Application Note

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Identification of Conjugation Sites in an Antibody Drug Conjugate

Using the Agilent 6545XT AdvanceBio LC/Q-TOF system

Suitable for Agilent
1290 Infinity III LC

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Abstract

This application note highlights the workflow of drug conjugation site identification in an antibody drug conjugate (ADC). The workflow uses an Agilent AssayMAP Bravo protein sample prep platform, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm 12.1 software. Twenty-six conjugation sites were confidently identified in a lysine-linked ado-trastuzumab emtansine (T-DM1) sample. The results also showed exceptional mass accuracy and consistent reproducibility.

Introduction

ADCs represent a cutting-edge class of biopharmaceuticals. ADCs are designed to deliver a cytotoxic payload specifically to a targeted site while minimizing off-target effects and enhancing therapeutic efficacy. ADCs are composed of a monoclonal antibody (mAb) linked to a potent cytotoxic agent using a chemical linker through a conjugation process. Since conjugation can occur at several available sites on lysine-based ADCs, multiple conjugation molecules can be present. Therefore, a lysine-conjugated ADC is a heterogenous mixture of conjugated biomolecules.[1]

Peptide mapping is a pivotal tool for the in-depth characterization of ADCs. Peptide mapping provides site-specific information about ADC conjugation sites. In this application note, the drug conjugation sites of a lysine-linked ADC, T-DM1, were characterized following the Agilent peptide mapping workflow. A 1290 Infinity II bio LC system and a 6545XT AdvanceBio LC/Q-TOF system were used in conjunction with an automated AssayMAP Bravo protein sample prep platform. Data analysis and mapping of conjugation sites were performed with MassHunter BioConfirm 12.1 software. The integrated workflow is illustrated in Figure 1.

Experimental

Materials

Urea, Trizma base, dithiothreitol (DTT), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma (Burlington, MA, USA). Formic acid (LC/MS grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The T-DM1 sample was purchased from Alliance Pharm (Singapore, Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Burlington, MA, USA).

Sample preparation

During sample preparation, 6.5 mg of TDM-1 were dissolved into 1.3 mL of denaturation buffer comprised of 8 M urea, 50 mM Tris (pH 8.0). Aliquots of 100 µL of the dissolved sample were transferred into the AssayMap sample plate wells. The digestion protocol was selected using the In-Solution Digestion: Single-Plate application. In this protocol, 10 µL of 100 mM DTT were added for sample reduction. The plate was incubated at 37 °C for two hours. For alkylation, 12 µL of 200 mM IAA were added, followed by incubation at room temperature in the dark for one hour. The sample was then diluted with 400 µL of water, and 20 µL of 0.5 µg/µL trypsin was added. After incubation at 37 °C overnight, the reaction was quenched by the addition of 60 µL of 10% TFA. The digested samples were subjected to LC/MS analysis.

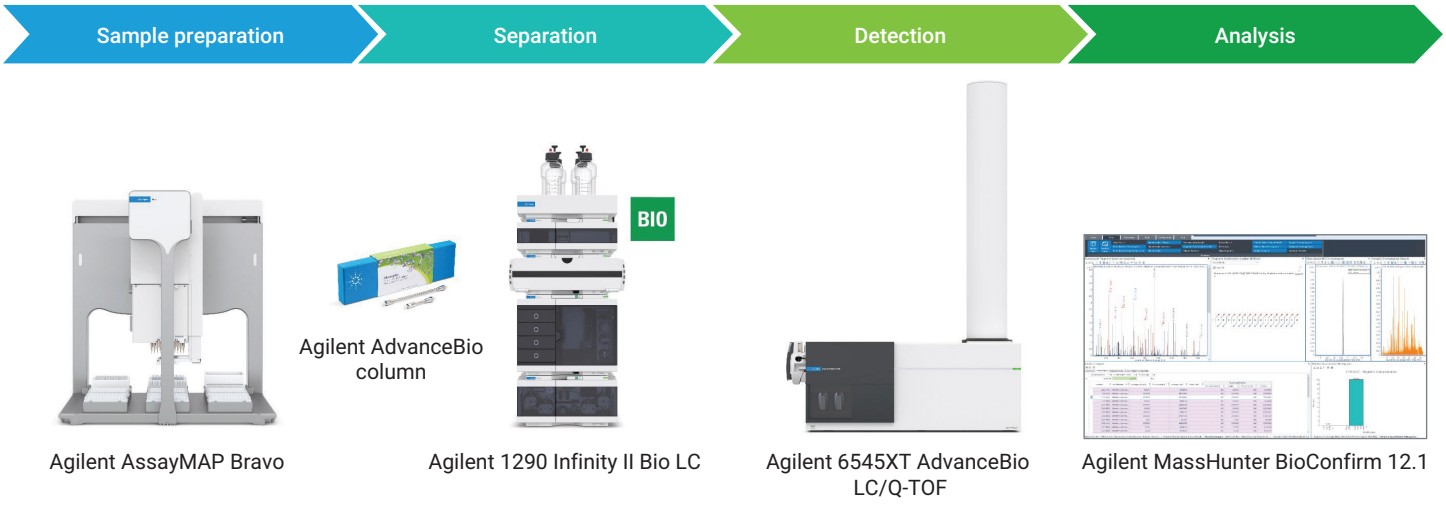

Figure 1. Agilent peptide mapping workflow.

Instrumentation

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- Agilent 1290 Infinity II bio LC system including:
  - Agilent 1290 Infinity II bio high-speed pumps (G7132A)
  - Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bioinert QuickConnect heat exchanger, standard flow (option #065)
  - Agilent 1290 Infinity II diode array detector (G7117B) with Agilent Max-Light cartridge cell, 10 mm
- Agilent 6545XT AdvanceBio LC/Q-TOF system with Agilent Dual Jet Stream ESI source

Software

- Agilent VWorks automation control software 14.1
- Agilent MassHunter data acquisition software 11.0
- Agilent MassHunter BioConfirm software 12.1

LC/MS analysis

Tables 1 and 2 list the parameters for LC and MS data acquisition used in the workflow.

Table 1. Liquid chromatography (LC) parameters.

LC Parameters	
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å (p/n 653750-902)
Thermostat	4 °C
Solvent A	0.1% Formic acid in H_2O
Solvent B	90% Acetonitrile and 0.1% formic acid in H_2O
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 3 1.0 3 70.0 45 71.0 90 73.0 90
Post Time	5 min
Injection Volume	20 µL
Column Temperature	60 °C

Table 2. Mass spectrometry (MS) data acquisition parameters.

Parameter	Value
Source	Agilent Dual Jet Stream ESI
Polarity	Positive
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 1,700
Acquisition Rate	8 Spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Minimum MS/MS Acquisition Rate	3 Spectra/sec
Isolation Width	Narrow (~*m/z* 1.3)
Max Precursor/Cycle	Top 10
Collision Energy	3.1 × (*m/z*)/100 + 1 for charge 2; 3.6 × (*m/z*)/100 – 4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 Counts and 0.001%
Dynamic Exclusion On	1 Repeat, then exclude for 0.2 min
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 Counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% Sstringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By charge state, then abundance; +2, +3, > +3

Data analysis

Data were processed following the Protein Digest workflow in MassHunter BioConfirm 12.1 software. The conjugation was defined as a new type of modification, MCC-DM1, in the Chemical Data Dictionary Tool. The modification adds 956.3644 Da in mass specifically to the lysine residue. The processing method parameters are listed in Table 3.

Table 3. Protein digest processing method parameters.

Parameter	Value
Condition	Reduced
Mods and Profiles	Alkylation (iodoacetamide), MCC-DM1
Enzyme	Trypsin
Find Peptides	Display biomolecules containing MS/MS scans
Match Tolerances	MS match tolerance: ± 20 ppm MS/MS match tolerance: ± 50 ppm Warn if score is < 3.00 Do not match if score is < 3.00 Allow missed cleavages up to 2 Peptide length range: 4 to 70 Allow terminal truncation Max number of modifications: 4

Results and discussion

The antibody backbone of T-DM1 is trastuzumab. The lysine amines of trastuzumab and the cytotoxic agent DM1 (emtansine) are conjugated by a nonreducible thioether linker, N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC).[2] The structure of T-DM1 is shown in Figure 2.

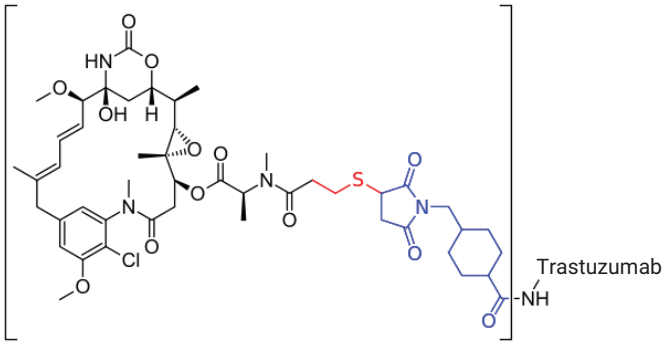

Figure 2. Molecular structure of T-DM1.

To locate the drug conjugation sites of T-DM1, the ADC was reduced, alkylated, and trypsin-digested using the In-Solution Digestion workflow on the AssayMAP Bravo platform. The digestion workflow was followed by LC/MS/MS analysis using the 6545XT LC/Q-TOF system coupled to the 1290 Infinity II bio LC system.

The DM1 payload has strong ultraviolet (UV) absorbance at 252 nm due to the presence of an aromatic ring and other chromophores in the structure. A comparison between UV chromatograms at 214 and 252 nm indicates that the main elution region of drug-conjugated peptides is from 36 minutes onwards (Figures 3A and 3B). This is further confirmed by the extracted ion chromatogram (EIC) of product ion *m/z* 547.22 (Figure 3C). This signature ion was produced from DM1 fragmentation during MS/MS. The conjugation of DM1 increases the overall hydrophobicity of the peptides, causing them to elute later in reversed-phase LC conditions.

An average sequence coverage of 94% was achieved for T-DM1. Figure 4 displays the overlaid biomolecule MS chromatograms of the peptides included in the coverage map from a single sample.

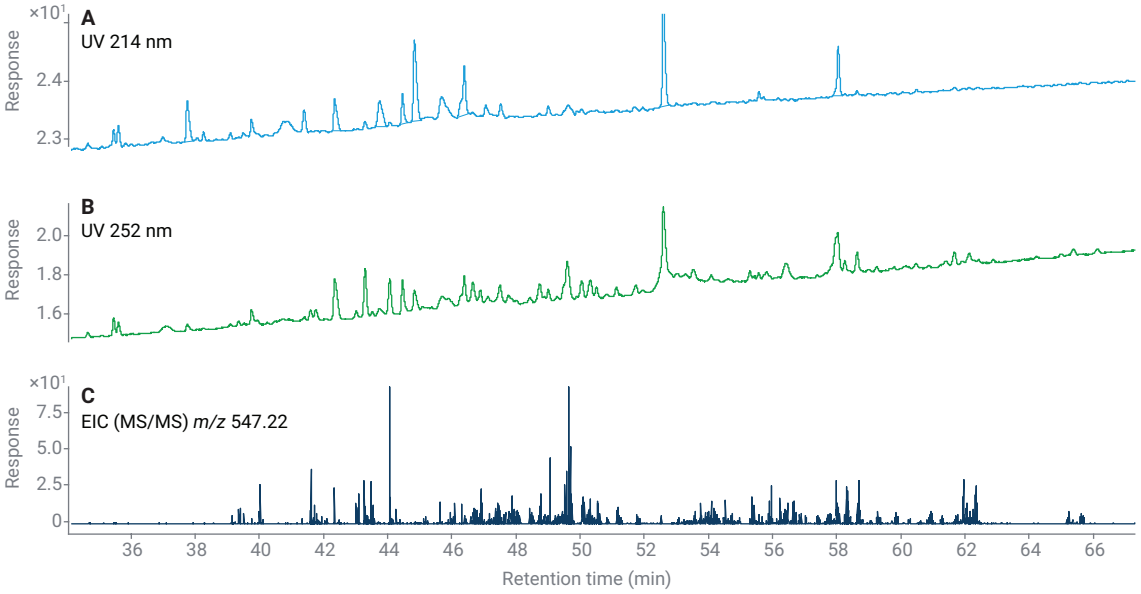

Figure 3. UV chromatograms of T-DM1 at (A) 214 nm and (B) 252 nm, and (C) the extracted ion chromatogram of T-DM1 product ion *m/z* 547.22.

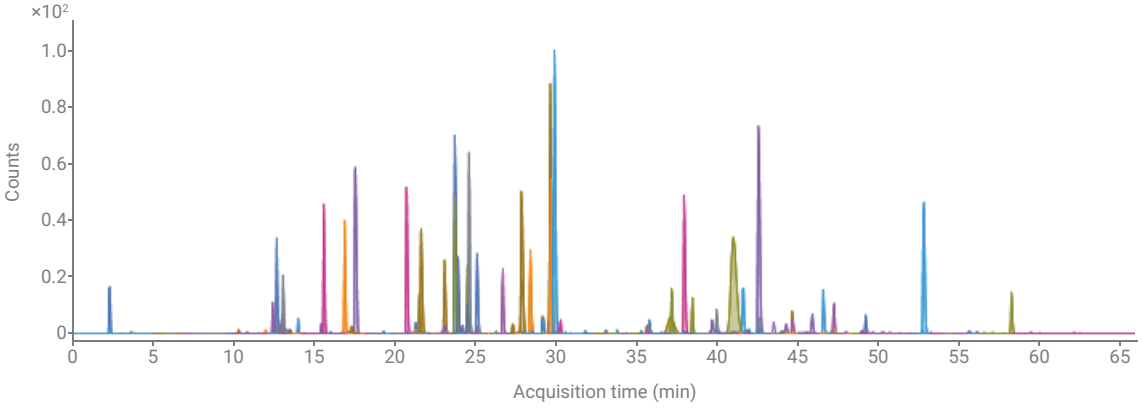

Figure 4. Overlaid biomolecule MS chromatograms of trypsin-digested T-DM1.

Among these peptides, BioConfirm software automatically identified 26 out of 44 lysine sites with conjugated MCC-DM1. The mass accuracy of all identified drug-conjugated peptides was within 3 ppm. The site locations and peptide sequences are listed in Table 4.

Table 4. MCC-DM1 conjugated peptides identified in T-DM1. The conjugation sites are marked with an asterisk (*).

No.	Chain	Site	Sequence	RT (min)	Delta Mass (ppm)
1	Light	K42	YQQKPGK*APK	41.73	0.70
2	Light	K107	VEIK*R	50.37	1.03
3	Light	K145	EAK*VQWK	51.27	1.11
4	Light	K188	ADYEK*HK	43.47	1.74
5	Light	K190	HK*VYACEVTHQGLSSPVTK	43.30	2.15
6	Light	K207	VYACEVTHQGLSSPVTK*SFNR	50.23	0.58
7	Heavy	K30	LSCAASGFNIK*DTYIHWVR	58.42	1.05
8	Heavy	K43	QAPGK*GLEWVAR	55.43	0.28
9	Heavy	K65	YADSVK*GR	49.53	1.32
10	Heavy	K76	FTISADTSK*NTAYLQMNSLR	55.9	1.96
11	Heavy	K136	GPSVFPLAPSSK*STSGGTAALGCLVK	59.34	0.63
12	Heavy	K208	ICNVNHK*PSNTK	41.86	0.76
13	Heavy	K213	ICNVNHKPSNTK*VDK	39.39	1.77
14	Heavy	K216	VDK*K	48.28	0.86
15	Heavy	K217	K*VEPK	47.03	0.93
16	Heavy	K225	SCDK*THTCPPCPAPELLGGPSVFLFPPKPK	56.47	1.05
17	Heavy	K249	THTCPPCPAPELLGGPSVFLFPPK*PK	62.15	0.16
18	Heavy	K251	LFPPKPK*DTLMISR	54.10	0.69
19	Heavy	K291	FNWYVDGVEVHNAK*TKPR	49.09	0.18
20	Heavy	K293	TK*PR	46.72	0.26
21	Heavy	K323	EYK*CK	46.75	0.93
22	Heavy	K325	CK*VSNK	46.08	0.84
23	Heavy	K329	VSNK*ALPAPIEK	52.66	0.31
24	Heavy	K337	ALPAPIEK*TISK	55.56	0.26
25	Heavy	K343	AK*GQPR	46.47	0.00
26	Heavy	K417	LTVDK*SR	50.48	0.81

Each identified sequence was verified through the following three criteria. LTVDKSR peptide with MCC-DM1 conjugated on lysine was used as an example in Figure 5 to illustrate the verification process.

- First, high quality MS/MS spectra with credible b and y ions were required to cover the peptide sequence. The fragment confirmation ladder feature in BioConfirm 12.1 software marks b and y ions based on their occurrence in the MS/MS spectra. This feature offers a rapid assessment of the quality of the MS/MS spectra for the biomolecule (Figure 5A). The enhanced peptide mapping algorithm in BioConfirm software also largely reduces artifacts for improved variable modification assignment, in this case MCC-DM1 modification.
- Second, the stereocenter in the DM1 molecule causes the drug-conjugated peptide to elute chromatographically as duplet peaks. The EIC of the peptide precursor ion confirms the presence of the stereoisomers (Figure 5B).
- Third, during peptide fragmentation, DM1 was also partially fragmented, generating DM1-associated ions. As shown in Figure 3C, *m/z* 547.22 is the most common and abundant fragment ion of DM1. Additionally, ions such as *m/z* 140.07, 453.19, and 485.22 are also present in the MS/MS spectra of the drug-conjugated peptides (Figure 5C). Furthermore, the loss of *m/z* 546.21 on y ions is commonly observed as partially fragmented DM1-linker-peptides.

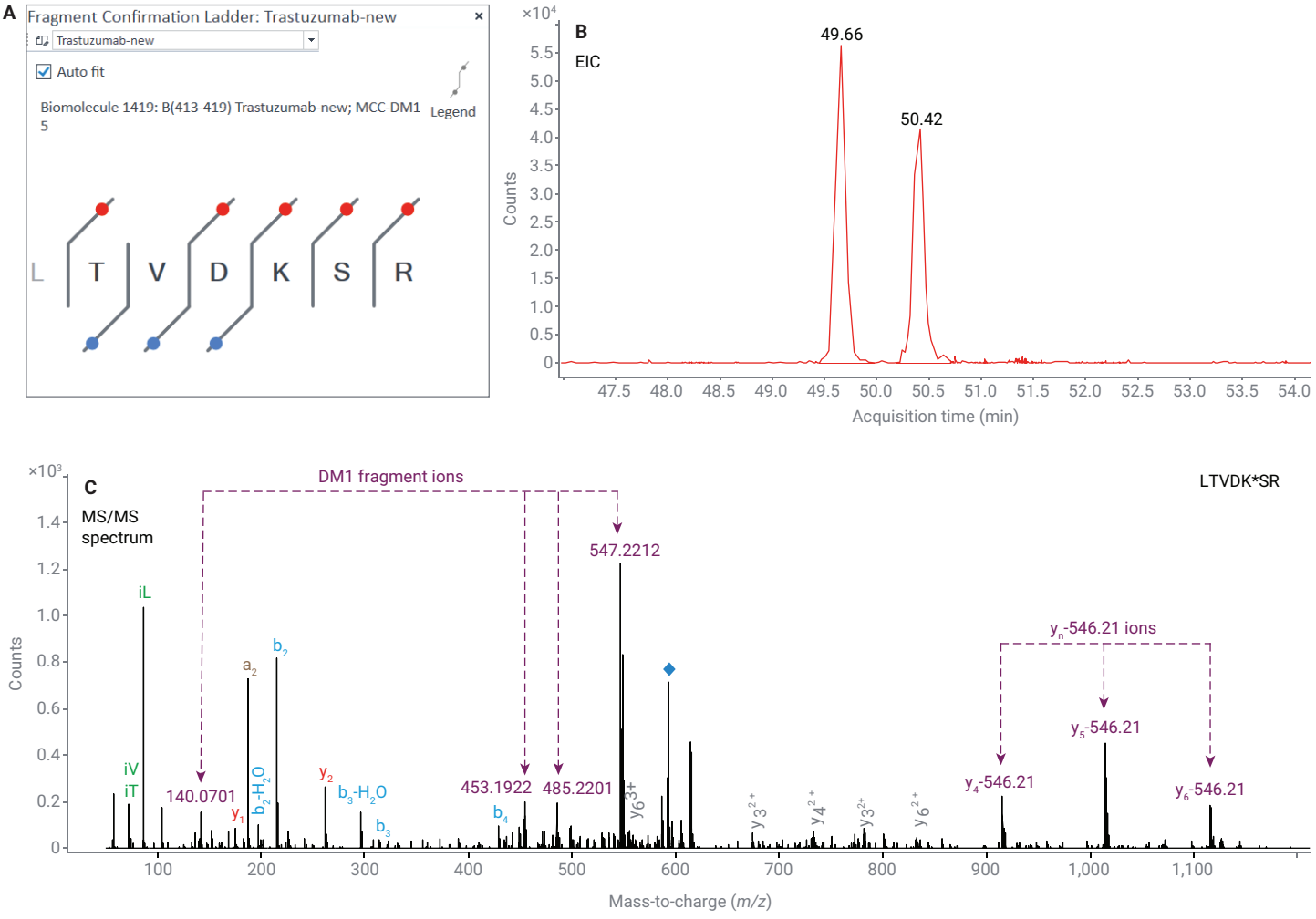

Figure 5. LTVDKSR peptide with MCC-DM1 modification on lysine. (A) The fragment confirmation ladder view in BioConfirm 12.1, (B) the extracted ion chromatogram (EIC) of precursor ion *m/z* 592.29^{3+}, and (C) the MS/MS spectra of LTVDKSR with MCC-DM1 modification.

The results also showed excellent reproducibility between seven replicated samples. Figure 6 shows overlaid biomolecule MS chromatograms of drug-conjugated peptides, including YQQKPGKAPK from the light chain and ALPAPIEKTISK from the heavy chain. Retention time (RT) relative standard deviations (RSDs) for both peptides were 0.27 and 0.03%, respectively. RSDs of abundance, in terms of signal volume, were 4.80 and 5.01%, respectively. This superior reproducibility is attributed to the automated sample preparation, reliable acquisition engine, and advanced software algorithm.

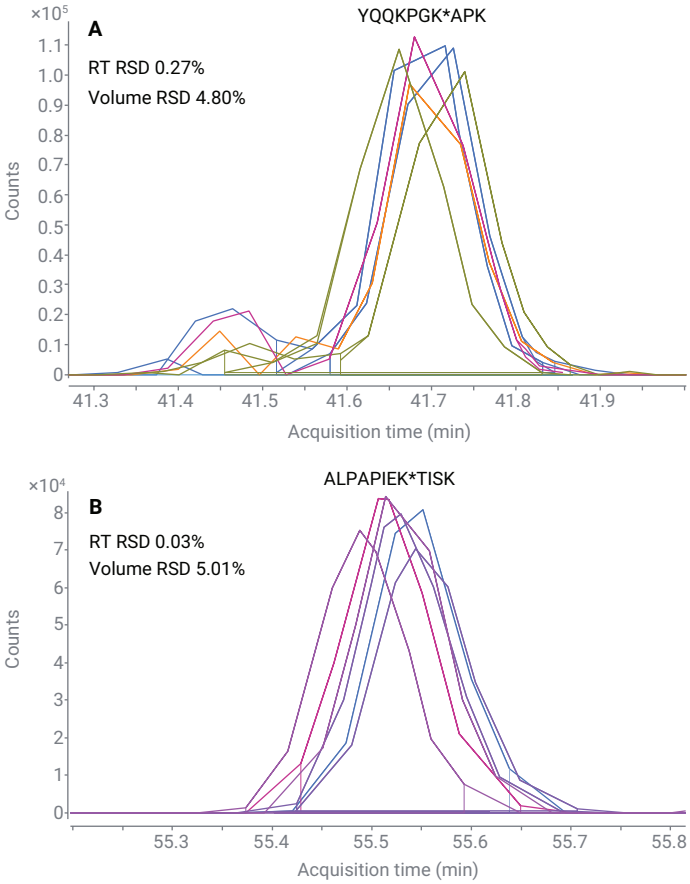

Figure 6. Overlaid biomolecule MS chromatograms of drug-conjugated peptides (A) YQQKPGK*APK and (B) ALPAPIEK*TISK.

Conclusion

In this application note, a lysine-linked ADC, T-DM1, was analyzed using the Agilent peptide mapping workflow to identify its drug conjugation sites. This workflow included an Agilent AssayMAP Bravo protein sample prep platform for automated sample preparation, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm software. Using this workflow, 26 lysines were confirmed to be drug-conjugated. These results demonstrate that the Agilent peptide mapping workflow enables accurate and reproducible identification of drug conjugation sites in ADCs.

References

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2. Lewis Phillips, G. D.; Li, G.; Dugger, D. L.; Crocker, L. M.; Parsons, K. L.; Mai, E.; Blättler, W. A.; Lambert, J. M.; Chari, R. V. J.; Lutz, R. J. Targeting HER2-Positive Breast Cancer with Trastuzumab-DM1, an Antibody–Cytotoxic Drug Conjugate. *Cancer Res*. **2008**, *68*, 9280–9290.

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