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應用手冊

Analysis of Deamidation and Oxidation in Monoclonal Antibody using Peptide Mapping with UPLC-MS^E

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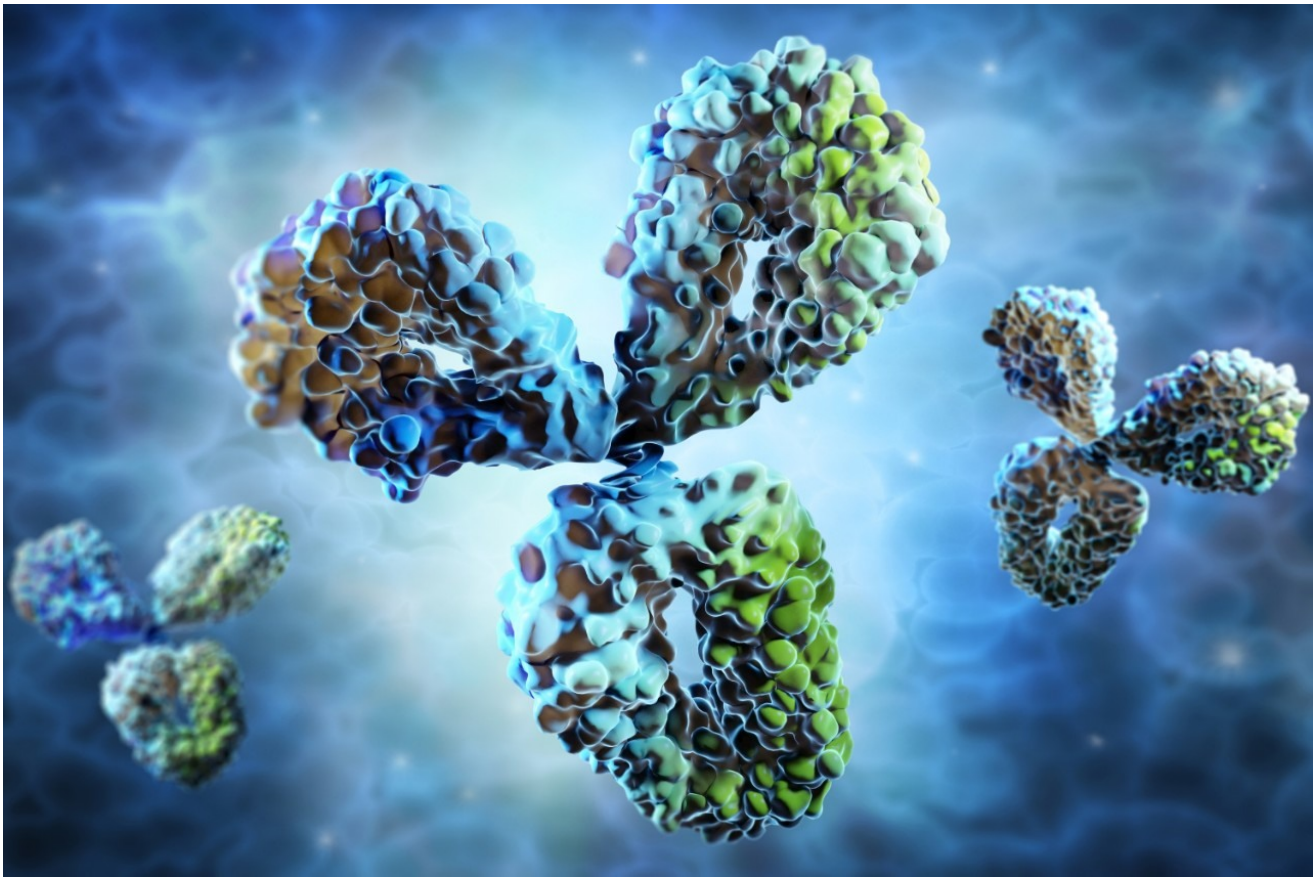

Abstract

This application note demonstrates that UPLC-MS^E is a suitable tool for characterizing PTMs in monoclonal antibodies. Using UPLC-MS^E with the ACQUITY UPLC and SYNAPT MS systems, the identification of peptide sequences and determination of site-specific moditications can be accomplished in a single LC run because of available peptide fragmentation information provided by MS^E.

Introduction

Monoclonal antibodies (mAb) are an important class of protein therapeutics. The mAb production in recombinant expression systems is often a source of post-translational modifications (PTMs), such as various degrees of glycosilation, oxidation, and deamidation.

Asparagine (N) deamidation and methionine (M) oxidation are two common modifications. Deamidation can diminish the activity and stability of an antibody.

Sulfur-containing methionine is a major target for oxidation. Studies[1-3] on mAb revealed oxidation during storage. In this application note, we demonstrate the use of data-independent acquisition mass spectrometry, MS^E, with UltraPerformance LC (UPLC) for profiling of N-deamidation and M-oxidation sites in mAb after prolonged storage.

Unmodified peptides were resolved from site-specific deamidated isoforms using a Waters ACQUITY UPLC System and identified by MS^E. The identities of modified "PENNY" peptides were further confirmed by spiking the sample with synthetic peptide standards. Relative quantitation of the modifications was estimated for both N-deamidation and M-oxidation identified from this antibody.

Experimental

Sample preparation and UPLC-MS^E experimental procedure were identical to a previous description.[4] Briefly, the antibody was digested with enzyme trypsin after reduction with dithiothereitol and alkylation with iodoacetamide. The resulting peptide mixture was separated using an ACQUITY UPLC System and detected by MS^E on a SYNAPT MS System. In order to profile substoichiometric modifications in the antibody, 120 pmol of freshly-prepared tryptic digest was injected for the UPLC-MS^E analysis.

The acquired data were processed by Identity^E Software of ProteinLynx Global SERVER 2.3. The processed data

[4,5] were searched against a database consisting of light and heavy chain sequences of the antibody, with trypsin specificity and one optional miscleavage. Cysteine (C) carbamidome-thylation, asparagine (N) deamidation, glutamine (Q) deamidation, and methionine (M) oxidation were allowed as optional modifications in the search.

UPLC-MS^E experiments for synthetic peptides (as listed in Table 1, purchased from Biomatic, Toronto, Canada) were performed using the same experimental conditions as for the digest. The identities of these peptides were confirmed by MS^E spectra. Their retention time (RT) is recorded in Table 1.

Synthetic Peptides[1]	RT (min)	Elution Order
A)		
GFYPSDIAVEWES NGQPENNYK	59.76	3
GFYPSDIAVEWES isoD GQPENNYK	59.36	1
GFYPSDIAVEWES DGQPENNYK	60.57	7
GFYPSDIAVEWES NGQPEisoD NYK	59.66	2
GFYPSDIAVEWES NGQPEDNYK	60.31	5
GFYPSDIAVEWES NGQPENDYK	60.14	4
GFYPSDIAVEWES NGEPENNYK	60.31	5
GFYPSDIAVEWES DGQPEDNYK	61.06	8
GFYPSDIAVEWES DGQPEDDYK	61.55	9
B)		
DIQMTQSPSSLSASVGDR	42.85	2
DIQMox TQSPSSLSASVGDR	36.07	1

[1] A) N/Q-deamidation, "PENNY" peptide T37 of heavy chain;
B) M-oxidation, T1 of light chain;
isoD - isoaspartic acid; Mox - oxidized M.

Table 1. UPLC elution order of synthetic peptides.

Results and Discussion

The features and operational aspects of UPLC-MS^E have been described previously.[4,5] Briefly, protein digest is separated by UPLC and on-line detected by MS^E. Two sets of MS data are collected in parallel: low-energy (MS) and elevated-energy (MS^E) chromatograms. The collected data are combined for identification of peptides with the help of sequence database searching analysis. The MS^E acquisition is data independent, which ensures sampling of low-abundance peptides and substoichiometric PTMs. The obtained spectra of such peptides allow for identification of peptide modifications. In this study, we focus on characterization of two major degradation

pathways: deamidation and oxidation in mAb the antibody.

In order to profile the modifications, the obtained UPLC-MS^E data were searched against light and heavy chain sequences of the antibdy with N/Q-deamidation (+0.98 Da) and M-oxidation (+16 Da) as optional modifications. The profiling returns 10 modified peptides, including eight deamidated and two oxidized peptides. The modification type, site, relative concentration, and RT of identified modified peptides are listed in Table 2. For comparison, the corresponding unmodified peptides are also included in the Table. All the MS^E spectra of modified peptides were successfully validated.

Protein	Peptide	Start	End	Modification Type	Sequence[1] & Modification Site	MH+	RT (min)	SC (%)[2]
Heavy-Chain	T6	51	59	Deamidation N55	IYPTNGYTR	1085.53	27.69	5.5
	T6	51	59	Deamidation N55	IYPTNGYTR	1085.53	28.8	46.2
	T6	51	59	No Modification	IYPTNGYTR	1084.55	26.96	48.3
	T10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	42.47	65.1
	T10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	45.36	13.4
	T10	77	87	No Modification	NTAYLQMNSLR	1310.66	43.7	21.5
	T23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.13	5.1
	T23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.78	9.3
	T23	278	291	No Modification	FNWYVDGVEVHNAK	1677.81	50.07	85.6
	T36	364	373	Deamidation N364	NQVSLTC*LVK	1162.61	42.85	1.6
	T36	364	373	Deamidation N364	NQVSLTC*LVK	1162.61	49.12	2.1
	T36	364	373	No Modification	NQVSLTC*LVK	1161.63	47.06	96.3
	T37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	59.36	39.2
	T37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	60.57	9.4
	T37	374	395	Deamidation N392 & Deamidation N389	GFYPSDIAVEWESNGQPENNYK & GFYPSDIAVEWESNGQPENNYK	2545.12	60.31	3.2
	T37	374	395	Deamidation N387 + N392	GFYPSDIAVEWESNGQPENNYK	2546.1	61.06	0.4
	T37	374	395	Succinimide Intermidate N387	GFYPSDIAVEWESNGQPENNYK	2527.1	61.38	1.3
	T37	374	395	No Modification	GFYPSDIAVEWESNGQPENNYK	2544.14	59.76	46.4
	T21	252	258	Oxidation M255	DTLMISR	851.43	28.23	4.7
	T21	252	258	No Modification	DTLMISR	835.43	32.76	95.3
Light-Chain	T3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	38.6	1.9
	T3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	41.69	32.6
	T3	25	42	No Modification	ASQDVNTAVAWYQQKPGK	1291	40.34	65.5
	T11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	67.01	2.51
	T11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	74.61	3.5
	T11	127	142	No Modification	SGTASVVC*LLNNFYPR	1797.9	72.05	93.99
	T14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	26.88	3.8
	T14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	28.11	3.2
	T14	150	169	No Modification	VDNALQSGNSQESVTEQDSK	2135.97	27.1	93

[1] C* - carbamidomethyl C

[2] SC - Stoichiometry in percentage, detected in freshly prepared sample

Table 2. Modification type, site, and relative concentration of modified peptides Identified from the antibody.

Of the two detected M-oxidations (Table 2), about 5% of M255 in the heavy chain was oxidized. The elution pattern and MS^E spectra of peptide T21 before and after M255 oxidation is shown in Figure 1. Peptide T21 with M255 oxidation elutes about 4 min earlier than the peptide without modification, because M-oxidation increases the hydrophilicity of peptides.

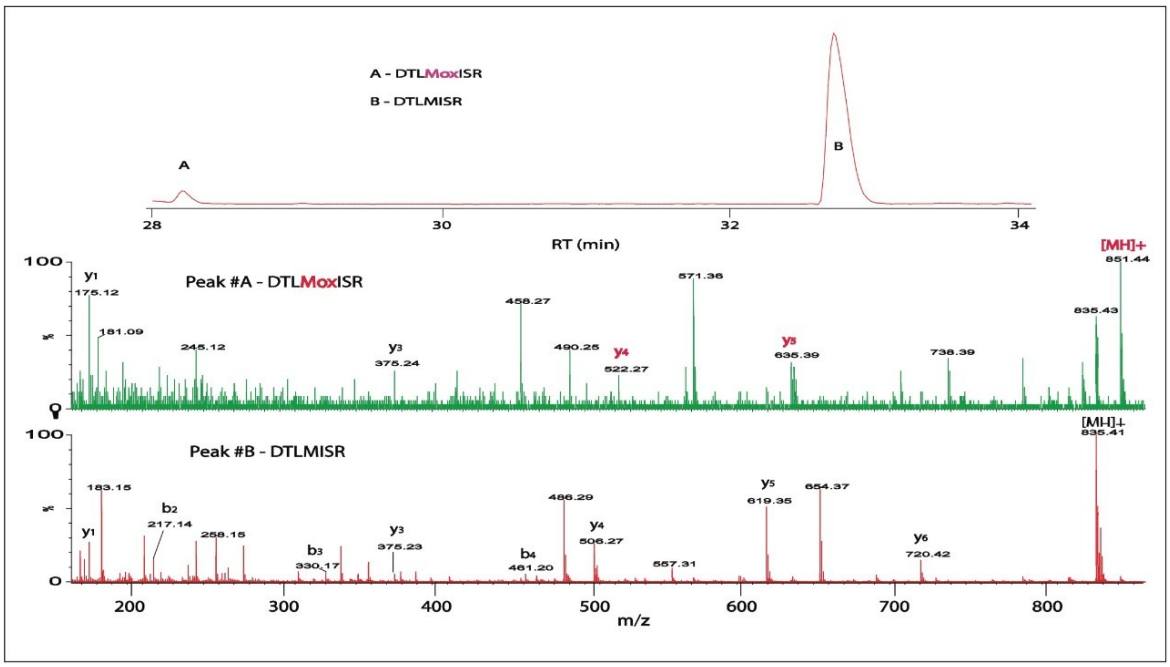

Figure 1. Elution pattern and MS^E spectra of peptide T21 in heavy chain before and after M255-oxidation. Y ions marked in red indicate the M-oxidation.

N-deamidations occur at different levels (Table 2). Each N-deamidation results in two isobaric modified isoforms, isoaspartic acid and aspartic acid, which can't be differentiated by MS. However, N-deamidated peptide with isoaspartic acid elutes earlier than its counterpart with aspartic acid, as confirmed by spiking the sample with synthetic isoaspartic and aspartic isoforms of deamidated peptide standards (see the elution order and RT in Table 1). The elution order is in agreement with literature data.

The peptide T37 of heavy chain with "PENNY" motif has been well studied in literature and suggested as the peptide most susceptible to deamidation in mAb.[6,7] The studies indicate that deamidation occurs on the first two N sites (N387 and N392). In this study, we show separation of six deamidated products (Figure 2). They are identified (see MS^E spectra in Figure 3) to be "PENNY" peptide with isoaspartic acid (isoD) (Peak 2), aspartic acid (D) (Peak 4) and succinimide intermediate (Suc) (Peak 6) of N387 deamidation, as well as newly-found products with deamidation on both N387 and N392 sites (Peak 5), and with deamidation on Q389 (Peak3). N392- deamidated product co-eluted with Q389-deamidated product. This was identified by examining the isotopic patterns of y-series ions in MS^E spectrum of peak 3 (data not shown), and further confirmed by RTs when spiking the corresponding synthetic standards in the sample (Table 1).

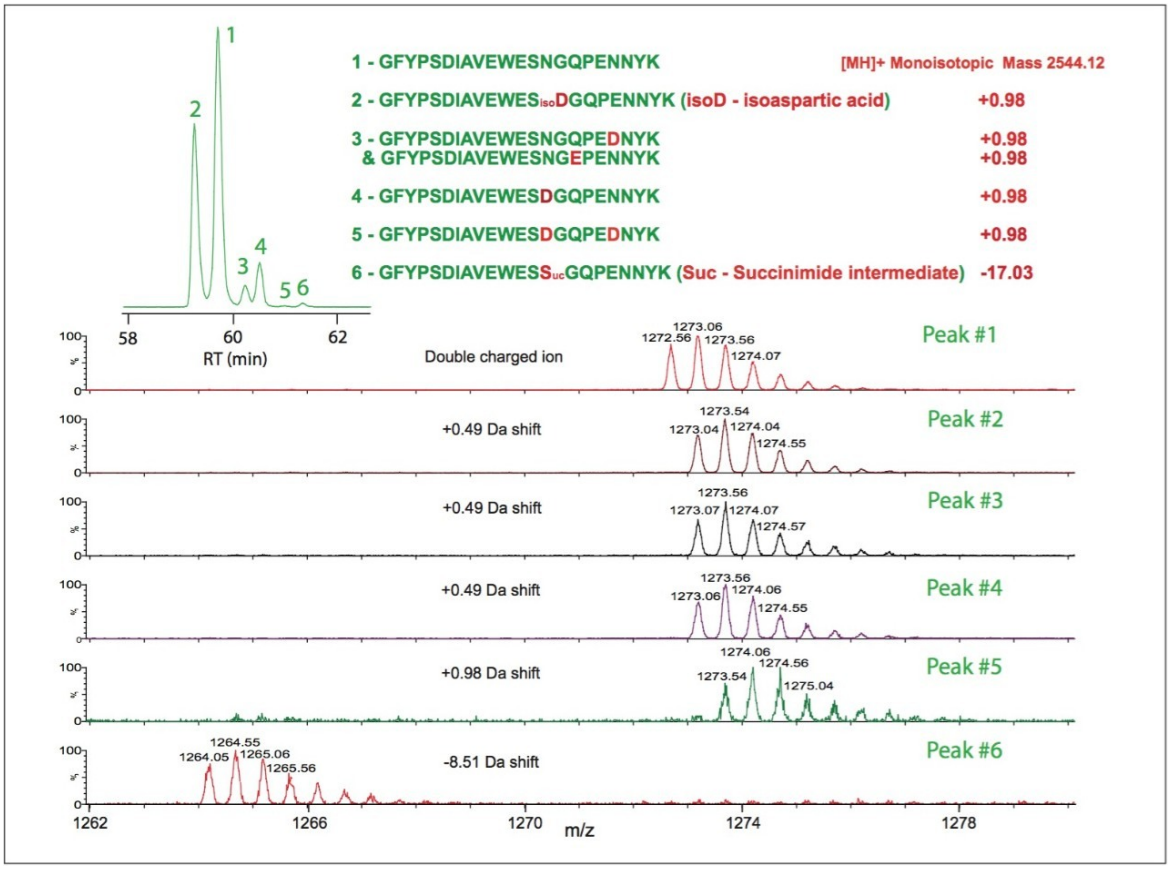

Figure 2. Elution pattern and MS spectra of "PENNY" peptide T37 in Heavy Chain before and after deamidation. Top – Elution pattern; Bottom – MS spectra.

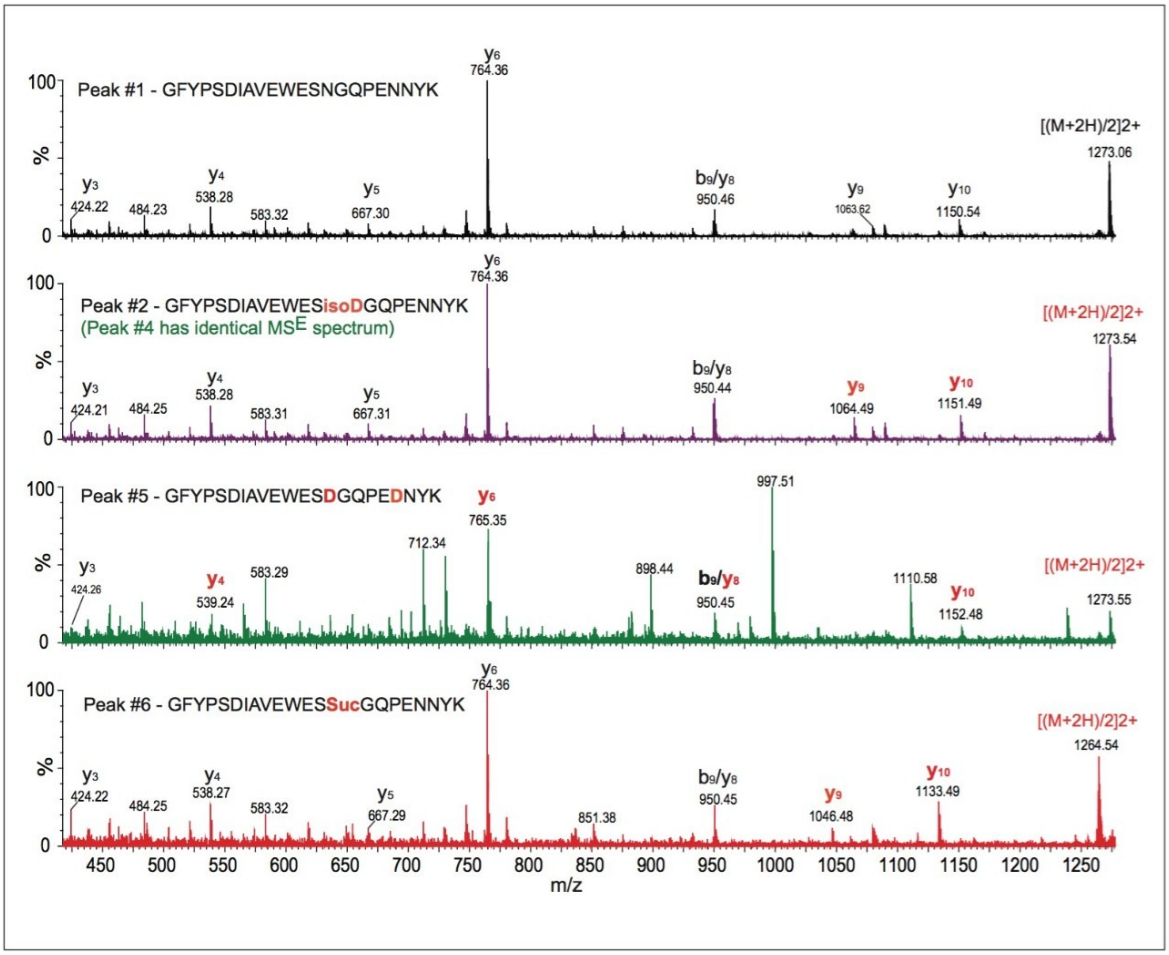

Figure 3. MS^E spectra of Peaks 1, 2 (or 4), 5, and 6 in Figure 2. Y ions marked in red confirm the sequence modifications.

The quantitative results listed in Table 2 show that N387 is the dominant deamidation site of the "PENNY" peptide in this antibody. The total deamidation rate of the "PENNY" peptide was ~ 53.6%.

Comparing the deamidation rate of the "PENNY" peptide with the deamidation of peptides T10 (~ 78.5%) and T6 (~ 51.7%) of heavy chain, we found deamidation on N387, N84, and N55 sites in heavy chain are the major degradation pathways of this antibody.

Conclusion

The results demonstrate that UPLC-MS^E is a suitable tool for characterizing PTMs in monoclonal antibodies. MS^E ensures sampling of low-abundance components and acquires indiscriminately MS^E spectra, enabling accurate identification of modified peptides in an unbiased, reproducible manner. The specific conclusions from this study:

1. UPLC-MS^E is capable of separating, identifying, and quantifying modified peptides and isoforms

2. The high mass resolution and high mass accuracy of the SYNAPT MS System ensures confident identification of modifications with small mass shift (e.g., N-deamidation with 0.98 Da mass difference) and modified isoforms

3. Synthetic peptides are helpful for determining modified isoforms and are required for confirmation

In a previous study[4], we have demonstrated that UPLC-MS^E is able to provide high sequence coverage mapping of mAb tryptic digest, with 97% sequence coverage for both light and heavy chains of the antibody. Therefore, UPLC-MS^E and SYNAPT MS system is an advanced platform for characterization of recombinant proteins, such as monoclonal antibodies.

In current LC-UV/MS peptide mapping methods, the identification of peptide sequences and determination of site-specific modifications typically require multiple tandem mass spectrometry experiments (either DDA MS/MS or targeted MS/MS). The methodology reported here achieves both goals in a single LC run because of available peptide fragmentation information provided by MS^E.

Peptide mapping with UPLC-MS^E improves the analytical efficiency of peptide characterization.

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