reported less artificial modifications within the online

approach. Altogether, these automated workflows rely on pre-separation of the antibody in the first dimension, followed by online fraction collection of the separated antibody variants. Hereafter, each collected fraction undergoes on-column reduction, tryptic digestion and RP-MS analysis. These online approaches are very powerful to selectively fractionate unknown peaks from quality control (QC) methods for further bottom-up characterization. However, one of the remaining challenges in on-line bottom-up approaches using mD-LC systems is trapping of small polar peptides in the RP-C18 column. This is a consequence of the high ACN content necessary to elute the reduced chains from the reduction-trap column [5]. Furthermore, many mD-LC platforms are based on non-commercial 2D-LC systems without integrated software. This requires a certain amount of expertise and experience making it very complex to implement mD-LC for routine characterization of quality attributes [15].

In the present study, we developed an on-line bottom-up strategy using a mD-LC-MS set-up for fast sample preparation and analysis of mAbs (reduction – tryptic digestion – RP-MS). The proposed mD-LC-MS approach relies on the direct injection of (formulated) mAb samples without any pre-separation and/or fractionation of antibody variants. The mD-LC set-up is based on a commercial Agilent 2D-LC system with easy-to-use hardware and software. The use of an immobilized trypsin column reduced the digestion time significantly and enabled the digestion in minutes instead of hours and without manual sample preparation time. Furthermore, various LC parameters such as 1D and 2D flow rate and column temperature were optimized providing better trapping of polar peptides resulting in higher sequence coverages.

3.3 Experimental section

3.3.1 Reagents and samples

Tris(hydroxymethyl) amino methane (TRIS) base (≥99.8%), DL-Dithiothreitol (DTT) (≥99%), hydrogen chloride (ACR reagent grade 37%), D-(+)-Glucose (≥99.5%), L-Histidine (≥99.5%), sucrose (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (Ultra LC-MS grade) was provided by Actu-All Chemicals (Randmeer, Oss, The Netherlands). Formic acid (≥99%) was purchased from VWR Chemicals (Radnor, PA). Calcium Chloride Dihydrate was obtained from J.T. Baker (99%) (Deventer, The Netherlands). Hydrogen peroxide (30%) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Tween-20 was provided by Roche Diagnostics (Mannheim, Germany). Deionized water was obtained from a Purelab ultra (ELGA Labwater, Ede, The Netherlands). Formulated, conventional and bispecific, mAbs were provided by F. Hoffmann-La Roche (Basel, Switzerland) and Roche Diagnostics (Penzberg, Germany).

3.3.2 Sample preparation

Formulated mAb1 (119 mg/mL in 5 mM L-histidine, 60 mM α,α-trehalose, 0.01%. polysorbate 20, pH 6.0) and BsAb1 (120 mg/mL in 20 mM His-acetate, 25 mM NaCl, 160 mM Sucrose, 7 mM L-methionine, 0.04% polysorbate 20, pH 5,5 ± 0,2) were simply diluted to 1 mg/mL before mD-LC-MS experiments with mobile phase A of the first dimension. mAb2 and BsAb2 (25.2 mg/mL) were buffer exchanged in a formulation buffer containing 240 mM sucrose, 0.02% polysorbate, 10 mM L-methionine in 20 mM His-HCl, pH 5.5. Prior mD-LC-MS experiments, the samples were diluted to 1 mg/mL with mobile phase A of the first dimension. Additionally, mAb2 was stressed under various stress conditions. Oxidative stress was performed by mixing mAb2 with hydrogen peroxide 0.2% (H_2O_2) (v/v). The mixture was incubated at room temperature for 24 hours. Forced deamidation was achieved by incubating mAb2 with a final concentration of 10 mg/mL with 200 mM Tris-HCl, pH 9 at 37°C for 3 and 7 days. Glycation was induced by incubating mAb2 with a final concentration of 12.5 mg/mL with 1M glucose at room temperature for 7 days. All stressed samples were kept at -20°C until analysis and diluted to 1 mg/mL with mobile phase A of the first dimension before mD-LC-MS experiments.

3.3.3 Instrumentation

mD-LC system Instrumentation

The system was completely configured using standard modules from the Agilent 1290 Infinity II 2D-LC system (Agilent Technologies, Waldbronn, Germany). The system consisted of the following modules: 1260 Bio Multisampler (G5668A), 1290 High Speed Binary Pump (G7120A), two 1260 Bio-Inert Quaternary Pump (G5654A), 1260 Multicolumn Thermostat (G7116A) configured with a 6 port/2 position valve (G1170A), two 1290 Multicolumn Thermostats (G7116B) configured with a 6 port/2 position valve (G5631) and a 6port/2position external valve (G1170A). The system was controlled by one OpenLab software package. An additional self-designed macro ''valve event plugin'' from ANGI (Gesellschaft für angewandte Informatik, Karlsruhe, Germany) was used to generate a start signal for the MS system. The mD-LC was directly connected to a QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an electrospray ionization (ESI) ion source. A detailed overview of the mD-LC set-up (gradients, flows and valve positions) can be found in **Table S1**.

First dimension: Trapping and Reduction

An AdvancedBio RP cartridge (2.1 I.D. x 12.5 mm) (Agilent Technologies) was used for subsequent trapping and reduction of 10 µg therapeutic antibody samples. Mobile phases were composed of water (A) and ACN (B) containing both 0.1% FA and 0.05% TFA. Mobile phase C was composed of 20 mM DTT in 50 mM Tris, pH 7.5. The column temperature was

set to 60°C. The antibody is first trapped onto the RP cartridge using 1% B and subsequently reduced using mobile phase C, resulting in heavy chains (Hc) and light chains (Lc). After reduction, the column was flushed with 1% B to remove any remaining DTT followed by the elution of mAb chains at 60% B at a flow rate of 45 µL/min. Next, a cleaning and reconditioning step was performed using 100% B for 5 minutes followed by 1% B for 5 minutes.

Second dimension: Tryptic digestion

During the elution step the valves located after the first and second dimension were set in-line allowing the transfer of reduced mAb chains from the AdvancedBio RP cartridge to a StyroZyme TPCK-Trypsin column (2.1 I.D. x 100 mm) (OraChrom, Massachusetts, USA). The column temperature was set to 37°C. Mobile phase A consisted of 50 mM Tris and 10 mM $CaCl_2$, pH 8 and mobile phase B of ACN. Mobile phase A was continuously introduced via a T-junction. The trypsin column was set in-line with 1D-RP and 3D-RP for 27 minutes using mobile phase A at a flow rate of 450 µL/min followed by a cleaning and reconditioning step. The column was flushed with 50% B at 1 mL/min for 5 minutes followed by 100% A at 1 mL/min for 5 minutes, this procedure was performed two times.

Third dimension: Peptide mapping

Tryptic peptide mixtures were separated on a RP XSelect CSH C18 column (Waters CSH RP-C18, particle size 3.5 µm, 2.1 mm I.D. x 100 mm) (Waters, Mildford, MA). The mobile phases were composed of water (A) and ACN (B) containing both 0.1% FA. The trapped tryptic peptides were first desalted using 1% B for 5 minutes. After desalting, the RP-C18 column was set in-line with the MS and the peptides were subsequently separated using a gradient from 1% to 35% B in 45 minutes and switching to 80% B for 6 minutes. The flow rate was set at 300 µL/min and the column temperature was set to 55°C.

Mass spectrometry

HyStar software (Bruker Daltonics) was used for the MS data acquisition. The acquisition of peptides was performed in positive ion mode in a *m/z* range of 150-2500. The in-source collision energy was 7 eV. The source parameters were set as follows: 500 V end plate offset, 4500 V capillary voltage, 5.5 L/min dry gas flow rate, 180°C dry gas temperature and 2.2 bar nebulizer gas pressure. The MS/MS spectra were generated by using fragmentation mode collision-induced dissociation (CID). The isCID energy and collision energy were set to 5 eV and 70 eV. MS data processing (*i.e.* assignment and peak integration) was manually performed using DataAnalysis (Bruker Daltonics) and Skyline 20.2 software (MacCoss Lab, U Washington).

3.4 Results and discussion

3.4.1 mD-LC-MS method development

In the present study, we developed an automated on-line bottom-up strategy using a mD-LC-MS system as shown in **Figure 1**. This on-line bottom-up strategy enables on-column reduction, tryptic digestion and peptide analysis by RP-MS.

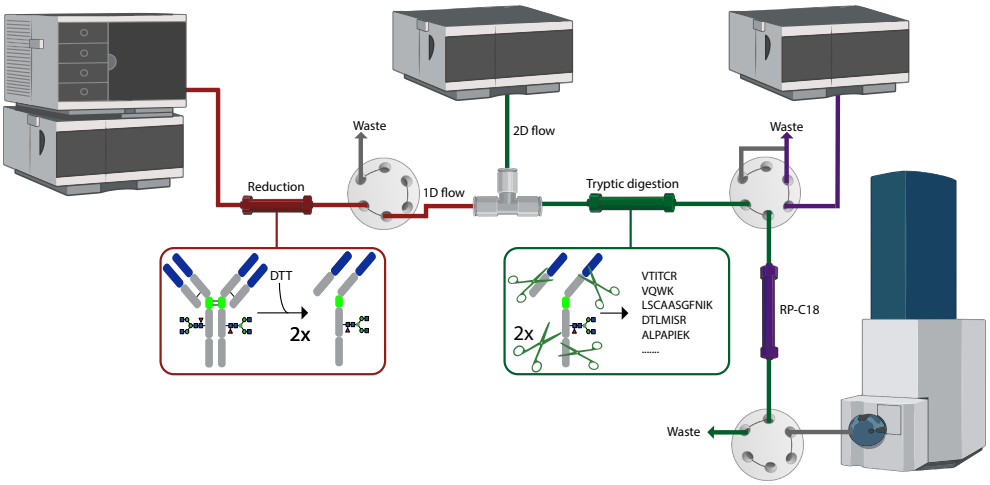

Figure 1. Schematic representation of the automated on-line bottom-up approach for mAbs using mD-LC-MS which enables on-line on-column reduction by RP-HPLC (1D), tryptic digestion (2D) and peptide mapping by RP-MS (3D).

One major challenge in bottom-up approaches using on-column reduction is the trapping of polar peptides on the RP-C18 column, since an increased concentration of acetonitrile (~60% ACN) is necessary for the elution of mAb chains from the reduction-trap column. As shown in earlier studies the amount of ACN was already lowered to ~11.6% but further dilution was limited due to the relatively low backpressure limit of the trypsin reactor (<200 bar) and high backpressure of the RP-C18 column (Waters BEH RP-C18, particle size 1.7 µm, 50 x 2.1 mm) [5]. Consequently, many polar peptides were not trapped on the RP-C18 column and therefore not detected during peptide mapping resulting in low sequence coverages (Light chain, Lc: 94% and Heavy chain, Hc: 86%). In this study, various parameters were optimized to improve trapping of small polar peptides. To address this challenge a column with optimized characteristics such as particle size, length and stationary phase was chosen. More precisely, the particle size was increased from 1.7 to 3.5 µm (Waters CSH RP-C18, particle size 3.5 µm, 2.1 mm I.D. x 100 mm) to get the possibility of reducing the amount of ACN even more while observing the pressure limit of the trypsin reactor. Besides, the column was longer (100mm) and had a CSH stationary phase to improve chromatographic

separation.

Furthermore, to improve chromatographic separation and trapping of polar peptides, the selected column was longer (50 mm versus 100 mm) and consisted of a different stationary phase (CSH versus BEH) which is more selective towards basic compounds [11]. This implementation increased the sequence coverage of mAb1 from 90% to 95% for the Hc, whereas the sequence coverage for the Lc remained at 96% (**Figure 2** and **Table S2**). The increased sequence coverage can be attributed to the improved trapping of polar peptides, including ''IYPTNGYTR'', ''ALPAPIEK'', as the applied column has a higher selectivity for basic compounds compared to the BEH RP-C18 column [11].

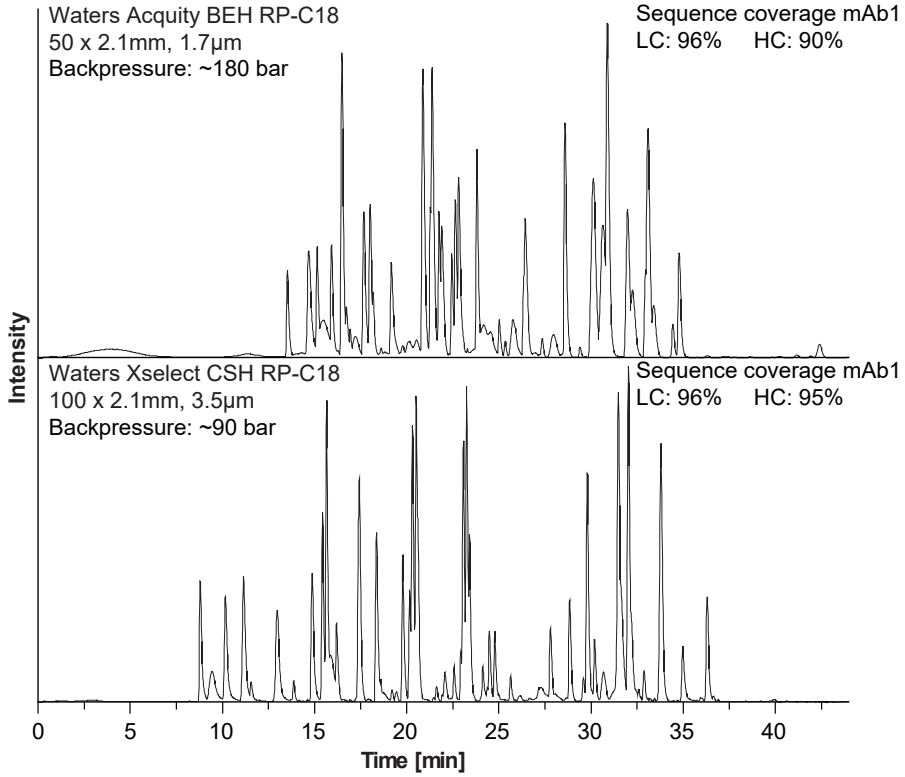

Figure 2. Comparison of two different RP-C18 columns in the mD-LC-MS set-up using mAb1, prior flow rate and temperature optimization. The Waters Acquity BEH RP-C18 column (particle size 1.7 µm, 50 x 2.1 mm) (Top) and the Waters XSelect CSH RP-C18 column (particle size 3.5 µm, 100 x 2.1 mm) (Bottom). A detailed overview of the identified peptides for both columns is shown in **Table S2.**

Moreover, the increased particle size of the CSH RP-C18 column led to a decreased backpressure (from ~180 bar to ~90 bar) when the column was set in-line with the first and second dimension. Lowering the backpressure of the RP-C18 column made the optimization of the ACN content possible. The dilution was optimized by varying the 1D- and 2D flow rates.

A decrease of the 1D flow rate from 60 µL/min to 50 or 45 µL/min resulted in a decrease of ACN from 11.6% to 10% or 9.2% (**Figure 3**). Dilution of the ACN content from 11.6% to 9.2% resulted in trapping of several polar peptides including the Fc oxidation at Hc-Met-252 (according to the Kabat numbering [16]). This oxidized peptide is one of the most polar peptides eluting early in RP-C18 and is particularly important because it decreases binding to the neonatal receptor (FcRn) reducing the half-life of the antibody [1]. To further dilute the amount of ACN, the 2D flow rate was increased from 250 to 350 or 450 µL/min which led to increased trapping of polar peptides. A flow rate of 450 µL/min lowered the ACN content from 9.2% to 5.5% and led to a sequence coverage of 97% and 96% for the Hc and Lc. Hence, this allowed the identification of the glycopeptide ''TKPREEQY**N**STR'', which plays an important role for complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) [17].

Additionally, the temperature dependence of polar peptides trapping in RP-C18 was investigated by performing the trapping and separation on the RP-C18 at 85°C, 75°C, 65°C and 55°C **(Figure 3)**. As expected, an increase of the temperature led to faster elution rather than trapping of polar peptides, as the retention factor is inversely proportional to the temperature [18], whereas a low temperature improved trapping of polar peptides without affecting the overall separation. For instance, the glycopeptide ''TKPREEQY**N**STR'' was better retained at 55°C compared to higher temperatures. Furthermore, lower temperatures enhance the column lifetime and decrease the risk of generating artificial PTMs during trapping. For this reason the temperature was set to 55°C.

In summary, the use of a different RP-C18 column (Waters CSH RP-C18, particle size 3.5 µm, 2.1 mm I.D. x 100 mm) together with reduction of ACN content (from 11.6% to 5.5%, respectively) and optimized column temperature (from 65 to 55°C) improved trapping of polar peptides on the RP-C18 column. Subsequently, the sequence coverage for the Hc increased from 90% to 97%, whereas the Lc remained at 96%. Besides, the optimized method allowed detection of polar Fc peptides, which play a critical role in effector functions and recycling. Only some very small polar peptides, containing less than six amino acid residues, on the Lc (*i.e.* GEC and FSGR) and Hc (*i.e.* QAPGK, VDK, VEPK, and K) were not detected. Note that these small peptides are often also missed in conventional offline bottom-up workflows.

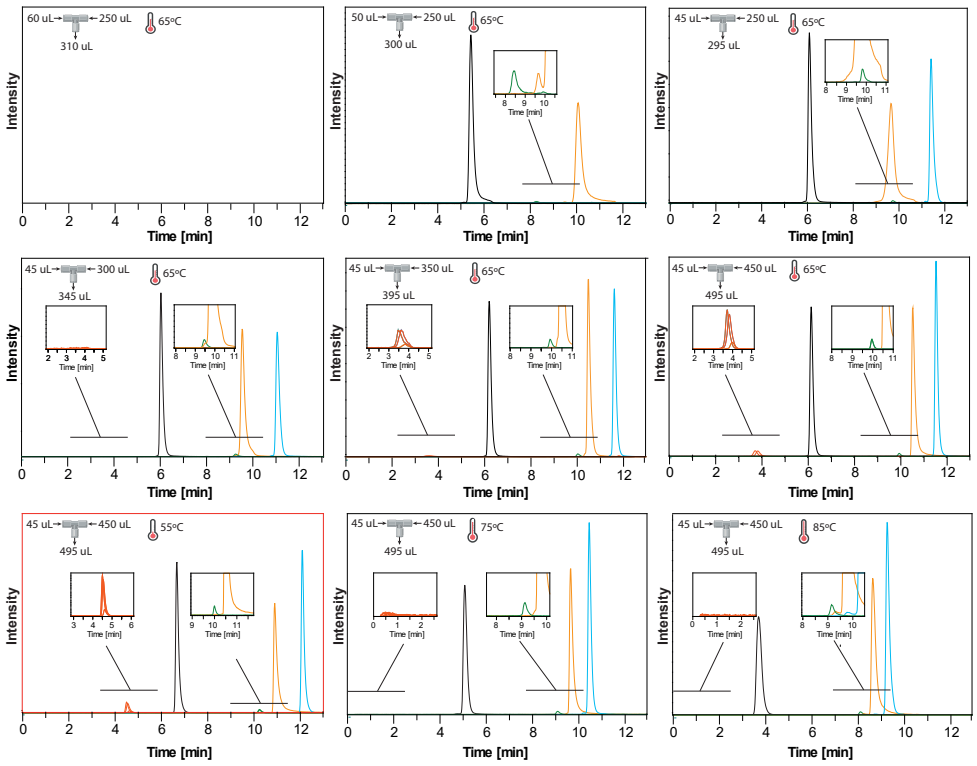

Figure 3. Optimization of the polar peptide trapping in the mD-LC-MS set-up using mAb1. The method was optimized by evaluating the 1D Flow (A) 2D Flow (B) and temperature in the 3D RP-C18 column (C). • TKPREEQY**N**STR$_{glycosylation}$ • DTL**M**ISR$_{oxidation}$ • VTITCR • AEDTAVYYCSR • VDNALQSGNSQESVTEQDSK. A detailed overview of all the optimization steps can be found in **Table S2.** Final conditions are highlighted in red.

3.4.2 Method evaluation for the direct analysis of formulated antibodies and new antibody-derived therapeutics

To demonstrate the performance of the mD-LC-MS set-up for the analysis of mAbs, both desalted and formulated mAb1 samples were analyzed. No differences in sequence coverage or separation (**Figure S1**) was observed between both samples indicating that formulation additives (*i.e.* polysorbate, saccharose) are not interfering with the analysis. Hence, injection of formulated mAbs can be performed directly without sample handling reducing total analysis time and the risk of inducing artificial modifications. To prove the generic applicability of the method for the analysis of diverse antibody formats, two conventional mAbs and two different bispecific mAb (BsAbs) were analyzed with the mD-LC-MS set-up. **Figure 4** shows the base peak chromatograms (BPCs) obtained for the four analyzed

antibodies. Comparable sequence coverages between 95-98% were obtained, highlighting the applicability of this approach for new antibody-derived therapeutics. Importantly, for all antibodies the Fc glycopeptides could be identified. The total analysis time, including on-column reduction, tryptic digestion and peptide analysis by RP-LC-MS was performed in less than 2 hours. Compared to conventional bottom-up strategies using manual sample preparation (offline approach), the total analysis time is considerably lower (<2 versus >12 hours), lower amount of protein is needed (10 µg versus 350 µg) and comparable sequence coverages are observed (≥95%) [8, 14, 19]. Furthermore, often mD-LC-MS bottom-up approaches are rather complex due to the use of non-commercial 2D-LC systems without integrated software [8, 11]. The proposed workflow is based on a commercial 2D-LC system and focuses on the analysis of the whole sample. Therefore, there is no need for online fraction collection of separated mAb variants which requires less modules (one pump and three valves less) and no plug-in. As a result, the mD-LC method consists of only three dimensions and is controlled by a single commercial software (OpenLAB CDS Software from Agilent) which makes implementation relatively easy and simplifies the work-around.

The method was further evaluated by assessing the repeatability (intra- and inter-day precision). To assess the intra-day precision, six replicates of formulated mAb1 were injected on one day using the same sample and mobile phases (**Figure S2**). The inter-day precision was assessed by the analysis of triplicates of formulated mAb1 on two different days using freshly prepared mobile phases and sample (**Figure S3**). For both intra- and inter-day precision, all measurements showed the same sequence coverage (Lc: 96% and Hc: 97%). The repeatability was further evaluated by the relative standard deviation (RSD) of the retention time of four polar peptides. Low intra- and inter-day variation was observed for the retention time (RSD 0.1-1.8%) of the four selected polar peptides (**Table S3**).

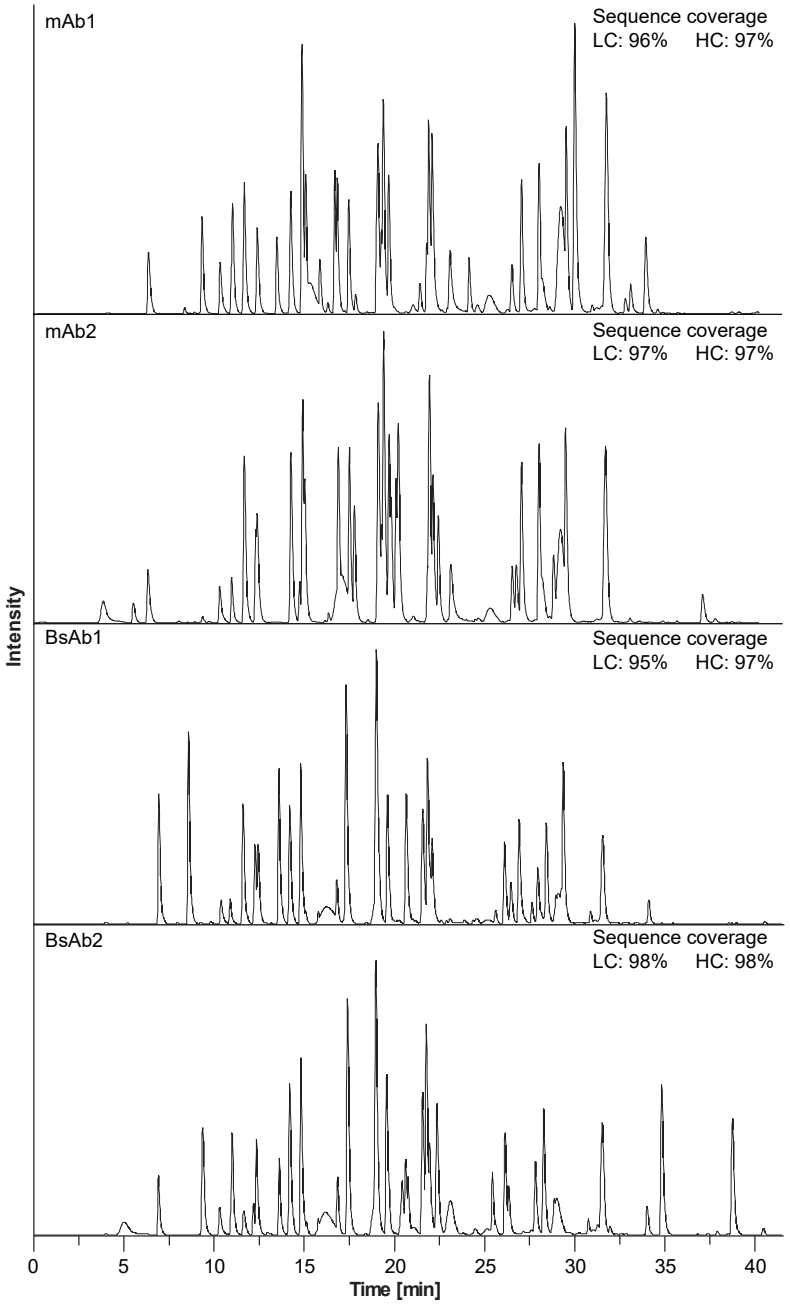

Figure 4. mD-LC-MS BPCs of formulated conventional mAbs (mAb1 and mAb2) and formulated bsAbs (BsAb1 and BsAb2) and their obtained sequence coverages, respectively. mAb1 (Lc: 96 % and Hc 97 %), mAb2 (Lc and Hc: 97 %), BsAb1 (Lc: 95% and Hc: 97%) and BsAb2 (Lc and Hc: 98%).

3.4.3 Analysis of stressed antibodies and PTM assessment

Next to sequence assessment, characterization of PTMs in therapeutic mAbs is highly important as these may alter the stability and/or affect the biological function of the protein [19]. PTMs such as deamidation of asparagine, methionine oxidation and lysine glycation are particularly important and can be considered as critical or non-critical quality attributes, which should be monitored carefully. To evaluate the capability of the established method to determine PTMs, a therapeutic antibody (mAb2) was exposed to various stress conditions (i.e. elevated pH, hydrogen peroxide, elevated glucose levels) [19] and analyzed with the proposed mD-LC-MS platform. The results were compared to a conventional off-line method as reported by Haberger et al. [19]. To induce deamidation, mAb2 was exposed to pH 9 at 37°C for 3 and 7 days. Analysis of the stressed samples revealed new signals eluting after their unmodified peptide with a mass increase of ~1 Da and were assigned as the deamidated variants and confirmed by MS/MS analysis. All four potential Asn sites were found deamidated (Lc-Asn-deamid-92, Lc-Asn-deamid-50, 52, 53, Lc-Asn-deamid-30, 31 and Hc-Asn-deamid-384, 389, 390) (**Figure S4**) with increasing intensities after 3 and 7 days (**Table S4**). Similarly as Haberger et al. [19], the most susceptible deamidation sites were found on Lc-Asn-92 and Hc-Asn-384, 389, 390, whereas Lc-Asn-50, 52, 53 and Lc-Asn-30,31 were less deamidation prone. Deamidation on Lc-Asn-92, which is particularly important as it is known to decrease the antigen binding site [19], increased from 15% to 36% after 7 days. Deamidation on Hc-Asn-384, 389, 390 and Lc-Asn-30, 31 resulted in multiple peaks as deamidation occurs on multiple amino acid positions in the respective tryptic peptide. Furthermore, methionine oxidation sites were assessed by the analysis of an H_2O_2 stressed mAb2 sample. The developed method was able to detect all six oxidation sites, including the oxidation at Hc-Met-252 (**Figure S5**). This peptide could not be detected with our previous mD-LC-MS method, due to the inability to trap polar peptides. However, Fc oxidation at Hc-Met-252 is of functional-relevance for the FcRn binding and therefore should be monitored carefully [20]. The most susceptible oxidation sites were found on Hc-Met-100c, Hc-Met-252 and Hc-Met-428 (from 1-2% to 97-98% after H_2O_2 stress), whereas Hc-Met-48 and Hc-Met-80 were less prone to oxidation and remained 1%, respectively (**Table S4**). In Addition, oxidation on Hc-Met-4, which is suspected to have an effect on the mAb2 target binding activity [19], increased from 1% to 58%, upon oxidative stress. Finally, lysine glycation was assessed by measuring a glucose stressed mAb2 sample. Depending on the mAb, glycation can have a detrimental effect on antigen binding as shown in several reports [21, 22]. Glycation on lysine residues affects the digestion pattern of trypsin, resulting in miss-cleaved peptides with an additional mass of +162 Da. As shown in **Figure S6**, both Lys-glycation sites (Hc-Lys-62, and Hc-Lys-33) were identified as miss-cleaved peptides as reported by Haberger et al. [19]. For Hc-Lys-33 the glycation level increased from 3% to 11%, whereas Hc-Lys-62 showed a minimal increase from 0.1% to 3%, respectively (**Table S4**). While these two glycation-sites show negligible to moderate effect on the binding activity of this mAb [19], it is still very important to be able to identify susceptible glycation-sites in the CDR region.

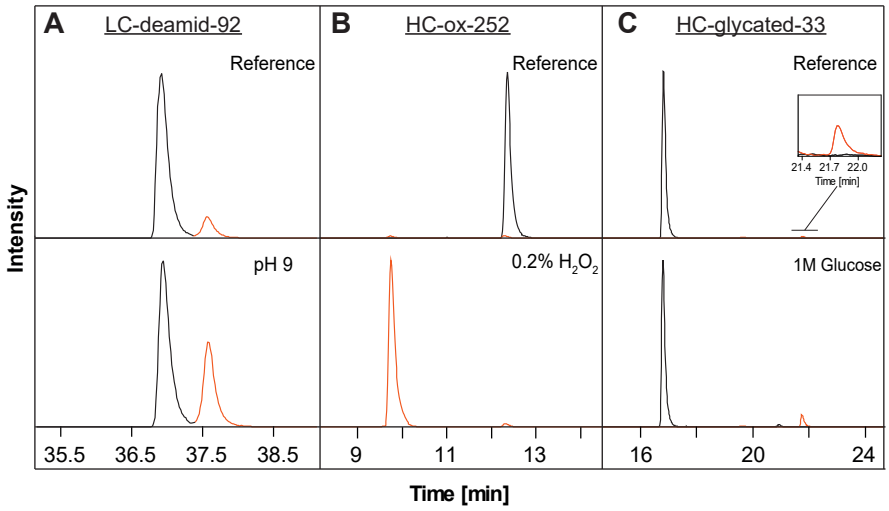

Figure 5. Extracted ion chromatograms (EIC) of A) Lc-Asn-92 (black) and deamidated Lc-Asn-92 (orange), B) Hc-Met-252 (black) and oxidized Hc-Met-252 (orange) and C) Hc-Lys-33 (black) and glycated Hc-Lys-33 (orange) of the reference material (upper panel) and stressed material (lower panel) of mAb2.

3.5 Conclusion

In this work we propose a new mD-LC-MS bottom-up strategy for the fast online characterization of (formulated) antibody-derived therapeutics (conventional and bispecific mAbs) without the need of any sample preparation. The method enables on-column reduction, tryptic digestion and peptide analysis by RP-MS. The easy-to-use hardware and software allow characterization of antibody-derived therapeutics in a straightforward manner. Compared to conventional off-line bottom-up approaches, the proposed workflow requires less hands-on time as no off-line steps need to be performed. Optimization of the method enabled trapping of polar peptides and improved the sequence coverage (95-98%) compared to previous mD-LC-MS methods (86-94%). Furthermore, the method permits to detect polar peptides, including Fc oxidation at Hc-Met-252 and Fc glycosylation at Hc-Asn-297, which could not be detected with previous mD-LC-MS methods. Furthermore, several PTMs (*i.e.* deamidation of asparagine, lysine glycation and methionine oxidation) can be detected and characterized within a single analysis. Therefore, the proposed mD-LC-MS bottom-up approach allows direct and fast characterization of antibody-derived therapeutics in less than 2 hours using only 10 µg of protein. Importantly, the proposed workflow is faster, requires lower amount of protein and obtains similar sequence coverages as conventional off-line bottom-up approaches. We believe that this method can considerably increase throughput for the characterization of antibody derived therapeutics in pharmaceutical labs.

3.6 Supporting information

Supplementary information is available free of charge on the Analytica Chimica Acta website: DOI: 10.1016/j.aca.2021.339015

3.7 Acknowledgments

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