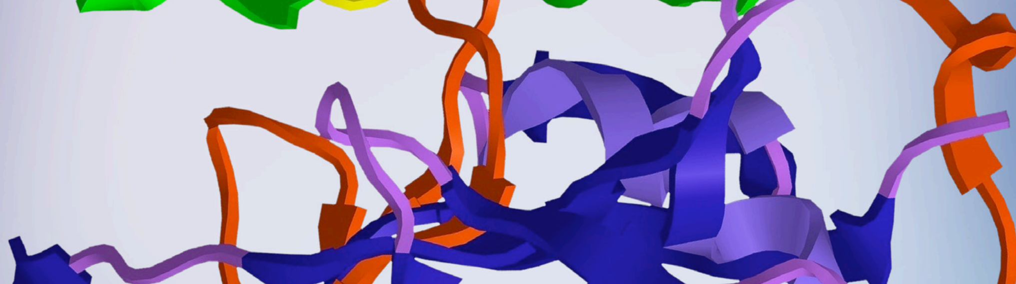


[Analyses of Biotherapeutic Peptides]

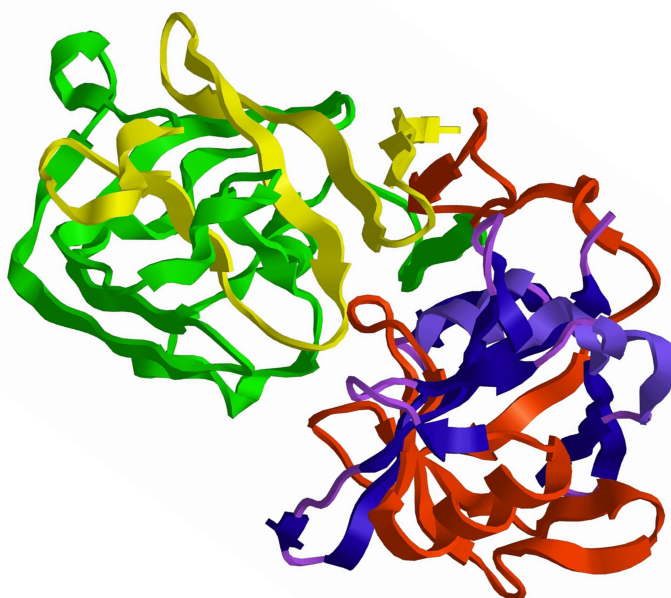
Chapter taken from Waters Peptide Separations Application Notebook (p/n: 720005801EN)

Introduction

The ability to effectively separate, identify, and quantitate peptides at substantially different concentrations is important in applications ranging from the analyses of biotherapeutic peptides and proteins to proteomics and biomarker investigations. To be successful, scientists acknowledge the importance of separation synergies that occur when a defined column, instrument, and method are assembled to address specific application needs.

The advent of UPLC® and various LC/MS column and instrumentation technologies have significantly enhanced the ability to obtain higher quality data for some of today's most challenging peptide separations and analyses. For many of these applications, LC coupled to (tandem) mass spectrometry (LC–MS or LC–MS/MS) is the preferred analytical technique as it allows very selective and sensitive measurements.

This document is a compilation of numerous published LC & LC–MS applications for the analyses of peptides.



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[APPLICATION NOTE]

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High Mass Loading of Peptides with Hybrid Particle C_{18} Columns and Acetic Acid Mobile Phases

Matthew A. Lauber, Stephan M. Koza, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Two hybrid particle column chemistries (BEH130 C_{18} and CSH130 C_{18}) with unique selectivities.
- BEH130 C_{18} and CSH130 C_{18} can produce narrower target peptide peaks and greater resolution when using mobile phases modified with optimal concentrations of HOAc versus those modified with 0.1% TFA. This can be exploited to obtain peptides with a pharmaceutically acceptable counter ion in fewer steps.
- BEH130 C_{18} and CSH130 C_{18} are QC tested with a tryptic digest of cytochrome *c*.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class Bio System

XSelect® CSH130 C_{18}, 5 µm

XBridge® BEH130 C_{18}, 5 µm

MassPREP™ Peptide Mixture

KEY WORDS

Reversed phase (RP), peptides, acetic acid (HOAc), charged surface hybrid (CSH), CSH130 C_{18}, BEH130 C_{18}, preparative chromatography, peptide separation technology (PST)

INTRODUCTION

Peptides have proven to be very useful as both therapeutic agents and markers in research. It is common practice to purify peptides for these purposes by preparative reversed-phase (RP) chromatography. In most instances, it is imperative that the purification process yield peptides of high purity. Contaminants can render results from biological assays ambiguous, and be a serious concern when present in active pharmaceutical ingredients. As a result, there is a need for high chromatographic resolution to minimize the co-elution of impurities that are often closely related chemically to the target peptide. There is also a need for column chemistries with excellent loadability to ensure that throughput and productivity are optimized. Typically, peptides are separated using mobile phases containing strong ion pairing agents, such as trifluoroacetic acid (TFA). However, when mobile phases containing TFA are employed, additional preparation steps are required. It is necessary to remove or exchange trifluoroacetate (TFA salt), because of its inherent toxicity.[1] Less toxic counter ions, notably acetate, are preferred. In fact, most peptide pharmaceuticals are either acetate salts or acetate-containing liquid formulations.[2-3] It would seem advantageous to avoid TFA mobile phases when possible, instead using acetic acid (HOAc) mobile phases. It has been previously shown that a peptide in a TFA solution, such as a crude synthetic peptide,[4-5] is more than partially converted to an acetate form after using HOAc mobile phases and isocratic RP chromatography.[6] Meanwhile, gradient separations with short wash steps involving a high concentration of an acetate buffer can be employed for more complete conversion of salt forms.[7] In the end, a streamlined purification process involving HOAc mobile phases would facilitate obtaining the desired peptide and counter ion with fewer steps.

This study investigates the use of both BEH C_{18} and CSH™ C_{18} columns for preparative peptide separations. BEH C_{18} is an organo-silica C_{18} stationary phase, based on ethylene bridged hybrid (BEH) technology, and is noted for its robustness and pH stability. Charged Surface Hybrid (CSH) C_{18} is an evolution of BEH C_{18} as it is modified to contain a low level positive surface charge under acidic conditions. The following data demonstrates that each of these stationary phases is amenable to high mass load peptide separations with both TFA- and HOAc-modified mobile phases. The following results also show that both BEH C_{18} and CSH C_{18} can yield narrower target peaks at high mass loads with optimized HOAc mobile phases than those containing 0.1% TFA.

EXPERIMENTAL

Sample description

MassPREP Peptide Mixture (p/n 186002337, shown in Table 1) was reconstituted with either 0.1% TFA or 0.1% HOAc (depending on the mobile phase employed) to a total peptide concentration of either 0.6 or 2.0 mg/mL (depending on the sample load). A low purity (<70%) preparation of the synthetic peptide, DFVGYGVKDFVGVGVK, was reconstituted in 0.1% TFA/0.1% HOAc to a concentration of either 1 or 4 mg/mL.

Method conditions (unless otherwise noted)

LC conditions

System: ACQUITY UPLC H-Class Bio System with a 20-cm Column Heater

Detection: ACQUITY UPLC TUV Detector with 500-nL Analytical Flow Cell

Xevo® G2 Q-Tof™ Mass Spectrometer

Wavelength: 214 and 250 nm

Scan rate: 2 Hz (filter time constant, 1 s)

Columns: XBridge BEH130 C_{18} 4.6 x 100 mm, 5 µm, Porous, 130Å (p/n 186003579)

XSelect CSH130 C_{18} 4.6 x 100 mm, 5 µm, Porous, 130Å (p/n 186007077)

Column temp.: 40 °C

Sample temp.: 10 °C

Injection volume: 50 to 1000 µL, sample loads noted below

Flow rate: 1 mL/min (split post-UV detector to *ca.* 20 µL/min for infusion into the MS source)

Mobile phases: See gradient tables

Vials: LCGC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)

Gradients: MassPREP Peptide Mixture

A: 0.1% (v/v) TFA in water

B: 0.1% (v/v) TFA in 90:10 acetonitrile (ACN)/water

or

A: 0.1% (v/v) HOAc in water

B: 0.1% (v/v) HOAc in 90:10 ACN/water

Time (min)	% A	% B
0	99.5	0.5
1	99.5	0.5
61	40.0	60.0
62	10.0	90.0
65	10.0	90.0
66	99.5	0.5
85	99.5	0.5

Focused Gradients for DFVGYGVKDFVGVGVK

A: 0.1% (v/v) TFA in water

B: 0.1% (v/v) TFA in 90:10 ACN/water

Time (min)	% A	% B
0.0	90	10
3.0	90	10
4.0	80	20
24.2	60	40
29.2	10	90
32.2	10	90
33.2	90	10
52.0	90	10

A: 0.1% (v/v) HOAc in water

B: 0.1% (v/v) HOAc in 90:10 ACN/water

or

A: 99:1 (v/v) water/HOAc – 1% HOAc

B: 90:9:1 (v/v) ACN/water/HOAc – 1% HOAc

Time (min)	% A	% B
0.0	90	10
3.0	90	10
3.3	87	13
23.5	67	33
29.2	10	90
32.2	10	90
33.2	90	10
52.0	90	10

MS conditions

Mass spectrometer:	Xevo G2 Q-Tof
Ionization mode:	ESI+
Analyzer mode:	Resolution
Capillary voltage:	3.00 kV
Cone voltage:	25 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Cone gas flow:	0.0 L/h
Desolvation gas flow:	800 L/h
Calibration:	NaI, 1 µg/µL from 50 to 2000 *m/z*
Acquisition:	50 to 1990 *m/z*, 2 Hz scan rate

Data management

MassLynx Software v4.1

RESULTS AND DISCUSSION

Loading studies of a nine-component peptide mixture

The application of CSH130 C_{18} and BEH130 C_{18} to analytical peptide separations, such as peptide mapping, has been extensively discussed in previous work.[8-9] Briefly, CSH130 C_{18}, with its novel positive surface charge, has been found to provide improved peak shapes and loadability compared to other peptide reversed-phase column chemistries. Significant increases in peak capacity, up to 90%, have been observed for analytical applications, particularly when mobile phases with little to no ion pairing are used. The positive surface charge of CSH130 C_{18} also provides unique selectivity and less retentivity compared to BEH130 C_{18}, which makes the two sorbents excellent companion column chemistries for peptide separations.

To investigate the performance of CSH130 C_{18} and BEH130 C_{18} in preparative separations, loading studies were performed with a number of different peptides and mobile phases that are typically used in manufacturing, namely those containing either TFA or HOAc. Analytical (4.6 mm I.D.) columns packed with 5 µm particles were employed for these method development experiments.

The MassPREP peptide mixture, containing nine different peptides shown in Table 1, was the first sample used to interrogate these columns. Figure 1 displays a set of chromatograms obtained for this mixture at semi-preparative loads using BEH130 C_{18} and CSH130 C_{18} as well as two different mobile phases, one containing 0.1% TFA and the other 0.1% HOAc. With 0.1% TFA, the average 4σ peak width for the BEH column was 0.8 minute. With 0.1% HOAc, this average peak width increased nearly two-fold to 1.5 minutes. HOAc is a much weaker acid than TFA, producing a less acidic mobile phase with significantly less ionic strength and ion pairing ability. Accordingly, peak shape for most C_{18} columns would be expected to be much worse when using HOAc instead of TFA. This assumption holds true for semi-preparative sample loads with BEH130 C_{18}, as shown in Figure 1. However, peak shapes were remarkably well maintained with the CSH130 C_{18} column when TFA was exchanged for HOAc. The average 4σ peak widths observed using the CSH130 column with 0.1% TFA and 0.1% HOAc mobile phases were 0.5 and 0.6 minutes, respectively. These peak width data are summarized in Figure 2, where individual peak widths for the peptides in the mixture are plotted according to column type and mobile phase condition. In addition to the data for the semi-preparative loads, data obtained for analytical sample loads are shown. This figure highlights that BEH130 C_{18} and CSH130 C_{18} produce similar peptide peak shapes under some conditions, including analytical sample loads with 0.1% TFA. However, under other conditions, such as semi-preparative loads with 0.1% HOAc, CSH130 C_{18} yields much narrower peaks. As has been demonstrated before,[8-9] CSH130 C_{18} tends to deliver markedly better peak shapes for peptides in acidic mobile phases with little to no ion pairing. These data also demonstrate that this becomes even more evident for sample loads 20 times greater than those routinely used for analytical separations.

	Peptide	Sequence
1	RASG-1	RGDSPASSKP
2	Angiotensin 1-7	DRVYIHP
3	Bradykinin	RPPGFSPFR
4	Angiotensin II	DRVYIHPF
5	Angiotensin I	DRVYIHPFHL
6	Renin Substrate	DRVYIHPFHLLVYS
7	Enolase T35	WLTGPQLADLYHSLMK
8	Enolase T37	YPIVSIEDPFAEDDWEAWSHFFK
9	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ

Table 1. MassPREP peptide mixture.

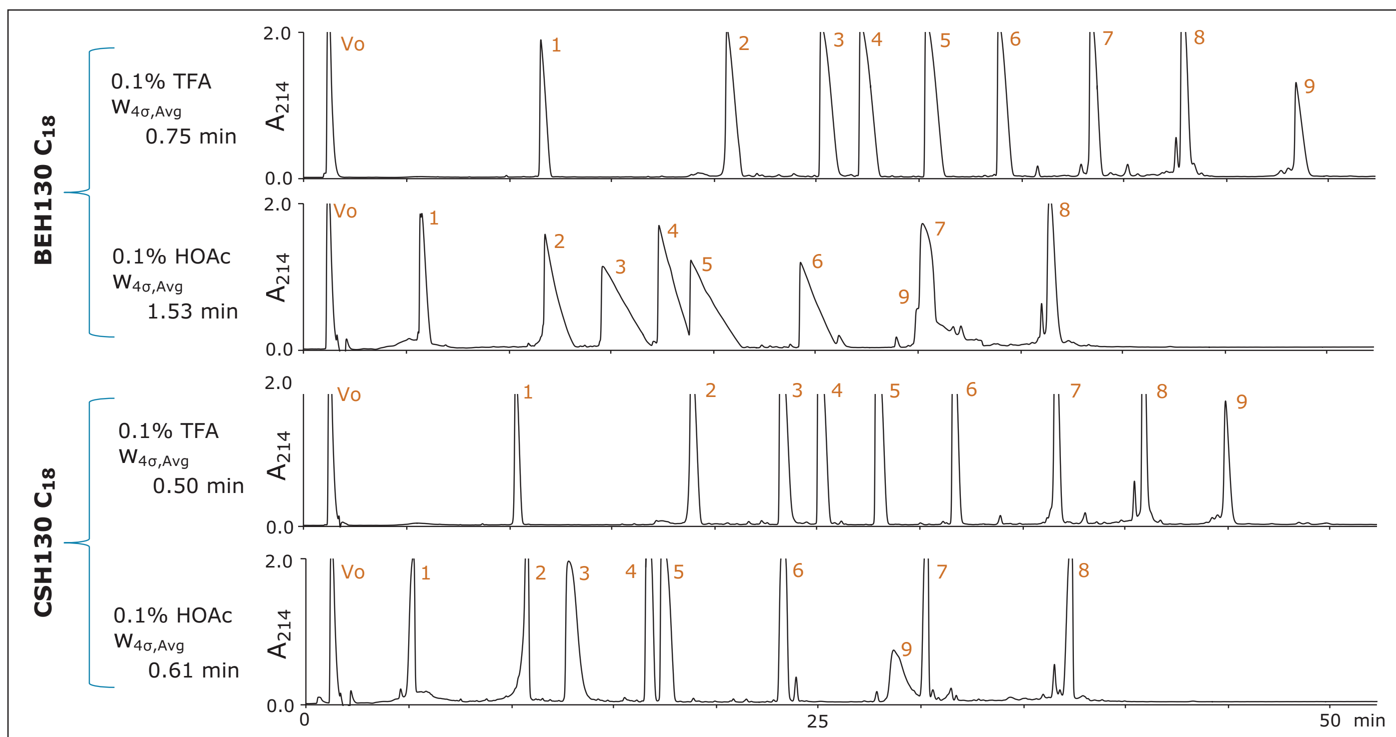

Figure 1. Analysis of the MassPREP peptide mixture at semi-preparative sample loads (500 µg of mixture) with BEH130 and CSH130 C_{18} 4.6 x 100 mm, 5 µm columns.

Figure 2. Peak widths of the individual species in the MassPREP peptide mixture observed at analytical (30 µg of mixture) and semi-preparative (500 µg of mixture) sample loads.

Low purity synthetic peptide

Preparative separations must often be performed at sample loads up to and sometimes more than 1000 times greater than those used for analytical separations. Sample loads below and within this regime were investigated using a synthetic, low purity peptide of the sequence DFVGYGVKDFVGVGVK, a neutral peptide (pI = 6) with a molecular weight of 1.7 kDa. Separations were performed on both the BEH and CSH columns using focused gradients to reduce run times, and low sensitivity wavelength (250 nm) detection to assess full peak shapes.

Semi-preparative and preparative sample loads with 0.1% HOAc modified mobile phases were analyzed first, as shown in Figure 3. A semi-preparative load (50 µg) on the BEH column produced the target peptide as a peak with the pronounced tailing that is consistent with commonly observed Langmuirian isotherms. Conversely, at a preparative load (1 mg), the target peptide eluted as a slightly fronting peak, typified by an anti-Langmuirian isotherm. Anti-Langmuirian isotherms are known to occur when peptides are present in zwitterionic form.[10] Consequently, the 0.1% HOAc modified mobile phase was not acidic enough to fully protonate the carboxyl groups of this synthetic peptide. The peptide was likely present in both cationic and zwitterionic forms. The relative amount of the zwitterion would be expected to increase with sample load, particularly when the concentration of the target peptide exceeds the protonation/buffering capacity of the mobile phase. This explains the dramatic change in peak shape with increasing load on the BEH column.

Interestingly, 0.1% HOAc, as seen in Figure 3, appears to be ideal for obtaining a narrow target peptide peak with BEH130 C_{18} at the preparative sample load. Under these conditions, the BEH column yielded a narrower target peptide peak than the CSH column. In fact, CSH130 C_{18} yielded fronting peaks at both sample loads with 0.1% HOAc. This is not surprising given that its positive surface charge minimizes the extent of tailing that occurs for peptides.[8-9] Thus, the fronting peak shape is more readily apparent. Because there is no peak tailing, the width of the target peak at the preparative sample load is actually greater on the CSH column than the BEH column.

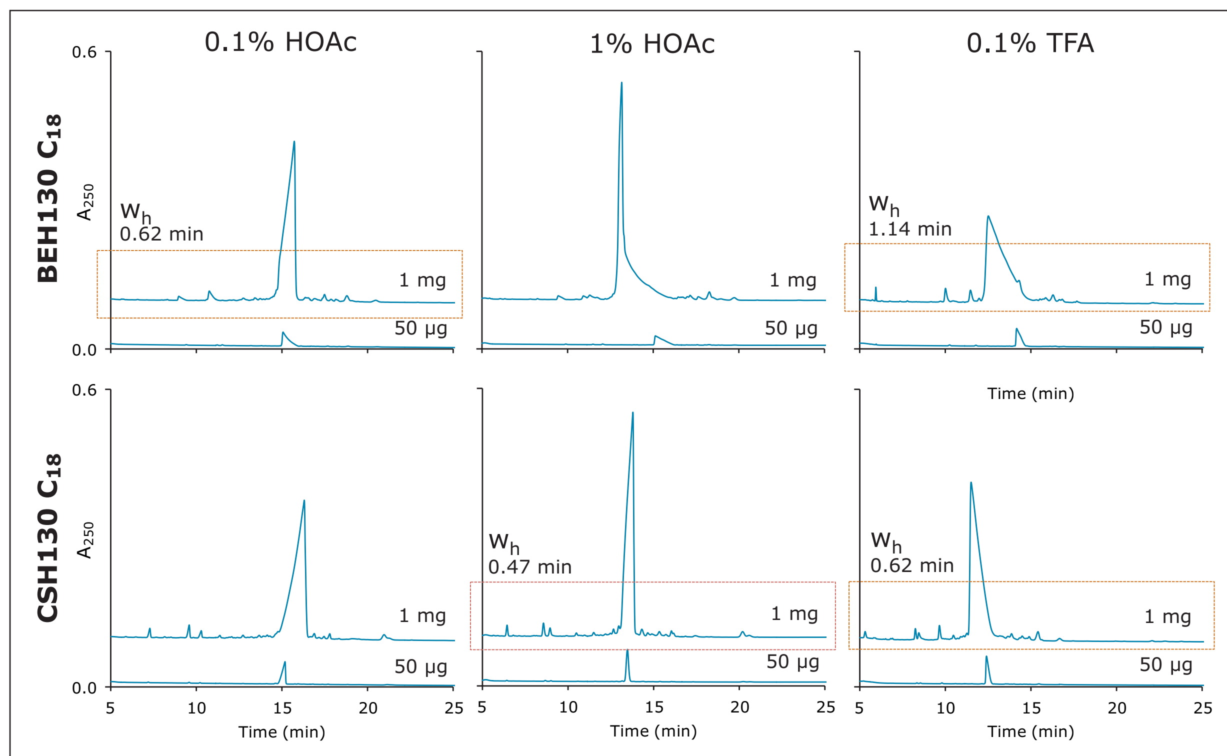

Figure 3. Chromatograms of low purity, synthetic peptide DFVGYGVKDFVGVGVK (pI 6, 1.7 kDa) obtained at semi-preparative (50 µg) and preparative (1 mg) sample loads. The outlined chromatograms are shown in Figure 4.

Based on this, CSH130 C_{18} and BEH130 C_{18} would be expected to produce optimal peak shapes under different mobile phase conditions. To this end, separations were also performed with a mobile phase modified with 10 times more acid (1% HOAc). Intermediate concentrations were not evaluated, although they may be of value for purification development. This change in the mobile phase composition significantly improved the peak shape for the CSH column, but actually worsened peak shape for the BEH column, as shown in Figure 3. With their optimal HOAc mobile phases, both the BEH (0.1% HOAc) and CSH (1% HOAc) columns produced narrow target peptide peaks (half height widths of 0.5 and 0.6 minutes, respectively). To benchmark these results, separations were also performed with 0.1% TFA as an ion-pairing agent, as seen in the right panel of Figure 3. With TFA, target peptide peak widths were 0.6 minute on CSH130 C_{18} and 1.1 minutes on BEH130 C_{18}. Regardless of column chemistry, the optimized HOAc mobile phases yielded significantly narrower peptide target peaks than those containing TFA. These results demonstrate that acetic acid mobile phases have utility for peptide preparative chromatography.

Narrower target peptide peaks often coincide with greater resolution of impurities and, in turn, provide the opportunity to collect higher purity fractions. The effect of column chemistry and mobile phase additive on the preparative loads of DFVGYGVKDFVGVGVK is shown again in Figure 4, where the baselines of the chromatograms and the capability of each separation to resolve a number of MS-identified impurities from the target peak are the focus. As previously noted, narrower target peaks were observed with the HOAc mobile phases. Figure 4 also demonstrates that co-elution of the monitored impurities was likewise minimized through the use of the HOAc mobile phases. In addition, it is apparent that through the use of the different mobile phase additives and the two different column chemistries, chromatographic selectivity between the target peptide and the impurities was dramatically altered. For the parameters screened in this loading study, the CSH column with a 1% HOAc mobile phase appeared to provide both the narrowest target peptide peak and the least co-elution with monitored impurities. Nonetheless, a comparable separation could be achieved with the BEH column and a 0.1% HOAc mobile phase. The availability of column chemistries with different selectivity and optimal additive concentrations can be of benefit when developing challenging preparative separations.

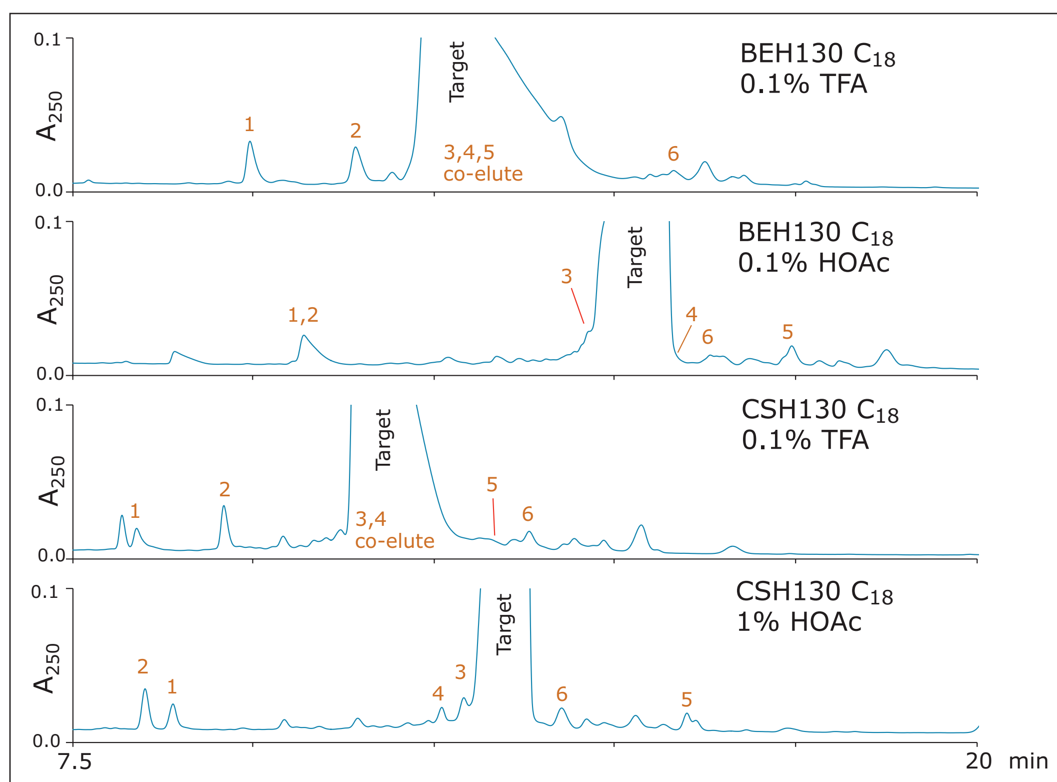

Figure 4. Chromatograms for a preparative sample load (1 mg) of DFVGYGVKDFVGVGVK obtained with BEH130 C_{18} and CSH130 C_{18} using either optimal HOAc mobile phases or mobile phases modified with 0.1% TFA. Retention times of the target peptide and the noted impurities were assigned by ESI-MS. The following mass shifts were observed for the assigned impurities: (1, -147.1 Da), (2, -263.1 Da), (3, -470.2 Da), (4, +548.4 Da), (5, -227.2 Da), and (6, +988.6 Da).

Thus far, results for only a moderate preparative sample load (1 mg) of the synthetic peptide, DFVGYGVKDFVGVGVK, has been discussed. Chromatograms acquired for 4 mg loads of this peptide are shown in Figure 5. This sample load would correspond to 0.5 g of material and, thus, very high productivity per injection on a larger 50-mm I.D. column. From these data, it is clear that both the CSH and BEH columns are amenable to high sample loads. It is notable, nevertheless, how strikingly consistent the peak profiles are for the CSH column from semi-preparative (50 µg) to preparative (4 mg) loads. Such predictability may prove useful when needing to develop an isolation method without consuming large amounts of sample.

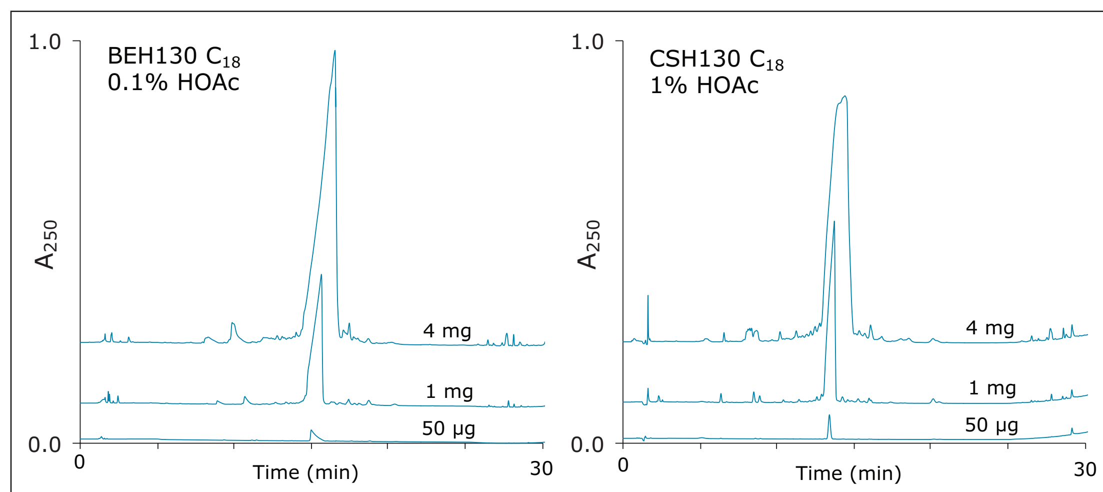

Figure 5. High mass loading (up to 4 mg) on BEH130 C_{18} and CSH130 C_{18} with optimized acetic acid mobile phases.

CONCLUSIONS

Based on loading studies with analytical bore columns, the use of 5-µm BEH130 C_{18} and 5 µm CSH130 C_{18} shows significant promise for facilitating preparative peptide separations with either TFA or HOAc containing mobile phases. Both BEH130 C_{18} and CSH130 C_{18} column chemistries have useful attributes. Under acidic conditions, CSH130 C_{18} tends to exhibit improved loadability and generally narrower target peaks compared to BEH130 C_{18}. Lower volume fractions can, therefore, be obtained with CSH130 C_{18}, which could be exploited to aid subsequent purification and solvent removal steps. BEH130 C_{18}, on the other hand, is perfectly suited to neutral/basic pH preparative chromatography, due to its longer term and higher temperature stability at such conditions. Finally, CSH130 C_{18} and BEH130 C_{18} also exhibit unique selectivity, making them useful companions for resolving challenging impurity/target peptide profiles.

Perhaps more interesting than these aforementioned attributes is the fact that each stationary phase optimizes with different concentrations of mobile phase additive and yields best peak shapes for preparative loads of an example synthetic peptide with optimized HOAc mobile phases, rather than those containing 0.1% TFA. This suggests an opportunity to leverage these hybrid particle C_{18} columns to streamline purification processes, since using HOAc mobile phases would mean that peptides with a pharmaceutically acceptable counter ion could be obtained in fewer steps.

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[APPLICATION NOTE]

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Mass-Directed Isolation of a Synthetic Peptide Using the ACQUITY QDa Detector

Jo-Ann M. Jablonski and Andrew J. Aubin
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The ACQUITY® QDa® Detector clearly identifies peptides whose monoisotopic or multiple charge state masses are between 30 and 1250 Da, reducing the ambiguity that often accompanies analysis and purification with UV-directed systems.
- Mass analysis helps in the identification of contaminant by-products present in the crude peptide mixture, ultimately assisting in the optimization of the peptide synthesis and cleavage protocol and leading to improved process efficiency.
- The AutoPurification™ systems configured with the ACQUITY QDa Mass Detector provide all the benefits of mass-directed purification including clear identification of target compounds from complex matrices, customized fraction collection and tracking, and automatic sample processing.

WATERS SOLUTIONS

AutoPurification System

ACQUITY QDa Detector

XBridge,® SunFire,® and XSelect®
CSH™ columns

Waters OBD™ Prep Columns

0.45 µm GHP Acrodisc 13 mm syringe filter

Column Selectivity Chart

KEY WORDS

Prep chromatography, peptide purification, peptide isolation, mass-directed purification, ACQUITY QDa Detector, OBD Prep Columns

INTRODUCTION

Peptides play a unique role in the development of new drug candidates and fill a specialized niche between traditional small molecule therapies and larger protein remedies. Because of their high specificity, stability, and safety, they are becoming more popular as a means for targeting many medical conditions.[1,2] Whether peptides are made step-wise using solid-phase peptide synthesis,[3] solution phase synthesis, or some combination of the two, almost all crude peptide mixtures are complex. While traditional peptide isolation is usually performed using UV-directed chromatography, mass-directed isolation makes the purification process easier with unambiguous discrimination between the target peptide and the contaminants formed during synthesis and cleavage. The AutoPurification System configured with an ACQUITY QDa Detector provides an alternative choice to UV-only systems, where mass detection not only improves isolation efficiency, but also helps in the identification of synthetic and cleavage by-products. This useful information suggests how the process can be improved and lead to greater target peptide yield. Here, we illustrate a simple mass-directed approach to peptide isolation and discuss the options for improving the efficiency of the purification protocol.

EXPERIMENTAL

Conditions

Analytical columns:	XBridge® C_{18}, 4.6 x 50 mm, 5 µm; SunFire C_{18}, 4.6 x 50 mm, 5 µm; XSelect CSH Phenyl-Hexyl, 4.6 x 100 mm, 5 µm
Analytical flow rate:	1.46 mL/min
Prep column:	XSelect CSH Phenyl-Hexyl OBD Prep, 19 x 150 mm, 5 µm
Prep flow rate:	25 mL/min
Mobile phase A:	0.1% trifluoroacetic acid in water
Mobile phase B:	0.1% trifluoroacetic acid in acetonitrile
Makeup solvents:	50:50 or 90:10 water:acetonitrile with 0.1% propionic acid
Cone voltage:	10 V
Probe temp.:	500 °C, 600 °C
Ionization mode:	ES+, continuum
Sampling frequency:	2 Hz
Scan range:	100–1200 amu
Wavelength:	220, 280 nm
Gradients and injection volumes:	as noted in figures
Sample:	Crude synthetic peptide comprised of the following 16 amino acid residues: 7 polar, 6 nonpolar, 1 acidic, and 2 basic; purity 56% by HPLC

Monoisotopic Peptide Mass: 1626.8 Da	
Target ions for charge states	***m/z***
$[M+2H]^{2+}$	814.4
$[M+3H]^{3+}$	543.3

Instrumentation

Waters AutoPurification System: 2545 Binary Gradient Module, 2767 Sample Manager, System Fluidics Organizer, 8–30 mL Flow Splitter, two 515 HPLC pumps, 2998 Photodiode Array Detector, ACQUITY QDa Detector

Figure 1. Waters AutoPurification System.

RESULTS AND DISCUSSION

Almost all crude peptides are challenging to isolate and purify due to the inherent complexity involved in synthesizing, deprotecting, and cleaving them from the resins used in solid phase peptide synthesis. Incomplete couplings, amino acid deletion sequences, incomplete cleavage impurities and adduct formations are among the myriad of by-products that complicate the isolation of the target peptide from the crude sample, yet the majority of them must be removed before the peptide candidate can be used in subsequent research. While UV-directed peptide purification is quite common, mass-directed purification removes the ambiguity associated with identifying the product of interest. Furthermore, developing a separation that encompasses both mass and UV detection ensures that more components will be revealed. Components that do not ionize will be detected by UV and, conversely, those that do not have a chromophore will be detected by mass. In addition, selecting an appropriate column and focusing the gradient[4] improve separations quickly and easily, and coupled with mass detection, make tracking the product straightforward.

For these studies, the crude synthetic peptide was dissolved in dimethylsulfoxide (DMSO) and filtered with a 13 mm Acrodisc GHP syringe filter. As shown in Figure 2A, the peptide product eluted at about 2.5 minutes on a short SunFire Column, but the fast screening gradient also revealed a coeluting impurity. Focusing the gradient (Figure 2B) appeared to move the coeluting impurity away from the product, but with a larger injection (Figure 2C) a small shoulder reappeared on the backside of the target product peak. Changing to a short XBridge C_{18} Column (Figure 2D) did not resolve the contaminant, but seemed to make it merge back under the tailing product peak.

Although the selectivities of the SunFire and XBridge C_{18} columns were different, it was evident that the separation could not be improved without using a column with a more dramatically different selectivity than the ones already tested. For this reason, and using the reversed-phase column selectivity chart as a guide, the XSelect CSH Phenyl Hexyl column was selected for the subsequent evaluation (Figure 3).

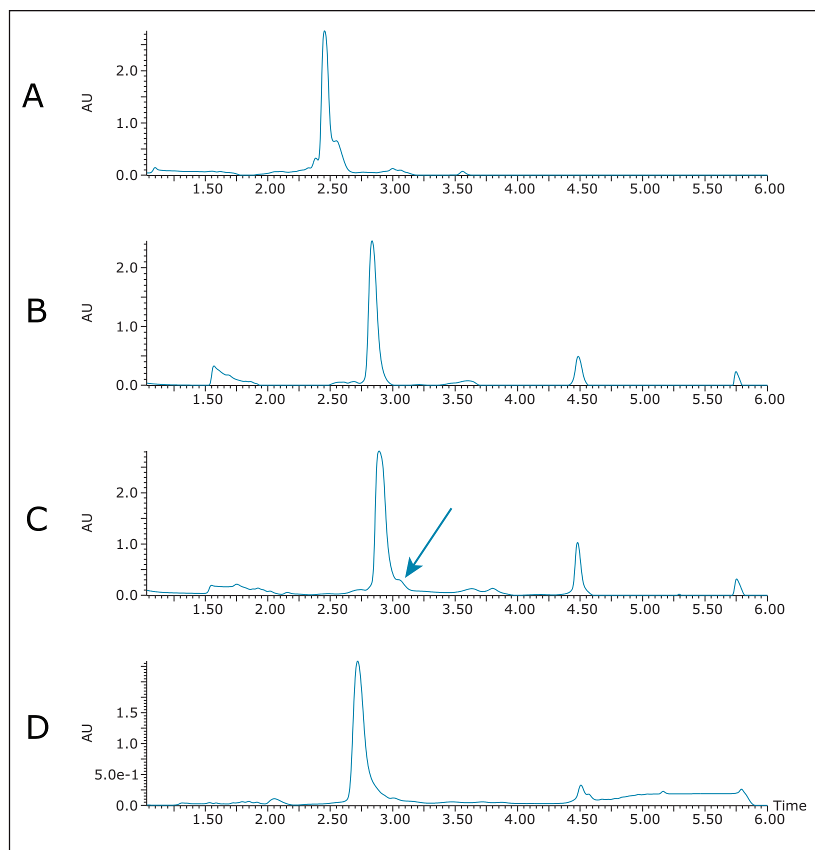

Figure 2. Screening and method development on the crude peptide sample (11.9 mg/mL) with UV detection at 220 nm on 4.6 x 50 mm, 5 µm columns.
A) SunFire C_{18}, 5–95% B in 6 min, 7.2% change/col vol; 10 µL
B) SunFire C_{18}, 23–31% B in 3 min, 1.27% change/col vol; 10 µL
C) SunFire C_{18}, 23–31% B in 3 min, 1.27% change/col vol; 20 µL
D) XBridge C_{18}, 23–31% B in 3 min, 1.27% change/col vol; 10 µL

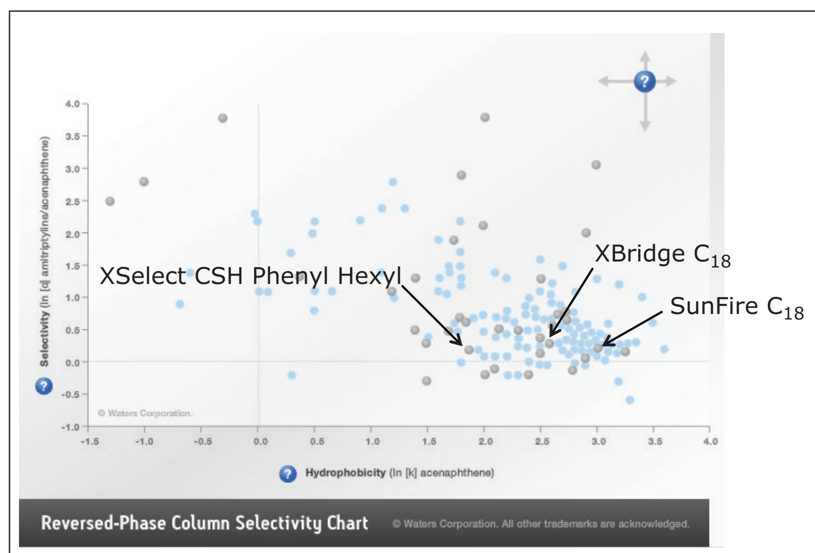

Figure 3. Reversed-Phase Column Selectivity Chart.

Although the slope of the gradient on the XSelect analytical column was slightly steeper, because the column was 50 mm longer and the packing had a different selectivity, the first scaled 20 µL injection revealed the emergence of 2 contaminant peaks (Figure 4A). As shown in Figure 4B, refocusing and reducing the rate of gradient change from 1.42%/column volume (cv) to 0.71%/cv almost fully resolved the contaminant peak. An even shallower gradient slope at 0.28%/cv fully resolved the contaminant (Figure 4C). In the interest of further improving the resolution between the peptide product and the contaminating by-product, the gradient was modified to run from 16–24%B, yet the slope was maintained at 0.28%/cv (Figure 4D). As expected, the impurity eluted later and the resolution between it and the peptide product was improved. Since the gradient slope was so shallow, it was unlikely that any further method development on this column would yield additional improvement in resolution.

More peptide sample was dissolved in DMSO and a loading study was performed on the 4.6 x 100 mm XSelect CSH Phenyl Hexyl column. A very conservative loading volume of 5 µL (Figure 5A) showed excellent separation between all of the sample components. A 10 µL injection revealed yet another slight shoulder eluting on the backside of the peptide product peak (Figure 5B), and the shoulder completely hiding under the product peak with a 15 µL loading (Figure 5C). The 10 µL loading was chosen for scale up because this would give a higher load on the prep column, yet still give enough resolution to effectively isolate pure product.

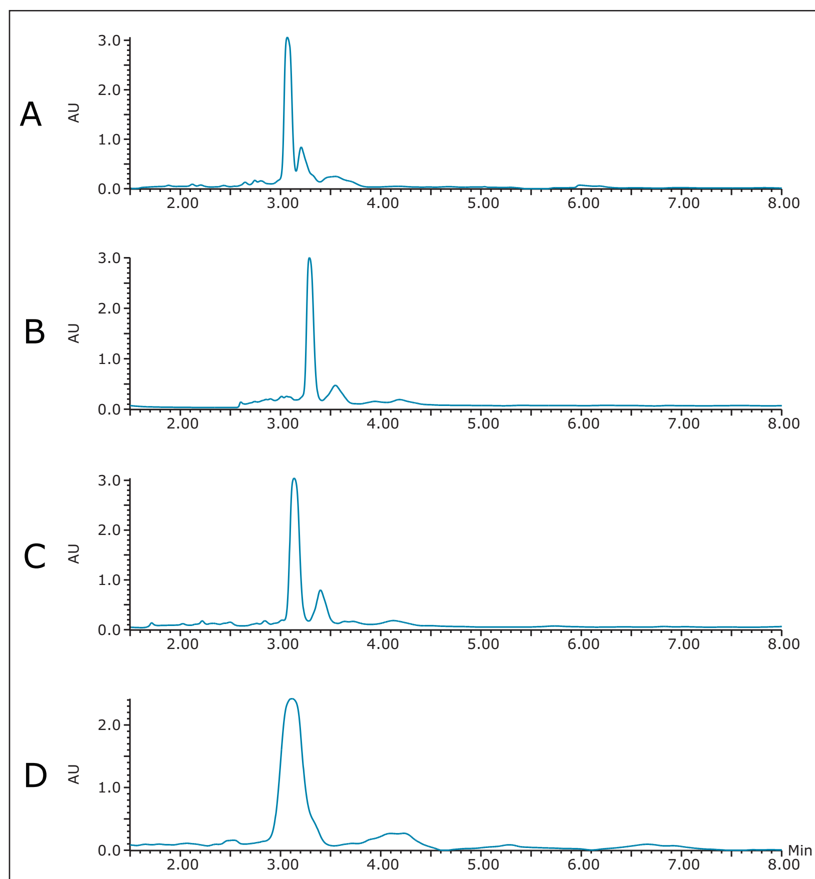

Figure 4. Method development on the XSelect CSH Phenyl Hexyl, 4.6 x 100, 5 µm column, 220 nm; 11.9 mg sample/mL; 20 µL.
A) 23–31% B in 5.36 min, 1.42% change/col vol
B) 20–28% B in 10.83 min, 0.71% change/col vol
C) 20–28% B in 27.30 min, 0.28% change/col vol
D) 16–24% B in 27.30 min, 0.28% change/col vol

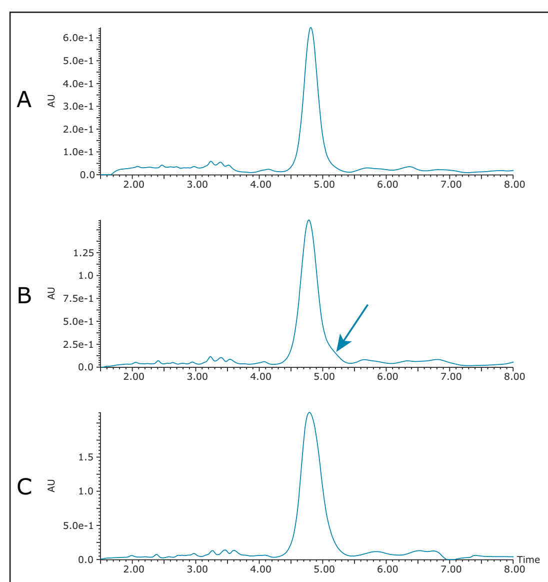

Figure 5. Loading on XSelect Phenyl Hexyl column, 4.6 x 100 mm, 16–24% B in 27.3 min, 220 nm; 19.5 mg sample/mL.
A) 5 µL
B) 10 µL
C) 15 µL

Geometric scaling from 10 µL on the 4.6 x 100 mm analytical column to 256 µL on the 19 x 150 mm preparative column gave identical chromatography at 220 nm, as expected (Figure 6A), shown here for reference. Mass-directed collection was used for fractionation with the mass triggers corresponding to the most abundant triply-charged and doubly-charged ions in the sample. Fractions 1 and 3 were triggered on the triply-charged ion $[M+3H]^{3+}$ with an *m/z* of 543.3 (Figure 6B), while fraction 2 was triggered on the doubly-charged ion $[M+2H]^{2+}$ with an *m/z* of 814.4 (Figure 6C). With the mass range of the ACQUITY QDa Detector spanning from 30 to 1250 Da, only peptides with multiple charges which fall within this range can be analyzed and isolated. In the interest of conserving time and solvent, the purification run was terminated shortly after the target peptide was collected. UV and mass analysis of the fractions showed the product to be very pure (Figure 7).

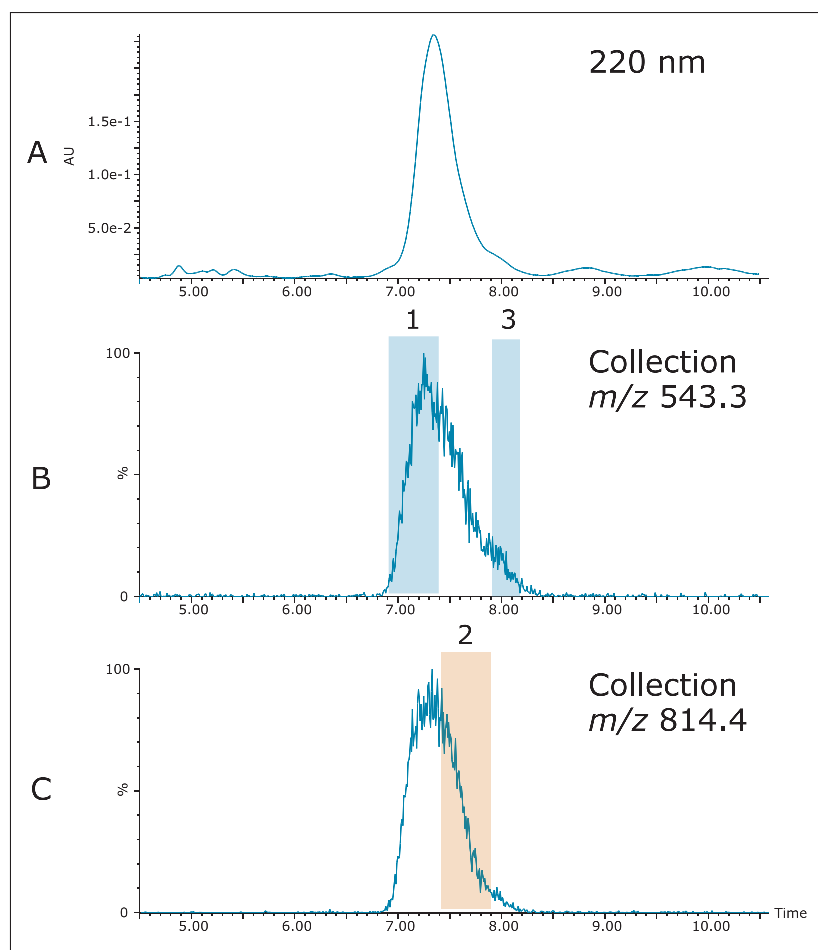

Figure 6. Isolation of peptide on the 19 x 150 mm XSelect CSH Phenyl Hexyl OBD Prep column, 5 µm; 256 µL. Gradient: 16–24% B in 40.9 minutes. The run was terminated after peptide collection. A = 220 nm, B = $[M+3H]^{3+}$ m/z 543.3, C = $[M+2H]^{2+}$ m/z 814.4. MS-directed collection. Fractions 1 and 3 triggered on the triply-charged ion, while fraction 2 triggered on the doubly-charged ion.

Makeup 50:50 Water:Acetonitrile:0.1% propionic acid CV 10, probe temperature 500°C, sampling frequency 2 Hz, ES+ Continuum, Detector gain 1, ESI+ capillary voltage 0.8

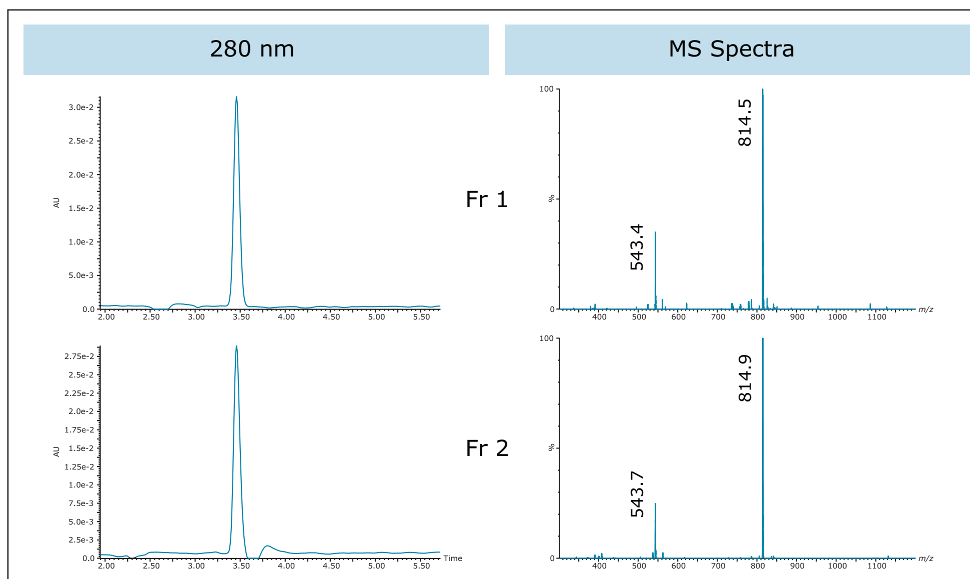

Figure 7. Fraction analysis on the XSelect CSH Phenyl Hexyl column, 4.6 x 100 mm; 40 µL. Gradient: 5–95% B in 12 min.

Mass detectors configured in purification systems almost always use a makeup solvent to dilute the tiny sample split from the main prep flow for analysis during the preparative run. The makeup solvent also simultaneously carries the sample to the detectors. Once the target ion is detected and it meets the collection parameters specified by the user, the detector signals that the peak is found and collection begins after the appropriate offset time to the fraction collector is reached. Trifluoroacetic acid (TFA) is commonly used as an ion-pairing reagent in peptide separations because it helps to improve chromatographic peak shape. Unfortunately, TFA also suppresses ionization in mass detection. For this reason, the makeup solvent for this isolation was 50:50 water:acetonitrile with 0.1% propionic acid. The propionic acid displaces the TFA and improves the ionization in the mass detector. Because of the very hydrophilic nature of the peptide used in these experiments, a second makeup solvent was evaluated – 90:10 water:acetonitrile with 0.1% propionic acid. As shown in Figure 8, both makeup solvents are acceptable for use in the isolation of this peptide. For peptides that are hydrophobic, the 50:50 Water:Acetonitrile makeup solvent is recommended to prevent sample precipitation. For the 90:10 water:acetonitrile composition, the probe temperature was 600 °C. Peak widths measured at half height were slightly narrower for the 90:10 water:acetonitrile composition.

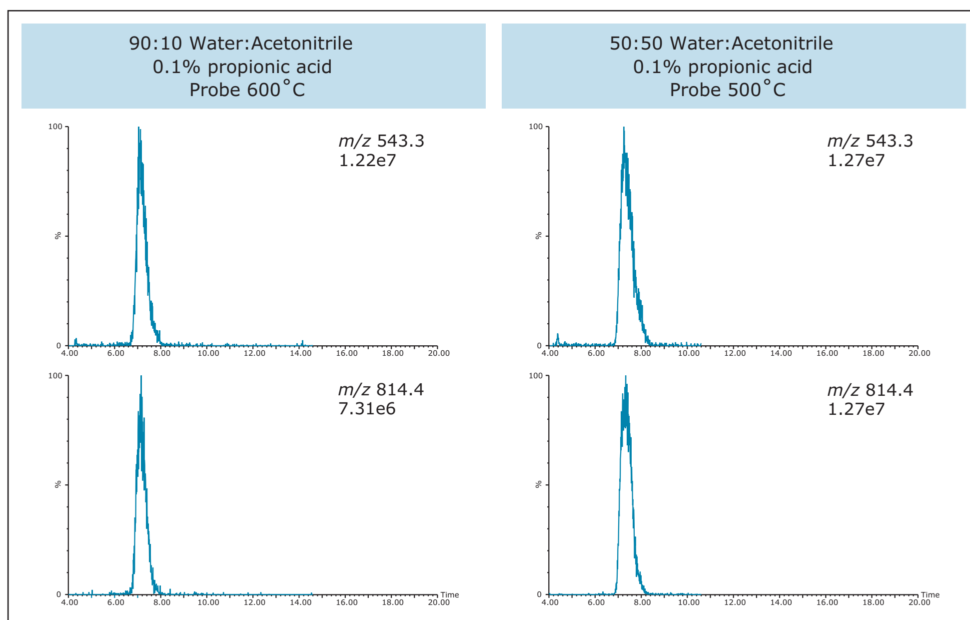

Figure 8. Comparison of preparative injections using different makeup solvents. Gradient: 16–24% B in 40.9 min; 256 uL.

Not only is an ACQUITY QDa effective in identifying and triggering the masses of the target product in peptide isolations and purifications, it is also useful for obtaining mass information about the other compounds that are present in the sample. As discussed earlier, the peptide synthesis and cleavage procedure has the potential to create many different types of impurities. With the chemist's knowledge of the synthetic protocol and with information obtained from the mass detector, assumptions can be made relating to how the synthesis and/or cleavage procedure can be improved. As shown in Figure 9, masses of impurities in the sample can be determined by looking at the mass spectra and mass chromatograms. Armed with this information, the chemist can then make prudent decisions for process enhancements.

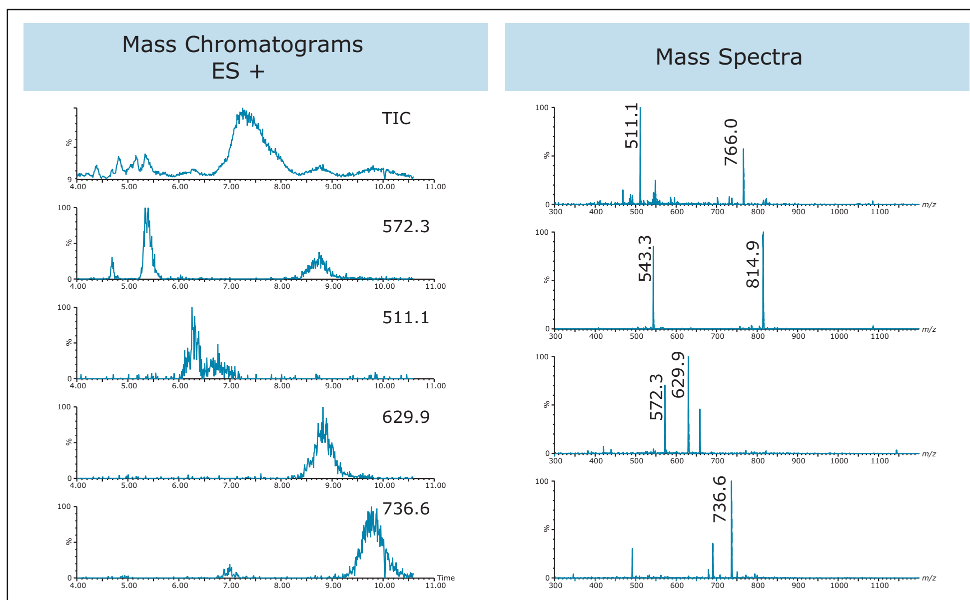

Figure 9. Mass assignments to impurities using the 19 x 150 mm XSelect CSH Phenyl Hexyl OBD Prep Column, 5 µm; 256 µL. Gradient: 16–24% B in 40.9 minutes.

CONCLUSIONS

- Isolation is easily accomplished using the ACQUITY QDa Mass Detector as configured in the Waters AutoPurification System, making it a lower cost alternative for purification of those peptides with monoisotopic masses or multiple charges which fall within the 30–1250 Da mass range of the detector.
- Columns with different selectivity are sometimes necessary for improving the separation of crude sample mixtures, which ultimately leads to the isolation of higher purity target peptide.
- Focusing the gradient improves the resolution of closely-eluting or co-eluting peptide impurities, contributing to the ease of target peptide isolation.
- The ACQUITY QDa Detector can be used to gather mass data on peptidic by-products, which may be useful for optimizing synthesis and cleavage protocols.

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OPTIMIZED CHROMATOGRAPHY FOR MASS-DIRECTED PURIFICATION OF PEPTIDES

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INTRODUCTION

Peptides play important roles in the physiology of organisms. They do, therefore, provide a major class of compounds for novel therapeutic products. Pure peptides are required to effectively study biochemical interactions in organisms as well as to understand structure-activity relationships in the development of peptide therapeutics.

Traditionally, preparative peptide separations are monitored using UV detection with the collection of many fractions over the course of the entire chromatographic program, necessitating labor-intensive post-run analysis and processing. Mass spectrometry (MS) has proven useful in quickly assessing peak identity and homogeneity in complex chromatograms, increasing sample throughput. Mass-directed isolation of compounds uses the mass spectrometer to recognize the target peak and deposit it in a fraction collector tube, reducing the number of processing steps.

The chemistry of peptides poses unique chromatographic challenges, including the selection of the appropriate column chemistry, the selection and adaptation of the mobile phase, and the optimization of the mode of detection.

In this study, we illustrate the development of general instrumental parameters and chemical conditions for mass-directed purification of peptides in the range of 8 to 25 residues. A refined protocol for translating a rapid pilot separation to an optimal preparative separation is demonstrated. Peptide compound isolation is more efficiently and effectively obtained using optimized chromatography and mass-directed purification.

EXPERIMENTAL

The purification of peptides poses specific challenges to the chromatographer. The first challenge is achieving high throughput while maintaining the shallow gradients necessary for peptide separation. Variable impurities, such as side products, deletions, and remaining reagents, can complicate and interfere with separations. Finally, the multiple charging of peptides often makes it difficult to predict the most abundant species in a given MS experiment.

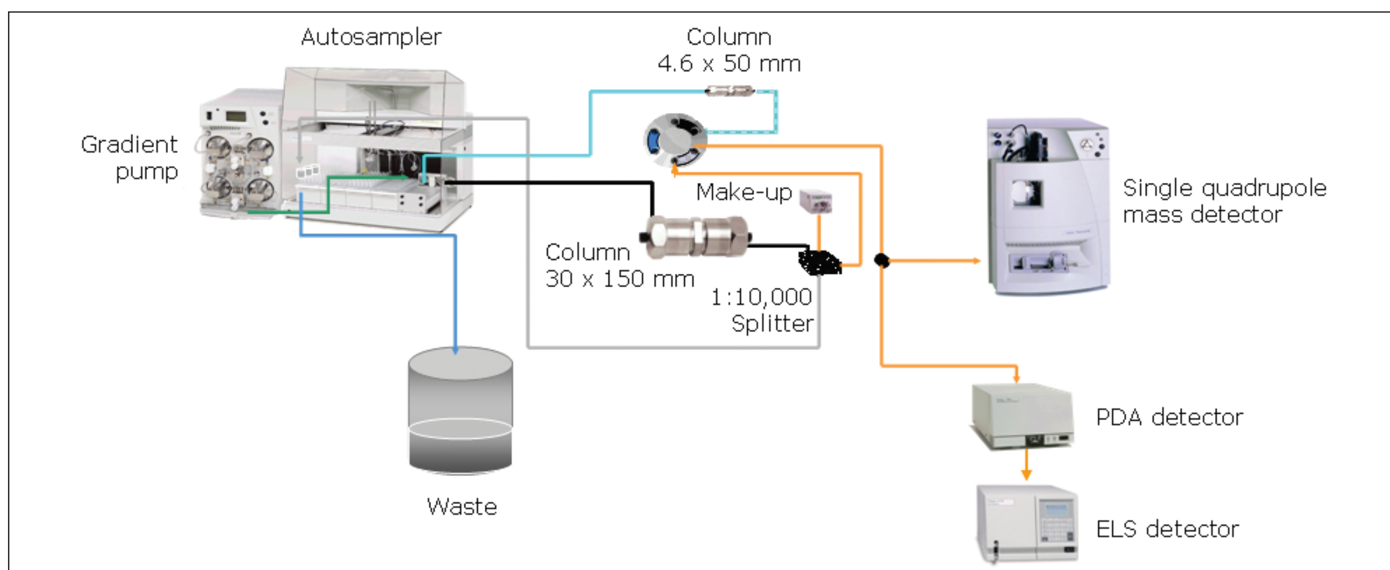

Figure 1. Configuration of the Waters Preparative Chromatography System used in these studies.

Waters Preparative Chromatography System

Throughout the experiments, a Waters Preparative Chromatography System consisting of a 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, ZQ™ Mass Spectrometer, 2 996 Photodiode Array Detector, 2420 Evaporative Light Scattering Detector, two 515 Pumps (for makeup/buffer delivery), LC Packings Flow Splitter (20 to 100 mL, 1:10,000 split), MassLynx™ Software and its FractionLynx™ Application Manager was used.

The analytical and preparative flow capabilities of the 2525 Binary Gradient Module allow the system to be used for both pilot and preparative-scale experiments.

For pilot-scale studies, a 4.6 x 50 mm Waters Symmetry® 300 C_{18} column with 5 µm particles is used.

For the preparative experiments, a 30 x 150 mm Waters Symmetry 300 C_{18} column with 7 µm particles is used.

Special consideration must be given to detection in preparative chromatography. The highly concentrated peaks exceed the linear range of common detectors. In addition, the high flow rates are not tolerated by detector hardware. Finally, some detectors used in this study (MS and evaporative light scattering) are destructive. It is, therefore, necessary to use a technique that reduces the flow and concentration reaching the detectors while minimizing loss of desirable material. A passive splitter is commonly used in the high ratio split and dilute technique, as shown in Figure 2.

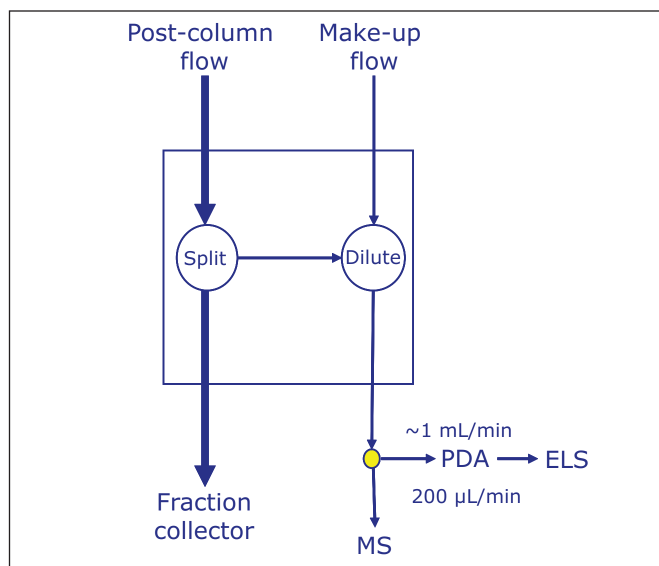

Figure 2. Flow path for the post-column sample flow. The passive flow splitter divides the prep flow 10,000:1, with 99.99% of the flow going to the fraction collector. The remaining 0.01% of the flow is transported to the detectors using the make-up flow.

Samples

The crude synthetic peptides were provided by Dr. Kelly Wasmund of Research Genetics, Inc.

(Huntsville, AL, U.S.). The two peptides were:

- NH_2-ISQAVHAAHAEINEAGR-COOH (abbreviated as ISQA)
- NH_2-SIINFEKL-COOH (abbreviated as SIIN)

Purification strategy

The strategy for the purification of the peptides is based on the use of pilot runs to define preparative conditions. From the retention time of the pilot runs, the percent organic mobile phase (%B) needed to elute each peptide is estimated. Assuming that the pilot scale separation is satisfactory, the conditions for the preparative chromatography are scaled based upon the ratio of column volumes. A shallow preparative gradient is then defined, ranging from 5% below to 3% above the calculated %B needed for peptide elution. The masses of the expected ions for possible charge states are used as mass triggers. The fractions are analyzed for purity using the same chromatographic conditions as the pilot run, with multiple detection modes to ensure purity.

System calibration

The relationship between time and actual organic mobile phase delivered to the column on the pilot-scale system is established using uracil (an unretained, UV-absorbing compound) in the organic mobile phase (B). The conditions for the system calibration are given below:

Solvent A: 100% water
Solvent B: 100% acetonitrile with 0.01 mg/mL uracil
Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 1. Calibration gradient. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

The absorbance trace resulting from the calibration run is given in Figure 3. From this run, the observed percentage of organic mobile phase (%B) is calculated using the following equation:1

$$\text{Observed \% B} = \frac{\text{Observed Absorbance}}{\text{Absorbance at 100\% Acetonitrile}} \times 95 + 5$$

Where:

- The absorbance at 100% acetonitrile is 600,000
- The observed absorbance is obtained from the absorbance trace at 258 nm
- Table 2 summarizes the results of the calibration.

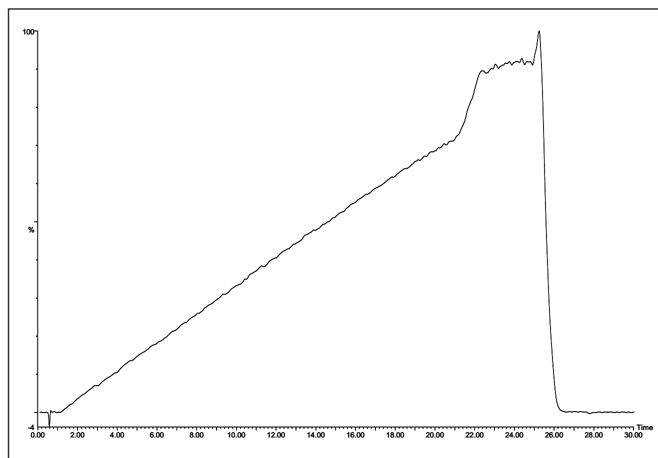

Figure 3. Absorbance trace for the system calibration.

Time	Observed Absorbance	Observed %B
0	1	
1	1	
2	24019	8.80
3	48966	12.75
4	70822	16.21
5	97858	20.49
6	121924	24.30
7	146816	28.25
8	172784	32.36
9	198040	36.36
10	221936	40.14
11	249464	44.50
12	272560	48.16
13	299896	52.48
14	320792	55.79
15	344572	59.59
16	370616	63.68
17	394568	67.47
18	416016	70.87
19	441728	74.94
20	460624	77.83

Table 2. Observed absorbance and calculated percentage of organic mobile phase with time.

Pilot-scale separations

Each peptide is individually wetted in 0.5 mL DMF and then diluted to 4.5 mL with water. The concentration of ISQA is estimated at 5 to 10 mg/mL and SIIN is estimated at 10 mg/mL. The conditions for the pilot-scale separations of each of the peptides are given in Table 3; the chromatographic results are presented in Figure 4.

Solvent A:	100% water
Solvent B:	100% acetonitrile
Injection vol.:	40 µL
Column:	4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate(mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 3. Gradient conditions used in the pilot-scale separations of ISQA and SIIN. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

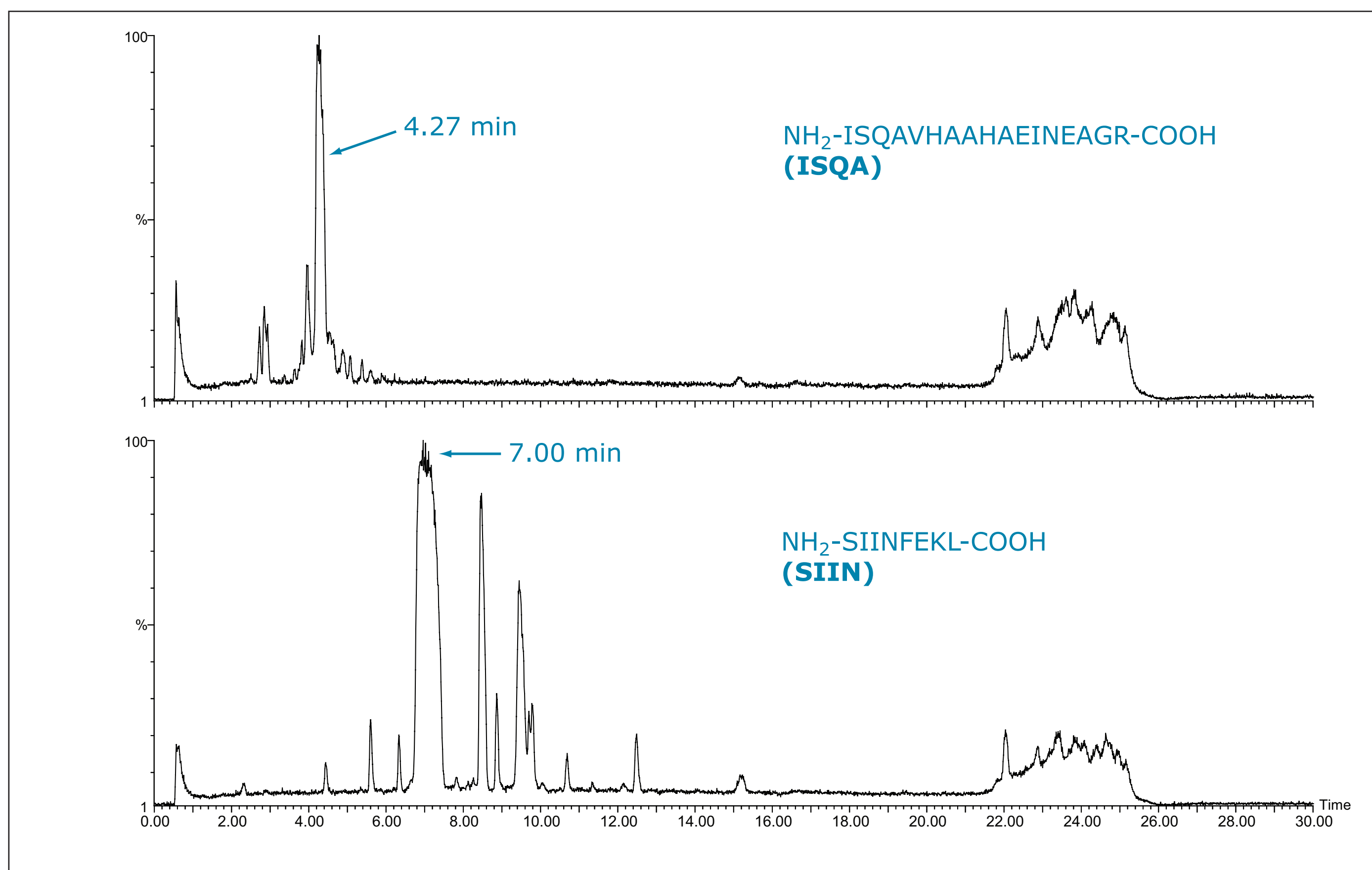

Figure 4. Total Ion Chromatograms (TIC) (ES+) for the pilot-scale separations of ISQA and SIIN.

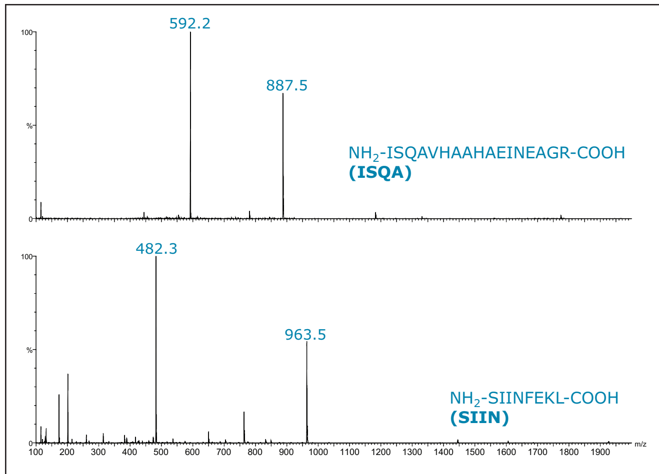

Figure 5. Mass spectra for ISQA and SIIN resulting from the pilot runs.

Defining the preparative gradient

Shallow preparative gradients create enhanced separations of the peptides from closely-eluting contaminants. In this case, a shallow gradient that ranges from 5% below to 3% above the calculated %B for peptide elution is used. The 4.27-minute retention time for ISQA indicates that it elutes near 17%B. This suggests that the preparative gradient for ISQA should start at 12%B and go to 20%B. Similarly, the retention time of SIIN at 7.00 minutes indicates that it elutes near 28%B. The shallow preparative gradient for this peptide should run from 23%B to 31%B.

Mass triggers

Target masses are selected to include the expected multiply-charged ions, Table 4.

Preparative chromatography

Purification of the ISQA and SIIN peptides is performed using shallow preparative gradients (Tables 5 and 6) for separation and fraction collection based on multiple target ions. The conditions for the preparative run of ISQA and SIIN are given in Tables 5 and 6, and the resulting chromatograms are shown in Figures 6 and 7, respectively.

NH_2-ISQAVHAAHAEINEAGR-OOOH (ISQA)	
Monoisotopic Mass	1772.9 Da
Target Ions for Charge States	m/z
$[M+H]^+$	1773.9
$[M+2H]^{2+}$	887.5
$[M+3H]^{3+}$	592.2
$[M+4H]^{4+}$	447.2
NH_2-SIINFEKL-COOH (SIIN)	
Monoisotopic mass	962.5 Da
Target ions for charge states	m/z
$[M+H]^+$	963.3
$[M+2H]^{2+}$	482.3
$[M+3H]^{3+}$	321.9

Table 4. Calculated target ions for various charge states of ISQA and SIIN.

ISQA

Solvent A: 100% water

Solvent B: 100% acetonitrile

Injection vol.: 5 mL

Column: 30 x 150 mm Symmetry 300, C_{18}, 7 µm

Target masses: $[M+H]^+$ = 1773.9, $[M+2H]^{2+}$ = 887.5, $[M+3H]^{3+}$ = 592.2, $[M+4H]^{4+}$ = 447.2

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	88.4	11.6
36.50	57.50	80.4	19.6
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 5. Shallow preparative gradient used for the purification of ISQA. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

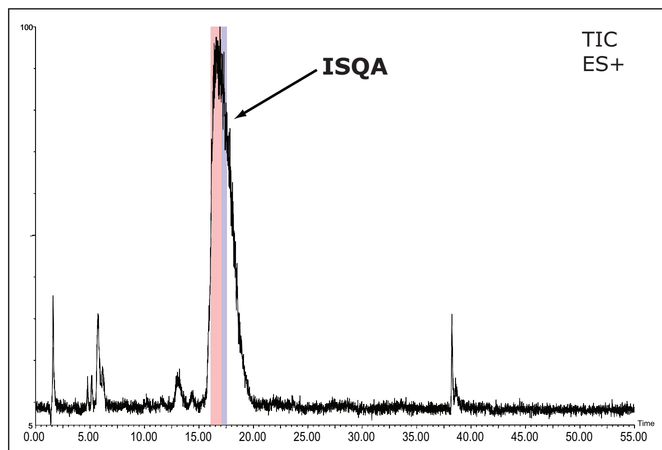

Figure 6. Preparative MS chromatogram for ISQA. The shaded bars represent fraction collection on m/z 592.2 in positive ion mode.

SIIN

Solvent A: 100% water

Solvent B: 100% acetonitrile

Injection vol.: 5 mL

Column: 30 x 150 mm Symmetry 300, C_{18}, 7 µm

Target Masses: $[M+H]^+$ = 963.5, $[M+2H]^{2+}$ = 482.3, $[M+3H]^{3+}$ = 321.9

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	77.1	22.9
36.50	57.50	69.1	30.9
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 6. Shallow preparative gradient used for the purification of SIIN. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

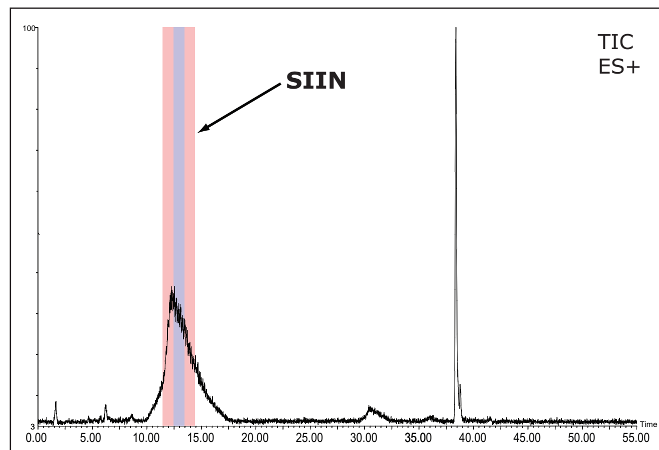

Figure 7. Preparative MS chromatogram for SIIN The shaded bars represent fraction collection on m/z 482.3 in positive ion mode.

Fraction analysis

The fractions resulting from the preparative runs are analyzed to assess purity. Multiple detection channels are used to monitor the analysis to provide more complete characterization of the fractions. The fraction analysis of both ISQA and SIIN peptides use the separation conditions in Table 7. The results of the fraction analysis are shown for ISQA and SIIN in Figures 8 and 9, respectively.

Solvent A: 100% water
Solvent B: 100% acetonitrile
Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 7. Gradient conditions for re-analysis of the ISQA and SIIN fractions. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

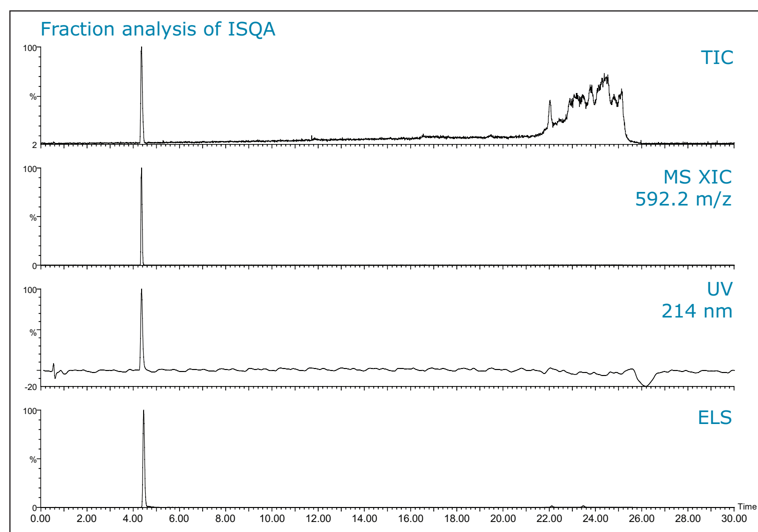

Figure 8. Fraction analysis of ISQA. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

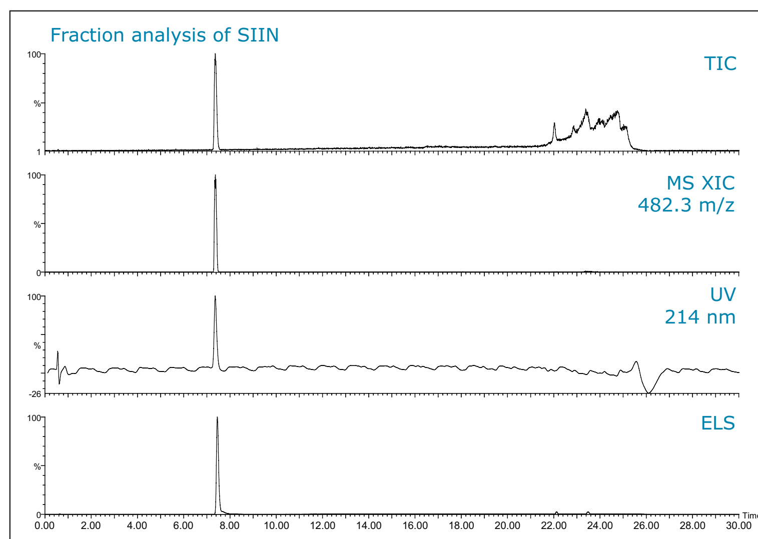

Figure 9. Fraction analysis of SIIN. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

CONCLUSION

The use of fast, pilot-scale runs provides a useful prediction of conditions for shallow-gradient, high-resolution preparative chromatography.

Notes

[1] The calibration gradient occurs over the 5% acetonitrile to 100% acetonitrile range. Five percent acetonitrile has an absorbance of zero because the UV detector zeroes at the start of the gradient.

At the end of the gradient, where 100%B is reaching the detector, the observed absorbance is 600,000.

The interval between 5% acetonitrile and 100% acetonitrile is 95%. This corresponds to the range between 0 and 600,000 on the absorbance scale.

To calculate the observed percentage of B in the middle of the 5% to 100% range as a percentage of the total range (0 %B to 100%B), the observed absorbance divided by the absorbance at 100% acetonitrile is multiplied by 95. Five percent is added to the value to account for the offset of the curve at the start of the run.

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[APPLICATION NOTE]

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Peptide Isolation Using the Prep 150 LC System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The Prep 150 LC System, an affordable, highly reliable system for preparative chromatography, is suitable for peptide purification.
- With knowledge of basic chromatography scaling principles, analytical methods can be predictably scaled for prep on the Prep 150 LC System, making it an ideal system for those laboratories where chemists prefer to process their own samples.
- Fraction simulation, a ChromScope™ Software tool, allows the user to visually set collection parameters on the chromatogram, saves time, sample, and solvent by eliminating trial and error injections in pursuit of the best collection method.
- The Prep 150 LC System, with its straightforward design and uncomplicated software control, facilitates users to more efficiently process samples and thereby increase productivity.

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Alliance® HPLC System

Prep 150 LC System

Waters OBD™ Prep Columns

ChromScope v1.4.1 Software

GHP Acrodisc Syringe Filters

KEY WORDS

Prep chromatography, peptide purification, peptide isolation, OBD Prep Columns

INTRODUCTION

Peptides are becoming more prevalent as diagnostics as well as therapeutics, mainly because of the technological advances in analytical methodology that have been made in recent years.[1] Analytical methods run on state-of-the-art instrumentation show improved sensitivity and higher resolution in a shorter amount of time, all of which lead to increased throughput. With higher efficacy, safety, and tolerability in humans as compared to small molecules, and with lower production complexity and cost than protein-based biopharmaceuticals, peptides are increasingly more attractive as potential drug candidates.[2] Whether peptides are made in solution, with solid phase strategies, or isolated from naturally-occurring sources, these potential products all require purification. The Prep 150 LC System is ideally suited for the isolation and purification of peptides with its reliable and robust instrumentation controlled by ChromScope Software. Although the crude peptide is commonly evaluated on an analytical HPLC system, with the knowledge of basic scaling principles, the larger scale isolation is straightforward and predictable with the Prep 150 LC System. Here, we demonstrate the analysis and UV-directed purification of a synthetic peptide using an Alliance HPLC System for screening and a Prep 150 LC System for isolation. This flexible strategy is applicable to many different types of samples which require purification. Methods can easily be optimized to save time and production cost.

EXPERIMENTAL

Conditions

Analytical columns:	XBridge® C_{18}, 4.6 x 100 mm, 5 µm
Analytical flow rate:	1.46 mL/min
Prep column:	XBridge C_{18} OBD Prep, 19 x 100 mm, 5 µm
Prep flow rate:	25 mL/min
Mobile phase A:	0.1% trifluoroacetic acid in water
Mobile phase B:	0.1% trifluoroacetic acid in acetonitrile
Wavelength:	220 nm, 280 nm
Gradients and injection volumes:	as noted in figures
Sample:	Crude synthetic peptide comprised of the following 20 amino acid residues: 7 polar, 8 nonpolar, 3 acidic, and 2 basic; 75% purity by HPLC

Instrumentation

Analytical:	Alliance HPLC System, 2998 PDA Detector
Prep:	Prep 150 LC System configured with a 2545 Binary Gradient Module, 2489 UV/Visible Detector, Prep Inject Module, and Waters Fraction Collector III controlled by ChromScope v1.4.1

Alliance HPLC System.

Prep 150 LC System with Waters Fraction Collector III.

DISCUSSION

Solid phase peptide synthesis[3] is a step-wise process where side-chain protected amino acids are sequentially added to a sequence by the formation of a peptide bond. Repeated cycles of coupling and deprotection with the addition of each amino acid continue until the desired sequence is assembled. After synthesis, the peptide is cleaved from the solid support and the side chain protecting groups are removed. As with any chemical reaction, incomplete deprotections, amino acid couplings, inadequate washing, or adduct formation during cleavage all contribute to impurity generation. Once the crude peptide is cleaved from the solid support and dried, it is analyzed and purified. In the present study, 14.4 mg of crude synthetic peptide was dissolved in 2 mL dimethylsulfoxide (DMSO) and filtered to give a final sample concentration of 7.2 mg/mL. HPLC analysis of the crude peptide on a 4.6 x 100 mm XBridge C_{18} Column showed that the purity was approximately 75% (Figure 1). The fast screening gradient ran from 5–50%B in 10 minutes, with a slope of 3.4% change per column volume (3.4%/cv). The peptide product eluted at approximately 33.5%B. Close examination of the chromatogram revealed the presence of several impurities eluting very closely to the main product peak.

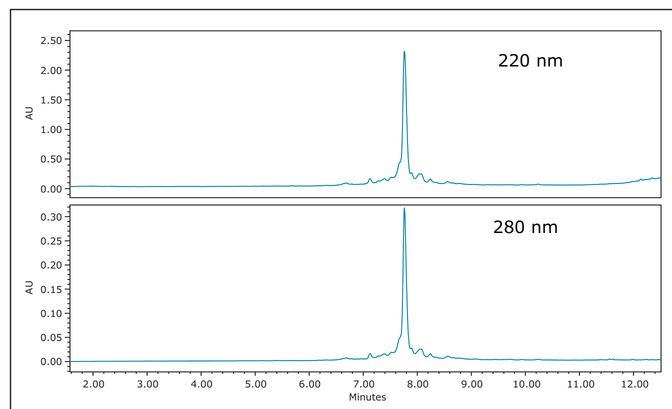

Figure 1. HPLC of the crude synthanalysis etic peptide on a 4.6 x 100 mm XBridge C_{18} 5 µm Column. Gradient: 5–50%B in 10 min, 3.4% change/cv, 5 µL.

As a general rule for focusing gradients,[4] one-fifth of the screening slope usually slows the rate of change of the gradient to effectively move closely-eluting peaks away from the desired target peak. As shown in Figure 2, one-fifth the rate of change at 0.67%/cv improved the chromatographic profile, but an even shallower slope of 0.33%/cv further increased the resolution between the large target peak and the two peaks eluting on either side of the product. Improved resolution ultimately leads to higher column loading and/or better product purity. For this reason, the focused gradient with the more shallow slope was selected for the isolation of the peptide.

With the resolution improved to almost baseline with the very shallow 0.33%/cv focused gradient, a loading study was performed using the 4.6 x 100 mm analytical column (Figure 3). While a 15 µL injection on the 4.6 mm I.D. column still showed acceptable resolution, the more conservative 10 µL loading was chosen for subsequent scaling because the distance between the main peak and the two by-products was greater. Note that as the injection volume increased, the peptide peak eluted slightly earlier, a normal phenomenon commonly observed with increased column loading.

Proper scaling[5] ensures that the chromatographic profile of a sample at the preparative scale will be identical to the chromatography obtained at the analytical scale. Geometric scaling of the flow rate and gradient to the 19 x 100 mm preparative column on the Prep 150 LC System showed the same chromatographic profile, as predicted (Figure 4). 85 microliters was injected on the prep column for the first run, equivalent to scaling from 5 µL on the analytical scale, for a more conservative approach toward determining the collection parameters.

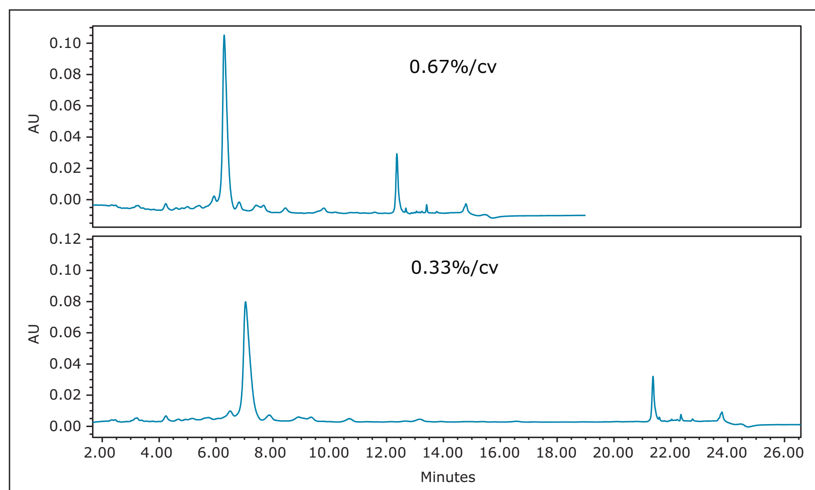

Figure 2. The effect of focusing the gradient used to analyze the crude peptide sample. Top trace: 28–36% B in 9 min, 5 µL. Bottom trace: 28–36%B in 18 min, 5 µL. Detection: 280 nm.

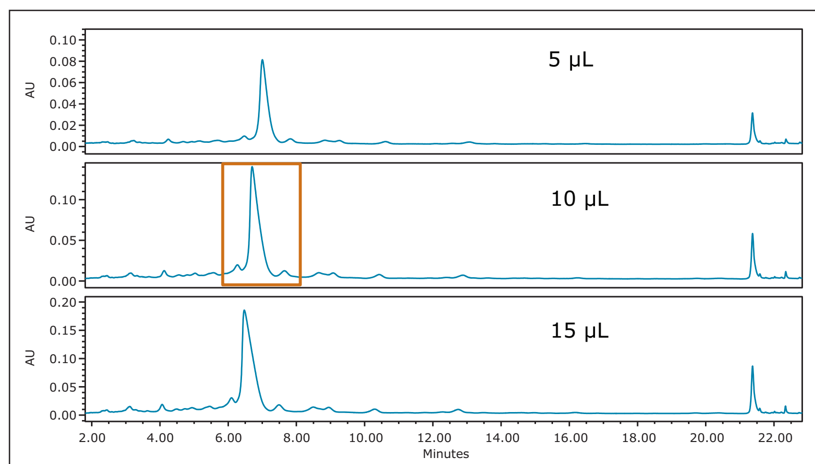

Figure 3. Loading study performed on the 4.6 x 100 mm column. Gradient: 28–36%B in 18 min with detection at 280 nm. The 10 µL loading was chosen for scaling to prep.

Injection No	Fraction #	Tube Location	Volume Collected In Tube (ml)
1	1	A:B	1.16
1	2	A:C	0.27
1	3	A:D	3.56
1	4	A:E	7.18

Figure 4. Fraction tracking shows the product tube location in the collection bed as well as the volume collected in each tube.

Three extra peaks were collected before the product peak eluted due to a wide collection window set in the method to ensure collection of the desired peptide. Although the peptide product was collected, a high threshold setting very conservatively heart-cut the product peak. Fraction tracking was clearly visible with collection bed tube location and volume readily available in the ChromScope display. The fraction simulation tool in the software was used to reset the collection parameters by visually adjusting the collection window and threshold (Figure 5) on the chromatogram. These optimized parameters were used for the subsequent prep isolations.

Two more preparative runs were performed, the first with an 85 µL injection to affirm the collection parameters, and the second with a 171 µL injection, the geometrically scaled injection volume as determined by the loading study on the 4.6 mm ID column. In the interest of saving time and solvent, the preparative runs were terminated after the target peak was collected and the column was manually washed and equilibrated. Figure 6 shows the chromatographic reproducibility of the prep runs, both of which were similar to the analytical profile obtained on the Alliance HPLC System. Knowledge of system volumes, identical column chemistry, and properly scaled injection volume, flow rate, and gradient time ensured predictable chromatography at the preparative scale.

The fractions were analyzed immediately after collection on the 4.6 x 100 mm XBridge C_{18} Column with the original fast screening gradient. The fractions were very pure (Figure 7).

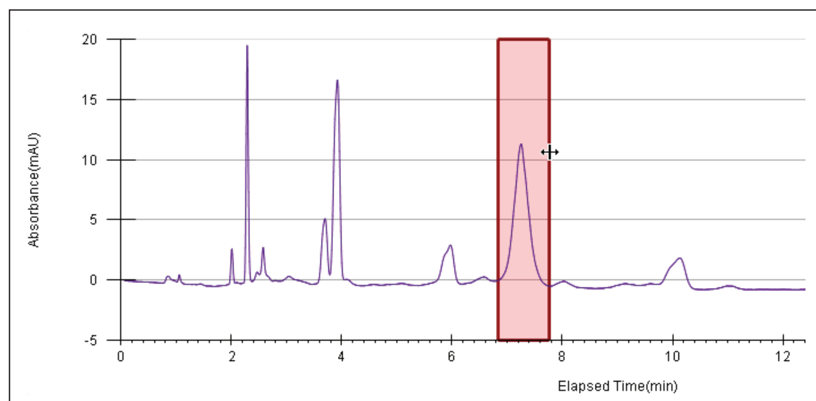

Figure 5. The fraction simulation tool was used to optimize fraction collection for the next preparative run.

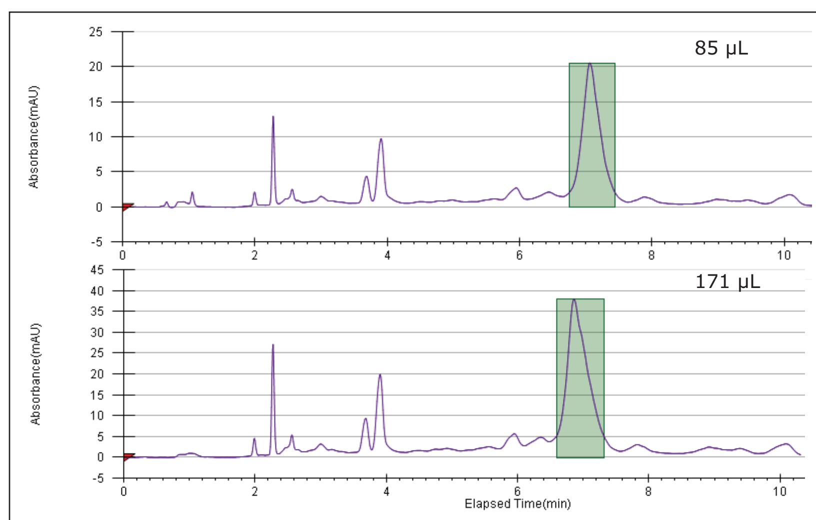

Figure 6. 85 and 171 µL injections on the 19 x 100 mm XBridge C_{18} Column using the newly defined collection parameters as determined using the fraction simulation tool. Detection: 280 nm.

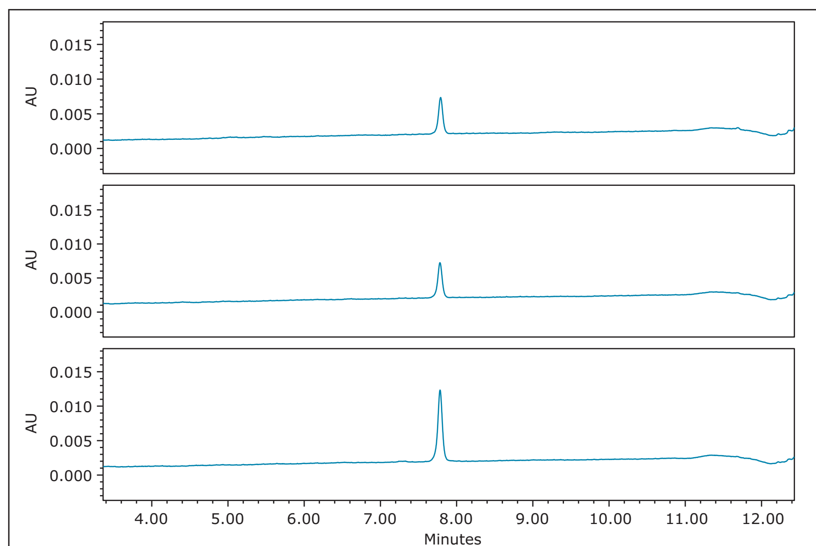

Figure 7. Analysis of the three collected fractions on the 4.6 x 100 mm XBridge C_{18} Column. Gradient: 5–50% B in 10 min, 20 µL. Detection: 280 nm.

CONCLUSIONS

We have shown that traditional peptide isolation performed by HPLC with UV detection is easily accomplished using the Prep 150 LC, a simple and robust chromatography system. While crude peptide analysis was performed on an Alliance HPLC System configured with a 2998 Photodiode Array Detector, with knowledge of system volumes and basic preparative scaling rules, the isolation of the target peptide was predictable and reproducible when the method was transferred to the Prep 150 LC System. Focusing the gradient was useful in improving the quality of the separation by increasing the resolution between closely-eluting by-products. ChromScope Software, with its intuitive and easy-to-use preparative features like fraction simulation, made setting collection parameters fast and uncomplicated. Fraction tracking information, including collection volume, was unambiguous with collection tubes clearly marked on the chromatogram and on the collection bed.

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[APPLICATION NOTE]

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Preparative Scale Chromatography of a Hydrophilic Peptide Using Hydrophilic Interaction Chromatography

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APPLICATION BENEFITS

BEH Amide columns are specifically designed to enhance the retention of polar compounds, making analysis, scaling, and isolation easier.

- Hydrophilic peptides that cannot be retained by reversed-phase may be retained in hydrophilic interaction chromatography using the BEH Amide column. Longer retention allows separation to occur.
- The BEH Amide bonded phase is more compatible with slightly higher aqueous content in mobile phases and sample diluents than unbonded HILIC stationary phases, making the BEH Amide column a more viable alternative for the purification of hydrophilic peptides.
- Improved mass loading of polar peptides on BEH Amide columns reduces the number of injections required to isolate the product, promoting process efficiency.
- Reproducible chromatograms at the analytical and preparative scales reduce ambiguity in the identification of target peptides and demonstrate BEH Amide column scalability.

WATERS SOLUTIONS

AutoPurification™ System

XBridge BEH Amide Columns

KEY WORDS

Polar compound isolation, BEH Amide columns, preparative LC, hydrophilic interaction chromatography, HILIC, hydrophilic peptides

INTRODUCTION

Reversed-phase columns are typically used for the analysis and isolation of peptides, however some hydrophilic peptides have little or no retention on C_{18} stationary phases. Insufficient interaction with the stationary phase leads to difficulties in the peptide isolation. Hydrophilic Interaction Chromatography (HILIC) is an alternative chromatographic technique useful in the isolation of compounds where analytes are separated based on a unique combination of liquid-liquid partitioning, adsorption, ionic interaction, and hydrophobic retention mechanisms. Compounds elute from the column as the gradient transitions from low aqueous to high aqueous mobile-phase composition.

The BEH Amide column, with a trifunctionally-bonded amide phase, was first introduced in 2009 with 1.7 µm particles for the analysis of polar compounds using the ACQUITY UPLC® System. Demand for a column capable of analyzing compounds such as hydrophilic synthetic peptides, saccharides, synthetic sugars, glycopeptides, and polar compounds from natural products has driven the development of a larger 5 µm particle for use in analytical and preparative HPLC applications. In this application note, we demonstrate the utility of the BEH Amide particle for the analysis and isolation of a hydrophilic peptide.

EXPERIMENTAL

LC Conditions

System:	Waters 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, 2996 Photodiode Array Detector, ZQ 2000 Mass Spectrometer, and 2420 ELSD Mass Detector
Columns:	XBridge BEH Amide, 5 µm, 4.6 x 150 mm, part number 186006595 XBridge BEH Amide, 5 µm, 19 x 150 mm, part number 186006605
Column Temp.:	40 °C
Mobile Phase A:	20/80 acetonitrile/ 10 mM ammonium formate pH 3
Mobile Phase B:	90/10 acetonitrile/ 10 mM ammonium formate pH 3
Weak Needle Wash:	90/10 acetonitrile/water
Strong Needle Wash:	20/80 acetonitrile/water
Seal Wash:	50/50 acetonitrile/water
Sample Diluent:	15/5/3 acetonitrile/ methanol/water
Flow Rate:	Reported in figures
Gradient:	Reported in figures
Injection Volume:	Reported in figures

SAMPLE PREPARATION

Analytical Scale

2.0 mg of polar peptide comprised of the following 20 residues: 4 basic, 11 polar and uncharged, 3 nonpolar, and 2 acidic, were dissolved in 1.15 mL of sample diluent, producing a concentration of 1.77 mg/mL peptide solution. The sample diluent was a mixture of 15/5/3 acetonitrile, methanol, and water. The crude peptide solution was vortexed and filtered through a 13 mm, 0.45 µm GHP syringe filter, part number WAT200516.

Preparative Scale

31 mg of polar peptide were dissolved in 2.3 mL of the sample diluent for a final concentration of 13.5 mg/mL. The sample mixture was vortexed and filtered.

RESULTS AND DISCUSSION

The analysis and isolation of polar peptides is often challenging because of the difficulty in ensuring the retention of very hydrophilic sequences on a reversed-phase column. Hydrophilic Interaction Chromatography (HILIC), is an orthogonal chromatographic separation technique which separates hydrophilic compounds by their interaction with a polar stationary phase. Liquid-liquid partitioning, adsorption, ion exchange, and hydrogen bonding mechanisms all contribute to the retention of the sample. Analytes are eluted from the column by increasing the polarity of the mobile phase. The selectivity and retentivity of compounds on different stationary phases is dependent upon the specific properties of the column packing. As shown in Figure 1, the elution profile of the analytes is unique for each of the three HILIC stationary phases when the column dimensions and the chromatographic method are held constant. The BEH Amide column shows the most retention for the various types of compounds and a different selectivity compared to the other two columns. Better retention of similar compounds often improves the resolution between them.

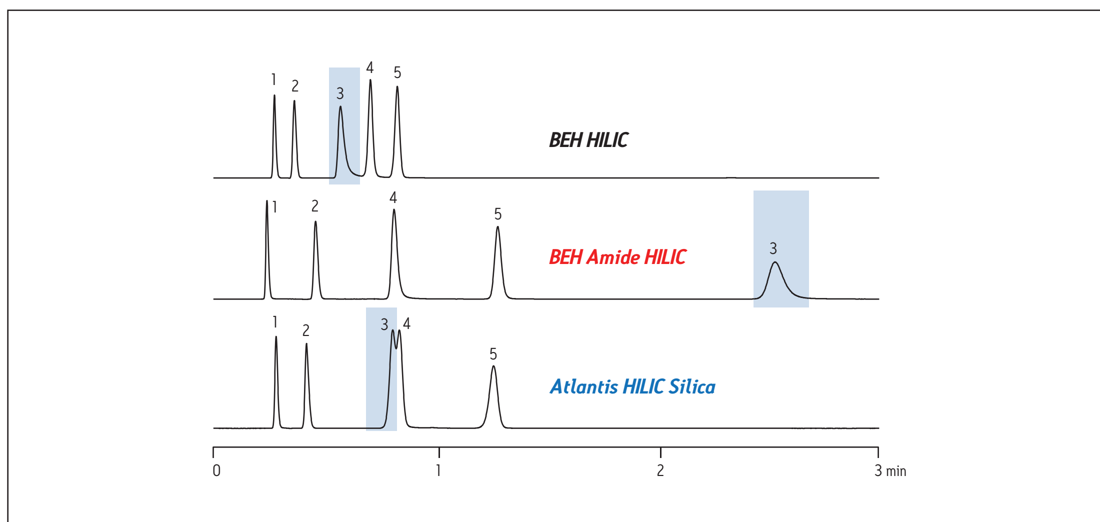


Figure 1. Here is a chromatographic representation of the differences in retentivity and selectivity of the 3 HILIC stationary phases. The Atlantis® HILIC Silica provides more retention and a different selectivity compared to the BEH HILIC (due to its higher surface polarity). The amide column exhibits the most retention for various types of compounds and a different selectivity compared to the other two columns. Conditions: Isocratic, 12 mM ammonium formate (pH 3) with 90% acetonitrile; UV at 254 nm. Compounds: (1) acenaphthene, (2) thymine, (3) 5-fluoroorotic acid, (4) adenine, (5) cytosine.

Although retention is crucial for effective separations, the nature of the target molecule must also be considered for a successful isolation of the compound. Deleted and failure sequences, adducts, and residual cleavage cocktail components contribute to the complexity of the crude sample mixture and complicate the isolation of the target peptide. The sample diluent also plays a role in retention, influencing solubility and peak shape. Traditional unbonded HILIC stationary phases usually require diluents and mobile phases with high organic concentration which limit the solubility of polar compounds at the high sample concentrations used in prep chromatography. Small amounts of water, even 10-20%, make the injection solvent incompatible with initial HILIC conditions on unbonded phases. Since the BEH Amide bonded phase tolerates mobile phases and injection solvents which are higher in aqueous content, polar peptides can be solubilized at concentrations amenable to preparative chromatography.

Because the amino acid sequence of the 20-mer in this study has no chromophores, peptide detection by UV is limited. Systems configured with alternate modes of detection identify target molecules with limited UV absorption or low ionization potential. Figure 2 shows the amino acid composition and the calculated monoisotopic mass and higher charge states for the peptide molecule used in this study. Since higher organic content mobile phases are typically used in HILIC, they are easily desolvated and provide an enhanced mass spectrometric response as well as faster fraction drying time. As shown in Figure 3, the peptide displays very little absorption at 220 nm due to the absence of chromophores in the amino acid sequence, but the ELSD and mass chromatograms have improved sensitivity, making isolation and analysis possible.

20 Amino Acids in Length

Amino acid composition:
4 basic
11 polar (uncharged)
2 acidic
3 nonpolar

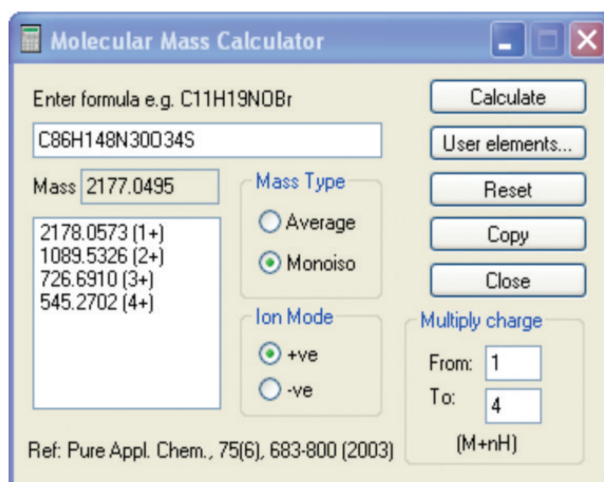
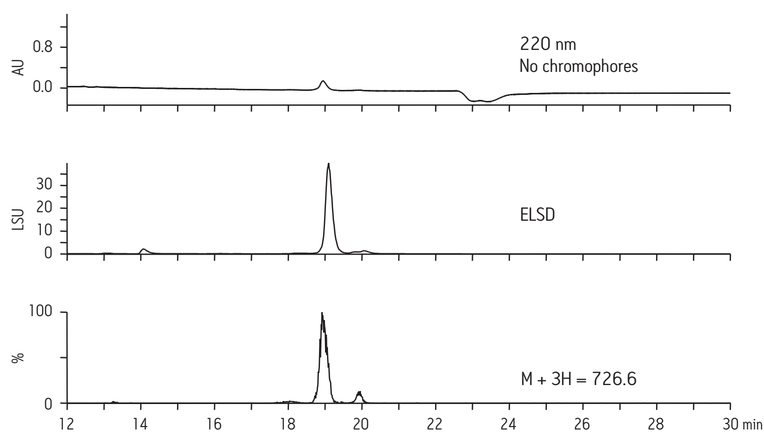


Figure 2. Amino acid composition and calculated monoisotopic and higher charge state masses for the hydrophilic peptide.



100% B-100% A in 18 min, hold 3 min, return to 100% B in 0.20 min, equilibrate 8.8 min.

Flow Rate: 1.46 mL/min
Mobile Phase A: 20 acetonitrile/80 10 mM ammonium formate pH 3
Mobile Phase B: 90 acetonitrile/10 10 mM ammonium formate pH 3
Strong Needle Wash: 20 acetonitrile/80 water
Weak Needle Wash: 90 acetonitrile/10 water
Sample Diluent: 75 acetonitrile/25 methanol with 150 µL water
Sample Concentration: 13.5 mg/mL

Figure 3. Comparison of the three modes of detection for the hydrophilic peptide on the 4.6 x 150 mm BEH Amide column.

Scaling separations requires matching column chemistry as well as appropriately scaled gradients. As laboratories explore options for increasing throughput in the purification process, fast screening gradients using UPLC® reduce the amount of time required for synthetic crude product analysis and, in some cases, fraction analysis. Since the BEH Amide column is available in sub-2-µm configurations, the synthetic crude peptide was analyzed using the ACQUITY UPLC. Maintaining the resolution between the UPLC and preparative scales requires the ratio of the length of the column to the diameter of the particle (or L/dp) remain constant. The UPLC, 2.1 x 50 mm, 1.7 µm column has an L/dp of about 29,400. The preparative, 19 x 150 mm, 5 µm column L/dp is 30,000, essentially equal to the L/dp ratio for the UPLC column. As expected, geometric scaling of the injection volume and chromatographic conditions produced a preparative chromatogram which is directly comparable to the sub-2-µm screening analysis done on the ACQUITY UPLC. Figure 4 illustrates the BEH Amide column scalability by comparing the chromatography using the fast screening gradient on UPLC and the larger scale chromatography used for the isolation.

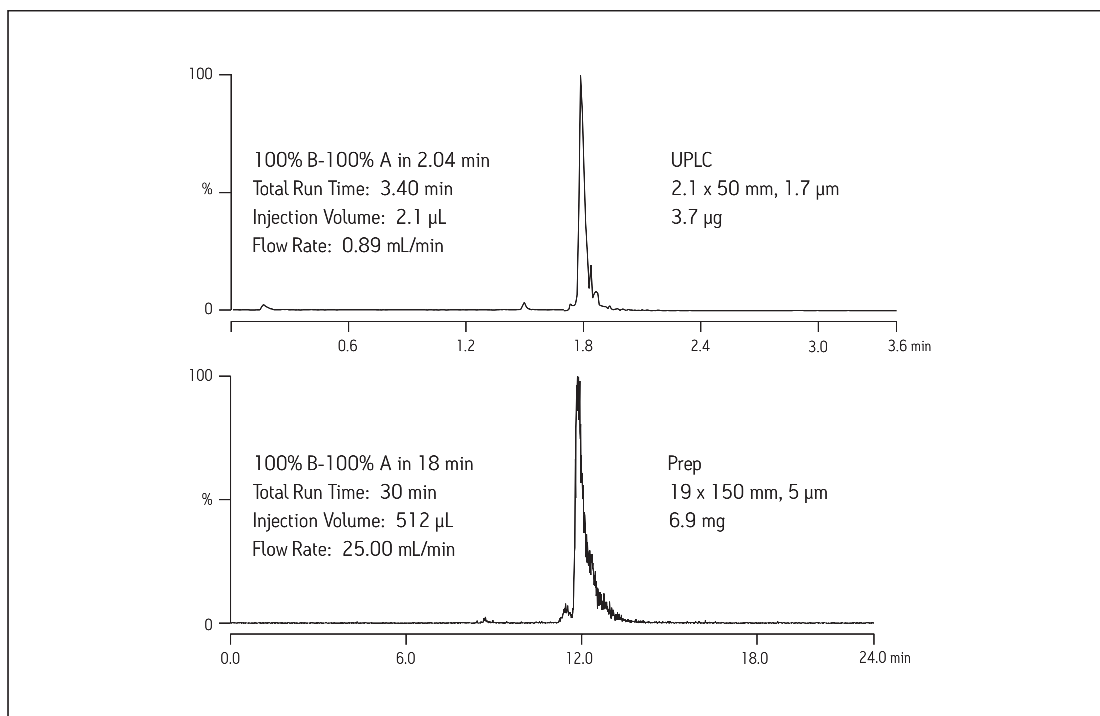


Figure 4. Hydrophilic peptide chromatograms at the UPLC and preparative scales.

CONCLUSIONS

- Hydrophilic peptides that cannot be retained by reversed-phase may be retained in hydrophilic interaction chromatography using the BEH Amide column. Longer retention allows separation to occur.
- The BEH Amide bonded phase is more compatible with slightly higher aqueous content in mobile phases and sample diluents than unbonded HILIC stationary phases, making the BEH Amide column a more viable alternative for the purification of hydrophilic peptides.
- Reproducible chromatograms at the analytical and preparative scales reduce ambiguity in the identification of target peptides and demonstrate BEH Amide column scalability.

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[APPLICATION NOTE]

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Small Scale Peptide and Impurity Isolation Using the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical Systems

Jo-Ann M. Jablonski and Andrew J. Aubin
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The ACQUITY UPLC® H-Class System configured with the Waters® Fraction Manager-Analytical (WFM-A) reproducibly separates and collects target peptide and its closely-eluting impurities, making isolation at the small scale fast and easy.
- Fast valve switching and movement between vessels, as well as a fraction divert valve with very low dispersion volume enables the collection of narrow peaks, increasing confidence in compound isolation.
- Small scale compound isolation saves time, solvent, and resources promoting faster research and development timelines.

WATERS SOLUTIONS

ACQUITY UPLC H-Class System

ACQUITY UPLC PDA Detector

Waters Fraction Manager-Analytical (WFM-A)

Empower® 3 Software

XBridge® Columns

0.45 µm GHP Acrodisc 13 mm syringe filter

KEY WORDS

Small scale prep chromatography, peptide purification, peptide isolation, ACQUITY UPLC H-Class, Waters Fraction Manager-Analytical (WFM-A), analytical purification

INTRODUCTION

As peptides become more popular in the development of new therapeutics, it is increasingly important to quickly optimize the synthetic and cleavage processes by isolating and identifying both the target peptide and its related impurities. Collecting and analyzing closely-eluting impurities while isolating the target peptide saves time and effort and provides additional information about steps that can be taken to improve the quality and yield of the peptide product. While peptide isolation is routine for groups involved in synthesis and cleavage, peptide isolation is also useful for scientists in research and discovery groups. Whether peptide studies are focused on how these complex molecules affect the body and are metabolized or on how peptides are isolated from naturally-occurring sources,[1] only small amounts are required for initial experiments. In this study, we illustrate the utility of the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical (WFM-A) Systems for the analysis and isolation of a synthetic peptide and its closely-eluting impurities. This instrument configuration can be adapted for the isolation of constituents from complex synthetic, metabolic, or natural product mixtures at the small scale.

EXPERIMENTAL

Conditions

Analytical column:	XBridge Peptide BEH C_{18}, 4.6 x 50 mm, 5 µm
Flow rate:	1.46 mL/min
Mobile phase A:	0.1% trifluoroacetic acid in water
Mobile phase B:	0.1% trifluoroacetic acid in acetonitrile
Wash solvent:	7:2:1 Acetonitrile/Methanol/Water
Purge solvent	9:1 Water/Methanol
Wavelength:	280 nm
Gradients and injection volumes:	as noted in figures
Column temperature:	30 °C
Sample:	Crude synthetic peptide comprised of the following 16 amino acid residues: 7 polar, 6 nonpolar, 1 acidic, 2 basic; purity 56% by HPLC

Instrumentation

ACQUITY UPLC H-Class System with an
ACQUITY UPLC PDA Detector and Empower 3 Software

Waters Fraction Manager-Analytical

Figure 1. ACQUITY UPLC H-Class System with Waters Fraction Manager-Analytical (WFM-A) System.

RESULTS AND DISCUSSION

The principles of scaling chromatography[2] remain the same whether the objective is to increase the amount of sample isolated at one time on a large column, or to decrease the amount of product based on the immediate need for material to perform experiments which answer pertinent questions quickly. For these studies, another new aliquot of the crude synthetic peptide sample used in previous work[3] was isolated using the same optimized and focused gradient,[4] this time at a much smaller scale – on a 4.6 x 50 mm XBridge Peptide BEH C_{18} Column using the ACQUITY UPLC H-Class System configured with a WFM-A fraction collector. The crude peptide (2.4 mg) was dissolved in dimethylsulfoxide (DMSO) and filtered using a 13 mm Acrodisc GHP syringe filter. Whereas 10 µL was the maximum injection volume that maintained resolution between the peptide and its impurities on the 4.6 x 100 mm XBridge C_{18} Column used in previous work (Figure 2), geometric scaling to the shorter 4.6 x 50 mm column for these experiments reduced the injection volume to 5 µL. Likewise, the reduction in column length automatically reduced the gradient run time from 18 minutes on the 100 mm column to 9 minutes on the 50 mm column. With the target peptide peak and its closely-eluting impurities eluting well before 36% B, the gradient conditions were adjusted to run from 28–32% B in 5 minutes, thus saving time in the method. Because of the complexity of crude synthetic peptide samples, shallow focused gradients with slopes of about 0.2–0.3% change per column volume are useful for resolving more sample constituents.

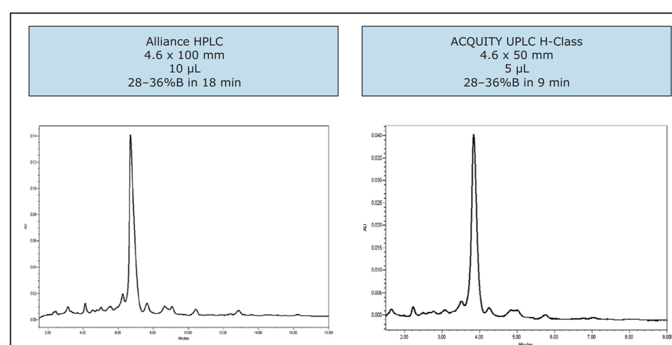
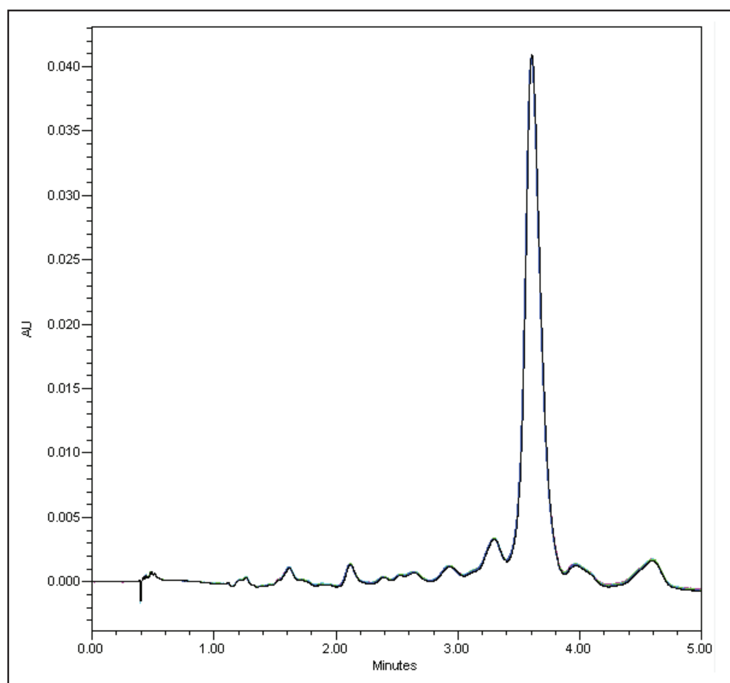


Figure 2. The peptide sample profile was very similar on two different systems. Geometric scaling principles were applied. The sample concentration for work done on the Alliance® HPLC System was 7.2 mg/mL while the sample concentration used on the ACQUITY UPLC H-Class System was 2.4 mg/mL. Column chemistry: XBridge C_{18}, 5 µm.

The ACQUITY UPLC H-Class System, with its low system dispersion,[5] exact control of solvent composition,[6] and accurate sample injection scheme,[7] provided excellent chromatographic reproducibility, as shown in Figure 3, where five peptide injections overlayed exactly. The WFM-A was specifically designed to minimize peak dispersion during collection. Figure 4 emphasizes the benefit of low peak dispersion in the fraction collection valve with the comparison of chromatographic profiles obtained with the WFM-A and with a traditional collector.



ABC peptide sample @ 2.4 mg/mL DMSO
5 µL injection Column temperature: 30 °C
28–32% B in 5 min
A = water with 0.1% TFA
B = acetonitrile with 0.1% TFA
Column: 4.6 x 50 mm XBridge Peptide BEH C_{18}, 5 µm; Flow rate = 1.46 mL/min; 280 nm

Figure 3. Overlay of 5 peptide injections on the 4.6 x 50 mm XBridge Peptide BEH C_{18} Column. Gradient: 28–32% B in 5 minutes, 5 µL injection, 280 nm.

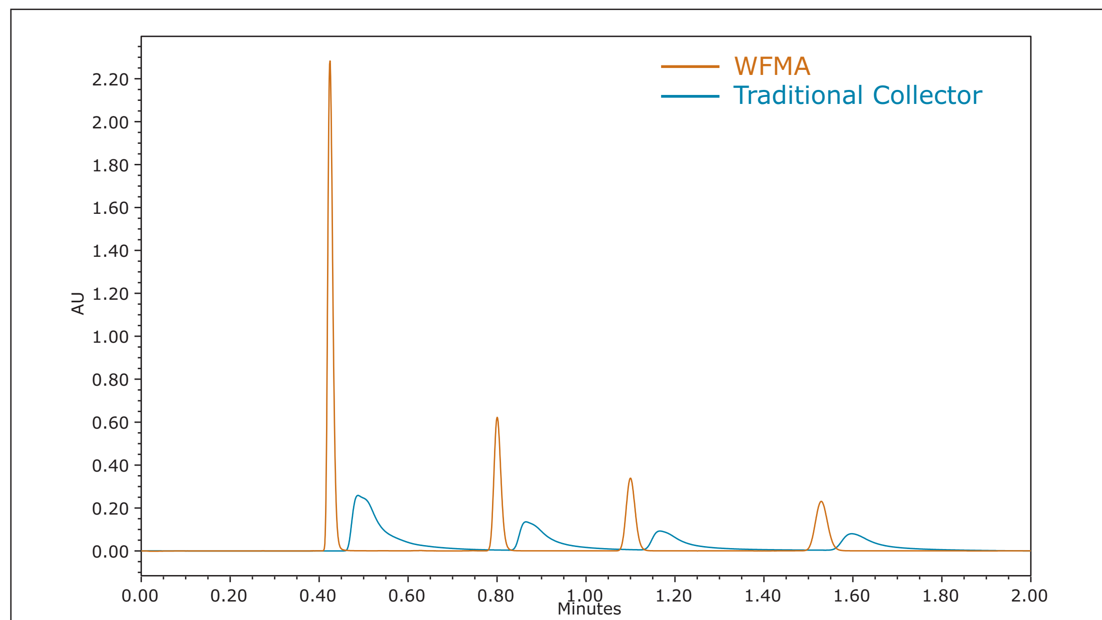


Figure 4. The WFM-A is specifically designed to address the challenge of peak dispersion. As shown, the WFM-A allows for the collection of concentrated narrow peaks of interest with the highest recovery possible.

Narrow, concentrated peaks are easily identified and collected with higher recovery when peaks are clearly defined. Fractions can be collected by time, slope, threshold, or any combination of the three. While the collection starting and ending times may be manually entered in the WFM-A method editor if desired, it is also possible to populate the WFM-A method automatically using the processed results from an analytical injection (Figure 5). Selecting the result displays the integrated peaks with their retention times, start times, and end times, which are then automatically filled into the collection event table (Figure 6).

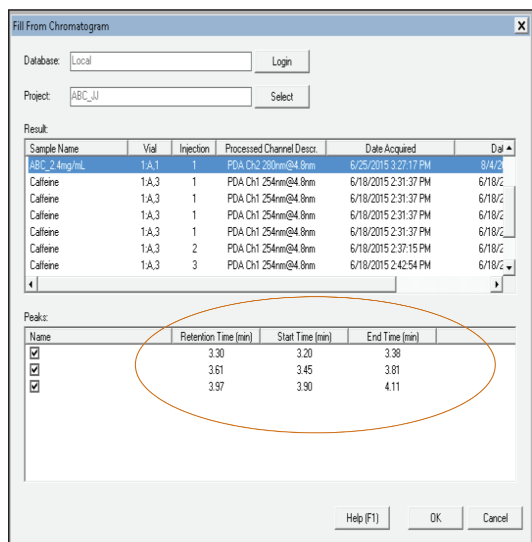


Figure 5. The sample result selected populates the peak table with the retention time as well as the start time and end time for each peak. Selecting OK fills in the Collection Event Table.

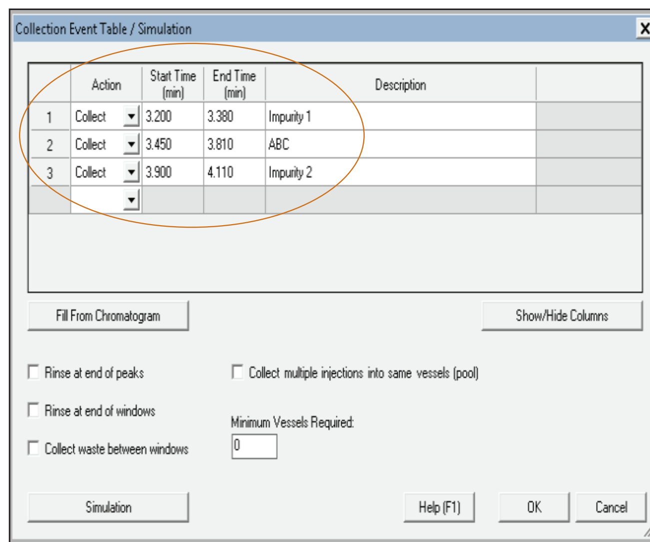


Figure 6. Collection Event Table in the fraction method.

With the collection method developed, the peptide and two closely-eluting impurities were isolated from the crude sample in a total of ten injections. Because the ACQUITY UPLC H-Class System is so reproducible, all of the sample purification chromatograms were identical. Figure 7 shows a representative chromatogram with the shaded areas indicating where fraction collection occurred. The fraction volumes were essentially identical for each of the compounds in each of the isolations (impurity 1, 0.29 mL; peptide, 0.55 mL; impurity 2, 0.33 mL). All of the fractions of each type were pooled. An aliquot of each pool was immediately analyzed using two different gradients.

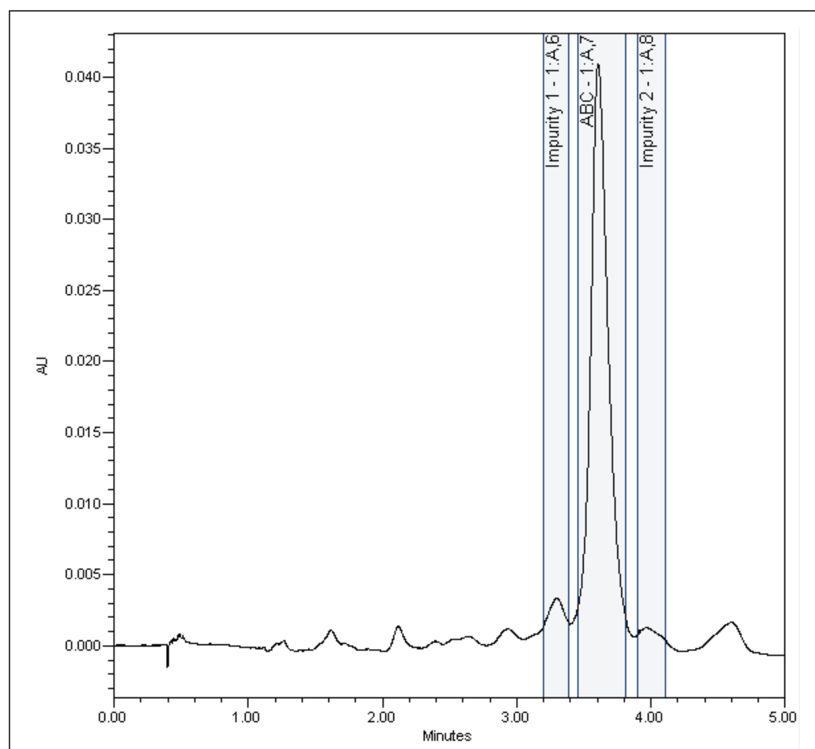


Figure 7. The peptide product and two closely-eluting impurities were collected into a 48-well plate containing 2 mL vials. Gradient: 28–32% B in 5 minutes on a 4.6 x 50 mm XBridge Peptide BEH C_{18} Column, 5 µm; 5 µL injection; 280 nm.

The peptide product purity was 100% as determined by both the fast gradient (Figure 8) and the shallower focused gradients (Figure 9) used for fraction analysis. Slight differences in the purities of both of the contaminant peaks were evident as shown by the two gradients. While the fast gradient (3.38% change per column volume) showed impurity 1 to be about 83% pure, the shallow focused gradient (0.30% change per column volume) resolved yet another coeluting peak and reduced the estimated purity to about 77%. Impurity 2 had a purity of 98% using the fast gradient and 80% using the focused gradient, again due to better resolution of compound constituents. If higher purity contaminant fractions were required for subsequent studies, further method development would likely be needed.

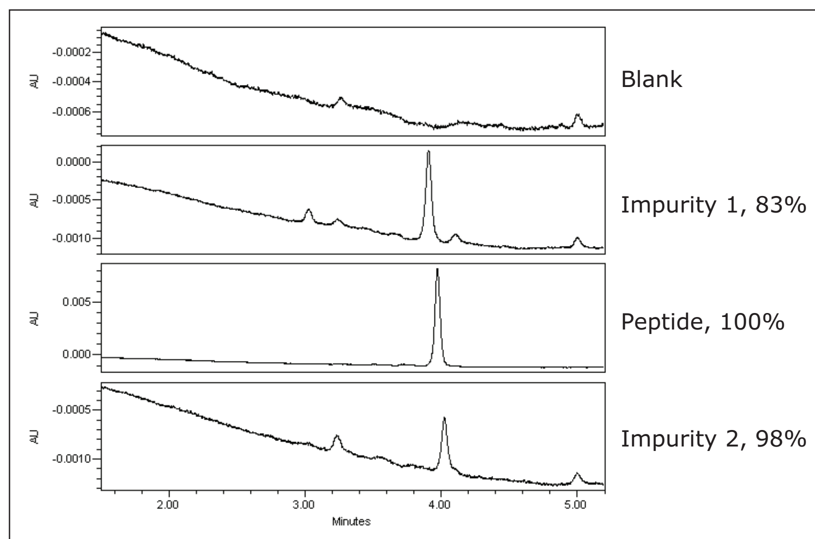


Figure 8. Approximate compound purities after subtracting the peaks present in the blank. Fraction analysis gradient: 5–50% B in 5 minutes, rate of gradient change 3.38%/column volume, injection volume 40 µL, 280 nm.

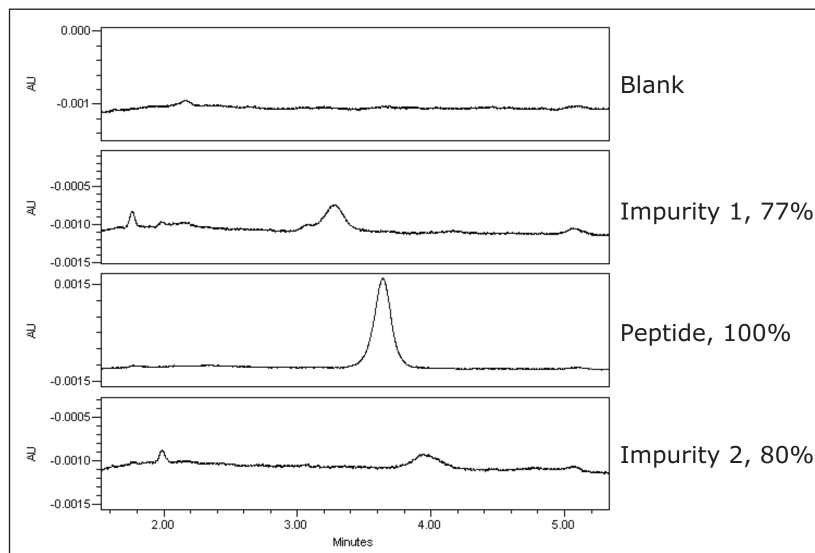


Figure 9. Approximate compound purities after subtracting the peaks present in the blank. Fraction analysis gradient: 28–32%B in 5 minutes, rate of gradient change 0.30%/column volume, injection volume 40 µL, 280 nm.

CONCLUSIONS

- The ACQUITY UPLC H-Class System configured with the Waters Fraction Manager-Analytical (WFM-A), with its very low system dispersion, exact control of solvent composition, accurate sample injection, and precise fraction collection, allows scientists to perform small scale peptide and impurity isolation with assurance.
- Fast valve switching and movement between vessels, as well as a fraction divert valve with very low dispersion volume, facilitates narrow target peak collection and increases confidence in compound isolation.
- Small scale peptide isolation saves sample, time, and resources, promoting efficiency in the purification process.
- The ACQUITY UPLC H-Class System configured with the WFM-A can be adopted for the isolation of compounds from complex synthetic, metabolic, or natural product mixtures at the small scale.

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November 2016 720005801EN IH-PDF