



Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution

Application Note

Biotherapeutics & Biosimilars

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Abstract

Protein biopharmaceuticals such as monoclonal antibodies and recombinant proteins are currently in widespread use for the treatment of various life-threatening diseases including cancer and autoimmune diseases. Protein therapeutics have a complexity far exceeding that of small molecule drugs, hence, unraveling this complexity represents an analytical challenge.

This Application Note demonstrates the potential of comprehensive two-dimensional liquid chromatography (LCxLC) using the Agilent 1290 Infinity 2D-LC Solution with DAD detection for the analysis of monoclonal antibody tryptic digests.



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Introduction

Therapeutic macromolecules produced by recombinant DNA technology are becoming increasingly important in the treatment of various diseases. It is estimated that, at present, the global protein therapeutics market (monoclonal antibodies (mAb) and other recombinant proteins) represents approximately 20 % of the total pharmaceutical market. Within the current decade, over 50 % of new drug approvals will be biologics¹.

During the development and lifetime of these molecules, an in-depth characterization is required. This Application Note describes a peptide

mapping method for identity and purity assessment of the monoclonal antibody trastuzumab. Trastuzumab, marketed as Herceptin, is a 150 kDa large molecule used in the treatment of HER2 positive metastatic breast cancer. Following trypsinolysis, over 100 peptides with varying physicochemical properties present in a wide dynamic concentration range are expected (Figure 1). This represents a complex analytical challenge, requiring the highest separation power. Comprehensive LCxLC is known to substantially increase the chromatographic resolution as long as the two dimensions are orthogonal and the separation obtained in the first

dimension is maintained upon transfer to the second dimension^{2,3}. This Application Note reports on the combination of strong cation exchange (SCX) and reversed-phase liquid chromatography (RPLC), two combinations shown to provide good orthogonality towards the analysis of peptides³. Fractions were transferred from the first to the second dimension using a dual loop interface maintaining the (first dimension) resolution. An Agilent 1290 Infinity 2D-LC Solution system was used. Some key results are highlighted and the performance of the system is demonstrated.

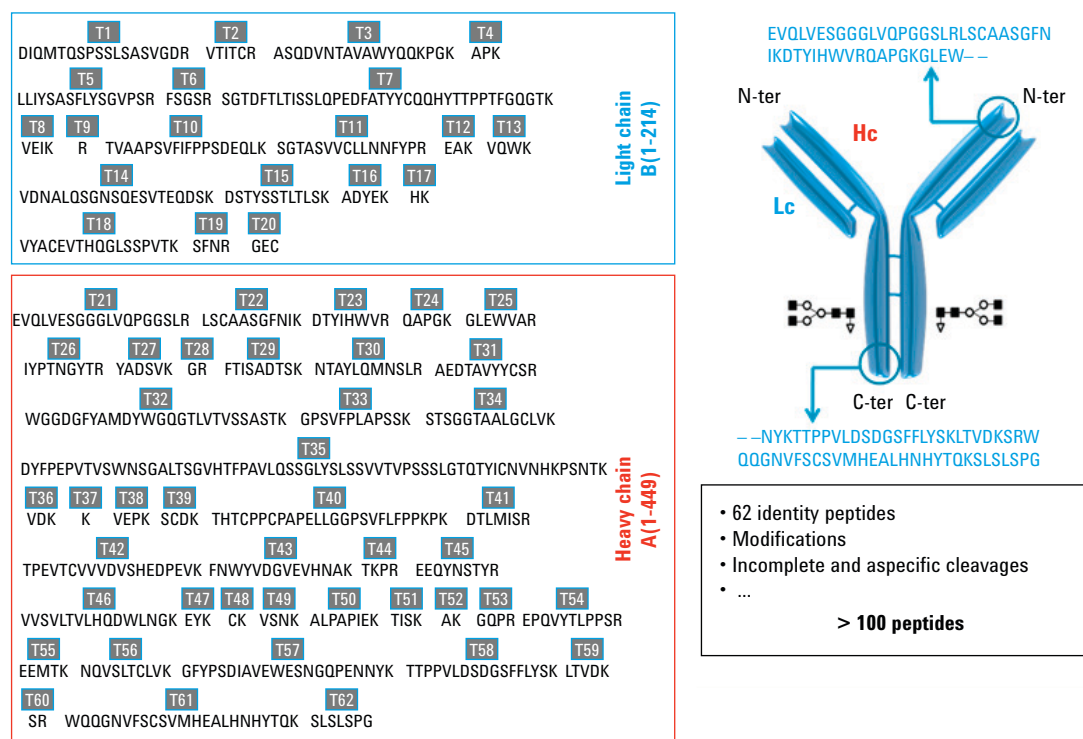


Figure 1. Structure and amino acid sequence of the protein. Identity peptides are labeled T1-T62.

Experimental

Solutions and Samples

All solvents were HPLC gradient grade from Biosolve B.V. (Valkenswaard, the Netherlands). Phosphoric acid and sodium chloride were from Sigma-Aldrich (Bornem, Belgium).

Sample Preparation

The following sample preparation procedure was used.

Sample	Volume corresponding to 100 µg protein
Add surfactant	0.1 % Rapigest in 100 mM Tris pH 7.5
Dilution	1 mM CaCl ₂
Reduction	5 mM dithiothreitol (DTT)
Alkylation	10 mM iodoacetamide (IAA)
Digestion	Trypsin (1:25 – w/w), 37 °C for 16 hours
Prepare for analysis	Lower pH (TFA), wait, centrifuge

The sample was also subjected to the following forced degradation conditions.

Oxidation stress	tert-butyl hydroperoxide (TBHP), 30 °C for 23 hours
pH Stress	Tris pH 9, 37 °C for 3 days

Instrumentation

An Agilent 1290 Infinity 2D-LC Solution was used. The configuration is shown below.

G4220A	Agilent 1290 Infinity Binary pump with seal wash option (1st dimension)
G4220A	Agilent 1290 Infinity Binary pump (2nd dimension)
G4226A	Agilent 1290 Infinity Autosampler
G1330A	Agilent 1290 Infinity Autosampler Thermostat
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector with standard flow cell
G1170A	Agilent 1290 Infinity Valve Drive
G4236A	2-position/4-port duo valve for 2D-LC

Software

- Agilent OpenLAB CDS Chemstation revision C.01.04 with 2D-LC add-on software
- GC Image LCxLC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

Method

1 st Dimension	
Column	MIC-15-Polysulfoethyl-Asp, 150 × 1.0 mm, 5 µm (PolyLC Inc.)
Solvent A	5 mM phosphate pH 3 in 5 % acetonitrile
Solvent B	5 mM phosphate pH 3 in 5 % acetonitrile + 400 mM NaCl
Flow rate	60 µL/min
Gradient	0 to 30 minutes : 3 to 25 % B 30 to 50 minutes : 25 to 45 % B 50 to 55 minutes : 45 to 100 % B 55 to 58 minutes : 100 % B Post-time : 13 minutes at 3 % B
Temperature	25 °C
2 nd Dimension	
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 µm (p/n 959943-902)
Solvent A	0.1 % phosphoric acid in water
Solvent B	acetonitrile
Flow rate	3.5 mL/min
Idle flow rate	0.5 mL/min
Initial gradient	0 to 0.43 minutes : 2 to 35 % B 0.43 minutes : 2 % B
Gradient modulation	35 % B at 0.43 minutes to 65 % B at 50 minutes to 100 % B at 51 minutes (Figure 2)
Temperature	55 °C
Modulation	
Modulation on	1 to 53 minutes
Loops	Two 40-µL loops, cocurrent configuration
Modulation time	0.50 minutes
Injection	
Volume	20 µL
Temperature	4 °C
Needle wash	6 seconds flushport (5 mM phosphate pH 3 in 5 % acetonitrile)
Detection	
Wavelength	Signal 214/4 nm, Reference 360/100 nm
Data rate	80 Hz

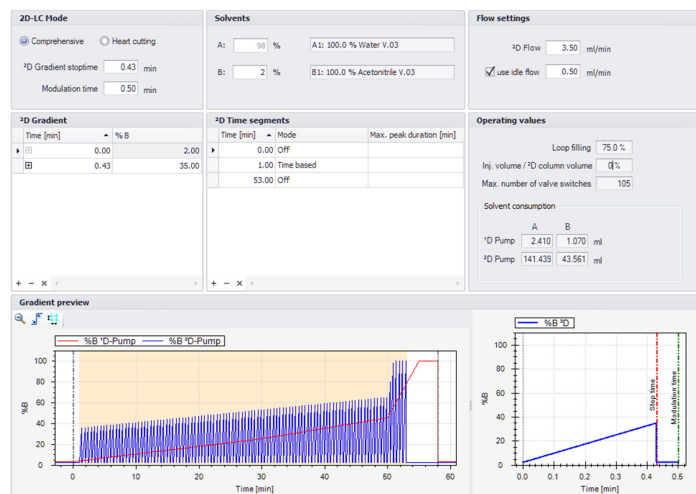


Figure 2. Screenshot of the 2D-LC method (OpenLAB CDS Chemstation).

Results and Discussion

Figure 3 shows the contour plot of a digest generated with the described method and the GC Image software. A good coverage of trastuzumabs sequence was obtained. The identification of the spots was based on an adapted method for combination with mass spectrometry (a G6530B QTOF system was used). The spots are randomly scattered across the surface indicating good orthogonality between both dimensions.

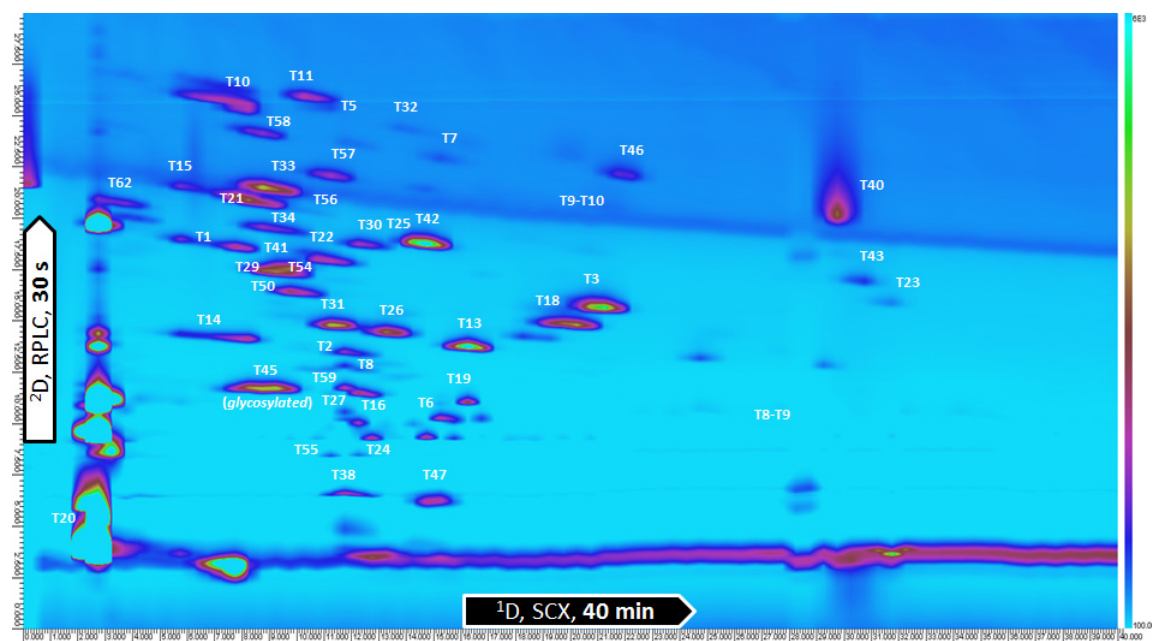


Figure 3. LCxLC contour plot for the analysis of a tryptic digest of trastuzumab.

The analysis was repeated five times to calculate the precision of the method. Table 1 summarizes the results for a selection of four peptides. The volume and retention time precision is very good, indicating that the method is useful for the comparison of different production batches and between originators and biosimilars.

Table 1. Precision data calculated on a selection of spots, n = 5.

%RSD	T3	T11	T43	T50
Volume	2.04	1.94	4.56	2.59
Height	0.85	1.78	2.15	0.54
RT 2D	0.09	0.05	0.25	0.15

The method can also be applied to detect impurities and modifications. To confirm this, a forced degradation study was carried out on a trastuzumab sample. The product was stressed under oxidative conditions and elevated pH conditions resulting in oxidation and deamidation, respectively. Figure 4 shows a detail of

an originator sample and an oxidation stressed sample. The stressed sample clearly contains additional spots. One particular spot was picked out to be identified by LC/MS/MS. The spot could be traced back to peptide T41. It is the result of an oxidation of methionine present in this fragment.

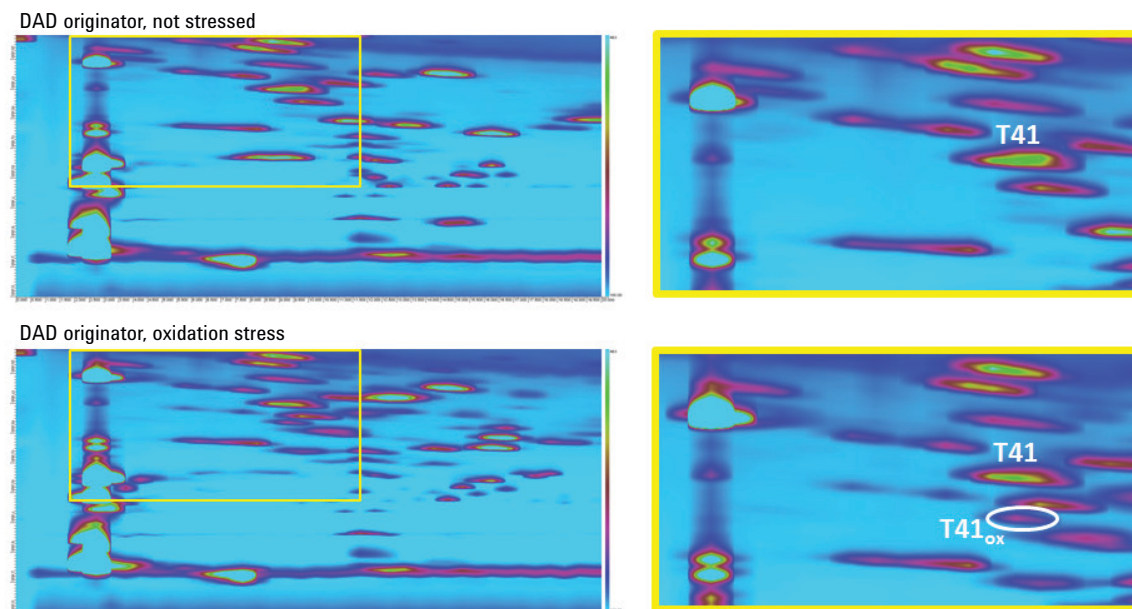
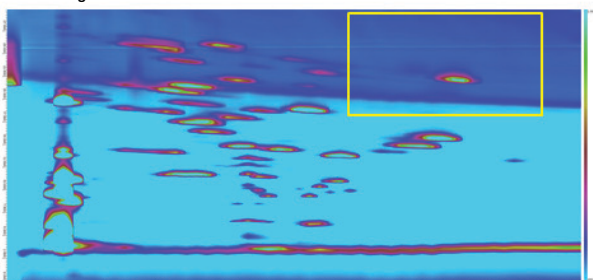


Figure 4. LCxLC contour plot for the analysis of a tryptic digest of non-stressed and oxidatively stressed trastuzumab. Note that the oxidation is already present at marginal levels in the non-stressed sample illustrating the strength of the methodology.

Figure 5 presents a similar example, where the result for a pH stressed sample is shown. Peptide 46 is known to be sensitive to deamidation because it contains asparagine. During the pH stress, asparagine is converted to aspartate. An additional spot is visible in the contour plot with identical retention time in the second dimension. This indicates that, under the applied conditions, it will be difficult to separate the identity peptide from its deamidated degradation product with reversed phase LC only. The identity of the modification was confirmed by LC/MS/MS using the adapted method.

DAD originator, not stressed



DAD originator, pH stressed

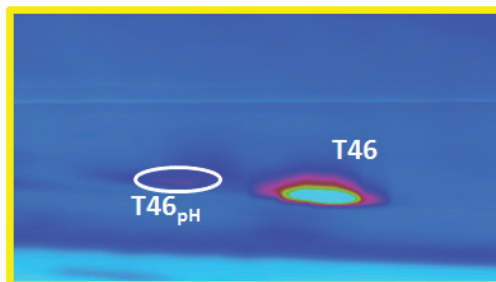
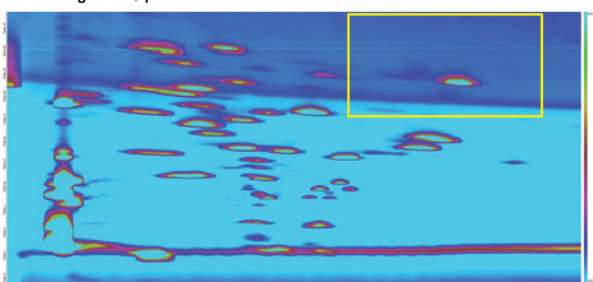


Figure 5. LCxLC contour plot for the analysis of a tryptic digest of nonstressed and pH stressed trastuzumab.

Conclusion

The Agilent 1290 Infinity 2D-LC Solution is an ideal solution for the analysis of complex samples such as protein biopharmaceuticals. This method demonstrated great potential for purity and identity assessment. The precision of the set-up was excellent allowing the use of the method for quality control and comparative studies between production batches and originators and biosimilars.

References

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