Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution
Part 2: HILIC × RPLC-MS

Application Note

Biotherapeutics & Biosimilars

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Abstract

Trastuzumab (marketed as Herceptin), is a monoclonal antibody used in the treatment of HER2 positive metastatic breast cancer. After tryptic digestion, over 100 peptides with varying physicochemical properties present in a wide dynamic concentration range are produced. High peak capacity is required for a detailed peptide mapping of such complex samples. Comprehensive two-dimensional liquid chromatography (LC×LC) is a very powerful tool to do this. This Application Note demonstrates the use of the Agilent 1290 Infinity 2D-LC Solution to perform a combination of hydrophilic interaction liquid chromatography and reversed-phase liquid chromatography. The system is coupled on-line with an Agilent 6530 Accurate-Mass Q-TOF LC/MS System.

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Introduction

Biopharmaceuticals such as therapeutic monoclonal antibodies (mAbs) and recombinant proteins are becoming increasingly important in the treatment of various diseases[1]. Peptide mapping is a commonly used technique for their comprehensive characterization and purity determination. The complexity associated with mAb digests demands the best separation power. Compared to one-dimensional separations, comprehensive two-dimensional LC (LC×LC) will drastically increase peak capacity 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 describes a two-dimensional comprehensive LC peptide mapping method for identity and purity assessment of the mAb trastuzumab (also known as Herceptin, Figure 1).

Hydrophilic interaction liquid chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) are recognized to provide orthogonal selectivity and, thus, yield complementary data[4]. HILIC and RPLC generally use a similar mobile phase, which facilitates the transfer of fractions from one mode to the other. Therefore, the combination of these two modes in an LC×LC setup for the analysis of peptides is promising, and results have already been reported in literature[3,5]. A previously published Application Note reported on LC×LC results obtained with a combination of strong cation exchange (SCX) and RPLC[6].

This Application Note describes the application of HILIC×RPLC to the analysis of tryptic digests of trastuzumab using the Agilent 1290 Infinity 2D-LC Solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS System. The details on the configuration for the on-line hyphenation of the second dimension separation to MS are shown, and the advantages of the combination of LC×LC coupled to a high-end mass spectrometer are illustrated with some representative results on stressed mAb samples.

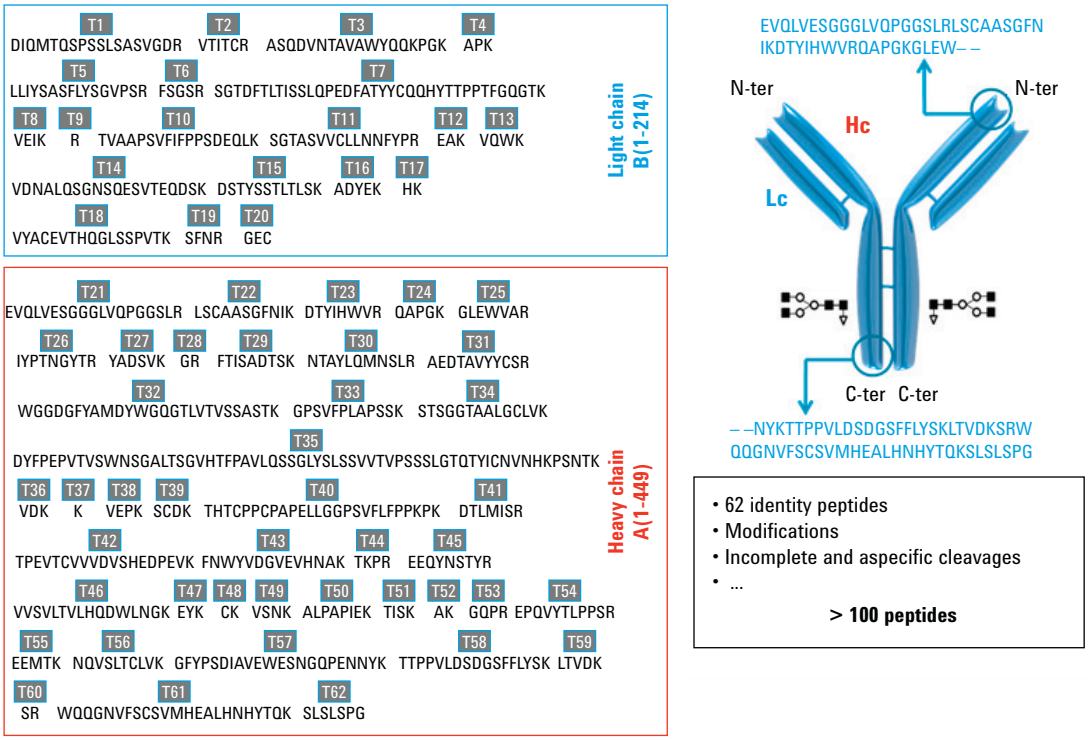


Figure 1. Structure and amino acid sequence of trastuzumab. Identity peptides are labeled T1-T62.

Experimental

Solutions and samples

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

Sample preparation

The sample preparation procedure is shown in Table 1.

Instrumentation

An Agilent 1290 Infinity 2D-LC Solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS System was used. The configuration is shown in Table 2, and a scheme of the setup is shown in Figure 2.

Software

- Agilent OpenLAB CDS Chemstation revision C.01.04 with 2D-LC add-on software
- Agilent MassHunter Software for QTOF revision B.04.00 Bioconfirm add-on software
- GC Image LCxLC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

Table 1. Sample preparation procedure.

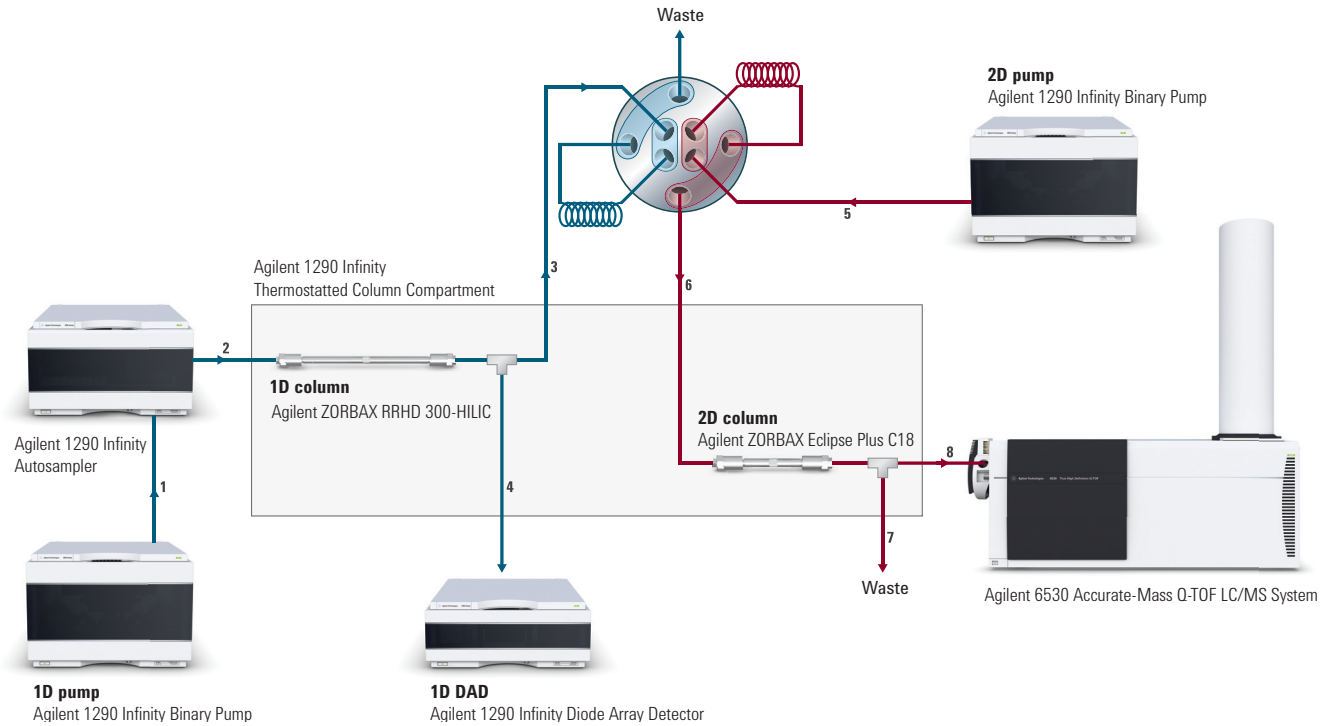
Step	Parameters
Sample	Volume corresponding to 100 µg protein
Add surfactant	0.1% Rapigest in 100 mM *Tris* pH 7.5
Dilution	1 mM $CaCl_2$
Reduction	5 mM Dithiothreitol (DTT), 60 °C for 30 minutes
Alkylation	10 mM Iodoacetamide (IAA), 37 °C for 60 minutes
Digestion	Trypsin (1:25 – w/w), 37 °C for 16 hours
Sample cleanup	Lower pH (TFA), wait, centrifuge
Dry sample	Dry in SpeedVac
Prepare for analysis	Redissolve in water/acetonitrile 40/60 (v/v)
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

Table 2. Instruments.

Instrument	Part no.
Agilent 1290 Infinity Binary Pump with seal wash option (1st dimension)	G4220A
Agilent 1290 Infinity Binary Pump (2nd dimension)	G4220A
Agilent 1290 Infinity Autosampler	G4226A
Agilent 1290 Infinity Autosampler Thermostat	G1330A
Agilent 1290 Infinity Thermostatted Column Compartment	G1316C
Agilent 1290 Infinity Diode Array Detector with standard flow cell (1st dimension)	G4212A
Agilent 1290 Infinity Diode Array Detector with standard flow cell (2nd dimension)	G4212A
Agilent 1290 Infinity Valve Drive	G1170A
Agilent 1200 Infinity Series 2 position/4-port duo valve for 2D-LC	G4236A
Agilent 6530 Accurate-Mass Q-TOF LC/MS System	G6530A

Method

1st dimension	
Column	Agilent ZORBAX RRHD 300-HILIC, 2.1 × 100 mm, 1.8 µm (p/n 858750-901)
Solvent A	15 mM ammonium formate in water, pH 4.5
Solvent B	15 mM ammonium formate in 90 % acetonitrile, pH 4.5
Flow rate	50 µL/min
Gradient	0–75 minutes, 90–45 % B 75–80 minutes, 45–0 % B 80–85 minutes, 0 % B Post-time 20 minutes at 90 % B
Temperature	30 °C
Detection DAD	
Wavelength	Signal 280/4 nm, Reference 400/100 nm
Data rate	10 Hz
2nd dimension	
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 µm (p/n 959943-902)
Solvent A	0.1 % formic acid in water
Solvent B	Acetonitrile
Flow rate	4 mL/min
Idle Flow rate	0.25 mL/min
Gradient	0–0.35 minutes, 0–60 % B 0.35 minutes, 0 % B
Temperature	30 °C
Modulation	
Modulation on	12–69 minutes
Loops	Two 40-µL loops, cocurrent configuration
Modulation time	0.45 minutes
Injection	
Volume	3 µL
Temperature	4 °C
Needle wash	5 seconds flushport (water/acetonitrile 25/75)
Detection MS	
Agilent Jet Stream technology source, positive ionization	
Drying gas temperature	340 °C
Drying gas flow	10 L/min
Nebulizer pressure	45 psig
Sheath gas temperature	400 °C
Sheath gas flow	11 L/min
Capillary voltage	3,500 V
Nozzle voltage	1,000 V
Fragmentor	175 V
Centroid data at 8 spectra/s	
Extended dynamic range (2 GHz), resolution 10,000 for *m/z* 1,000	



1	1D-Pump to Autosampler	Calibration Capillary (G1312-67500)
2	Autosampler to 1D-Column (1.6 µL heat exchanger)	SS, 0.17 mm
3	Tee 1 to 2D-LC valve	SS, 0.12 mm × 200 mm
4	Tee 1 to 1D-DAD	SS, 0.12 mm × 140 mm
5	2D-Pump to 2DLC valve	SS, 0.17 mm
6	2D-LC valve to 2D-Column (1.6 µL heat exchanger)	SS, 0.12 mm × 270 mm
7	Tee 2 to waste	SS, 0.12 mm × 340 mm
8	Tee 2 to detector (AJS source or DAD 2D)	SS, 0.075 mm × 340 mm (5067-4783)

Figure 2. Scheme of the used configuration.

Configuration details

A combination of HILIC in the first dimension and RPLC in the second dimension should provide good orthogonality for compounds such as peptides in an LC×LC setup. The orthogonality and complementarity has been proven in various reports, but mostly using a stop-flow or an off-line LC×LC approach. In the stop-flow approach, the flow of the first dimension is interrupted while the transferred fraction is analyzed in the second dimension. This can be done several times per analysis. For off-line applications, peptide fractions are collected after a first-dimension separation, and these fractions are re-injected (often after some treatment for solvent removal and/or sample concentration) on the second dimension column. There are two major reasons these techniques are used.

The first is that there is a historical lack of robust, user-friendly, and commercially available multidimensional LC instrumentation and software. This void has been filled with the introduction of the 1290 Infinity 2D-LC Solution and its full control in the OpenLab CDS Chemstation.

A second and equally significant obstacle is that the apparent easy mobile phase transfer from a HILIC separation to a RPLC separation (or *vice versa*) is not as straightforward as it might seem. Although both modes use very similar mobile phase components, their chromatographic principles are very different. This leads to beneficial orthogonality, but also gives rise to some restrictions in combining both modes.

RPLC can be used for analytes with a broad range of polarities and functionalities and is generally carried out using mobile phases that contain a substantial amount of water and a miscible organic solvent (generally methanol or acetonitrile). Conversely, HILIC is a technique that is applied to increase retention for highly polar and

hydrophilic compounds that show less retention in RPLC. The mobile phase also contains an organic (acetonitrile in most cases) and aqueous component, but the fraction of the latter is generally much lower compared to RPLC. In this Application Note, HILIC was used as the first dimension. This means that the modulator loops were primarily filled with acetonitrile containing fractions that were sequentially injected onto a very fast RPLC gradient. Consequently, there was a significant loss of separation power in the second dimension and polar compounds typically analyzed with HILIC will elute completely or partially in the void due to the high elution strength and volume of the injection solvent. To reduce the loss of material in the void, several modifications have to be made to the original one-dimensional separations. In this Application Note, the flow rate of the first dimension was decreased to 50 µL/min (below the optimal flow rate for a 2.1-mm column) and an additional split was carried out before the modulator valve to even further decrease the flow and the amount of acetonitrile transferred to the second dimension column. The split was achieved by a zero dead volume T-piece that split the flow to the modulator and to a DAD (Figure 2). Because of the low pressure generated in the first dimension (as a consequence of the low flow rate and relatively high acetonitrile content), an additional restriction had to be added by means of a Calibration Capillary (Figure 2).

The second dimension was operated at a high flow rate (4 mL/min) and gradients starting at 100 % aqueous conditions were applied. This meant that the first dimension acetonitrile containing effluent was significantly diluted in water when injected onto the second dimension, minimizing the breakthrough of the more polar peptides. Additionally, the temperature for the second dimension was set to only 30 °C to increase retention.

Although the split, after the first dimension, adds complexity to the configuration, it also has a significant advantage, namely the ability to fully monitor the first dimension separation when the split is directed to a detector instead of waste. The low flow rate requires a detector with a small cell volume to reduce extra column band broadening. In this configuration, an Agilent 1290 Infinity Diode Array Detector (G4212A) was installed with a standard flow cell. This cell had an internal volume of only 1 µL for a 10-mm path length.

The complexity of the digest justifies the application of LC×LC for a detailed peptide mapping. In a routine environment, this analysis would preferably be carried out using UV detection. It has previously been shown that this is possible with the instrumental setup[6]. During method development and for in-depth and early phase studies on these types of samples, the use of high-end mass spectrometry is mandatory.

Hyphenating LC×LC with MS poses some additional difficulties. The high flow rate applied in the second dimension cannot be directly transferred to the MS source since such amounts of liquid will not be evacuated properly. For that reason, an additional split was installed after the second dimension column and before the source. A 75-µm capillary was used to connect the T-piece to the MS inlet (Figure 2). This created enough restriction to split the flow ca. 1/10. Band broadening was minimized because of the low volume of this capillary.

This capillary was connected directly to the electrospray needle; the diverter valve was bypassed to eliminate its contribution to the band broadening. This implied that the complete sample composition would enter the source, including matrix and sample preparation material. To keep the source clean and obtain a better stability over a sequence, the modulation could be switched on and off. In this application, modulation was carried out only between 12 and 69 minutes. When the modulation was switched off, the first dimension effluent was not transferred to the second dimension, reducing the amount of contaminating material that entered the MS source. An additional advantage was that the second dimension flow rate could be reduced during the period where no 2D-LC was carried out. In this case, the idle flow rate was set to 0.25 mL/min instead of the 4 mL/min during modulation. Taking into account the 20-minute re-equilibration time between analyses, this option saved nearly 200 mL of second dimension mobile phase per analysis.

Results and Discussion

The LC×LC peptide map of trastuzumab is shown in Figure 4. The contour plot was generated with the MS total ion current data using the GC Image software. The spots were identified by matching the experimentally acquired data on the theoretical trastuzumab sequence at high mass accuracy (< 5 ppm) using the MassHunter Bioconfirm Software. The resulting separation shows good orthogonality between both dimensions, and provides complementary data to the previously reported SCX×RPLC analyses[6]. Figure 4 also shows the first dimension separation detected after the split. This first dimension chromatogram is useful to monitor, for example, the quality of the first dimension separation.

The applicability of the developed method was evaluated with stressed and nonstressed samples. Trastuzumab was subjected to forced degradation conditions (pH or oxidation) prior to digestion (see analytical conditions). Comparing stressed with nonstressed data should reveal degradation products. This indicates if the method is capable to detect impurities and modifications of, for example, quality control of mAb samples.

Figure 5 shows the extracted ion plots for peptide T41 and its oxidation product. From the comparison of nonstressed and oxidized samples, it is clear that the nonstressed trastuzumab already contains small amounts of the oxidation product (oxidation of methionine present in this fragment). The inserts in Figure 5 show the mass spectra taken from the spots using the GC Image software.

Figure 6 illustrates a deamidation event caused by stressing trastuzumab at high pH. The extracted ion plots are shown for peptide T46 (905.507 *m/z*) and its degradation product T46pH (905.999 *m/z*).

The deamidation converts asparagine into aspartate in this peptide. The retention time in the second dimension is almost identical, which indicates that under the applied second dimension conditions, the degradation product is difficult to separate. The first dimension HILIC is necessary to isolate the deamidated peptide from its intact originator peptide. This a good example of the advantage of orthogonality of the two dimensions.

Conclusion

The Agilent 1290 Infinity 2D-LC Solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS system shows great potential for the comprehensive analysis of monoclonal antibody digests. The possibilities of LC×LC coupled to a high-end mass spectrometer for detailed peptide mapping analyses are illustrated with some representative results on stressed and nonstressed trastuzumab digests.

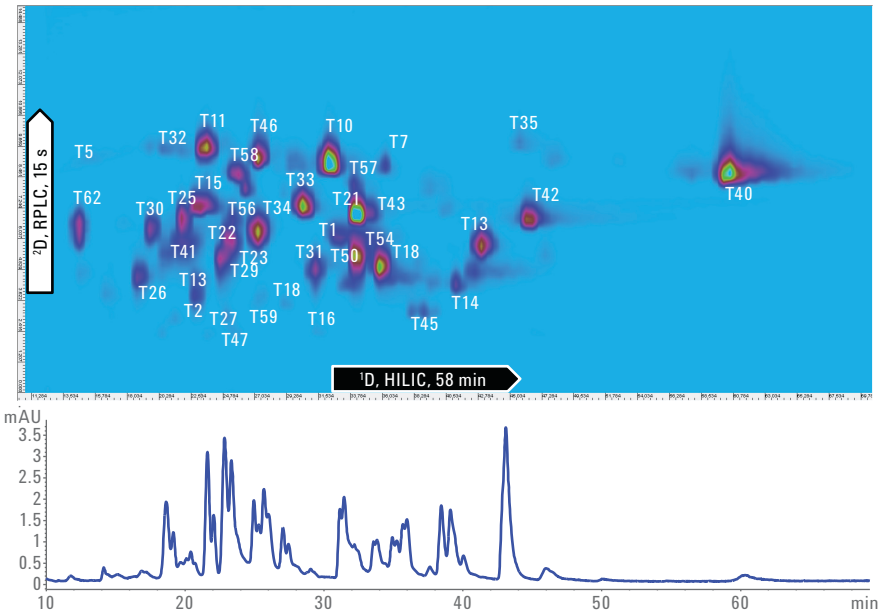


Figure 4. LCxLC contour plot for the analysis of a tryptic digest of trastuzumab. The first dimension separation from the split before the modulation is shown in the chromatogram below (DAD data, 280 nm).

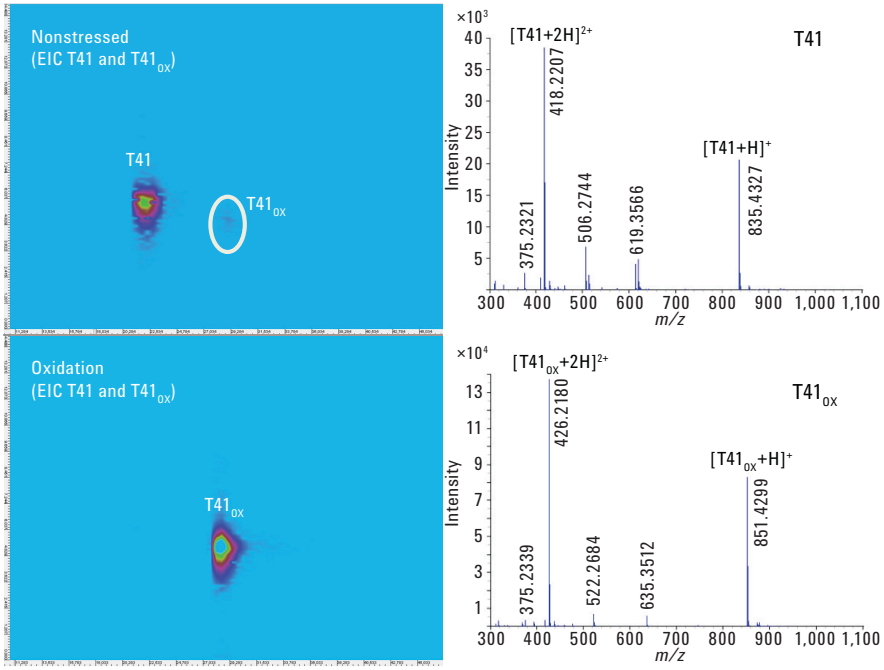


Figure 5. LCxLC extracted ion contour plot for the analysis of a tryptic digest of nonstressed and oxidatively stressed trastuzumab. The oxidation is already present at trace levels in the nonstressed sample illustrating the strength of the methodology (T41 = 418.220 m/z, $T41_{ox}$ = 426.218 *m/z*).

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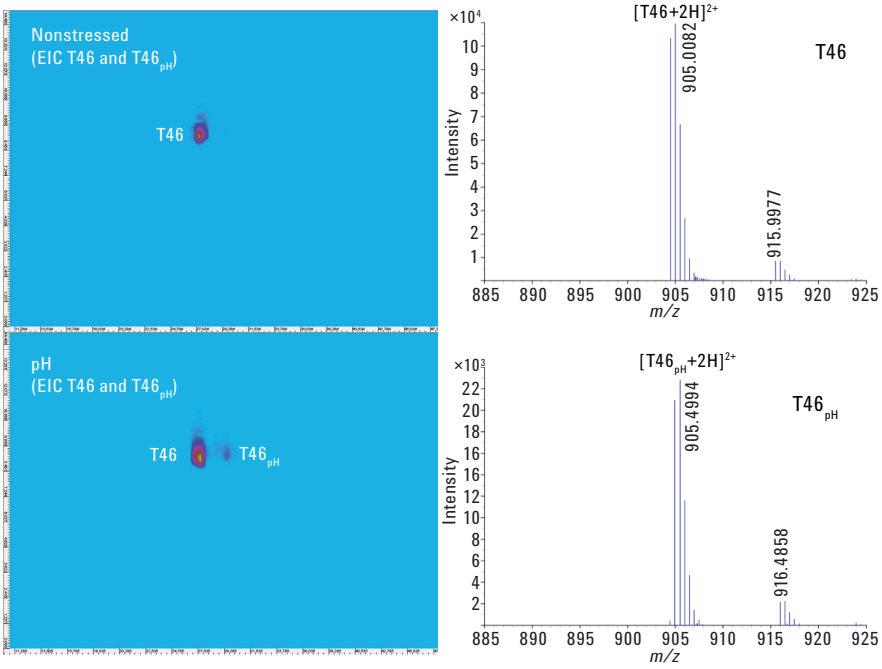

Figure 6. LCxLC extracted ion contour plot for the analysis of a tryptic digest of nonstressed and pH stressed trastuzumab (T46 = 905.507 m/z, $T46_{pH}$ = 905.999 *m/z*).

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