Comparison of Reversed-Phase Column Types for Peptide Mapping Separations

Technical Overview

Introduction

LC/MS-based peptide mapping has become an essential tool for the characterization of biomolecules. Reversed-phase (RP) column selection for LC/MS analysis plays a crucial role in facilitating peptide separation, as the complex tryptic digest contains a mixture of peptide characteristics. It is well documented that LC/MS peptide mapping separations under formic acid conditions produce broader peak shapes, lower peak capacity, and poor resolution of closely related peptides. Therefore, the correct choice of RP column is critical in achieving a successful peptide mapping separation.

This study evaluates the commercially available RP peptide column types of different particle and pore sizes. LC/MS peptide mapping separation with a formic acid mobile phase was performed using a monoclonal antibody (mAb) tryptic digest. The quality attributes of peptide mapping such as retention time, peak width, and peak capacity were evaluated to assess column performance. The results demonstrate the advantage of Agilent AdvanceBio Peptide Plus columns for robust peptide mapping separations.

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Materials and Methods

Trastuzumab was bought from a local pharmacy and stored according to the manufacturer's instructions. DL-Dithiothreitol (DTT), iodoacetamide (IAA), formic acid, and LC/MS grade solvents were from Sigma-Aldrich. High-quality sequence grade trypsin (p/n 204310) was obtained from Agilent Technologies, Inc.

Trypsin digestion

Before the digestion of the mAb Trastuzumab with trypsin, the disulfide bonds were reduced (DTT) and alkylated (IAA) under denaturing conditions (guanidine-HCl). This pretreatment ensured complete mAb denaturation and solubilization, allowing efficient access of the protease to the target substrate. Following reduction and alkylation, the pH of the solution was adjusted to pH 7–8, and trypsin digestion (20:1 protein to protease w/w) was performed overnight at 37 °C. The samples were immediately analyzed using LC/MS, or stored at –80 °C until use.

Extracted compound chromatograms were used to calculate the peak capacity values according to the following formula:

Peak capacity (Pc) =

$$1 + \left(\frac{2.35}{4}\right)\left(\frac{\text{Gradient time (t)}}{\text{Average peak width } (W_h)}\right)$$

Instrumentation

LC system

Agilent 1290 Infinity II LC

MS system

Agilent 6530 accurate-mass quadrupole time-of-flight (Q-TOF) system with Agilent Jet Stream ion source

Columns

Vendor	Inner diameter (mm)	Length (mm)	Particle size (µm)	Pore size (Å)
Agilent AdvanceBio Peptide Plus	2.1	150	2.7	120
Vendor A	2.1	150	2.2	120
Vendor B1	2.1	150	2.6	100
Vendor B2	2.1	150	5.0	100
Vendor C	2.1	150	2.7	160
Vendor D1	2.1	150	2.5	130
Vendor D2	2.1	150	2.7	90
Vendor D3	2.1	150	1.7	130

LC/MS Conditions

Parameter	Agilent 1290 Infinity II LC
Injection volume	1 µL (1 µg/µL)
Sample thermostat	5 °C
Mobile phase A	0.1 % FA in water
Mobile phase B	0.1 % FA in ACN
Gradient	At 0 minutes ⟶ 3 %B At 1 minute ⟶ 3 %B At 31 minutes ⟶ 40 %B At 33 minutes ⟶ 95 %B At 34 minutes ⟶ 95 %B At 34.1 minutes ⟶ 3 %B
Stop time	34.1 minutes
Post time	5 minutes
Column temperature	55 °C
Flow rate	0.5 mL/min

Parameter	Agilent 6530 accurate-mass quadrupole time-of-flight
Ion mode	Positive ion mode, dual AJS ESI (profile)
Drying gas temperature	250 °C
Drying gas flow	12 L/min
Sheath gas temperature	250 °C
Sheath gas flow	11 L/min
Nebulizer	35 psi
Capillary voltage	3,500 V
Fragmentor voltage	175 V
Skimmer voltage	65 V
Oct RF Vpp	750 V
Acquisition parameters MS mode	Data were acquired at 2 GHz Extended Dynamic Range MS mass range 100–2,000 *m/z* MS/MS mass range 50–2,000 *m/z* MS scan rate (spectra/second): 8 MS/MS scan rate (spectra/second): 3 Ramped collision energy Charge state Slope Offset 2 3.1 1 3 and >3 3.6 –4.8
Data analysis	Agilent BioConfirm software B.08

Results and Discussion

Figure 1 shows a comparison of the LC/MS chromatograms produced by the eight RP columns for the mAb tryptic digest using a formic acid mobile phase. All the RP column types showed diversity in peptide separation, and the selectivity difference across these columns is apparent. Table 1 summarizes the comparison results. One of the important column criteria during peptide mapping method development is the ability to accommodate a large number peaks under a given gradient elution condition.

Under the same column dimensions (2.1 × 150 mm), vendor D identified the most peaks. However, the AdvanceBio Peptide Plus column provided higher sequence coverage (99.3 %) with almost as many peaks. In addition, the lowest %RSD values for sequence coverage demonstrate the reproducibility of the coverage maps produced using the AdvanceBio Peptide Plus column.

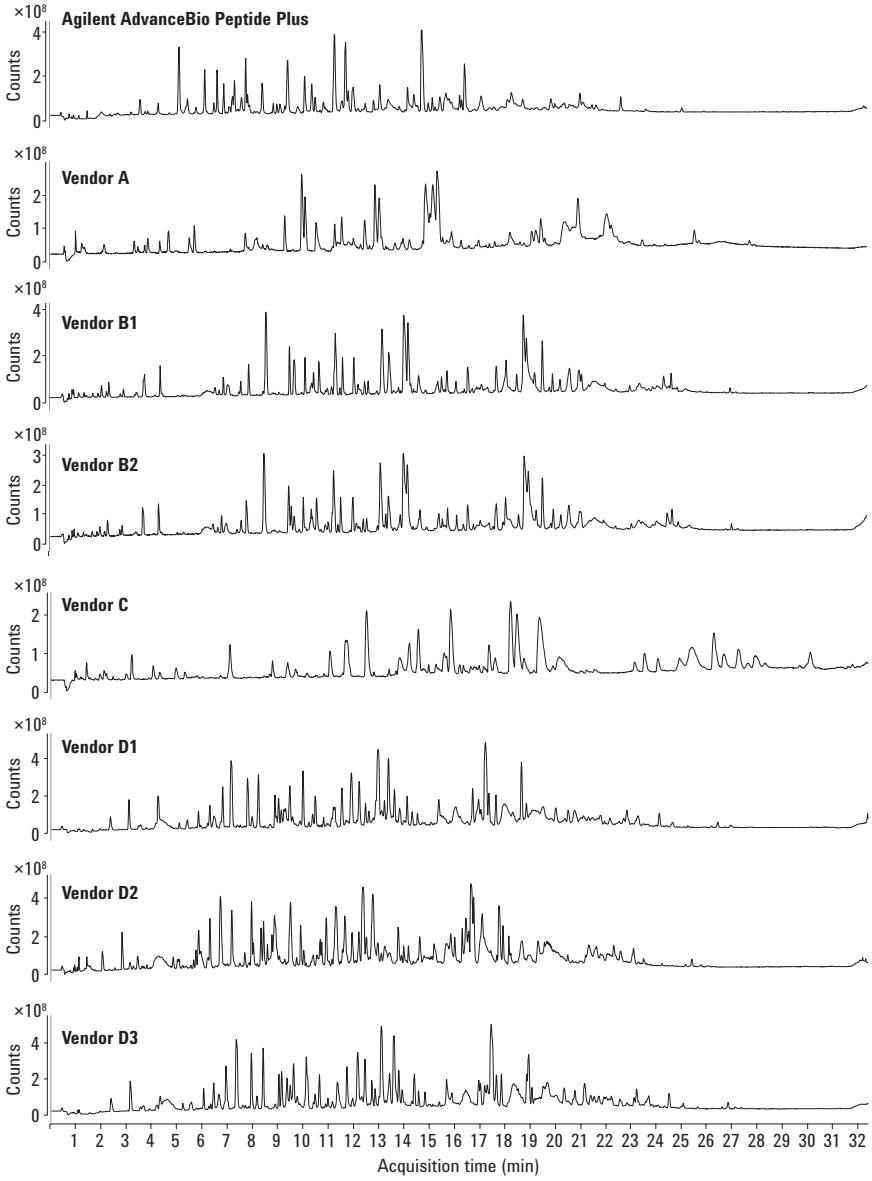


Figure 1. Comparison of mAb peptide mapping profiles of eight RP column types.

Table 1. Number of peaks and sequence coverage comparison of eight RP columns (n = 5).

Vendor	Average number of peaks	Average sequence coverage	Sequence coverage %RSD	Maximum pressure (bar)
Agilent AdvanceBio Peptide Plus	96	99.3 %	0.52	366
Vendor A	63	83.2 %	3.95	450
Vendor B1	92	85.9 %	2.26	385
Vendor B2	85	86.6 %	7.96	389
Vendor C	61	85.0 %	1.60	337
Vendor D1	107	80.3 %	5.78	495
Vendor D2	101	87.1 %	8.05	307
Vendor D3	100	87.5 %	5.04	790

Reproducibility, narrow peak shapes, and high peak capacity are key quality attributes of peptide mapping. Figure 2 compares the LC/MS peptide maps produced by eight RP columns. The profiles are overlays of five consecutive injections. The AdvanceBio Peptide Plus column showed highly consistent performance with no observable shift in the chromatographic profile between the replicate injections. Figures 3 and 4 show the retention time and peak width reproducibility data. Twenty extracted compound chromatogram (ECC) peaks across the chromatographic run were selected to evaluate the column reproducibility. For AdvanceBio Peptide Plus, all the retention time average percent RSD values were ≤0.07 %. The lower standard deviations for peak width (FWHM) obtained with the AdvanceBio Peptide Plus column demonstrate the superior performance for narrow peaks. Figure 5 shows the peak capacity comparison results. The AdvanceBio Peptide Plus and vendor B columns performed comparably concerning peak capacity. There was a 3.5 % increase in peak capacity values for the AdvanceBio Peptide Plus column. The vendor C column produced broader peak shapes, lower peak capacity values, and suffered from poor retention time reproducibility. The AdvanceBio Peptide Plus column showed improved performance over the sub-2 µm column (vendor D3) with less than half the backpressure.

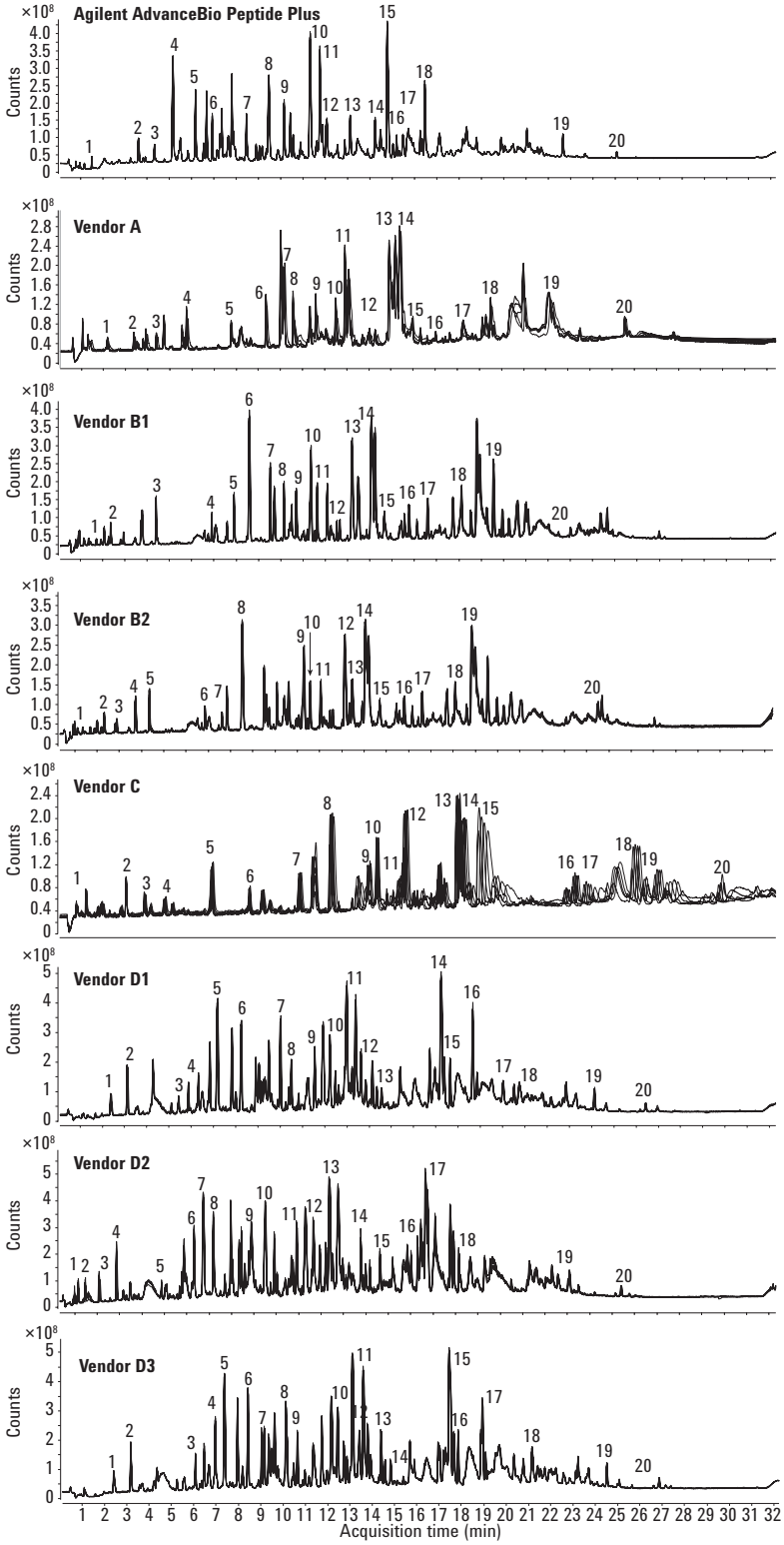


Figure 2. Overlaid total ion chromatograms of mAb tryptic peptide map (five replicate injections) using different RP column types. Peaks identified with numbers are used for retention time and peak width reproducibility calculations.

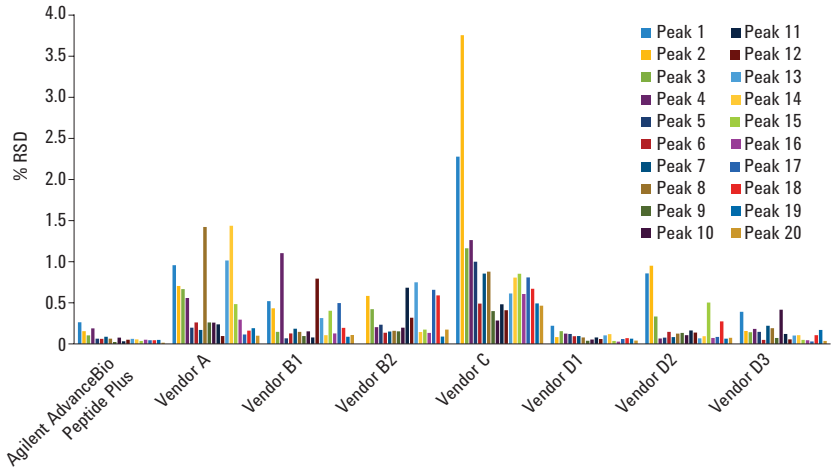


Figure 3. Comparison of retention time reproducibility for eight RP columns.

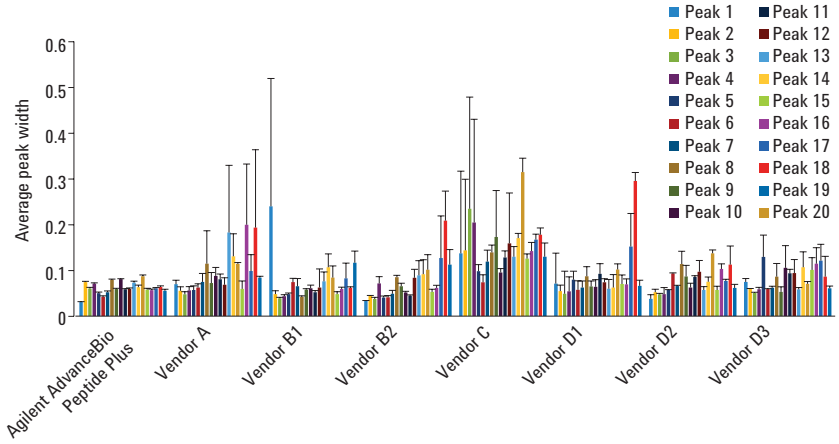


Figure 4. Comparison of peak width (FWHM) reproducibility for eight RP columns.

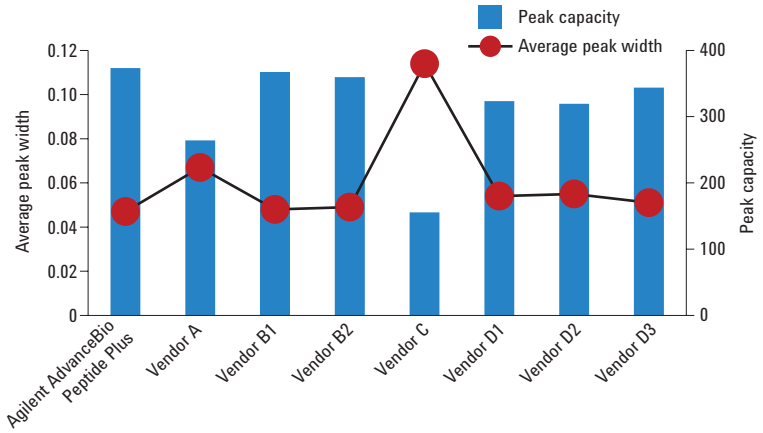


Figure 5. Peak capacity and peak width comparison for eight RP columns.

To further evaluate the column performance, heavy chain and light chain complementarity-determining region (CDR) peptides were observed. CDRs are antigen-binding regions, and the peptide sequence corresponding to CDRs are susceptible to modifications. Therefore, it is important to observe the CDRs of mAbs. Figure 6 shows the ECC for six CDR peptides with each column. All six CDR peptides were identified with the AdvanceBio Peptide Plus, vendor A, and C columns. The narrow peak shape and baseline resolution of these peptides with the AdvanceBio Peptide Plus demonstrate the column's separation power.

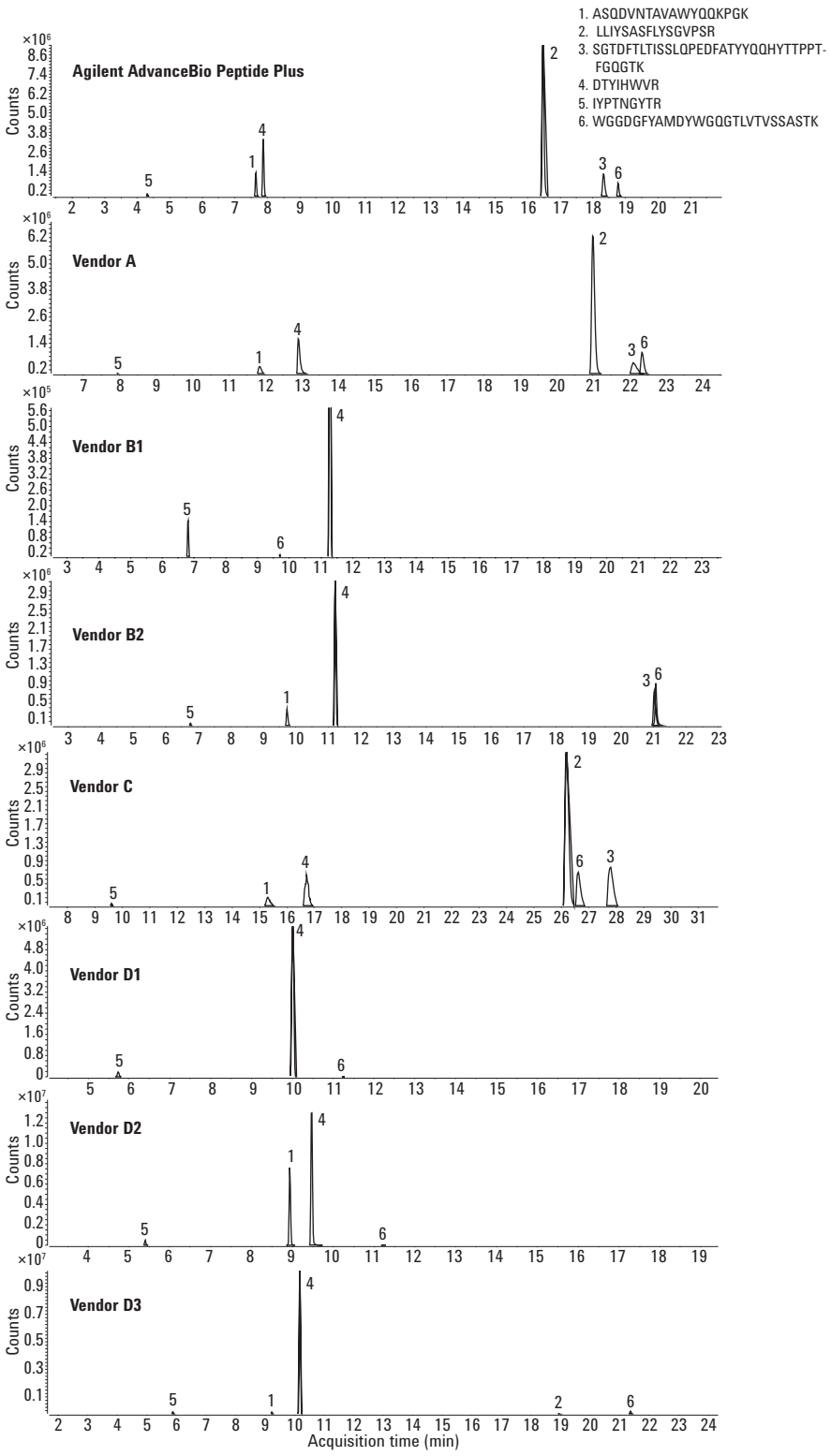


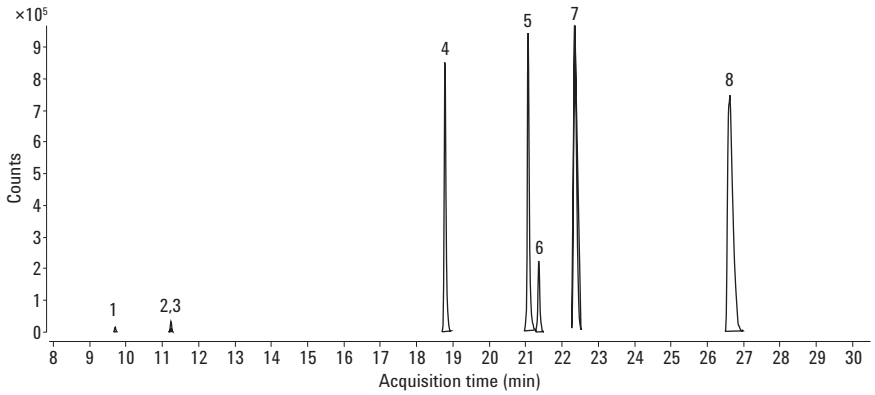
Figure 6. Comparison of separation of CDR peptide sequence using eight RP columns. Six overlaid ECCs.

Figure 7 compares the ECC of one of the heavy chain CDR peptides, proving the narrow LC/MS peak shape with stronger signal-to-noise ratio achieved using the AdvanceBio Peptide Plus column.

Conclusions

The Agilent AdvanceBio Peptide Plus column demonstrates:

- Reproducible peptide mapping
- Higher peak capacity values
- High-efficiency peptide separation with narrow peak shapes
- Best performance for LC/MS peptide mapping under formic acid mobile phase conditions



Peak	Column	FWHM
1	Vendor B1	0.033
2	Vendor D2	0.043
3	Vendor D1	0.035
4	Agilent AdvanceBio Peptide Plus	0.051
5	Vendor B2	0.057
6	Vendor D3	0.055
7	Vendor A	0.089
8	Vendor C	0.159

Figure 7. Monitoring heavy chain of CDR3 peptide. Overlaid ECC from eight RP columns. CDR peptide sequence: WGGDGFYAMDYWGQGTLVTVSSASTK.

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