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Evaluating 2D-RP/RP Fractionation Capabilities of the ACQUITY UPLC M-Class System with 300-µm I.D. Configuration

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Abstract

In this application note, we demonstrate that the ACQUITY UPLC M-Class System with 2D Technology (300 µm I.D., high capacity configuration) is capable of providing high chromatographic resolving power required for the quantitative HCP analysis and the separation of other complex peptide samples.

Benefits

To provide the experimental procedure used for testing the chromatographic performance of the ACQUITY UPLC M-Class System with a 300-µm ID configuration.

Introduction

The ACQUITY UPLC M-Class System with 2D Technology (300-µm I.D., high capacity configuration) has been

recently used for the analysis of low-abundance host cell proteins (HCPs) that are typically present in the concentration range of 1–100 ppm (ng/mg) in a highly-purified biotherapeutic protein.[1-5] To successfully quantify these levels of HCPs, the dynamic range of the separation needs to be extended. In order to extend the dynamic range of the separation to detect and quantify low level HCPs, an orthogonal 2D reversed-phase (RP/RP) approach is used where sample is loaded at pH 10 and separated at pH 2.5.[6]

The microscale 2D-LC separation system consists of a 1st dimension reversed-phase (RP) column (XBridge Peptide BEH, C_{18}, 300Å, 5 µm, 1.0 x 50 mm) for fractionation of the peptide mixture under basic conditions (pH 10), an ACQUITY UPLC M-Class Symmetry C_{18}, 2D HCP Trap Column (5 µm, 300 µm x 25 mm) for retaining the peptides eluted in each fraction from the 1st dimension column, and an ACQUITY UPLC M-Class HSS T3 Column (100Å, 1.8 µm, 300 µm x 150 mm) analytical column operating under acidic conditions (pH 2.5) for high-resolution peptide separations (Figure 1). Fractions are eluted from the 1st dimension column using discontinuous step gradients of increasing organic concentration.

While a typical 2D-LC experiment involves 3, 5, or 10 fractionation steps, the sample complexity determined the number of elution steps required. As peptides elute from the 2nd dimension, a quadruple time-of-flight mass spectrometer is used to detect the peptides and their fragments by alternating collision cell energy between a low and elevated energy state (MS^E data acquisition mode).

Here we demonstrate the chromatographic performance of the ACQUITY UPLC M-Class System with 2D Technology for the five-step fractionation of two peptide mixtures: MIX-1 MassPREP Digestion Standard and ENL Digestion Standard.

Experimental

LC conditions

2D-LC system:	ACQUITY UPLC M-Class System with 2D Technology (300-µm ID, high capacity configuration) using on-line dilution ACQUITY UPLC M-Class 300-µm scale tubing kit

	(p/n 205001432)
First LC dimension (operating at pH 10.0)	
Column:	XBridge Peptide BEH C_{18}, 300Å, 5 µm, 1.0 x 50 mm (p/n 186003615)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18} 2D HCP Trap Column, 5 µm, 300 µm x 25 mm (p/n 186007499)
Flow rate:	10 µL/min
Mobile phase A:	20 mM ammonium formate in DI water (pH=10)
Mobile phase B:	Acetonitrile (ACN)
Diluting solution:	0.1% TFA in DI water, 90 µL/min flow rate
Step-elution gradient:	A 5-step elution gradient was used for the fractionation of the peptide mixture at pH 10. The percentages of ACN in each step were: 11.4, 14.7, 17.4, 20.7, and 50.0%, respectively.

Example of the step elution gradient (Fraction 1)

Time(min)	Flow (µL/min)	%B
0.0	10	0.0
0.5	10	0.0

Time(min)	Flow (µL/min)	%B
1.0	10	11.4
6.0	10	11.4
7.0	10	0.0
20.0	10	0.0

Second LC dimension
(operating at pH 2.5)

Column:	ACQUITY UPLC M-Class HSS T3 Column, 100Å, 1.8 µm, 300 µm x 150 mm (p/n 186007472)
Column temp.:	60 °C
Flow rate:	10 µL/min
Mobile phase A:	0.1% FA in DI water (pH=2.5)
Mobile phase B:	0.1% FA in ACN

Gradient elution table:

Time(min)	Flow (µL/min)	%B
0	10	3
40	10	45

Time(min)	Flow (µL/min)	%B
41	10	90
42	10	90
43	10	3
50	10	3

MS conditions

MS system:	SYNAPT G2-S HDMS Mass Spectrometer equipped with a narrow bore ESI probe (p/n 186007529)
Ionization mode:	ESI+
TOF resolution:	Resolution mode (~22,000)
Capillary voltage:	2.6 kV
Cone voltage:	40 V
Source offset:	60 V
Source temp.:	100 °C
Desolvation temp:	150 °C
Desolvation gas flow:	500 L/h

Lockspray:	0.2 µM GFP in 50% ACN, 0.1% FA, sampled every 4 min	
Acquisition parameters:	*m/z* range: 100–1990 0.5 sec scans, 50 min runtime	
MS^E settings	Trap	Transfer
Low energy scans (precursors)	4V	4V
High-energy fragmentation scans	20-45V ramp	10V

**Observation: A Xevo G2-XS Mass Spectrometer using similar MS conditions is suited for this application as well.*

Data management

MassLynx Software v4.1 (SCN 916)

Sample preparation

A tryptic digest mixture derived from four different proteins (MassPREP Digestion Standard MIX-1, p/n 186002865) was diluted with 1 mL of 20 mM ammonium formate (pH 10) to prepare a solution containing 1 µM yeast alcohol dehydrogenase (ADH), 1 µM rabbit glycogen phosphorylase b (PHO), 0.4 µM yeast enolase 1 (ENL), and 0.6 µM bovine serum albumin (BSA). In addition, a stock solution containing 1 µM of the ENL MassPREP Digestion Standard (p/n 186002325), was prepared by adding 1 mL of 20 mM ammonium formate to the ENL glass vial (1 nmol). The digest was then diluted 10 fold to prepare a solution containing 100 nM ENL digest. The injection volume for each sample was 50 µL.

Results and Discussion

Two samples, a tryptic digest mixture derived from four different proteins (MassPREP Digestion Standard MIX-1) and an enolase digest (ENL MassPREP Digestion Standard) were studied using 2D-RP/RP chromatography with the configuration shown in Figure 1.

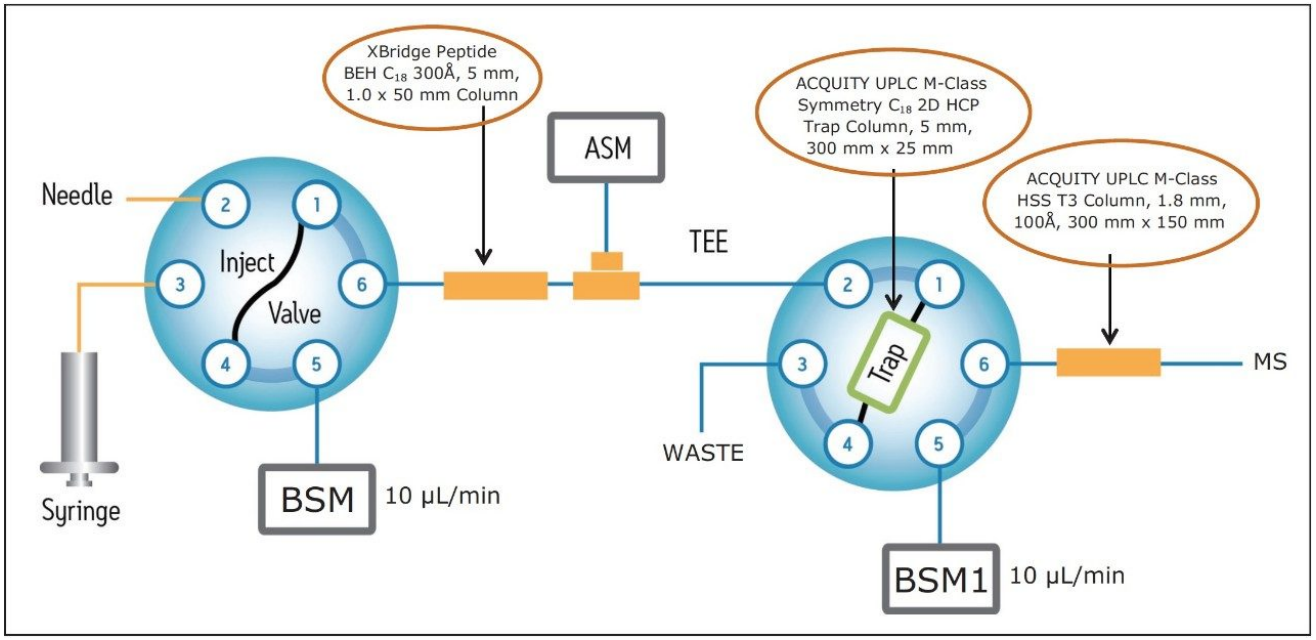

Figure 1. Fluidic configuration of the two-dimensional high-pH/low-pH RP/RP ACQUITY UPLC M-Class System (300 µm ID configuration) employing on-line dilution.

Figure 2 displays the base peak chromatograms (2A) and several extracted mass chromatograms (2B) recorded for the 2D-LC RP/RP five-step fractionation experiment performed on the MIX-1 digest using the parameters listed in the Experimental section.

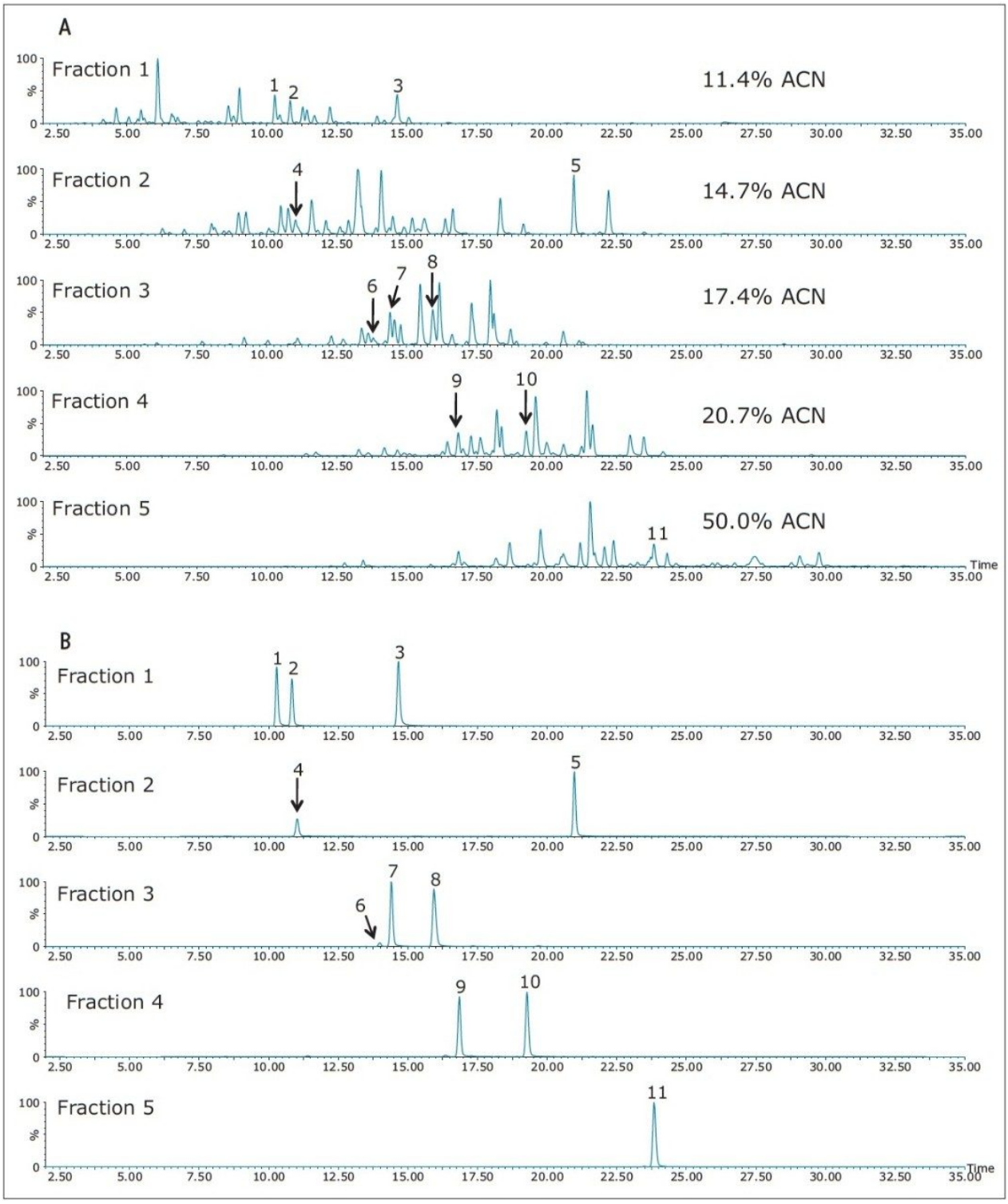

Figure 2. 2D-LC/MS^E analysis of MIX-1 digest using a five-step fractionation experiment: (A) Base peak

chromatograms from the 2nd dimension separation of the 5 fractions eluted with increasing percentages of ACN from the 1st dimension separation: 11.4% (Fraction 1), 14.7% (Fraction 2), 17.4% (Fraction 3), 20.7% (Fraction 4) and 50.0% ACN (Fraction 5); (B) Extracted mass chromatograms generated for 11 peptides from MassPREP Digestion Standard MIX-1 eluting in different fractions. Table I contains a list of the 11 peptides annotated in this figure.

The elution times and peak widths of 11 peptides (from four different proteins) highlighted in this figure are listed in Table I along with peptide sequences and their corresponding monoisotopic precursor ions. The same 2D-LC fractionation experiment was repeated for the ENL digest and the base peak chromatograms produced for this sample are presented in Figure 3. The retention times and peak widths of 12 ENL peptides eluting in different fractions are shown in Table II.

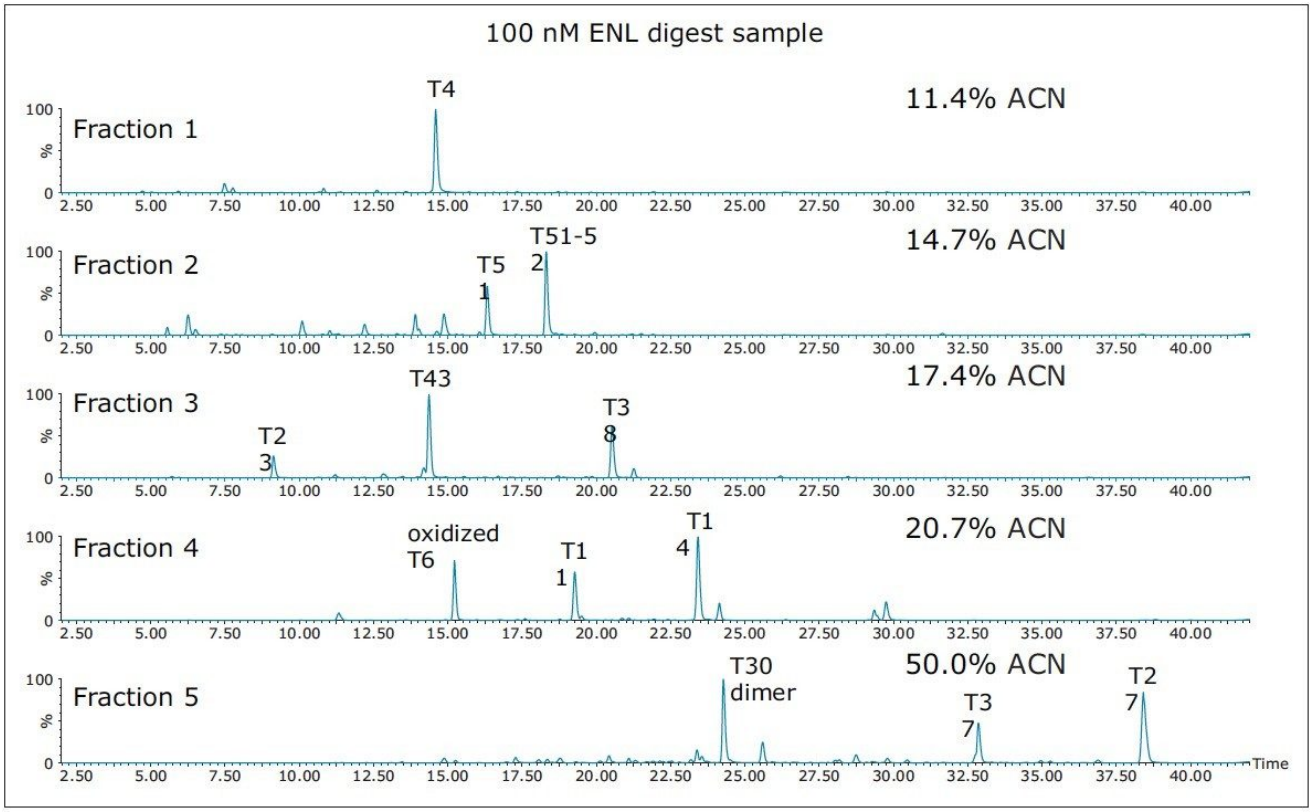

Figure 3. 2D-LC/MS^E analysis of 100 nM ENL digest using a five-step fractionation experiment. Base peak chromatograms recorded for the 2nd dimension separation for 5 peptide fractions eluted with increasing percentages of ACN from the 1st dimension separation: 11.4% (Fraction 1), 14.7% (Fraction 2), 17.4% (Fraction 3), 20.7% (Fraction 4), and 50.0% ACN (Fraction 5) are displayed. Table II contains a list of the 12 peptides annotated in this figure.

Based on the peak width values recorded at 10% of peak height for the peptides listed in Table I and II, the average peak capacity of the 2nd dimension separation is estimated to be 200. Considering that the 2D-LC experiment is comprised of a five-step fractionation process, the theoretical peak capacity of the 2D-LC HCP setup is 1,000, clearly demonstrating the capability of this 2D-LC system to generate a high resolution separation.

Peak number	Peptide ID	Peptide sequence	Precursor / (charge)	Retention time (min)	Peak width (sec)	2D Fraction
1	PHO	T92	GYNAQEYYDR	639.77 / (+2)	10.3	11.0 1
2	BSA	T54 (alkylated)	EYEATLEECCAK	751.81 / (+2)	10.8	10.5 1
3	ENL	T4	GNPTVEVELTTEK	708.86 / (+2)	14.6	12.5 1
4	ADH	T14	VLGIDGGEGK	472.76 / (+2)	11.0	13.0 2
5	PHO	T63	IGEEYISDLDQLR	775.89 / (+2)	21.0	11.0 2
6	BSA	T57	HLVDEPQNLIK	653.36 / (+2)	14.0	11.0 3
7	ENL	T43	VNQIGTLSESIK	644.86 / (+2)	14.4	12.0 3
8	ADH	T5	ANELLINVK	507.30 / (+2)	15.9	12.5 3
9	ADH	T23	SISIVGSYVGNR	626.34 / (+2)	16.8	12.0 4
10	ENL	T11	NVNDVIAPAFVK	643.86 / (+2)	19.3	12.5 4
11	PHO	T94	QIIEQLSSGFFSPK	790.92 / (+2)	23.8	13.5 5

Table I. List of peptides from the MassPREP Digestion Standard MIX-1 that were identified (labeled) in the chromatograms shown in Figure 2.

Peptide ID	Peptide sequence	Precursor(s) / (charge)	Retention time (min)	Peak width (sec)	2D Fraction
T4	GNPTVEVELTTEK	708.86/(+2)	14.6	12.5	1
T51	IEEELGDNAVFAGENFHHGDK	582.77/(+4) and 776.69/(+3)	16.3	12.0	2
T51-52	IEEELGDNAVFAGENFHHGDKL	611.04/(+4) and 814.39/(+3)	18.3	12.5	2
T23	IGSEVYHNLK	580.31/(+2)	9.1	12.0	3
T43	VNQIGTLSESIK	644.86/(+2)	14.4	12.0	3
T38	TAGIQIVADDLTVTNPK	878.48/(+2) and 585.99/(+3)	20.5	13.0	3
oxidized T6	SIVPSGASTGVHEALEM(ox)R	619.64/(+3)	15.2	11.5	4
T11	NVNDVIAPAFVK	643.86/(+2)	19.3	12.5	4
T14	AVDDFLLSLDGTANK	789.91/(+2)	23.4	14.0	4
T30 disulfide dimer	IGLDCASSEFFK	658.31/(+4) and 877.42/(+3)	24.3	12.5	5
T37	YPIVSIEDPFAEDDWEAWSHFFK	943.43/(+3)	32.9	13.0	5
T27	YGASAGNVGDEGGVAPNIQTAEEALDLIVDAIK	1086.54/(+3) and 815.16/(+3)	38.4	19.5	5

Table II. List of peptides from the ENL digest labeled as peaks 1–12 in the chromatograms presented in Figure 3.

Conclusion

Here we demonstrate that the ACQUITY UPLC M-Class System with 2D Technology (300 µm I.D., high capacity configuration) is capable of providing high chromatographic resolving power required for the quantitative HCP analysis and the separation of other complex peptide samples.

References

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