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## Abstract

For peptide separations, selectivity can be changed by altering mobile phase pH. In this application note, we show the selectivity differences when separating peptide standards and selected NISTmAb™ critical quality attribute (CQA) tryptic peptides on an XBridge™ Premier Peptide BEH™ C<sub>18</sub> 130 Å Column and an XSelect™ Premier Peptide CSH™ C<sub>18</sub> Column, using mobile phase pH ranging from 2.7 to 9.2. Extracted Ion Chromatograms (XIC) obtained from BioAccord™ LC-MS System and waters\_connect™ show distinct selectivity difference between selected peptides and their deamidated forms.

## Benefits

- Mobile phase pH can change selectivity of reversed-phase peptide separations, helping to separate difficult critical pairs
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## Introduction

It has been shown that pH of the mobile phase can alter the selectivity of reversed-phase separation. High degree of orthogonality was achieved when varying the pH of the peptide separation.<sup>1</sup> In this application note, the selectivity difference is shown in separating a peptide standard mixture as well as a mAb tryptic digest sample under mobile phase pH of 2.7, 3.1, 3.8, 5.5, 6.5, and 9.2. As an example, separation of a tryptic peptide and its deamidated forms is shown in Extracted Ion Chromatograms (XIC) obtained from BioAccord LC-MS System

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and waters\_connect. The selectivity is drastically different under various mobile phase pH separation, which indicates altering mobile phase pH could be beneficial in separating difficult critical pairs.

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## Experimental

### Sample Description

MassPREP™ Peptide Mixture (p/n: 186002338 <<https://www.waters.com/nextgen/global/shop/standards--reagents/186002338-massprep-peptide-mixture-5-pk.html>> ) and mAb Tryptic Digestion Standard (p/n: 186009126 <<https://www.waters.com/nextgen/global/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html>> ) were reconstituted in 100 µL and 80 µL of 0.1% formic acid, respectively.

### LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS (Total system bandspread $5\sigma \leq 7 \mu\text{L}$ )
Detection:	ACQUITY BioAccord MS detection, TUV @ 214 nm
Column(s):	XBridge Premier Peptide BEH C <sub>18</sub> 130 Å, 2.5 µm, 2.1 x 150 mm Column (p/n: 186009835) XSelect Premier Peptide CSH C <sub>18</sub> 130 Å, 2.5 µm, 2.1 x 150 mm Column (p/n: 186009906)
Column temperature:	60 °C
Sample temperature:	10 °C
Injection volume:	15 µL, 10 µL
Flow rate:	0.2 mL/min

## Mobiles phases

A: 0.1% Formic acid in H<sub>2</sub>O (pH 2.7)

B: 0.1% Formic acid in Acetonitrile

A: 0.1% Formic acid, 10 mM Ammonium Formate in H<sub>2</sub>O (pH 3.1)

B: 0.1% Formic acid, 10 mM Ammonium Formate in 80% Acetonitrile

A: 10 mM Ammonium Formate in H<sub>2</sub>O (pH 3.8)

B: 10 mM Ammonium Formate in 80% Acetonitrile

A: 10 mM Ammonium Acetate in H<sub>2</sub>O (pH 5.5)

B: 10 mM Ammonium Acetate in 80% Acetonitrile

A: 10 mM Ammonium Acetate in H<sub>2</sub>O (pH 6.5)

B: 10 mM Ammonium Acetate in 80% Acetonitrile

A: 10 mM Ammonium Formate in H<sub>2</sub>O (pH 9.2)

B: 10 mM Ammonium Formate in 80% Acetonitrile

## Gradient Table for MassPREP Peptide

### Mixture, mobile phase pH 2.7\*

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.2	99.5	0.5	Initial
2.0	0.2	99.5	0.5	6
22.0	0.2	45	55	6
25.0	0.2	5	95	6
26.0	0.2	5	95	6
28.0	0.2	99.5	0.5	6
40.0	0.2	99.5	0.5	6

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*\*: For mobile phase pH 3.1 to pH 9.2, the gradient goes from 0.6% B to 68.8% B due to 80% ACN in mobile phase B.*

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## Gradient Table for mAb Tryptic Digestion

Standard, mobile phase pH 2.7\*\*

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.2	99.5	0.5	Initial
2.0	0.2	99.5	0.5	6
62.0	0.2	50	50	6
65.0	0.2	5	95	6
66.0	0.2	5	95	6
68.0	0.2	99.5	0.5	6
80.0	0.2	99.5	0.5	6

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**\*\*:** For mobile phase pH 3.1 to pH 9.2, the gradient goes from 0.6% B to 62.5% B due to 80% ACN in mobile phase B.

## ACQUITY RDa Detector Settings

Mode:	Full scan with fragmentation
Mass range:	50–2000 <i>m/z</i>
Polarity:	Positive
Sample rate:	5 Hz
Cone voltage:	30 V
Fragmentation cone voltage:	60 V–120 V
Capillary voltage:	1.20 kV
Desolvation temperature:	350 °C

## Data Management

LC/MS software:

waters\_connect

## Results and Discussion

UV traces of a MassPrep Peptide Standard Mixture separation (Table 1: Components) under various mobile phase pH on two Premier Peptide C<sub>18</sub> columns are shown in Figure 1. The peaks were identified based on their mass obtained from BioAccord RDa Mass Detector and waters\_connect. While selectivity is somewhat different on the two columns used, the more significant difference in selectivity is observed due to altering mobile phase pH. It is worth noting that at pH >5.5, the recovery of peaks 9 (enolase T37) and 10 (melittin) decreased. Although the reason was not studied, it is suspected that the solubility of the large and low pI enolase T37 peptide was reduced, and the ionic retention was increased for the large and high pI (>12) melittin peptide in the higher pH mobile phases. Nonetheless, caution should be used when separating large peptides under more basic conditions. In addition, method reproducibility should be carefully assessed when using high pH mobile phases as small changes in mobile phase pH can alter selectivity significantly.

Peak #	Component name	Retention time *(minutes)	Molecular weight **(g/mol)	pKa	Peptide sequence
1	Allanatoxin (VO marker)	0.76	158.0440	–	–
2	RASG-1	0.48	1000.4938	9.34	RGDSPASSKP
3	Angiotensin frag. 1–7	9.59	898.4661	7.35	DRVYIHP
4	Bradykinin	12.04	1059.5613	12.00	RPPGFSPFR
5	Angiotensin II.	13.06	1045.5345	7.35	DRVYIHPF
6	Angiotensin I.	14.92	1295.6775	7.51	DRVYIHPFHL
7	Renin substrate	16.82	1757.9253	7.61	DRVYIHPFHLLVYS
8	Enolase T35	19.18	1871.9604	7.34	WLTGPQLADLYHSLMK
9	Enolase T37	21.55	2827.2806	3.97	YPIVSIEDPFAEDDWEAWSHFFK
10	Melittin	24.93	2845.7381	12.06	GIGAVLKVLTTGLPALISWIKRQQ

*Table 1. A list of peptides (MassPREP Peptide Mixture, p/n: 186002338) that are separated under various pH conditions using a gradient of increasing acetonitrile concentration (please see Figure 1).*

## Premier peptide BEH C<sub>18</sub> 130Å

## Premier peptide CSH C<sub>18</sub>

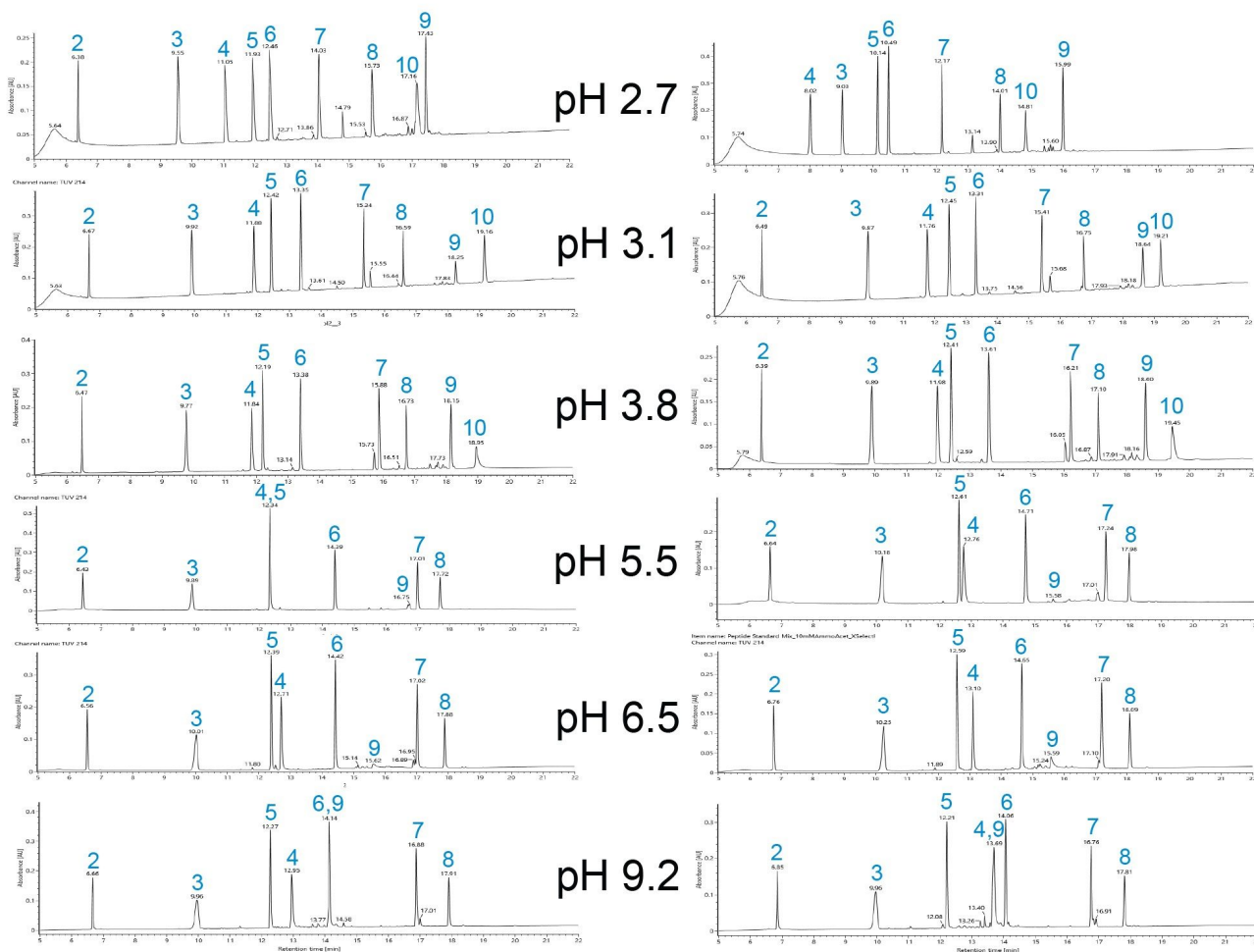


Figure 1. UV traces of a peptide standard mixture separation under various mobile phase pH on two Premier Peptide C<sub>18</sub> columns: XBridge Premier Peptide BEH C<sub>18</sub> 130 Å, 2.5 µm, 2.1 x 150 mm Column (p/n: 186009835) and XSelect Premier Peptide CSH C<sub>18</sub> 130 Å, 2.5 µm, 2.1 x 150 mm Column (p/n: 186009906) using a gradient of increasing acetonitrile concentration.

Figure 2a shows the Base Peak Intensity (BPI) of NISTmAb tryptic digest separation on the XBridge Premier Peptide BEH C<sub>18</sub> 130 Å Column under various mobile phase pH using a gradient of increasing acetonitrile concentration. Arrows with the same color represent the same tryptic peptide identified by waters\_connect. Heavy chain T37 peptide (GFYPSDIAVEWESNGQPENNYK) is highlighted in red. Figure 2b shows the Extracted

Ion Chromatograms (XIC) of heavy chain T37 peptide and its deamidated forms. The selectivity is drastically different under different mobile phase pH conditions. Figure 2c shows correlation plots of retention time of 42 peptides under various pH verses the retention time of these peptides under pH 2.7. The correlation ( $R^2$ ) decreases as the mobile phase pH increases. This shows that higher pH conditions exhibit higher orthogonality (lower  $R^2$ ) to the low pH condition (pH 2.7), consistent with previous reports.<sup>1</sup> Based on the ten most intense peptide ions, the average response for pH 9.2 ammonium formate was approximately one-third of that observed for 0.1% formic acid (pH 2.7).

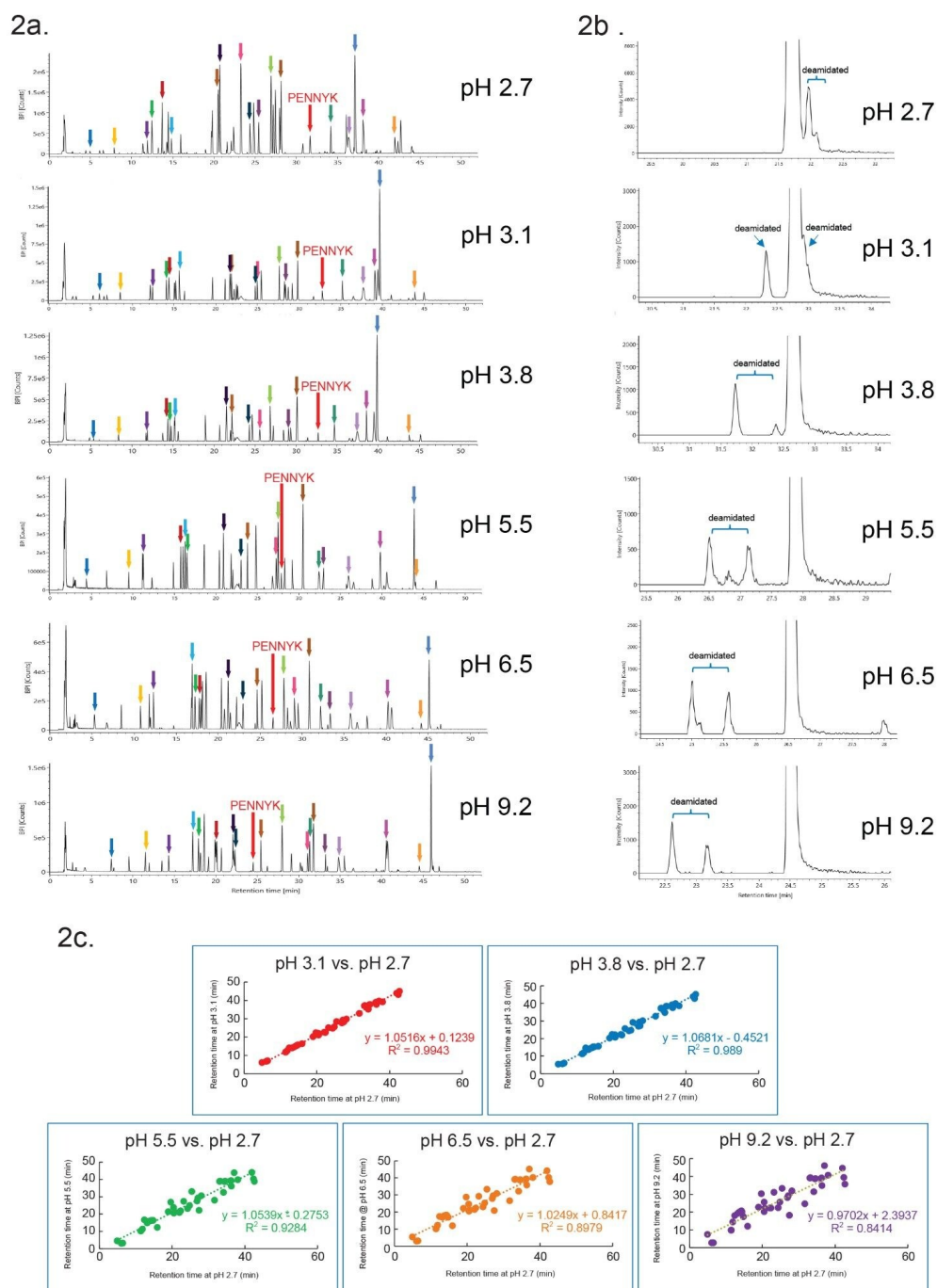


Figure 2. Separation of NISTmAb tryptic peptides on an XBridge Premier Peptide BEH  $C_{18}$  130 Å 2.5  $\mu$ m 2.1 x 150 mm Column under various mobile phases pH. a. Base Peak Intensity (BPI) chromatograms obtained from a BioAccord LC-MS System. Arrows with



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*the same color represent the same peptide identified by waters\_connect. HC T37 peptide (GFYPSDIAVEWESNGQPENNYK) is highlighted in red. b. Extracted Ion Chromatograms (XIC) of HC T37 peptide and its deamidated forms under various mobile phase pH. c. Correlation of retention time under various mobile phase pH.*

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## Conclusion

Reversed-phase separations of peptide standard mixtures and mAb tryptic digest samples were performed on two Premier Peptide C<sub>18</sub> columns under various mobile phase pH ranging from pH 2.7 to pH 9.2. Significant differences in selectivity were observed using different mobile phase pH using a gradient of increasing acetonitrile concentration, which can be helpful in method development in separating difficult peptide critical pairs. Generally, a low pH formic acid (*e.g.*, 0.1% FA for MS Detection) or trifluoroacetic acid (*e.g.*, 0.1% TFA for Optical Detection) reversed-phase eluent additive will be used for routine synthetic peptide and peptide mapping separations. However and as shown, deploying a high pH separation as part of a heightened characterization comparability study can be beneficial because the different peptide separation selectivity obtained (*i.e.* orthogonality) provides a more thorough assessment. In addition, due to the high pH stability of the hybrid-silica based BEH and CSH peptide columns, low and high pH methods can be run on the same column within the same sample set.

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## References

1. Gilar M., Olivova P., Daly A.E., Gebler J.C. Development of Orthogonal Separation Methods for 2D-HPLC (MS/MS) Analysis of Peptides. Waters Poster, 2005.
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