An Alternative Approach for Characterization of Impurities and Site-specific Modifications in Protein Drugs

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INTRODUCTION

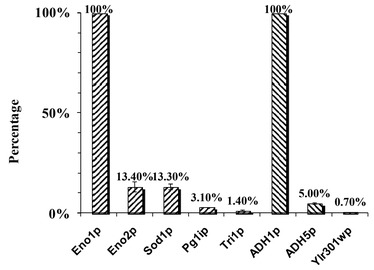
Sequence variants and posttranslational modifications (PTMs) such as glycosylation, deamidation, and oxidation are common in recombinant protein pharmaceuticals. They potentially affect the safety, activity and stability of protein drugs. Effective monitoring of such impurities and PTMs requires sensitive and reproducible methods.

In this study, we have applied an Ultra Performance Liquid Chromatography–Data Independent Acquisition Mass Spectrometry (UPLC/MS^E) approach for characterization and quantification of impurity proteins and site-specific PTMs in protein drugs. The method was evaluated using tryptic digests of yeast enolase, alcohol dehydrogenase (ADH), and a humanized monoclonal antibody (IgG1). Synthetic peptides were used to 1) further confirm the deamidations in the IgG1 "PENNY" peptide, and distinguish aspartyl and isoaspartyl isoforms; 2) exclude potential Met oxidation artifacts produced in the MS ion source; and 3) test ionization efficiency of modified peptides.

ADVANTAGES OF LC/MS^E

- **Unbiased data acquisition**
- **Unbiased sampling of low-abundance peptides**
- **Improved detection sensitivity**
- **Suited for quantification via MS signal intensities**
- **Excellent reproducibility**
- **Improved analytical speed and efficiency**

Relative concentration of impurity proteins identified by UPLC/MS^E analysis and Hi3 quantitation of Enolase and ADH (Normalized to Eno1p or ADH1p)

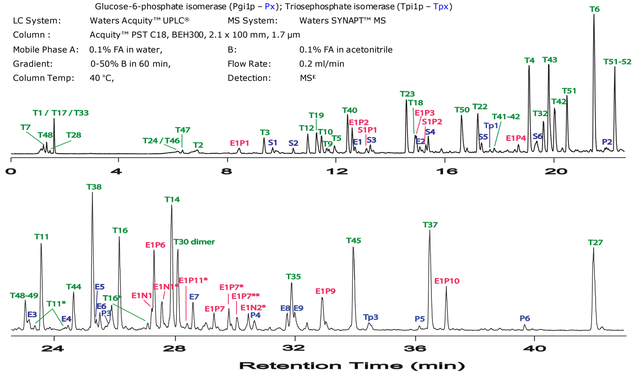


Peptides identified by UPLC/MS^E covered 97% of Enolase protein sequence. MS^E data also identified peaks corresponding to protein impurities , partial/Non-tryptic peptides, and modified peptides.

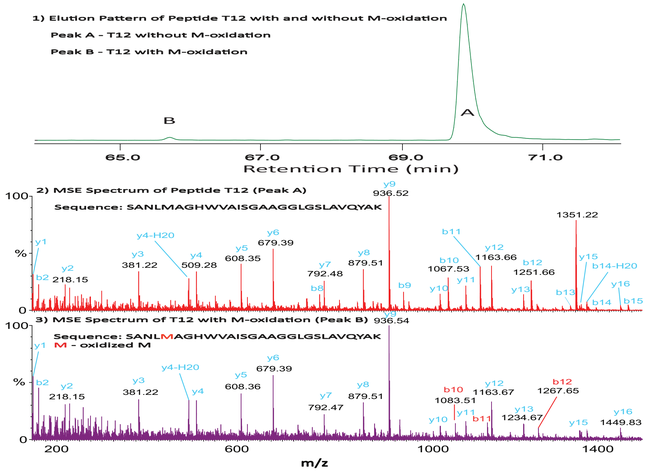
UPLC/MS^E Characterization of Enolase Peptide Map of Tryptic Digestion

Target Protein – Enolase 1 (Eno1p - Txx, trytic peptides; E1Px/E1Nx, partially/Non-tryptic peptides, *- Peptides with deamidation)
Impurity Proteins – Enolase 2 (Eno2p – Ex); Cu-Zn Superoxide dismutase (Sod1p – Sx, S1Px);
Glucose-6-phosphate isomerase (Pgi1p – Px); Triosephosphate isomerase (Tpi1p – Tpx)

LC System:	Waters Acquity™ UPLC®	MS System:	Waters SYNAPT™ MS
Column :	Acquity™ PST C18, BEH300, 2.1 x 100 mm, 1.7 µm		
Mobile Phase A:	0.1% FA in water,	B:	0.1% FA in acetonitrile
Gradient:	0-50% B in 60 min,	Flow Rate:	0.2 ml/min
Column Temp:	40 °C,	Detection:	MS^E



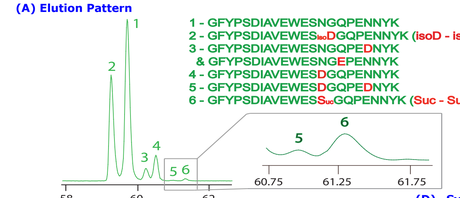
UPLC/MS^E Separation and identification of an ADH peptide and it's modified form (Met oxidized)



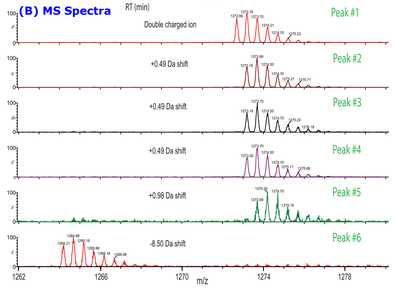
UPLC/MS^E Identification of "PENNYK" peptide (HT37) modifications in a Humanized IgG1 map

(Kept at 4°C for one year). (A) Elution Pattern, (B) MS Spectra, (c) MS^E Spectra, (D) RT of Synthetic Peptides

(A) Elution Pattern



(B) MS Spectra

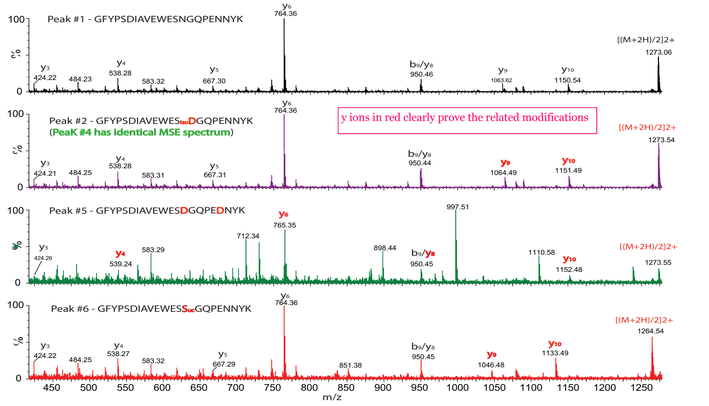


(D) Synthetic peptides were used to confirm RT of HT37 deamidations, and confirm in-sample LT1 Met-oxidation

Synthetic Peptides[1]	RT (min)	Elution Order
A)		
GFYPSDIAVEWESNGQPENNYK	59.76	3
GFYPSDIAVEWESisoDDGQPENNYK	59.36	1
GFYPSDIAVEWESDGQPENNYK	60.57	7
GFYPSDIAVEWESNGQPEisoDNYK	59.66	2
GFYPSDIAVEWESNGQPEDNYK	60.31	5
GFYPSDIAVEWESNGQPENDYK	60.14	4
GFYPSDIAVEWESNGEPENNYK	60.31	5
GFYPSDIAVEWESDGQPEDNYK	61.06	8
GFYPSDIAVEWESDGQPEDDYK	61.55	9
B)		
DIQMTQSPSSLSASVGDR	42.85	2
DIQMoxTQSPSSLSASVGDR	36.07	1

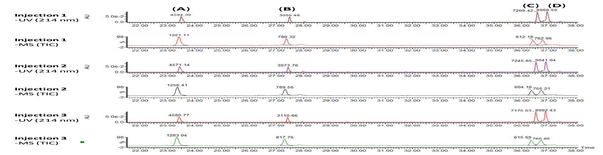
[1] A) N/Q-deamidation, "PENNY" peptide T37 of heavy chain; B) M-oxidation, T1 of light chain;
isoD - isoaspartic acid; Mox - oxidized M.

(c) MSE Spectra



Ionization efficiency (by LC/MS^E analysis) of Synthetic HT37(PENNYK) and LT1 and Peptides

1) UPLC/UV (214 nm) and UPLC/MS(TIC) Chromatograms. Peak A - DIQ*Mox*TQSPSSLSAVGDR, Peak B- DIQ*M*TQSPSSLSAVGDR, Peak C - GFPSDIAVEWESNGQPENNYK, Peak D - GFPSDIAVEWES*D*GQPENNYK



2) Ionization Efficiency of Tested Modified Peptides (IE-modified/unmodified) Calculated by

IE-modified/unmodified = (MS(modified)/MS(unmodified))/(UV(modified)/UV(unmodified)) * 100%

Injection	MS(A)*	MS(B)	MS(C)	MS(D)	UV(A)	UV(B)	UV(C)	UV(D)	MS(A)/MS(B)	UV(A)/UV(B)	IE-oxidized/Unmodified**	MS(D)/MS(C)	UV(D)/UV(C)	IE-deamidated/Unmodified
1	1251.11	780.32	612.18	762.96	4594.39	3050.48	7209.42	8960.59	1.6033	1.5061	106.45	1.2463	1.2429	100.27
2	1256.41	789.56	604.16	755.21	4571.44	3073.76	7245.65	9041.04	1.5913	1.4872	106.99	1.2500	1.2478	100.18
3	1283.00	817.76	615.69	760.86	4580.78	3110.66	7175.03	8902.43	1.5689	1.4726	106.54	1.2358	1.2408	99.60
Average ± SD											106.66 ± 0.29			100.02 ± 0.36

* MS(X) - peak area of MS total ion chromatographic peak X; UV(X) - peak area of UV (214 nm) chromatographic peak X.

Modification Type, Site and Stoichiometry of Modified Peptides Identified from the Humanized IgG1

Protein	Peptide	Start	End	Modification Type	Sequence & Modification Site	MH+	RT (min)	SC (%)[1]
Heavy-Chain	HT6	51	59	Deamidation N55	IYPTNGYTR	1085.53	27.69	5.5
	HT6	51	59	Deamidation N55	IYPTNGYTR	1085.53	28.8	46.2
	HT6	51	59	No Modification	IYPTNGYTR	1084.55	26.96	48.3
	HT10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	42.47	65.1
	HT10	77	87	Deamidation N84	NTAYLQMNSLR	1311.64	45.36	13.4
	HT10	77	87	No Modification	NTAYLQMNSLR	1310.66	43.7	21.5
	HT23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.13	5.1
	HT23	278	291	Deamidation N289	FNWYVDGVEVHNAK	1678.79	51.78	9.3
	HT23	278	291	No Modification	FNWYVDGVEVHNAK	1677.81	50.07	85.6
	HT26	305	320	Deamidation N318	VVSVLTVLHQDWLNGK	1809.01	65.9	21
	HT26	305	320	Deamidation N318	VVSVLTVLHQDWLNGK	1809.01	67	8.5
	HT26	305	320	No Modification	VVSVLTVLHQDWLNGK	1808.01	66.1	70.5
	HT36	364	373	Deamidation N364	NQVSLTC*LVK[2]	1162.61	42.85	1.6
	HT36	364	373	Deamidation N364	NQVSLTC*LVK	1162.61	49.12	2.1
	HT36	364	373	No Modification	NQVSLTC*LVK	1161.63	47.06	96.3
	HT37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	59.36	39.2
	HT37	374	395	Deamidation N387	GFYPSDIAVEWESNGQPENNYK	2545.12	60.57	9.4
	HT37	374	395	Deamidation N392 & Deamidation N389	GFYPSDIAVEWESNGQPENNYK & GFYPSDIAVEWESNGQPENNYK	2545.12	60.31	3.2
	HT37	374	395	Deamidation N387 + N392	GFYPSDIAVEWESNGQPENNYK	2546.1	61.06	0.4
	HT37	374	395	Succinimide Intermidate N387	GFYPSDIAVEWESNGQPENNYK	2527.1	61.38	1.3
	HT37	374	395	No Modification	GFYPSDIAVEWESNGQPENNYK	2544.14	59.76	46.4
	HT21	252	258	Oxidation M255	DTLMISR	851.43	28.23	4.7
	HT21	252	258	No Modification	DTLMISR	835.43	32.76	95.3
Light-Chain	LT1	1	18	Oxidation M4	DIQMTQSPSSLSASVGDR	1894.88	43.41	in-source
	LT1	1	18	No Modification	DIQMTQSPSSLSASVGDR	1878.88	43.41	
	LT3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	38.6	1.9
	LT3	25	42	Deamidation N30	ASQDVNTAVAWYQQKPGK	1291.98	41.69	32.6
	LT3	25	42	No Modification	ASQDVNTAVAWYQQKPGK	1291	40.34	65.5
	LT11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	67.01	2.51
	LT11	127	142	Deamidation N137	SGTASVVC*LLNNFYPR	1798.88	74.61	3.5
	LT11	127	142	No Modification	SGTASVVC*LLNNFYPR	1797.9	72.05	93.99
	LT14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	26.88	3.8
	LT14	150	169	Deamidation N158	VDNALQSGNSQESVTEQDSK	2136.95	28.11	3.2
	LT14	150	169	No Modification	VDNALQSGNSQESVTEQDSK	2135.97	27.1	93

[1] SC - Stoichiometry in percentage, detected in freshly prepared sample; [2] C* - Carbamidomethyl C

CONCLUSIONS

1. **UPLC/MS^E analysis of protein tryptic digests yielded qualitative and quantitative information about protein impurities and the presence of protein modifications.**
2. **Unbiased data acquisition with UPLC/MS^E provides high sequence coverage, especially for peptides with low-stoichiometry modfications (<0.5%).**
3. **Reproducible high resolution UPLC peptide separations ensured confident identification of isobaric modifications with sub Da mass differences (e.g., single and double Asn-deamidated peptides)**
4. **Synthetic peptides were helpful for distinguishing modified peptides isoforms, and evaluating the ionization efficiency of peptides and their modified forms.**

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