

Poster Note PN-36

## Towards the fast and increasingly simplified analysis of trisulfide formation in biopharmaceutical antibodies

### Introduction

Chemical artifacts, such as deamidation events or disulfide structure modification, can alter the structure of bio-pharmaceuticals. A particularly challenging artifact is the formation of trisulfides. High trisulfide levels interfere with certain conjugation chemistries in the development of antibody drug conjugates. It is best to address those potential issues early in the development pipeline to avoid costly remediation downstream.

We have previously developed a fully automated method for the identification of disulfide structures in biopharmaceuticals based on an LC-MALDI-TOF/TOF workflow.

In this work we evaluated the extension of this workflow with regards to the additional detection of trisulfides.

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Scrambling	
Trisulfides	

Methods

A monoclonal IgG1 antibody (mAb) sample with elevated levels of trisulfide formation (Pfizer) and a selection of mAbs on the market (IgG1, IgG2 and IgG4: adalimumab, panitumumab and natalizumab) were digested using Lys-C/ trypsin under non-reducing conditions. Peptides were separated by nanoLC and spotted on a MALDI target in 10 sec intervals. MS and MS/MS spectra were obtained using a rapifleX MALDI-TOF/TOF at high speed employing a 10 kHz smartbeam 3D-laser (Bruker). Fully automated data analysis using the DisulfideDetect software (Bruker) provided for the detection and identification of the expected disulfide bonds incl. scrambled disulfides. Subsequent manual analysis of the same LC-MALDI dataset yielded species 32 Da higher than the corresponding disulfide – containing peptides (DSB-peptides). These species were deduced from the identified peptides to contain trisulfides (TSB-peptides). For an even easier and quicker analysis, we developed a new method using stepwise elution from ZipTips (Millipore) to determine the trisulfide content in a single MALDI mass spectrum.

Results

Automated disulfide bond analysis using rapifleX instrumentation and DisulfideDetect software determined the disulfide bond state of the mAbs as shown in **Fig.1** for adalimumab. All asymmetric dipeptides with one disulfide bond were analyzed by this method. The inter-chain disulfides between the LC and the HC are known to be especially susceptible to trisulfide formation and were further evaluated manually. In human IgG1, this peptide is a short and hydrophilic peptide (1261.5 Da), which elutes at low acetonitrile concentration in a RP-LC gradient. In human IgG2 and IgG4, the LC–HC dipeptide is larger (2039.94 Da) and elutes later in the gradient. In all 4 antibodies, trisulfide dipeptides were detected and identified using MS/MS. Quantitative results are listed in **Table 1**.

Table 1: Quantitation results on the LC-HC dipeptides of a Pfizer mAb and other IgG1, IgG2 and IgG4 antibodies.

mAb	Method	Trisulfide content
Pfizer IgG1 mAb	LC-MALDI	13.5 ± 2.6 %
Pfizer IgG1 mAb	ZipTip	9.5 ± 0.75 %
Pfizer IgG1 mAb	ESI-qTOF	11.3 %
Adalimumab	LC-MALDI	0.6 %
Adalimumab	ZipTip	0.65 %
Panitumumab	LC-MALDI	1.9 %
Natalizumab	LC-MALDI	0.4 %

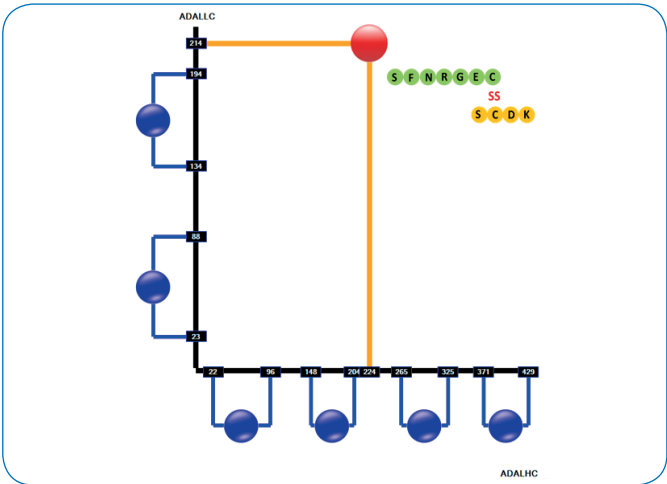


Fig.1: Resulting disulfide structure printout of a human IgG1 antibody (adalimumab) in the DisulfideDetect software. The disulfide bond is highlighted that links LC and HC. This particular site – together with the hinge di-peptide – is susceptible to trisulfide formation.

The IgG1 mAb (Pfizer) showed increased trisulfide formation in the LC-HC dipeptide (**Fig.2**) – 11.3 % in agreement with previous LC-ESI-qTOF experiments (**Table 1**). Three LC-MALDI replicates with 3 MS measurements each revealed an average trisulfide amount of 13.5 ± 2.6% (CV=19%). For the 3 therapeutic mAbs we determined trisulfide rates between 0.4 and 1.9% from single LC-MALDI experiments.

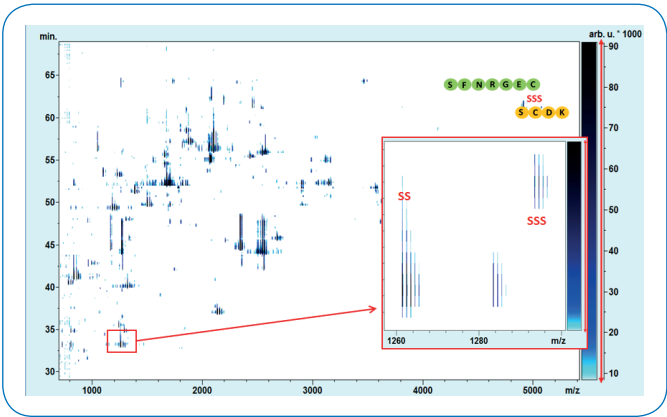


Fig.2: LC-MALDI-MS analysis of the IgG1 mAb (Pfizer) reveals increased trisulfide formation. The regular LC-HC linkage DSB-peptide (SS) and the corresponding TSB-peptide (SSS) are marked accordingly. The disulfide and trisulfide dipeptides elute with a difference of 2 min and were identified by MALDI-TOF-MS/MS.

In addition to the LC-MALDI analysis, we developed a simple method to quickly check – e.g. in screening scenarios – trisulfide formation of LC-HC dipeptide in human IgG1 antibodies. The Lys-C digest was bound to a C18 ZipTip and LC-HC dipeptides were eluted using 10% ACN, 0.1 % TFA and further analyzed by MALDI-TOF-MS (**Fig. 3**). Result from 21 replicates: average trisulfide content is 9.53 ± 0.75% (CV=8%).

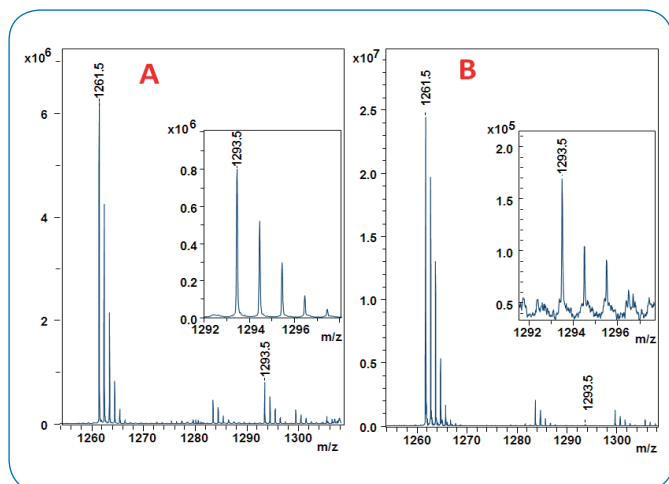


Fig.3: MALDI-TOF analysis of 2 human IgG1 antibody digests using the 10% acetonitrile ZipTip fraction for trisulfide quantitation. A: IgG1 (Pfizer) with 9.5% TSB-peptide content (from Fig.2) B: adalimumab with only 0.65 % of TSB-peptide.

The dipeptides were identified by MALDI-TOF-MS/MS in both, LC-MALDI and ZipTip analyses. (**Fig.4**).

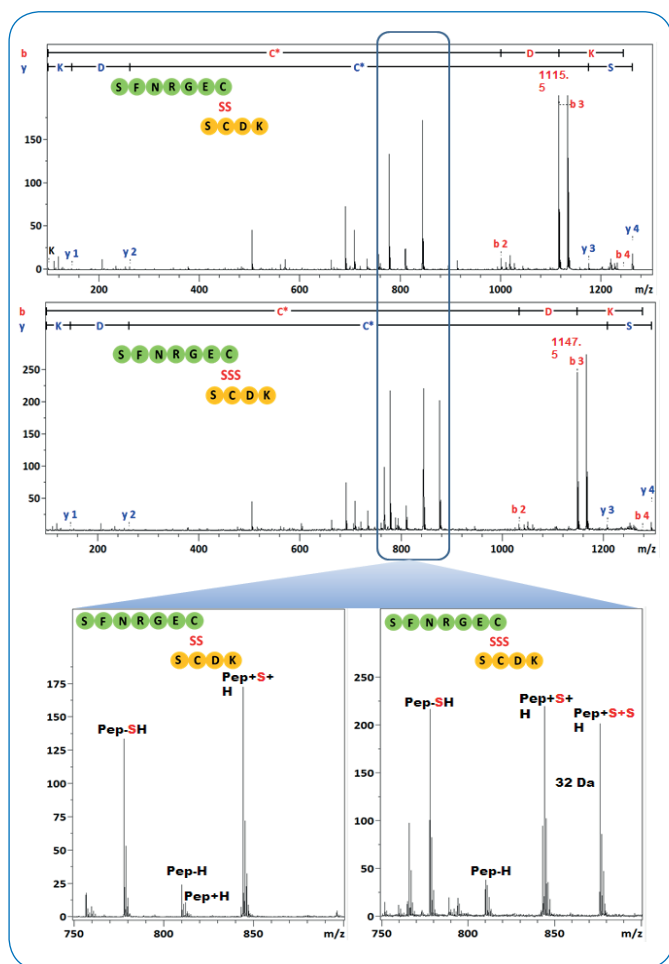


Fig.4: MS/MS spectra from the DSB- and TSB-peptides in Fig. 3. The characteristic fragment pattern for both types of peptides are highlighted, they are used to assign the DSB/TSB peptide type

## Summary

MALDI-MS was investigated as a new approach to identify and quantify trisulfides in biopharmaceuticals via:

1. An LC-free screening-compatible high-speed method which provided quantification with CV < 10 %
2. An LC-MALDI-MS/MS method that yielded results comparable to LC-QTOF data with CVs < 20 %.
3. The 10-bit digitizer in the rapifleX TOF/TOF enabled the quantitation up to 3 orders dynamic range.

The quality of the MS/MS data was very high and compatible with a straightforward detection and identification of trisulfides even without dedicated software support.

## Conclusions

- Disulfide – bonded peptide were automatically identified
- In addition, trisulfides in mAbs were manually identified at +32 Da/ +1-3 min later in the LC-MS datasets
- Trisulfide peptides show a specific fragment ion pattern useful for their safe detection by MALDI-MS/MS
- Quantification in the 0.1-1% range was demonstrated (dynamic range ~ 10<sup>3</sup>)



### Breaking the Rules

The new DisulfideDetect workflow from Bruker

Scan the QR Code or click on the link to watch our Youtube Video: [www.youtube.com/BrukerDaltonics](http://www.youtube.com/BrukerDaltonics)

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