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Selecting a Reversed-Phase Column for the Peptide Mapping Analysis of a Biotherapeutic Protein

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APPLICATION BENEFITS

- Side-by-side performance of ten reversed-phase columns for the separation of peptides
- Performance evaluated in 0.1% trifluoroacetic acid (TFA) or 0.1% formic acid (FA)
- High peak capacities, retention, and distinct selectivities for both UPLC,® UHPLC, and HPLC reversed-phase LC separations of peptides are discussed
- Recommendations for peptide mapping column selection

WATERS SOLUTIONS

Peptide BEH C_{18} Column, 130Å, 1.7 µm

Peptide CSH C_{18} Column, 130Å, 1.7 µm and 2.5 µm

Peptide BEH C_{18} Column, 300Å, 1.7 µm

Peptide HSS T3 Column, 100Å, 1.8 µm

Reversed-Phase CORTECS® and CSH™ Phenyl Hexyl Columns

Xevo® G2 Q-Tof Mass Spectrometer

MassPREP™ Peptide Mixture

KEY WORDS

Peptide mapping, UPLC, UHPLC, HPLC, proteins, reversed phase, IgG

INTRODUCTION

The peptide mapping of proteins is a fundamental tool that has been applied to both the proteomics-based discovery of new biotherapeutic proteins and for monitoring the modification and degradation of those proteins as they are developed and commercialized.[1] When developing a reproducible and informative peptide map, the enzymatic digestion protocol and the separation of the resultant peptides need to be optimized. In this note, we will focus on reversed-phase (RP) column selection considerations for peptide mapping applied to the routine characterization and analysis of biotherapeutic proteins. These analyses that are based on either optical (UV absorbance) detection, mass spectrometric (LC-MS) detection, or both. Many of these column selection considerations can also be directly applied to the selection of columns for LC-MS based proteomics studies and synthetic peptide purity analyses.

In this study, ten distinctly different RP column types, representing variations in ligand characteristics, base particle composition, and particle size, were evaluated using acetonitrile gradients and either 0.1% trifluoroacetic acid (TFA) or 0.1% formic acid (FA) ion-pairing reagents. Separations of both a peptide standard mix and the tryptic digest of a reference monoclonal antibody were used for these comparisons. The performance metrics for this evaluation included both peak capacity and peptide retention. In addition, several of the selectivity differences observed between selected columns are also highlighted.

EXPERIMENTAL

Sample description

MassPREP Peptide Mixture Sample was reconstituted in 0.1% formic acid to a concentration of ~15 µg/mL per peptide. National Institute of Standards and Technology (NIST) monoclonal antibody (NISTmAb) reference material, RM 8671 was reduced, alkylated (iodoacetamide), and then digested with trypsin. The sample was acidified prior to analysis with 1:9 ratio of 1% formic acid. The final concentration of injected mAb sample was ~0.1 mg/mL.

LC conditions

LC system: ACQUITY UPLC® H-Class Bio

Detection: ACQUITY UPLC TUV Detector with 5 mm titanium flow cell

Wavelength: 214 nm

Columns:

ACQUITY UPLC Peptide BEH C_{18}, 130Å, 1.7 µm, 2.1 x 150 mm (p/n: 186003556)

ACQUITY UPLC Peptide CSH C_{18}, 130Å, 1.7 µm, 2.1 x 150 mm (p/n: 186006938)

XSelect Peptide CSH C_{18} ***XP***, 130Å, 2.5 µm, 2.1 x 150 mm (p/n: 186006943)

ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 x 150 mm (p/n: 186003687)

ACQUITY UPLC Peptide HSS T3, 100Å, 1.8 µm, 2.1 x 150 mm (p/n: 186008756)

ACQUITY UPLC CSH Phenyl-Hexyl, 130Å, 1.7 µm, 2.1 x 150 mm (p/n: 186005408)

CORTECS C_{18}, 90Å, 2.7 µm, 2.1 x 150 mm (p/n: 186007368)

CORTECS UPLC C_{18}+, 90Å, 1.6 µm, 2.1 x 150 mm (p/n: 186007117)

CORTECS C_{18}+, 90Å, 2.7 µm, 2.1 x 150 mm (p/n: 186007398)

CORTECS UPLC T3, 120Å, 1.6 µm, 2.1 x 150 mm (p/n: 186008500)

Column temp.: 60 °C

Sample temp.: 10 °C

Injection volume: 15 µL for MassPREP Peptide Mixture and 50 µL for NIST mAb digest

Flow rate: 0.2 mL/min

Mobile phases:

A: 0.1% (v/v) trifluoroacetic acid (TFA) or 0.1% (v/v) formic acid (FA) in water

B: 0.1% (v/v) trifluoroacetic acid (TFA) or 0.1% (v/v) formic acid (FA) in acetonitrile

Gradient: (Peptide standard)

Time (min)	%A	%B
Initial	99.5	0.5
2	99.5	0.5
32	50.0	50.0
35	5.0	95.0
36	5.0	95.0
38	99.5	0.5
50	99.5	0.5

Gradient: (mAb tryptic digest)

Time (min)	%A	%B
Initial	99.5	0.5
2	99.5	0.5
62	50.0	50.0
65	5.0	95.0
66	5.0	95.0
68	99.5	0.5
80	99.5	0.5

ASR standard:	MassPREP Peptide Mixture (p/n: 186002337)
Sample vials:	Polypropylene 12 x 32 mm Screw Neck Vial with cap and PTFE/silicone septum, 300 µL volume (p/n: 186002640)

Data management

Chromatography software:	MassLynx® (v4.1) and UNIFI® (v1.8)

MS conditions

Mass spectrometer:	Xevo G2 Q-Tof
Ionization mode:	ESI+
Analyzer mode:	Resolution
Scan rate:	2 Hz
Capillary voltage:	3.00 kV
Cone voltage:	62 V
Source temp.:	150 °C
Desolvation temp.:	450 °C
Cone gas flow:	2.0 L/h
Desolvation gas flow:	600 L/h
Calibration:	NaI 2 µg/µL from 100 to 2000 *m/z*
Acquisition:	100 to 2000 *m/z*, 10 Hz scan rate

RESULTS AND DISCUSSION

PEAK CAPACITY EVALUATIONS

Background

Peptide mapping separations can be exceedingly complex. As an example, the tryptic peptide map of the NIST monoclonal antibody reference material (NISTmAb) is comprised of over 50 predicted peptides of three amino acid residues or more. In addition, there are numerous , lower abundance modified or degraded peptides, incompletely and non-specifically digested peptides, and autolytic trypsin peptides. This can result in a separation with well over a hundred components many of which represent critical attributes of the protein structure and must be detected at low abundances in the presence of closely eluting peptides at nearly two orders of magnitude higher mass loads. Also problematic in these separations are the minimal differences in molecular structure that can exist between variants of a given peptide, such as the deamidation of a single amino acid residue in a peptide with more than 20 residues.[2]

Given these challenges, a column that provides the narrowest chromatographic peaks for the peptides separated over the length of the gradient elution provides the greatest probability of success in these complex separations. This performance characteristic for a separation is referred to as "peak capacity" (PC) which is a dimensionless value determined by dividing the average peak width of the separation by the length of the gradient, in a general sense PC can be thought of as being proportional to the average resolution of the separation.[3,4] In this study 4σ peak capacities were determined based on the following equation:

$$P_{c,4\sigma} = 1 + \left[\left(\frac{2.35}{4}\right)\left(\frac{t_{gradient}}{W_{h,avg}}\right)\right]$$

Where $t_{gradient}$ is the time of the gradient and $w_{h,avg}$ is the average peak width at half-height.

PC values were determined for a set of columns (Table 1) having the same 2.1 mm I.D. and 150 mm length.

Table 1. Columns evaluated in study.

Column	Pore Size (Å)	Particle Size (µm)	Ligand	Particle Type
ACQUITY UPLC Peptide BEH C_{18}	130	1.7	C_{18}	Hybrid, FP
ACQUITY UPLC Peptide BEH C_{18}	300	1.7	C_{18}	Hybrid, FP
ACQUITY UPLC Peptide CSH C_{18}	130	1.7	C_{18}	Charged Surface Hybrid, FP
XSelect Peptide CSH C_{18} ***XP*** Column	130	2.5	C_{18}	Charged Surface Hybrid, FP
ACQUITY UPLC CSH Phenyl-Hexyl	130	1.7	Phenyl-Hexyl	Charged Surface Hybrid, FP
ACQUITY UPLC Peptide HSS T3 C_{18}	100	1.8	C_{18}	Silica, FP
CORTECS C_{18}	90	1.6	C_{18}	Silica, SC
CORTECS UPLC C_{18}+	90	1.6	C_{18}	Charged Surface Silica, SC
CORTECS C_{18}+	90	2.7	C_{18}	Charged Surface Silica, SC
CORTECS UPLC T3	120	1.6	C_{18}	Silica, SC

FP: fully porous, SC: solid core

Peak capacity values were determined for all of the columns evaluated using a mixture of peptide standards (MassPREP Peptide Mixture) which was comprised of the components shown in Table 2. This mixture contains peptides of varied sizes and charges. The use of peptide standards facilitated the determination of PC by providing well resolved and predominantly pure peptides for which suitability parameters such as peak width can be more readily determined using UV absorbance data. Further assessments of the performance of the columns were also carried out by visually evaluating the tryptic digest of the NIST reference mAb (IgG_1). Selected columns were also more quantitatively interrogated by determining the PC for the extracted ion mass chromatograms (XIC) of selected peptides (Table 3) from the tryptic digest of the NIST reference mAb. These peptides represent an even broader range of molecular weights than the peptides present in the MassPREP Peptide Mixture.

Table 2. MassPREP Peptide Standards.

Peak ID	Peptide	Molecular Weight (monoisotopic)	pI	Sequence
1	RASG-1	1000.49	9.34	RGDSPASSKP
2	angiotensin frag. (1-7)	898.47	7.35	DRVYIHP
3	bradykinin	1059.56	12.00	RPPGFSPFR
4	angiotensin II	1045.53	7.35	DRVYIHPF
5	angiotensin I	1295.68	7.51	DRVYIHPFHL
6	renin substrate	1757.93	7.61	DRVYIHPFHLLVYS
7	enolase T35	1871.96	7.34	WLTGPQLADLYHSLMK
8	enolase T37	2827.28	3.97	YPIVSIEDPFAEDDWEAWSHFFK
9	melittin	2845.74	12.06	GIGAVLKVLTTGLPALISWIKRKRQQ

Table 3. Selected NIST mAb tryptic peptides.

Peptide	Molecular Weight (monoisotopic)	pI	Sequence
H31	447.27	9.35	TISK
H10	699.43	9.35	NQVVLK
H14	1321.65	8.63	STSGGTAALGCLVK
L15	1501.75	6.18	DSTYSLSSTLTLSK
H23	1676.79	5.53	FNWYVDGVEVHNAK
H38	1872.91	4.10	TTPPVLDSDGSFFLYSK
H37	2543.12	3.96	GFYPSDIAVEWESNGQPENNYK
L7	4482.98	3.94	FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK
H15	6713.29	7.46	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK

H: heavy chain derived peptide, L: light chain derived peptide

Peptide Standard Results

Comparisons of the chromatograms and PC values obtained for the ten columns for both 0.1% TFA or 0.1% FA-based separations of the MassPREP Peptide Mixture are shown in Figures 1 through 4. TFA and FA are the predominant ion-pairing reagents used in peptide mapping experiments. The advantages of TFA in terms of chromatographic performance (PC, retention, and baseline noise) and the advantages of FA with regards to MS sensitivity have been well documented, as has the capability of chromatographic phases using BEH (ethylene bridged hybrid) particles with a positively charged surface chemistry, which Waters® refers to as a CSH (charged surface hybrid) particle, to provide high PC separations in FA and mixed FA plus TFA mobile phases.[4,5]

All of the columns evaluated provide high PC peptide separations in both TFA and FA containing mobile phases as shown by the charts in Figures 2 and 4. However, in both mobile phases the Peptide CSH C_{18}, 130Å columns in 1.7 µm and 2.5 µm particle sizes, and the CSH Phenyl-Hexyl, 130Å, 1.7 µm particle size columns provide the highest average PC. This performance advantage is significantly more differentiated in the FA-containing mobile phase as compared to the TFA containing mobile phase.

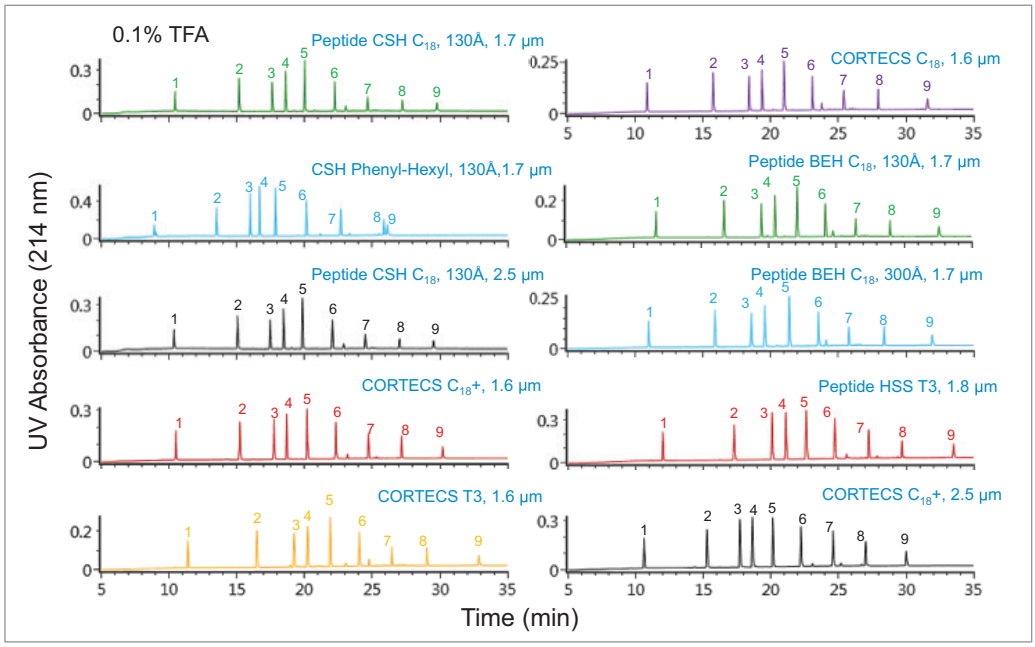


Figure 1. Comparison of chromatographic results for MassPREP Peptide Standards using 0.1% TFA in water/acetonitrile mobile phases. Peak identities are provided in Table 1.

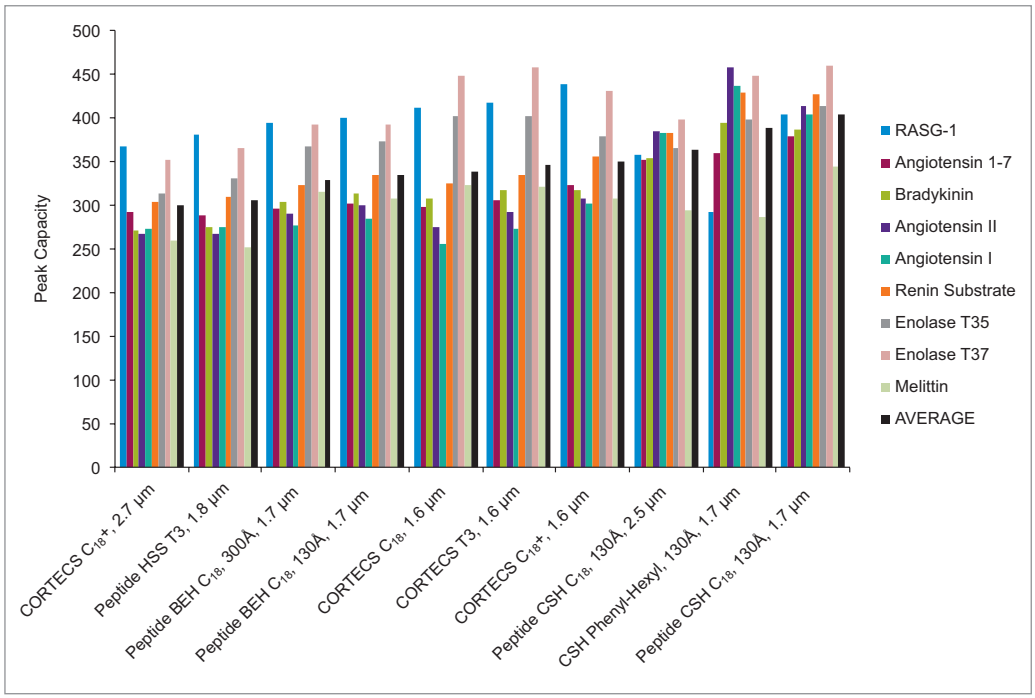


Figure 2. Comparison of 4σ peak capacity results determined for MassPREP Peptide Standards using 0.1% TFA in water/acetonitrile mobile phases.

The PC values for individual peptides varied from column to column. As a clear example, the PC results for the peptide melittin with a FA mobile phase (Figure 3) is significantly higher for the Peptide BEH C_{18}, 300Å and Peptide BEH C_{18}, 130Å columns than is for the Peptide CSH C_{18}, 130Å, 1.7 µm column. However, the PC for the other seven peptides is significantly higher for the latter. In this case, melittin is a fairly unusual peptide in that it contains a sequence motif of four strongly basic residues (KRKR). It is reasonable to hypothesize that this highly basic set of residues causes there to be significant charge repulsion on the CSH Column such that effective pore size is reduced, restricted diffusion effects are increased, and PC is diminished for what is a fairly large peptide (2854 Da). That slightly higher PC values were observed for melittin and the enolase T37 peptide (2827 Da) on the 300Å pore size BEH column in comparison to the 130Å pore size BEH particle suggest that restricted diffusion effects can in fact be observed for a 3 kDa peptide. We will further examine pore size effects in the NIST mAb peptide mapping results presented later in this application note.

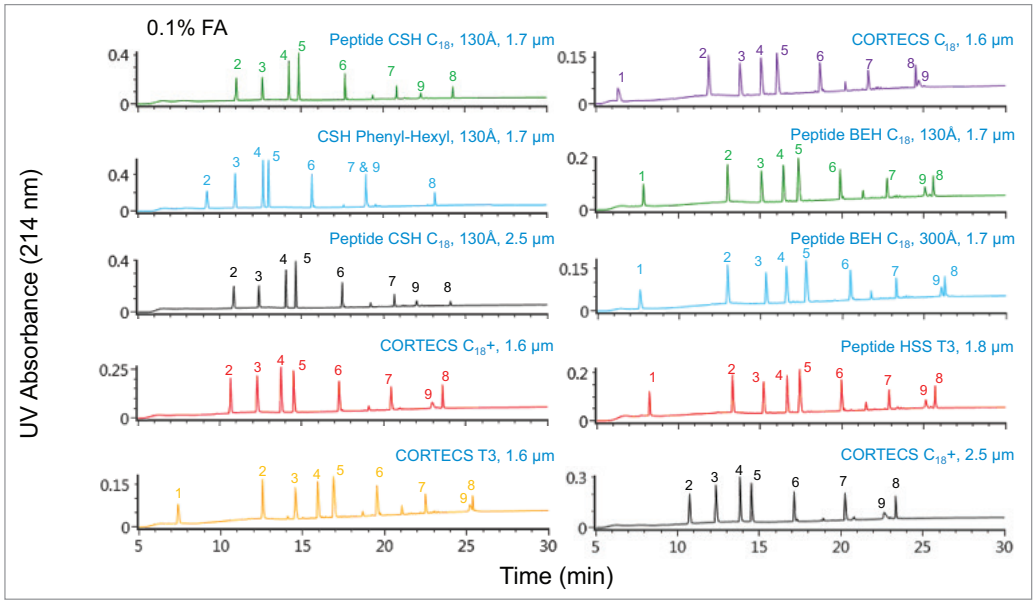


Figure 3. Comparison of chromatographic results for MassPREP Peptide Standards using 0.1% FA in water/acetonitrile mobile phases. Peak identities are provided in Table 1.

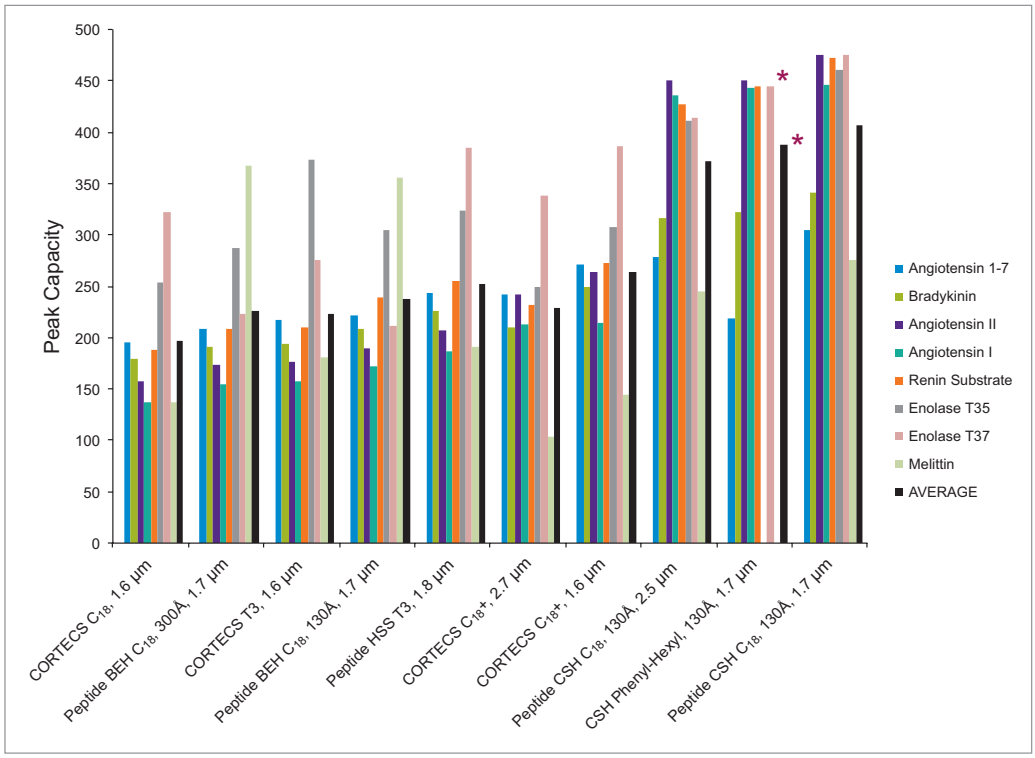


Figure 4. Comparison of 4σ peak capacity results determined for MassPREP Peptide Standards using 0.1% FA in water/acetonitrile mobile phases. Missing PC values (*) for melittin and enolase T35 peptides for CSH Phenyl-Hexyl Column due to co-elution of those peptides.

In other instances, these variations in individual peptide PC between different columns can be due to the characteristics of the peptide such as charge, size, and hydrophobicity, in addition to characteristics of the phase such as pore size, particle morphology, and ligand characteristics. As an example, the unusually low PC observed for melittin on the CORTECS T3, 120Å Phase when using a FA mobile phase may be predominantly the result of secondary ionic interactions between the particle surface and this strongly basic (pI =12.1) peptide. It is important to emphasize that these column chemistry differences can also be used to an advantage when developing an optimal peptide separation.

Particle size is also a key component in improving PC, which has an inverse square-root dependence on particle size. Within the set of columns evaluated there are two pairs of columns that are packed with different sized particles but have comparable pore size and surface chemistries. One of the column pairs is the fully-porous Peptide CSH C_{18}, 130Å column in 1.7 µm and 2.5 µm particle sizes, and the other column pair is the solid-core CORTECS C_{18}+, 90Å Column in 1.6 µm and 2.7 µm particle sizes. The individual peptide PC values (Figures 2 and 4) followed similar trends for both column pairs; therefore, we can assess the effect of particle size using the average PC values. For the CSH C_{18} Columns, increases in average PC of 11% and 16% were observed in TFA and FA when comparing the 1.7 µm to the 2.5 µm particle size columns. Similarly, for the CORTECS C_{18}+ Columns, increases in average PC of 17% and 15% were observed in TFA and FA when comparing the 1.6 µm to the 2.7 µm particle size columns. In both cases, the increase in PC was below the predicted values based on particle size of 21% and 30% for the CSH C_{18} and the CORTECS C_{18}+ Columns. These discrepancies may be partly attributed to the effect of post-column dispersion volume (tubing and detector flow-cell) on the small volume peaks (~20 µL) generated in this study, variations in peptide behavior and separation conditions, and column packing efficiencies. Nonetheless, these data indicate that smaller particle sizes can provide significant gains in PC. Additionally, it can also be surmised from the comparison of the CSH C_{18} and CORTECS C_{18}+ chromatograms that while the use of solid-core particles may have some advantages over fully-porous particles, other factors in this case, such as surface chemistry, can have a greater influence on chromatographic performance.

mAb Peptide Mapping Results

To specifically address the effect that particle surface charge and pore size can have on PC for a peptide map, we will evaluate selected peptides (Table 3) from the trypsin-digested peptide maps of reduced and alkylated NIST mAb. A comparison of the UV absorbance based peptide maps generated for this sample using either TFA or FA ion-pair mobile phases are presented in Figures 5 and 6. Consistent with previous observations for the peptide standard separations (Figures 1–4), all of the columns provide functional peptide separations with adequate peak capacity and a broad range of selectivity in both TFA and FA mobile phases. Nevertheless, due to the complexity of this sample, it is through extracted ion chromatograms (XIC) of selected peptides that the PC values of the various separations can be best elucidated. By using this selective detector, the complications that unresolved peptides can have on PC determinations can be minimized.

Comparisons of the XIC-based PC results in both TFA and FA mobile phases for the Peptide CSH C_{18}, 130Å, 1.7 µm column and Peptide BEH C_{18}, 130Å, 1.7 µm column (Figures 7 and 8) demonstrate the usefulness of the controlled charge applied to the CSH particle surface. These two C_{18} RP chemistries are comparable in both particle size and pore size, and are based on the same bridged ethyl and silica hybrid particle (BEH). Of note in this comparison is that both columns provide high PC separations for a broad range of peptide sizes and charge. With TFA as an ion pair, the CSH-based column had 10% greater PC in comparison to the BEH-based column; and, with FA as an ion-pair, the improvement in PC for the CSH Column was 20%. The improved loading of peptides on the CSH particle relative to the BEH particle provides for this increased PC.[5]

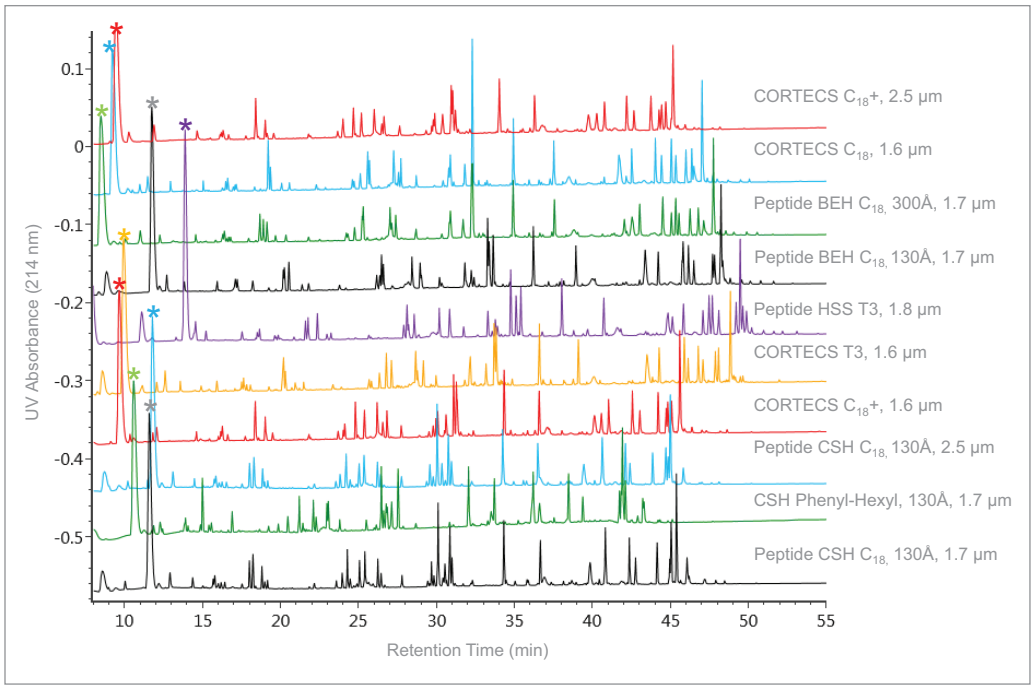


Figure 5. Comparison of chromatographic results for tryptic digest of reduced and alkylated NIST mAb using 0.1% TFA in water/acetonitrile mobile phases. Peak identities are provided in Table 1. Peak identified with asterisk (*) is produced by the reduction/alkylation procedure and is not protein related.

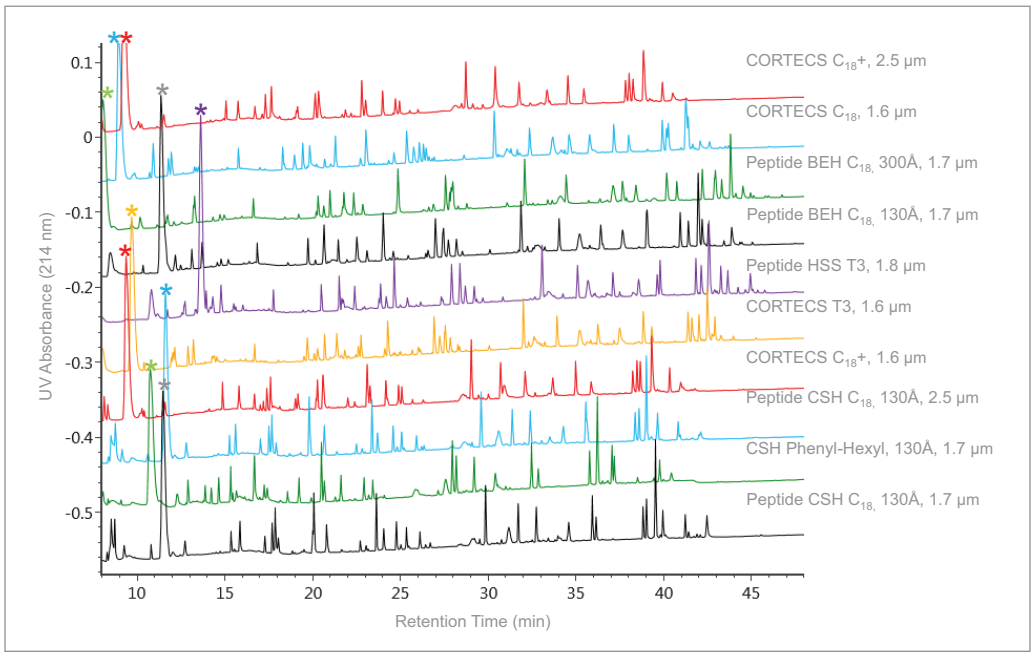


Figure 6. Comparison of chromatographic results for tryptic digest of reduced and alkylated NIST mAb using 0.1% FA in water/acetonitrile mobile phases. Peak identities are provided in Table 1. Peak identified with asterisk (*) is produced by the reduction/alkylation procedure and is not a peptide .

To further evaluate the effect of pore size on peptide separations, XICs for the Peptide BEH C_{18}, 130Å, 1.7 µm and the Peptide BEH C_{18}, 300Å, 1.7 µm columns were compared. An overlay of these XICs is presented in Figure 9, and a chart of the results is presented in Figure 10. In this comparison, peptides with a molecular weight greater than 1000 Da were considered.

In comparing the selected PC results of the two columns (Figure 10), it was observed that peptides with molecular weights greater than 1.8 kDa benefited from the 300Å pore size column with a corresponding modestly higher PC. This improvement in chromatography for the larger mAb-derived peptides may result from a combination of reduced restricted diffusion effects and the increased accessible surface area afforded by the 300Å pore-size phase.

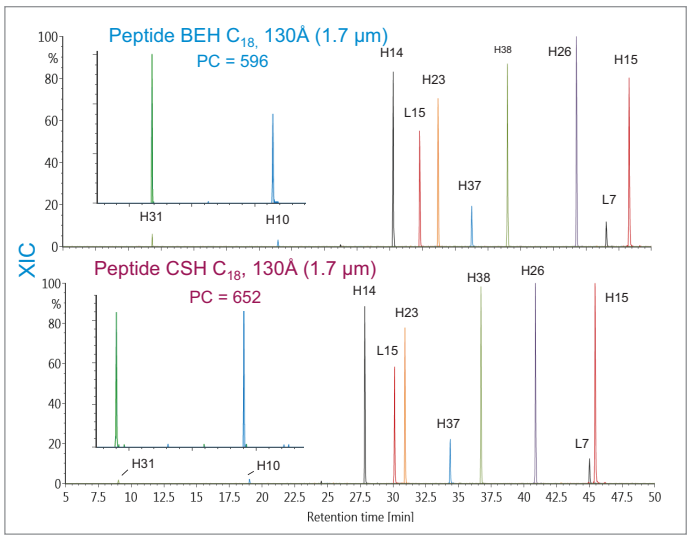


Figure 7. The effect of surface charge on XIC derived peak capacities for selected NIST mAb tryptic peptides with 0.1% TFA in water/acetonitrile mobile phases. Peak identities are provided in Table 3.

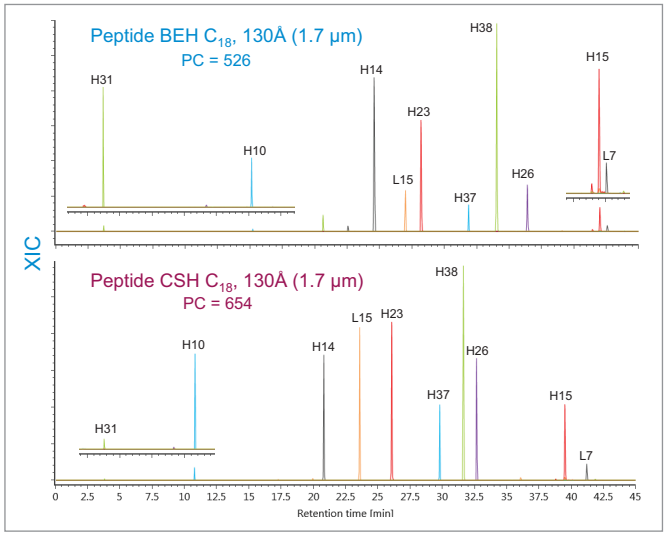
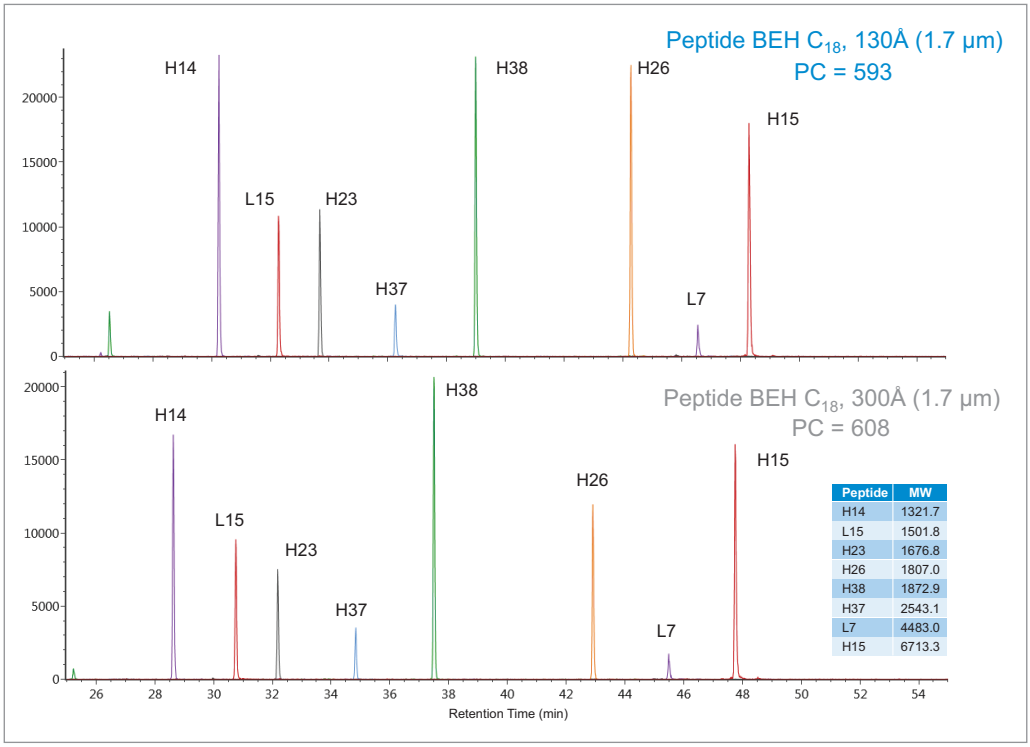


Figure 8. The effect of surface charge on XIC derived peak capacities for selected NIST mAb tryptic peptides with 0.1% FA in water/acetonitrile mobile phases. Peak identities are provided in Table 3.



Peptide	MW
H14	1321.7
L15	1501.8
H23	1676.8
H26	1807.0
H38	1872.9
H37	2543.1
L7	4483.0
H15	6713.3

Figure 9. XIC and average peak capacities for 130Å versus 300Å pore size, 1.7 µm particle size BEH C_{18} particles. Shown are selected XIC of NIST mAb tryptic peptides with 0.1% TFA in water/acetonitrile mobile phases. The MW values for the peptides (Da) are provided in the table inset. Peak identities are provided in Table 3.

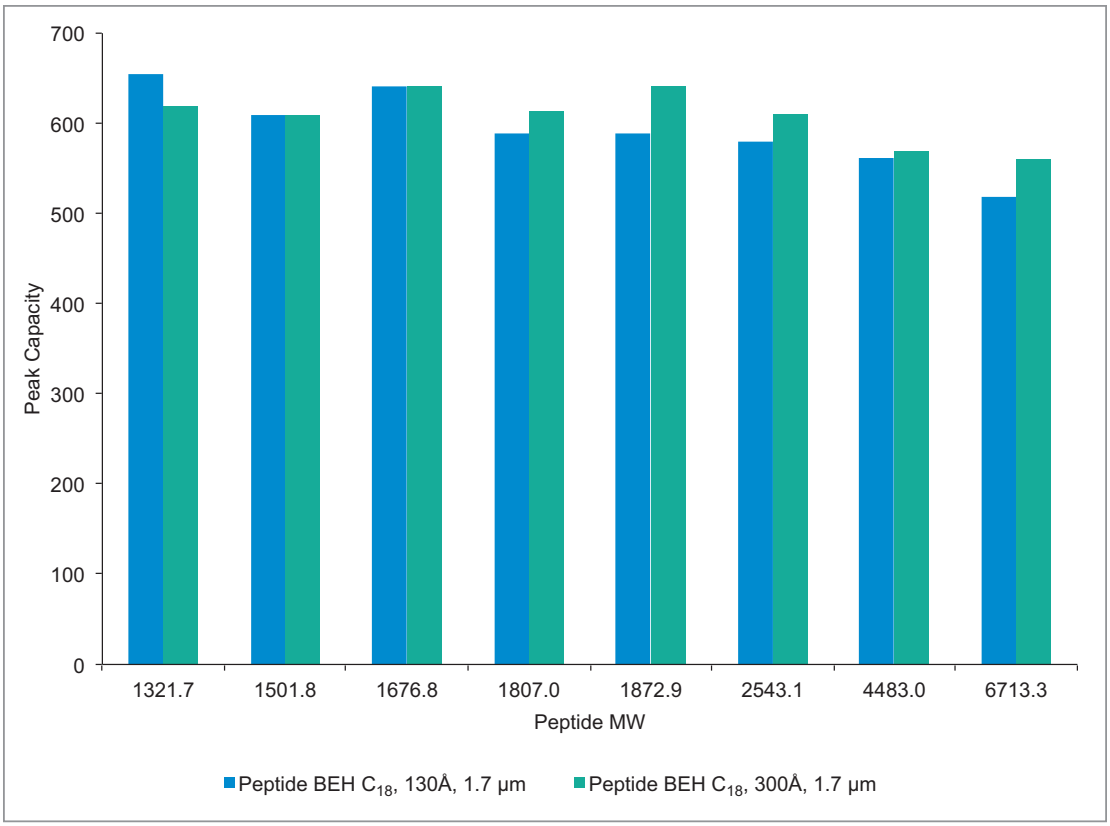


Figure 10. The effect of pore size, 130Å (BEH130) versus 300Å (BEH300), on XIC derived peak capacities (Figure 9) for selected NIST mAb tryptic peptides with 0.1% TFA in water/acetonitrile mobile phases.

PEPTIDE RETENTION EVALUATIONS

The percentage of the amino acid sequence that can be monitored during the peptide mapping of a therapeutic protein (i.e., coverage) standard is an important method development consideration. It has been proposed in USP General Chapter <1055> "Biotechnology-Derived Articles – Peptide Mapping" that 95% or greater sequence coverage be the target for a validated peptide map. Therefore, a RP column that provides greater peptide retention can be used to advantage if the protein digest results in numerous hydrophilic peptides, particularly if some of those peptides must be quantified in order to monitor a critical quality attribute (CQA).

For this evaluation, we calculated the concentration of acetonitrile at which the four least hydrophobic peptides in the MassPREP Peptide Standard Mixture eluted from each column of interest. These data are shown for both 0.1% TFA (Figure 11) and a 0.1% FA (Figure 12) containing mobile phases. The Peptide HSS T3, 100Å column provided the highest retentivity of the columns tested in both TFA and FA. This phase is manufactured with an intermediate ligand density, an approach that has been shown to promote retention.[6] All of the columns evaluated provided effective retention in TFA, with some retention of the RASG-1 peptide. However, the retentivity of the CSH Phenyl-Hexyl, 130Å Column was significantly lower than all of the other phases. Retention of the RASG-1 peptide is of note because despite its size (1000.49 Da) it has hydrophobicity indexes (HI, SSRCalc, v Q, ©2006–2015, Manitoba Centre for Proteomics & Systems Biology) of only 4.40 and 0.54 in TFA and FA for the C_{18}, 100Å column. The hydrophobicity index is the predicted concentration of acetonitrile at which the peptide will have a retention factor (k') of 10.[7] In evaluating the retention in FA, the Peptide HSS T3, 100Å is again the most retentive phase, while the Peptide BEH C_{18}, 130Å, Peptide BEH C_{18}, 300Å, and CORTECS T3, 100Å Columns also provide adequate retention for RASG-1 under these conditions. For the other columns tested, RASG-1 is not adequately retained for analysis.

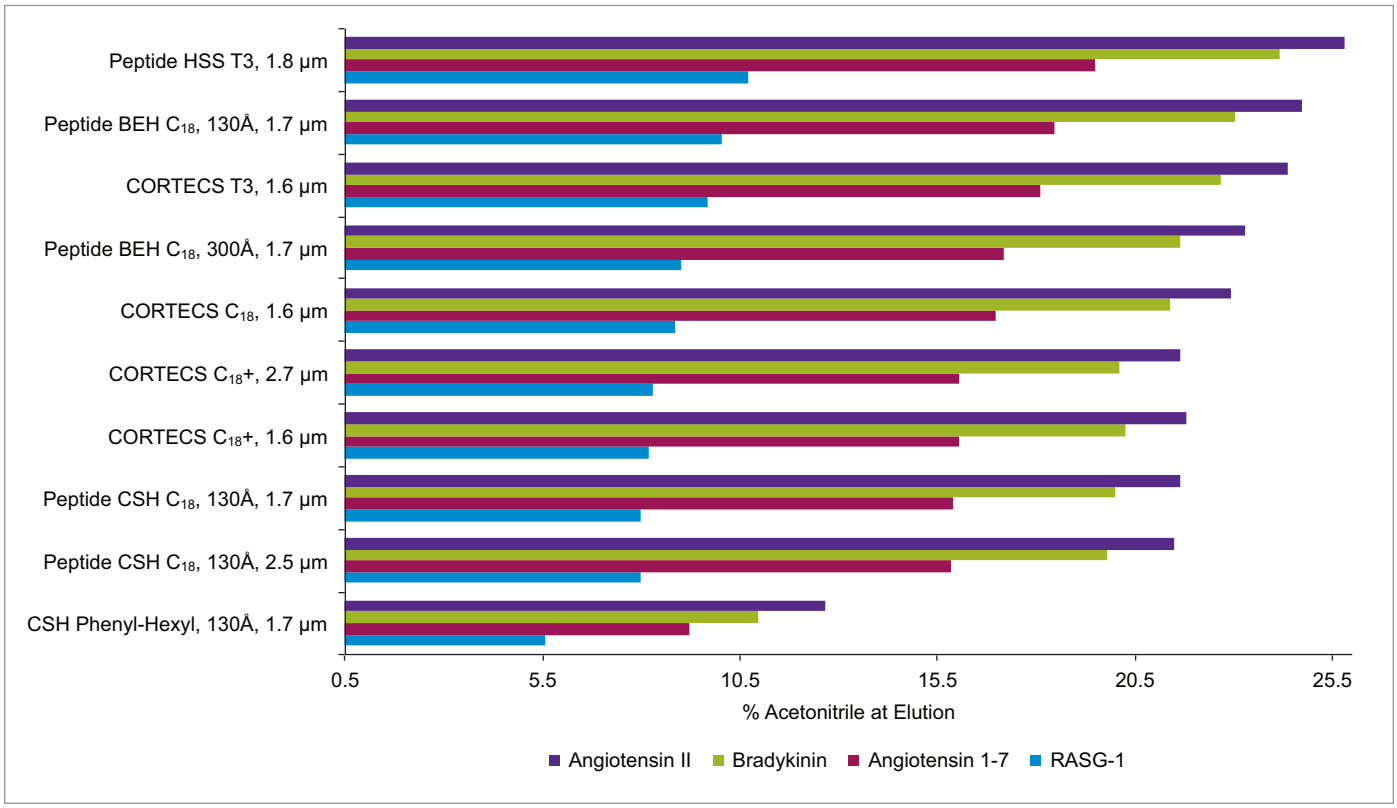


Figure 11. Comparison of calculated peptide retention results determined for MassPREP Peptide Standards using 0.1% TFA in water/acetonitrile mobile phases.

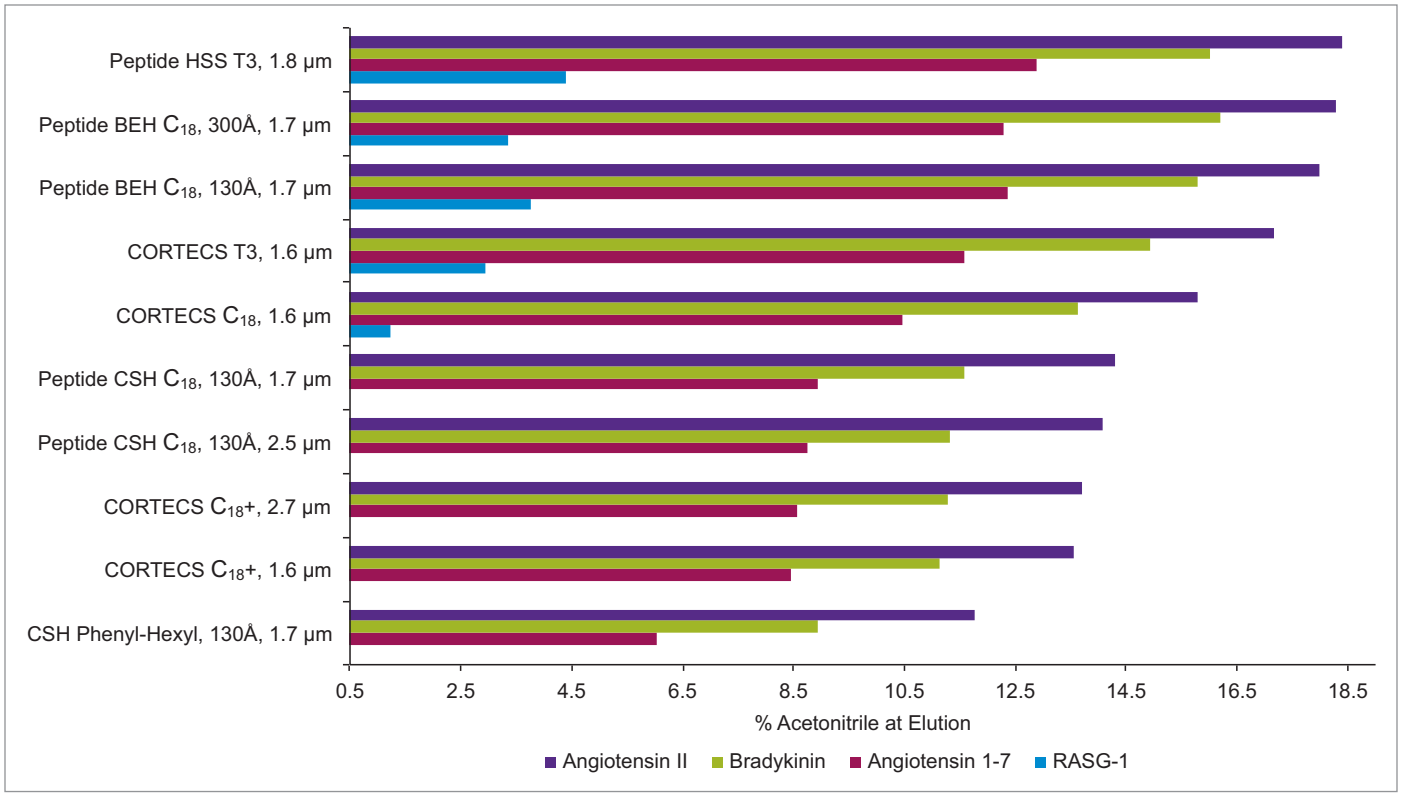


Figure 12. Comparison of calculated peptide retention results determined for MassPREP Peptide Standards using 0.1% FA in water/acetonitrile mobile phases.

When developing a peptide mapping method for the analysis of a therapeutic protein, the selection of an appropriate reversed-phase column with appropriate retentivity requires one to consider separation conditions (e.g., mobile phase and temperature). However, another important consideration is the nature of the protein digest. In particular, if the proteolytic enzyme selected yields a large number of smaller hydrophilic peptides, the use of a more retentive phase can be advantageous. However, if the proteolytic digest yields, larger more hydrophobic peptides, then using a more retentive phase may not provide a significant benefit.

PEPTIDE SELECTIVITY CONSIDERATIONS

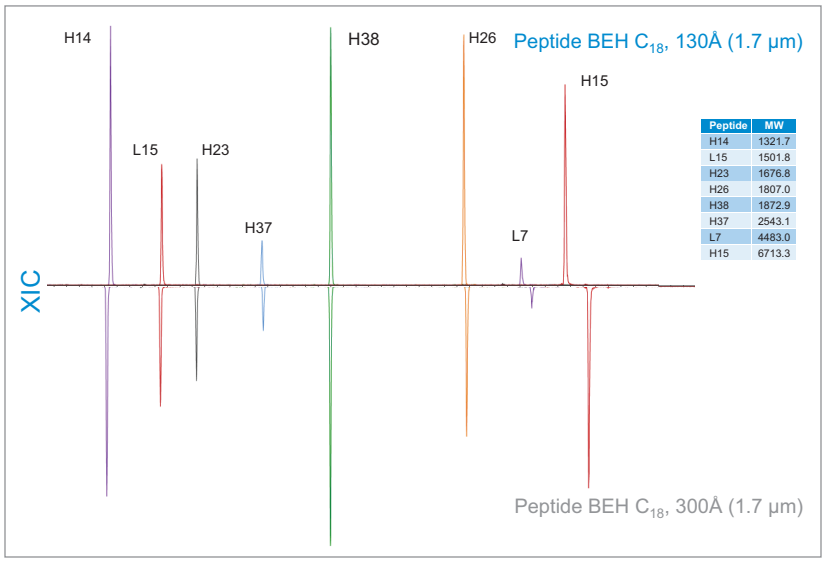
Selectivity differences among different phases can also be used to an advantage to improve a peptide mapping separation method, namely by manipulating the resolution of a critical pair of closely eluting peptides. It is important to note, however, that increasing the selectivity for a given critical pair of peptides in these complex peptide mapping separations may result in a loss of selectivity between other pairs. Selectivity differences can be observed in many of the data presented, and may be the result of differences such as ligand type, ligand density, particle surface charge, and particle pore size. Of these variables, a change in ligand type can have the most significant impact on selectivity. As an example, Figure 1 compares the separation of the MassPREP Peptide Standard Mixture as obtained with several C_{18} phases. The separation achieved on the CSH Phenyl-Hexyl, 130Å Phase shows a change in elution order between Peak 8 (enolase T37) and Peak 9 (melittin). The greater relative retention of enolase T37 on the CSH Phenyl-Hexyl Phase is likely the result of the large number of aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine). In particular, an amino acid motif of sequential phenylalanines contained in the T37 peptide can form strong π–π interactions with the phenyl ligand of that phase.

More subtle differences in selectivity are observed for phases with the same ligand; however, changes in ligand density, in addition to the characteristics of the base particle such as surface charge and pore size, can influence selectivity. Examples of these differences can be observed in comparisons of the XIC results for selected peptides from the trypsin-digested peptide maps of reduced and alkylated NIST mAb. For these comparisons, the Peptide BEH C_{18}, 130Å column was compared to the Peptide BEH C_{18}, 300Å, Peptide CSH C_{18}, 130Å, and Peptide HSS T3, 100Å Columns. To better visualize these selectivity differences, the time axes of the chromatograms have been aligned on a selected peptide (H38).

The primary differences between the Peptide BEH C_{18}, 130Å and the Peptide BEH C_{18}, 300Å phases is pore size and surface area (185 m^2/g and 90 m^2/g). The ligand (C_{18}) and ligand densities (3.1 µmole/m^2) are comparable. As a result, as peptide size increases, a selectivity difference that is contributed to by a size exclusion effect is observed. This effect is more pronounced for the two largest peptides, L7 and H15, which are predicted to have significantly restricted pore access with a 130Å pore-size media.

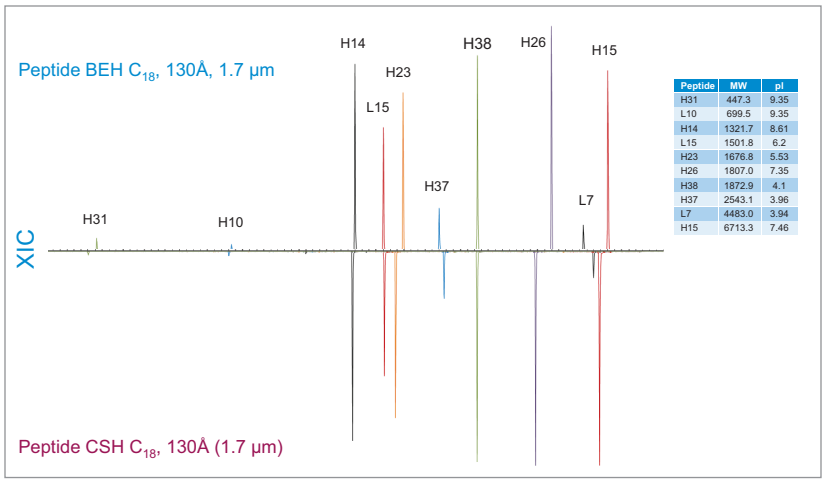
Comparisons of the Peptide BEH C_{18}, 130Å, 1.7 µm separation to the separations obtained for like particle size Peptide CSH C_{18}, 130Å and Peptide HSS T3, 100Å Columns are shown in Figures 14 and 15. The differences in selectivity observed between the Peptide BEH C_{18}, 130Å and Peptide CSH C_{18}, 130Å Columns may be due to several factors. The primary difference between these two particles is the applied positive charge on the surface of the Peptide CSH C_{18} phase, though there are also slight differences in C_{18}-ligand density with the Peptide CSH C_{18} phase being approximately 25% lower (2.3 versus 3.1 µmole/m^2). Most notably, it is the significant surface charge difference between these two phases that can provide a potentially advantageous selectivity difference in the separation of peptides, particularly those that have substantial differences in net charge.

More subtle differences in selectivity are observed in the comparison of the Peptide BEH C_{18},130Å and the Peptide HSS T3, 100Å columns. These columns differ significantly in base particle composition (BEH versus silica), ligand density (3.1 and 1.6 µmole/m^2), pore size (130Å and 100Å), and surface area (185 m^2/g and 230 m^2/g). This range of differences makes it difficult to predict peptide selectivity changes, therefore an empirical comparison, like the one shown here, is typically warranted.



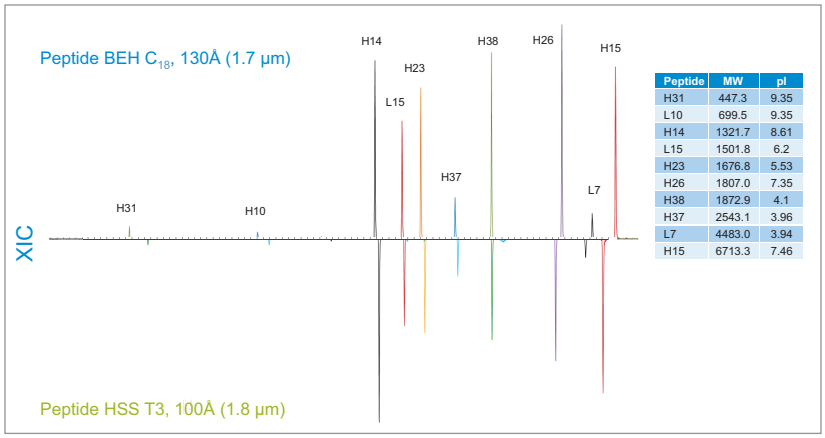
Peptide	MW
H14	1321.7
L15	1501.8
H23	1676.8
H26	1807.0
H38	1872.9
H37	2543.1
L7	4483.0
H15	6713.3

Figure 13. XIC profiles demonstrating the effect of pore size on selectivities for selected NIST mAb tryptic peptides using 0.1% TFA in water/acetonitrile mobile phases. Chromatograms have been aligned based on the retention time of the H38. The MW values for the peptides (Da) are provided in the table inset. Peak identities are provided in Table 3.



Peptide	MW	pI
H31	447.3	9.35
L10	699.5	9.35
H14	1321.7	8.61
L15	1501.8	6.2
H23	1676.8	5.53
H26	1807.0	7.35
H38	1872.9	4.1
H37	2543.1	3.96
L7	4483.0	3.94
H15	6713.3	7.46

Figure 14. XIC profiles demonstrating the effect of positive surface charge on selectivities for selected NIST mAb tryptic peptides using 0.1% TFA in water/acetonitrile mobile phases. Chromatograms have been aligned based on the retention time of the H38. The MW and pI values for the peptides (Da) are provided in the table inset. Peak identities are provided in Table 3.



Peptide	MW	pI
H31	447.3	9.35
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H14	1321.7	8.61
L15	1501.8	6.2
H23	1676.8	5.53
H26	1807.0	7.35
H38	1872.9	4.1
H37	2543.1	3.96
L7	4483.0	3.94
H15	6713.3	7.46

Figure 15. XIC profiles demonstrating the effect of particle surface chemistry (BEH and silica) on selectivities for selected NIST mAb tryptic peptides using 0.1% TFA in water/acetonitrile mobile phases. Chromatograms have been aligned based on the retention time of the H38. The MW and pI values for the peptides (Da) are provided in the table inset. Peak identities are provided in Table 3.

CONCLUSIONS

A chromatographic peptide mapping method must provide adequate resolution and recovery of a broad set of peptides if it is to be used in the quality analysis of a biotherapeutic protein. The three principal attributes of a RP column that can be used to advantage for these complex separations are peak capacity, retention, and selectivity. Selecting a column for a peptide mapping separation is decidedly difficult, as there are a multitude of columns provided by Waters and numerous other manufacturers. In addition, one must also take into account the many varied properties of peptides, such as size and charge, and mobile-phase conditions (TFA or FA). In reality, there is no single column that will provide an optimal separation for every peptide pair under any condition. As a result, column screening may be a necessary approach. All of the Waters RP columns listed in Table 4, along with a brief description of their attributes, are potentially capable of producing an effective peptide mapping separation and could be screened during method development. However, screening all of these columns along with selected columns from other manufacturers is not feasible. Therefore, a subset of four of these Waters RP particle technologies that generally provide peak capacity, retention, and selectivity differences to enable the successful development of RP peptide mapping separations have been identified as peptide chemistries (Table 4). These peptide column particles are also quality tested to provide additional assurance of performance reproducibility for peptide separations.

Table 4. Particle Technology Summary.

		Particle Technology	UPLC and HPLC Particle Sizes (µm)	Comments
Fully porous	Most recommended	Peptide CSH C_{18}, 130Å	1.7, 2.5	Highest PC in both TFA and FA
				Significant selectivity differences compared to BEH C_{18} phases
				Lower retention than HSS T3 and BEH C_{18} phases
		Peptide BEH C_{18}, 130Å	1.7, 3.5	High PC in both TFA and FA
				High peptide retention
		Peptide BEH C_{18}, 300Å	1.7, 3.5	High PC in TFA, moderate-to-high PC in FA
				Increased PC for larger MW peptides
				Moderate-to-high peptide retention
		Peptide HSS T3, 100Å	1.8, 2.5	Highest peptide retention
				High PC in both TFA and FA
				Diminished PC for larger MW peptides
	Alternatives	CSH Phenyl-Hexyl, 130Å	1.7, 2.5	High PC in both TFA and FA
				Significant differences in selectivity
				Peptide retention is significantly lower than other phases tested
Solid core		CORTECS T3, 120Å	1.6, 2.7	High PC in TFA ,moderate-to-high PC in FA
				High peptide retention
		CORTECS C_{18}+, 90Å	1.6, 2.7	High PC in TFA and FA
				Low peptide retention
				Smaller pore size not as amenable to larger peptides
		CORTECS C_{18}, 90Å	1.6, 2.7	High PC in TFA, but had the lowest PC in FA
				Moderate peptide retention
				Smaller pore size not as amenable to larger peptides

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