DIONEX
Passion. Power. Productivity.

UHPLC-MS/MS to Monitor Modifications in Biopharmaceuticals

Evert-Jan Sneekes, Remco Swart, and Wim Decrop
Dionex Corporation, Amsterdam, The Netherlands

ANTIBODY STABILITY ANALYSIS

Peptide mapping is used in various stages of the development and production of biopharmaceutical proteins to ensure product integrity and stability. Stability of monoclonal antibody therapeutics is an important aspect of characterization programs. The most reliable information can only be obtained by long-term monitoring of the stability at the normal storage conditions throughout the shelf-life of a product. However, to obtain information of the most susceptible degradation pathways and the most vulnerable sites of the molecules, stability of molecules can be assessed under extreme conditions, such as elevated temperatures, exposure to chemicals, light, or any combination of these.

Typically, enzymatically-derived peptides are separated by reversed-phase ion-pairing with acidic conditions. Modifications to biopharmaceutical proteins may include truncation, oxidation, reduction, glycosylation, isomerization, deamidation, and clipping.

These changes in the product are revealed by changes in the retention times and by mass-spectrometric detection. In the case of monoclonal antibody analysis, this challenging task is further augmented by the presence of numerous other peptides. Shallow solvent gradients can be applied as a means to increase resolution but the resulting elongated analysis times are disadvantageous.

Here, we have applied UHPLC column technology for peptide mapping of biopharmaceutical proteins. The effects of gradient LC conditions and column technology on peak capacity and analysis time were investigated. Parameter validation has been performed for stressed samples that represent relevant molar differences between native and modified peptides.

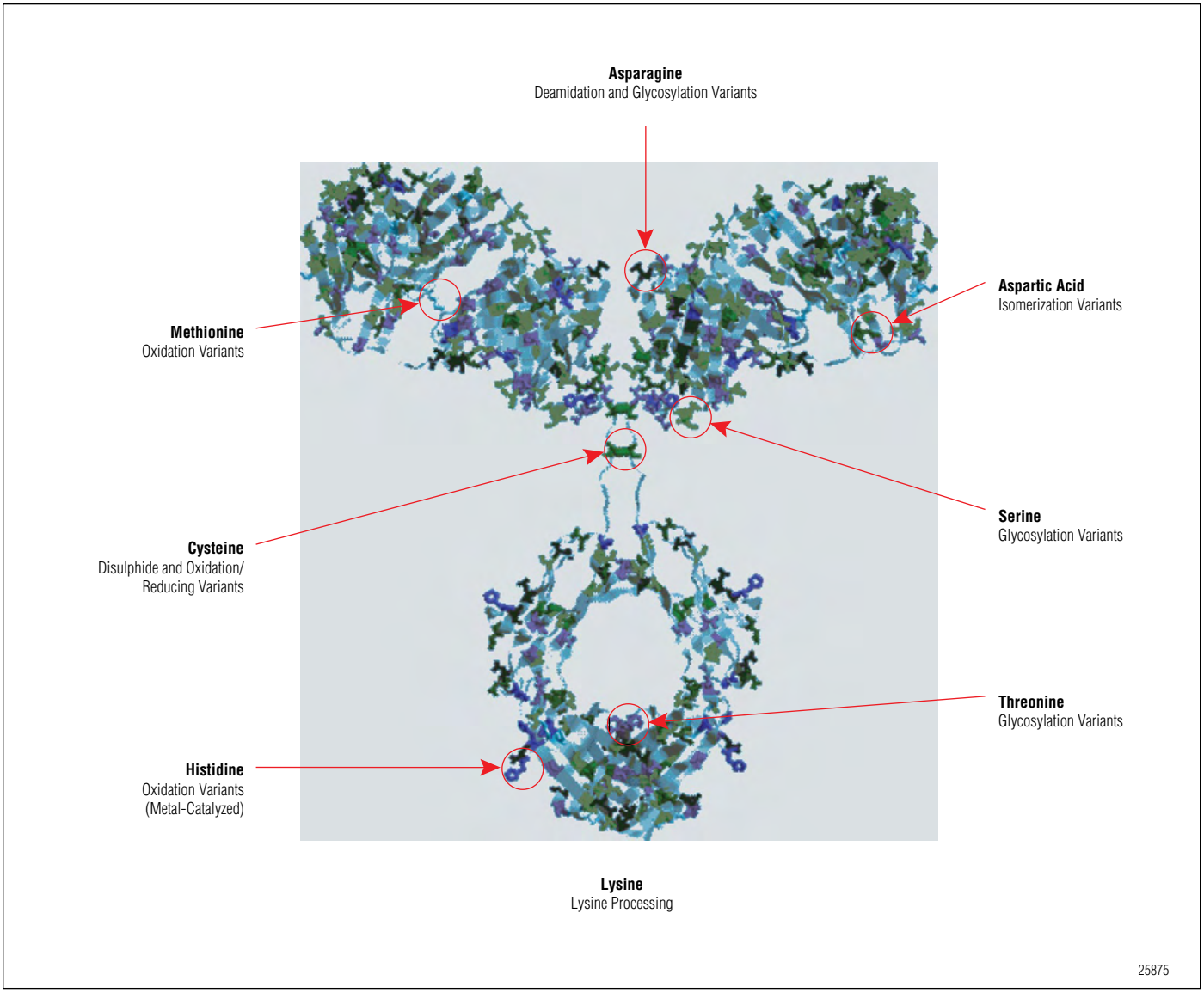

Figure 1. Challenges to characterize and analyze MAb: possible variations in a MAb.

INSTRUMENTATION AND COLUMNS

HPLC experiments were carried out using an UltiMate® 3000 RSLC system (Dionex, Germany) equipped with:

- SRD-3600 Membrane Degasser
- HPG-3400RS High Pressure Gradient Binary Rapid Separation Pump
- TCC-3000RS Thermostatted Column Compartment
- WPS-3000TRS Rapid Separation Well Plate Sampler
- VWD-3400RS Variable Wavelength Detector equipped with a 2.5 µL flow cell

Dionex Acclaim® RSLC C18 columns with a dimension of 100 × 2.1 mm i.d. and packed with particles sizes of 2.2, 3, and 5 µm, 120 Å were used, unless mentioned differently.

All separations were carried out using acetonitrile as an organic modifier and trifluoroacetic acid as ion pair in a concentration of 0.05% v/v.

INFLUENCE OF PARTICLE SIZE ON TOTAL ANALYSIS TIME AND PEAK CAPACITY

Faster monoclonal antibody peptide mapping can be achieved by reducing the particle size. When using columns of the same dimensions and reducing the particle size from 5 to 3 and 2.2 µm, while maintaining the optimum linear velocity in the column for each particle size respectively, results in increasing peak capacity (nc is inversely proportional to the square root of the plate height), and shorter analysis time (Figure 2). Average peak capacities of 290, 350, and 380 were found for 5, 3, and 2.2 µm particulate columns respectively.

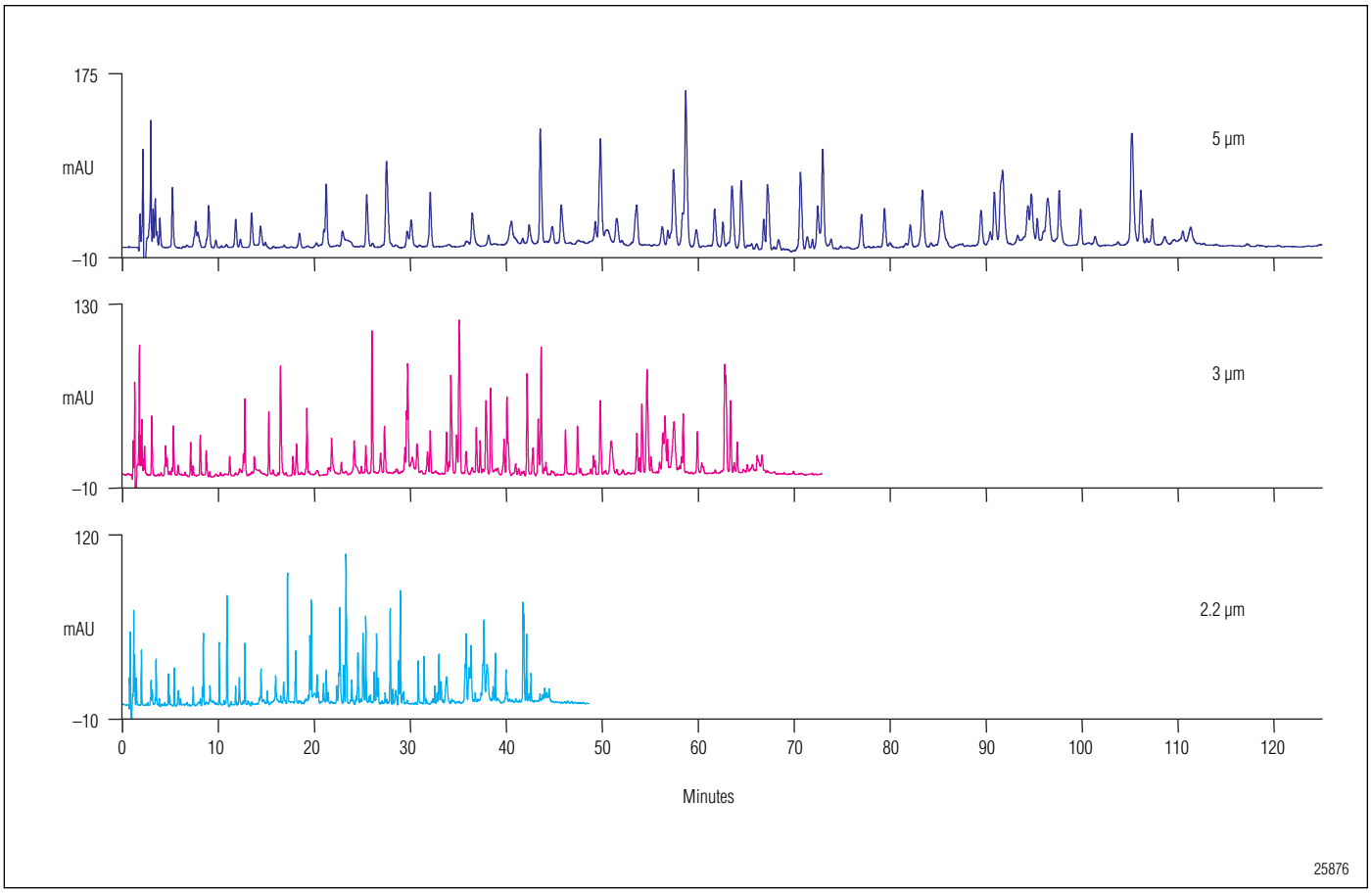

Figure 2. Separation of tryptic digest of monoclonal antibody on Acclaim C18 stationary phases with various particle sizes. Column dimensions: 2.1 × 100 mm.

The total analysis time was reduced by changing the particle size from 5 over 3 to 2.2 µm, while maintaining the same linear velocity.

Keeping the flow rate constant and increasing the column length causes the total analysis time and the system backpressure to increase proportionally. When combining the effect of a smaller particle size stationary phase (with their higher optimum velocity) and an increased column length, a considerable increase in efficiency can be expected. The tryptic digest of a MAb was separated on a 200 mm long column (two 100 mm columns in series) packed with 2.2 µm C18 particles (Figure 3). The calculated average peak capacity was found to be 570, significantly higher than for the 100 mm long columns

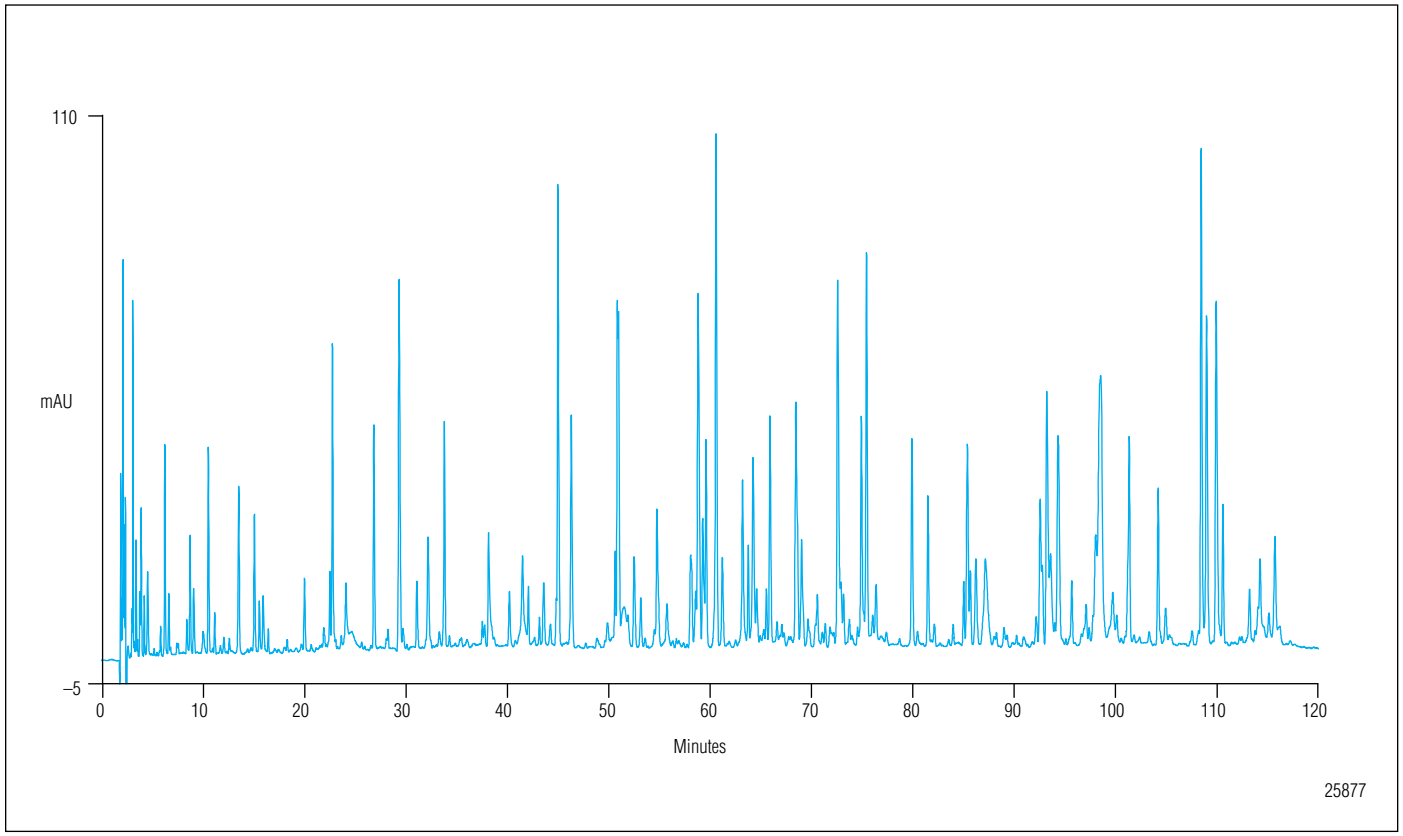

Figure 3. Separation of tryptic digest of monoclonal antibody on Acclaim RSLC C18 stationary phase with a 2.2 µm particle size, applying a 120 min gradient. Column dimensions: 2.1 × 200 mm.

RETENTION TIME PRECISION

For the comparison of different batches of antibody, e.g., stability batches or stressed samples, it is of utmost importance to have high retention time precision. This allows comparing the chromatograms of normal antibodies with modified antibodies in great detail and also evaluating the peptide mapping data visually. Also for MS detection, high chromatogram precision yields better comparability of different datasets. An example of 6 consecutive MAb peptide maps illustrating the high repeatability of the UltiMate 3000 HPLC platform is shown in Figure 4. The retention time precision for most peptides was below 0.05% RSD.

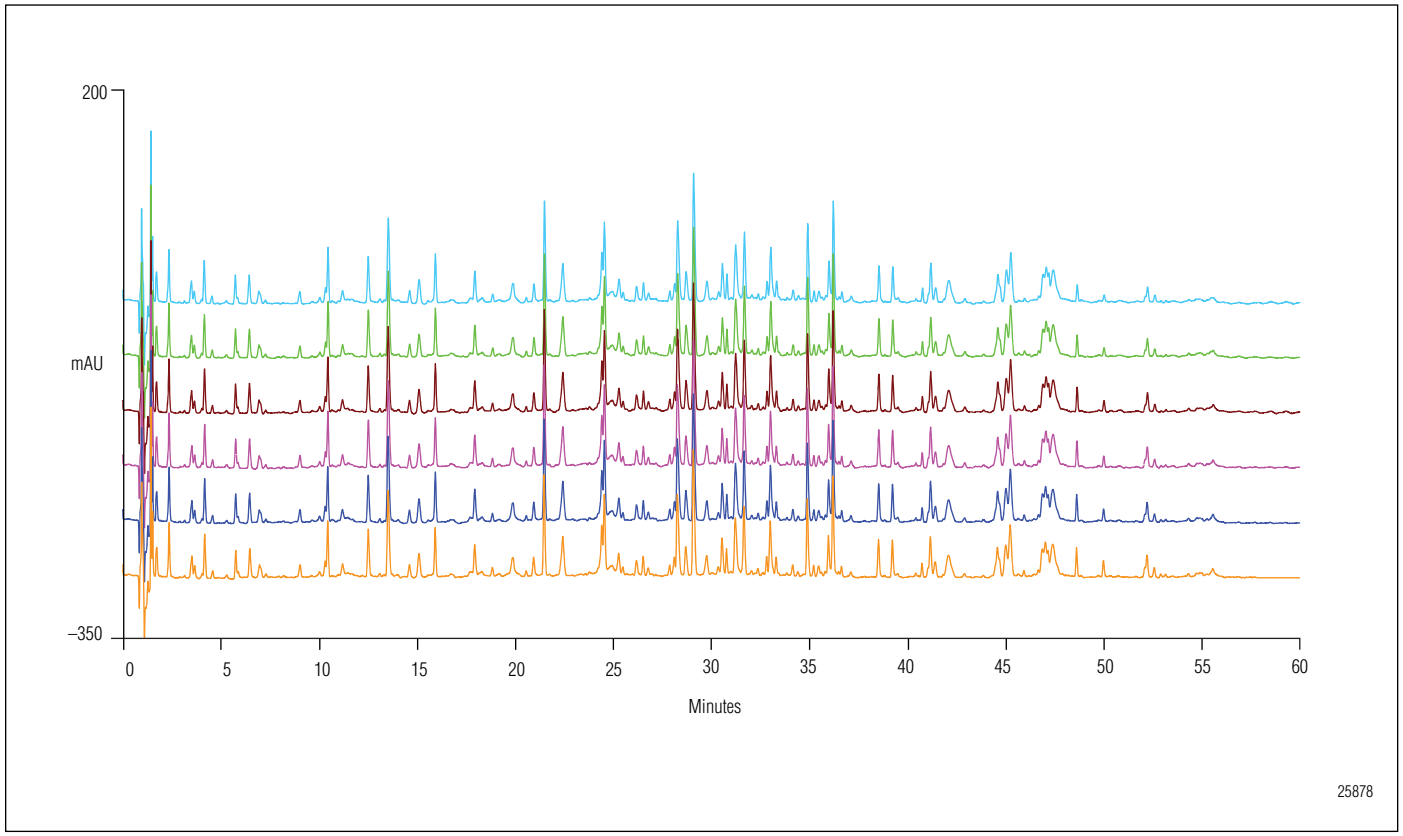

Figure 4. Repetitive peptide maps of a digested monoclonal antibody on the Acclaim RSLC C18, 2.2 µm column.

IDENTIFICATION OF PROTEIN MODIFICATIONS

Susceptible degradation sites of therapeutic proteins are routinely assessed under accelerated conditions, such as exposure to chemicals or incubation at elevated temperature or a combination of both. In this study, deamidation of asparagine in the MAb (see Figure 5) was induced by thermally stressing a monoclonal IGg1 antibody protein at pH 7.4 at 37 °C for one week in PBS. (Deamidation protocol was adapted from Huang et al., Anal. Chem. 2005, 77, 1432–1439). The control antibody and the deamidated antibody were then subjected to tryptic digestion followed by RPLC-MS/MS analysis.

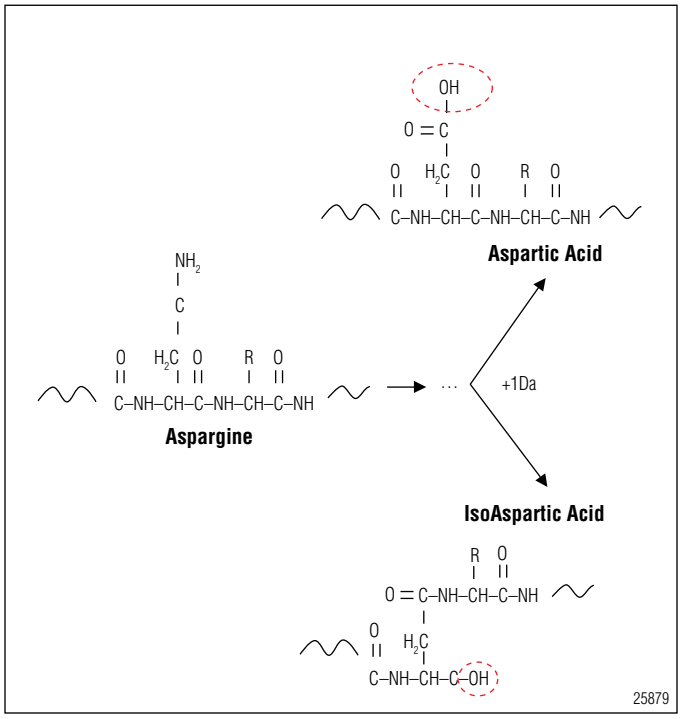

Figure 5. Deamidation of asparagine via a succinimide intermediate is a common degradation.

In this study, several deamidation sites were found, including one for a peptide related to the antibody light chain (SGTASVVCLLNNFYPR). This deamidation was expressed in the MS data by a shift of 1 Da and a retention time shift of 0.2 min at approximately 47 min in the chromatogram.

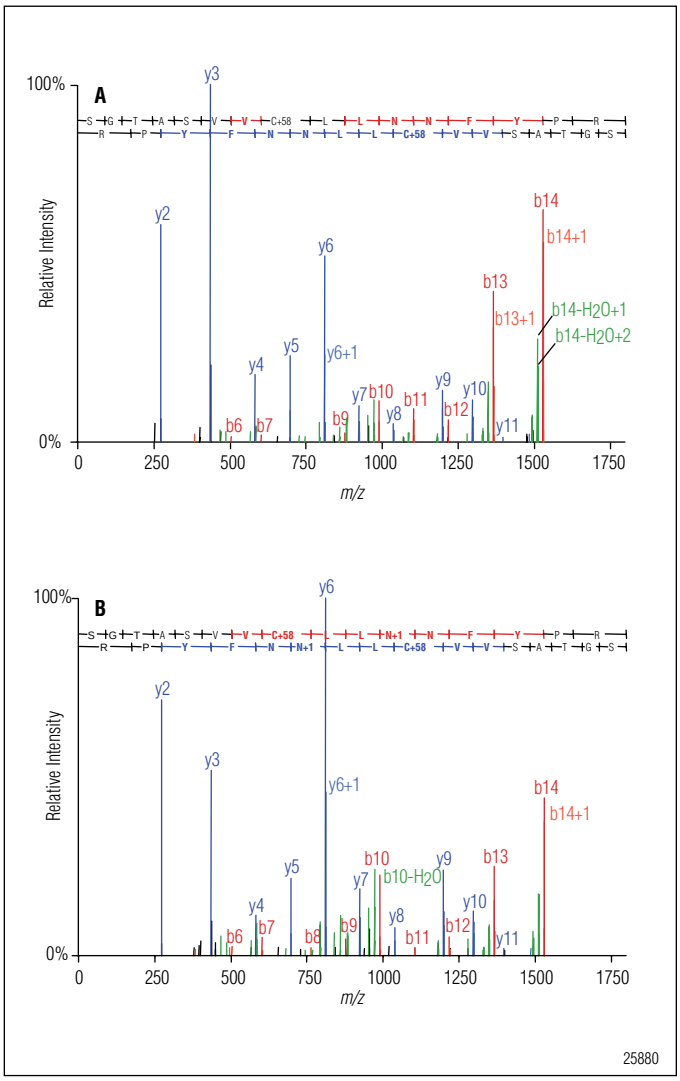

Figure 6. A) MS/MS spectrum of a tryptic peptide in the antibody digest with sequence SGTASVVCLLNNFYPR (t_R = 47.09 min). B) Corresponding peptide in the light chain of the stressed antibody (t_R = 46.88 min) featuring deamindation of asparagines and a shift in mass of 1 Da.

UV detection can be applied to quantify these protein modifications. As a result, to the high resolution small modifications can be resolved and eventually quantified. Deamidation was quantified to be 4% in the normal sample and 30% in the stressed antibody (Figure 7).

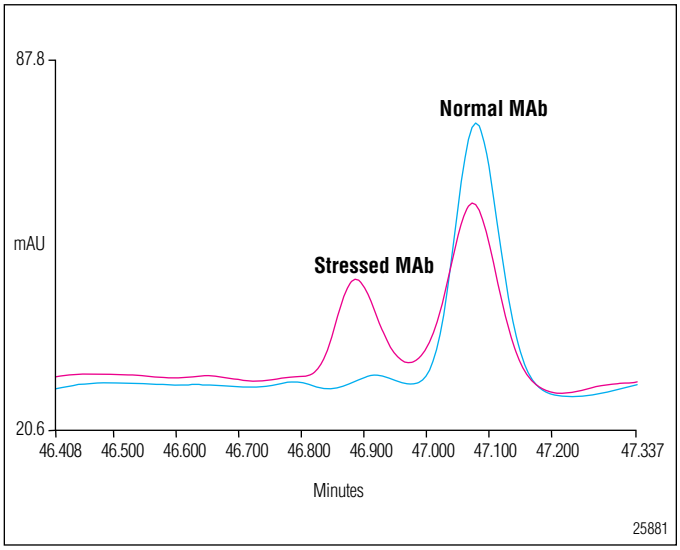

Figure 7. Overlayed chromatograms of the normal and the stressed antibody for one of the deamidated peptides.

CONCLUSIONS

- The Acclaim RSLC C18 2.2 µm stationary phase is ideally suited for peptide mapping, e.g., the analysis of biologics.
- High-resolution peptide maps with peak capacities up to 570 can be obtained on 200 mm long columns applying shallow gradients.

Passion. Power. Productivity.

DIONEX

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768; (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

LPN 2519-01 7/10