

# Neutral pH Reversed Phase LC-MS Methodology for the Recovery and Characterization of Highly Acidic Peptides of Enbrel

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## Introduction

### Challenge:

- Some acidic peptides such as phosphopeptides and highly sialylated glycopeptides experience significant or complete analyte loss in conventional reversed phase liquid chromatography (LC).
- Conventional RP-LC mobile phase conditions are acidic in nature, causing residual metal oxides on the surfaces in the LC flow path to have non-specific chelation reactions with negatively charged species.

### Solution:

- We employ a neutral pH RPLC-MS method with post-column addition of acid, which allows us to detect and characterize these previously “unrecoverable” O-glycopeptides in conventional RPLC-MS methods. Herein, we demonstrate recovery of Enbrel™ highly sialylated T20 peptide which has previously only been detected after sialic acids have been removed<sup>1</sup>.

## Experimental

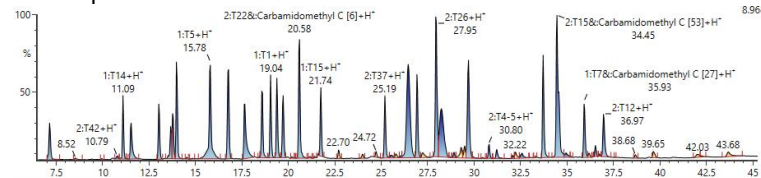
- LC-MS System: ACQUITY™ H-Class coupled to Xevo™ G2-XS
- ACQUITY Premier CSH C18 130Å, 1.7µm, 2.1 x 100mm Column
- MPA: 10mM Ammonium Acetate, pH 7.5
- MPB: 100% Acetonitrile
- Column Temp: 65 °C
- Gradient: 1-45%B in 50 min
- Load: 1-2 µg NIST mAb or Enbrel tryptic digest

- Post-Column Infusion of Formic Acid from integrated MS fluids (1:10 ratio to LC flow rate)

## Results

### NIST mAb Tryptic Digest

#### Acidic pH Mobile Phase



#### Neutral pH Mobile Phase

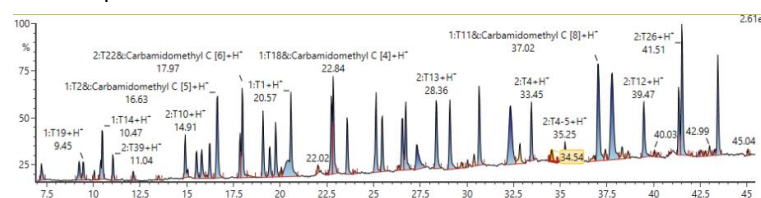


Figure 1. TICs for NIST mAb tryptic digest in acidic vs neutral pH mobile phase conditions. 94% sequence coverage is achieved in both—all peptides matched in acidic conditions are also detected in neutral pH.

### Enbrel T20 Highly Sialylated O-glycopeptides

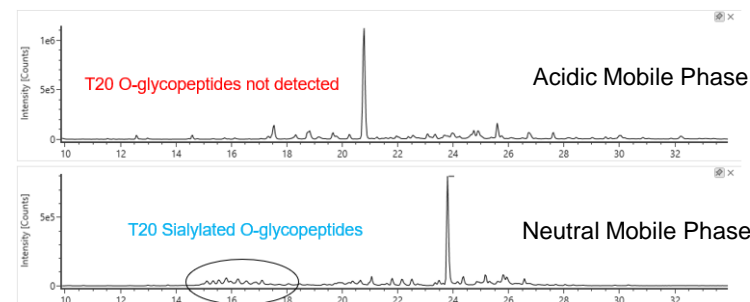


Figure 2. Extracted ion chromatogram of high energy channel of Enbrel tryptic digest in acidic (top) vs neutral (bottom) pH conditions, extracting for m/z 292 & 274, common oxonium ions for glycans containing sialic acid. Sialic acid-containing O-glycopeptides of T20 are only detected in the neutral pH condition.

Peptide #	Peptide	# HexNAc	# Hex	# NeuAc	Observed m/z	Relative Abundance
T1	LPAQVAFPTYAPEPGSTCR	0	0	0	688.00 (3+)	91.8%
		1	1	1	906.75 (3+)	4.5%
		1	1	2	1003.78 (3+)	3.6%
T16-18	PGTETSDVCKPCAPGTFSS NTTSSTDICRPHQICNVVAIP GNASMDAVCTSTSPTR (De-N-glycosylated)	0	0	0	1203.14 (5+)	39.4
		1	1	0	1276.17 (5+)	1.5
		1	1	1	1334.39 (5+)	46.2
		1	1	2	1392.60 (5+)	12.9
T19	SMAPGAVHLQPVSTR	2	2	2	987.44 (3+)	79.2%
		2	1	1	836.39 (3+)	7.6%
		2	2	1	890.42 (3+)	2.6%
		1	1	1	768.70 (3+)	8.5%
		2	1	0	739.36 (3+)	2.1%
T20	SQHTQPTPEPSTAPSTSFL PMGSPPAEGSTGDEPK	7	7	6	1613.65 (5+)	4.0%
			6	7	1639.47 (5+)	3.1%
			7	7	<b>1671.87 (5+)</b>	<b>9.8%</b>
			7	8	<b>1730.09 (5+)</b>	<b>17.3%</b>
			7	9	<b>1788.31 (5+)</b>	<b>17.4%</b>
			7	10	<b>1846.53 (5+)</b>	<b>12.9%</b>
			7	11	1904.75 (5+)	4.2%
			6	6	1581.03 (5+)	1.2%
			6	8	1697.69 (5+)	2.2%
			6	9	1755.91 (5+)	1.7%
			T22-23	THTCPPCPAPELLGGPSVF LFPPKPK	6	6
5	7	1566.25 (5+)				1.9%
6	7	<b>1598.65 (5+)</b>				<b>6.4%</b>
5	8	1624.46 (5+)				1.3%
6	8	<b>1656.86 (5+)</b>				<b>7.2%</b>
6	9	<b>1715.08 (5+)</b>	<b>6.3%</b>			
T22-23	THTCPPCPAPELLGGPSVF LFPPKPK	0	0	0	711.873 (4+)	93.6%
		1	1	1	875.9321 (4+)	4.1%
		1	1	2	948.7058 (4+)	2.3%

Table 1. List of ALL O-glycopeptides in Enbrel tryptic digest using neutral pH reversed phase chromatography. The previously “unrecoverable” T20 peptide was found with 6-7 Core 1 occupied sites, with a total of 6-11 sialic acids among them.

## Conclusions

Neutral pH reversed phase methodology is successful in mitigating chelation effects and improve recovery of highly sialylated peptides of Enbrel, while maintaining expected sequence coverage for typical peptide mapping experiments. We are currently investigating alternative fragmentation techniques such as ECD for site localization of the O-glycans.