Increasing Efficiency of Peptide Separations by Decreasing Particle Size and Column Dimension

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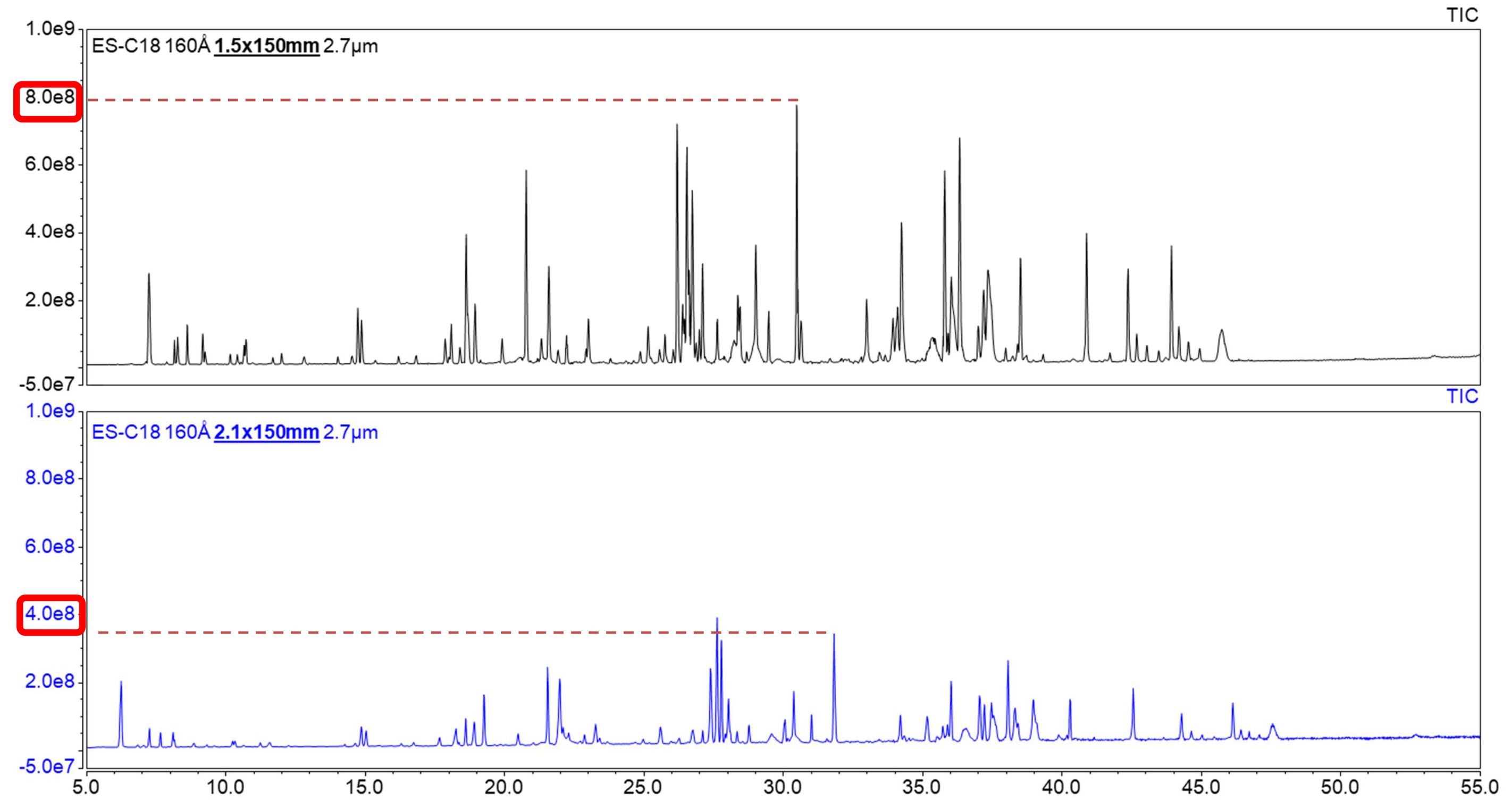
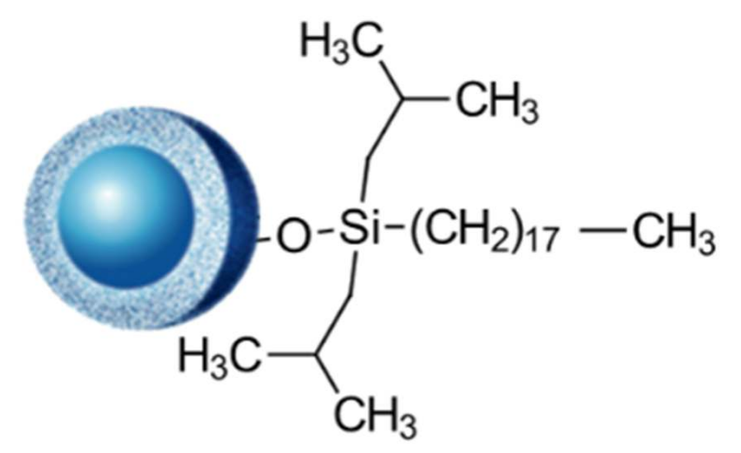
Introduction

With more complex biopharmaceutical drugs being developed each year, the testing and confirmation of these drugs becomes more important for patient safety. The chromatographic and MS separation demands of these drugs also becomes more challenging which requires improved column technology. By decreasing particle size, column efficiency/resolution can be increased to enable better peak identification. Higher peak capacity can be obtained using advanced column technology. By separating a tryptic digest of trastuzumab screening multiple peptide columns ranging in column ID and particle size, an increase in sensitivity, decrease in peak widths, and marked decrease in solvent usage is demonstrated using standard LCMS systems which was only previously realized with specialized microflow systems.

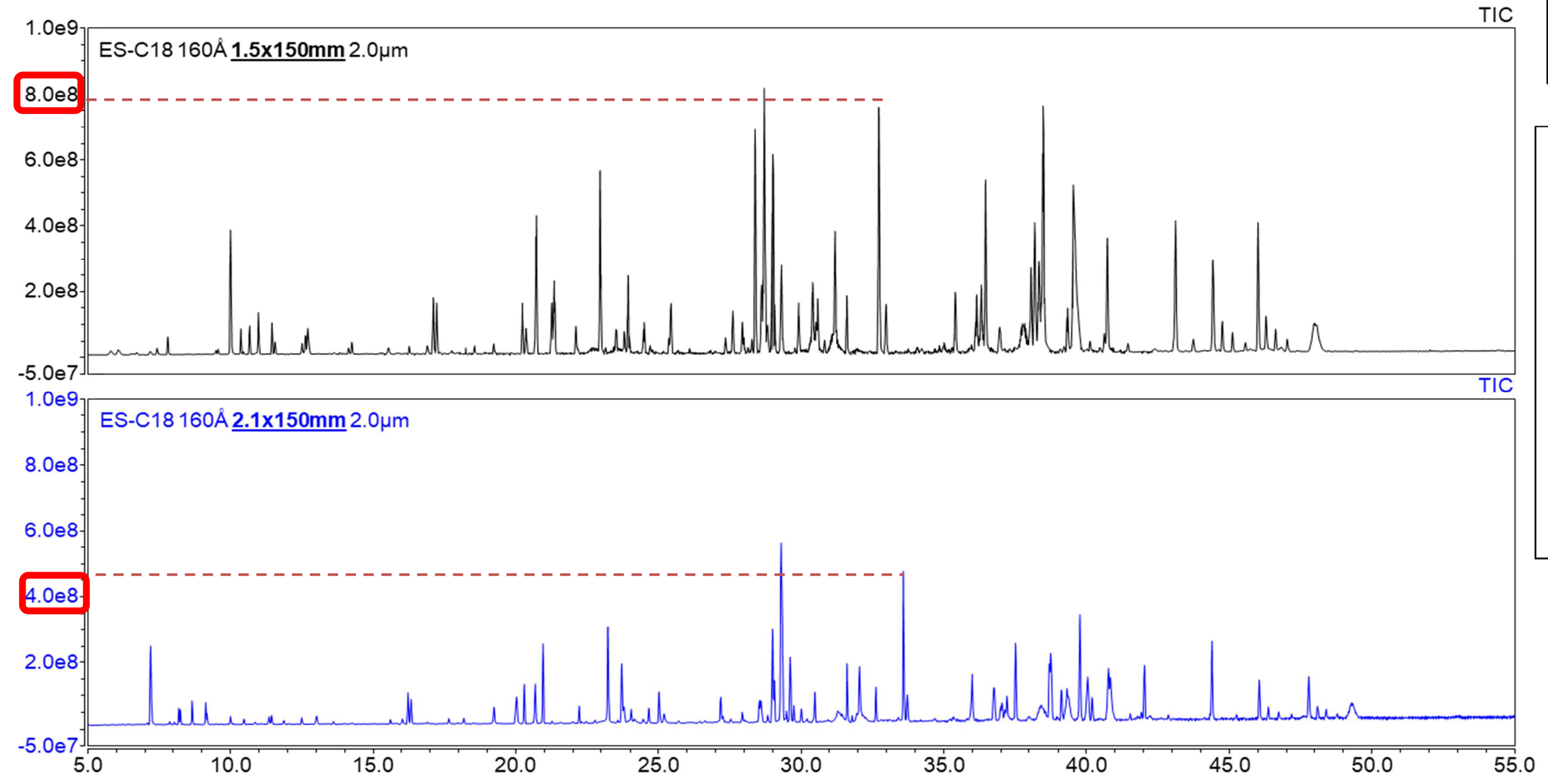


Trastuzumab Fab Region



ES-C18 Structure

A separation of Trastuzumab tryptic digest is performed on a HALO 160 Å ES-C18 column using a ThermoFisher Qexactive HF. By switching from a 2.1 mm ID to a 1.5 mm ID column there was 2x increase in total ion current observed along with a reduction in solvent consumption of 14mL. Extra column volume was reduced by optimizing the tubing from the column outlet to the MS source. The use of a 1.5 mm ID column delivers an increase in sensitivity and reduces solvent usage without having to invest into a specialized micro flow HPLC system.
This increase in sensitivity is also observed on smaller particle sizes which can be seen in the MS traces below. The smaller 2.0 µm SPP particle can achieve a sensitivity increase over its larger counterparts by encouraging the analytes to follow similar paths creating narrower peaks and increasing sensitivity. With the addition of a reduced column internal diameter, the increase in sensitivity becomes even more apparent.



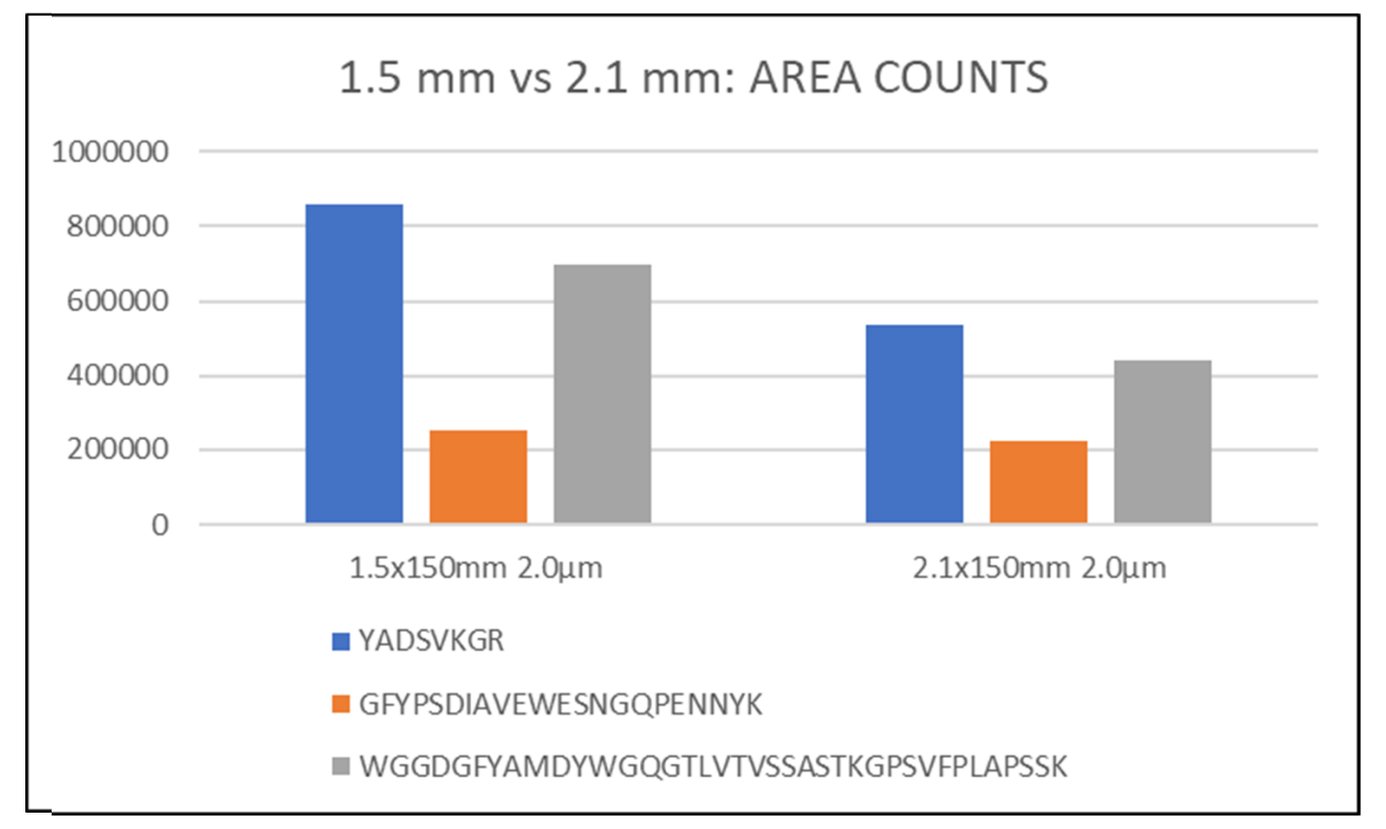
TEST CONDITIONS:
Column: HALO 160 Å ES-C18 , 2.0 µm, 1.5 x 150 mm
Column: HALO 160 Å ES-C18 , 2.0 µm, 2.1 x 150 mm
Mobile Phase A: Water, 0.1% DFA
Mobile Phase B: Acetonitrile, 0.1% DFA
Gradient:

Time	%B
0.5	2
60.5	50
61.0	70
65.0	70
65.5	2
70.0	Stop

Flow Rate: 0.2 mL/min for 1.5mm
0.4 mL/min for 2.1mm
Temperature: 60 °C
Injection Volume: 1 µL
Sample: 1mg/mL Trastuzumab tryptic digest
Sample Solvent: 1.5M Guanidine HCl/0.5% Formic Acid/~50mM Tris pH 7.8
LC System: Shimadzu Nexera X2

Tubing Optimization:
50µm x 600mm Column to Diverter Valve
50µm x 350mm Diverter Valve to Ground
50µm x 100mm Ground to Source

MS CONDITIONS:
System: ThermoFisher Q Exactive
Spray Voltage (kV): 3.8
Capillary temperature: 320 °C
Sheath gas: 35
Aux gas: 10
RF lens: 50

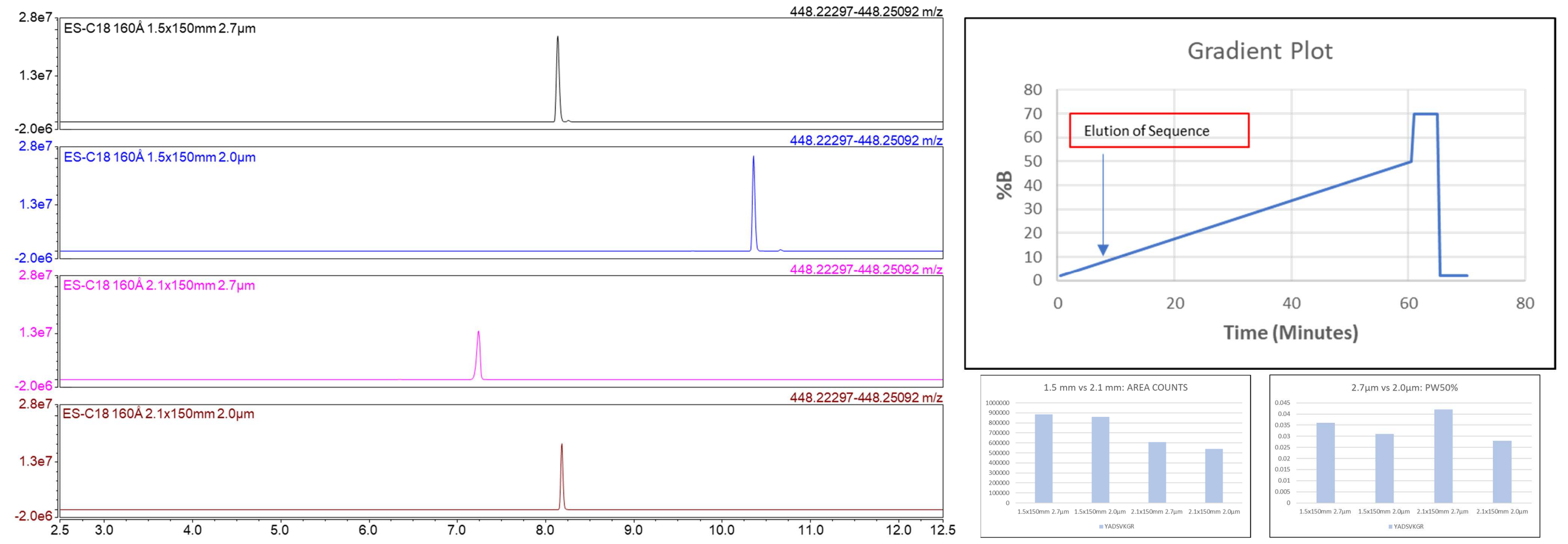


Above compares the two different column ID's against area counts of three different known peptide sequences.

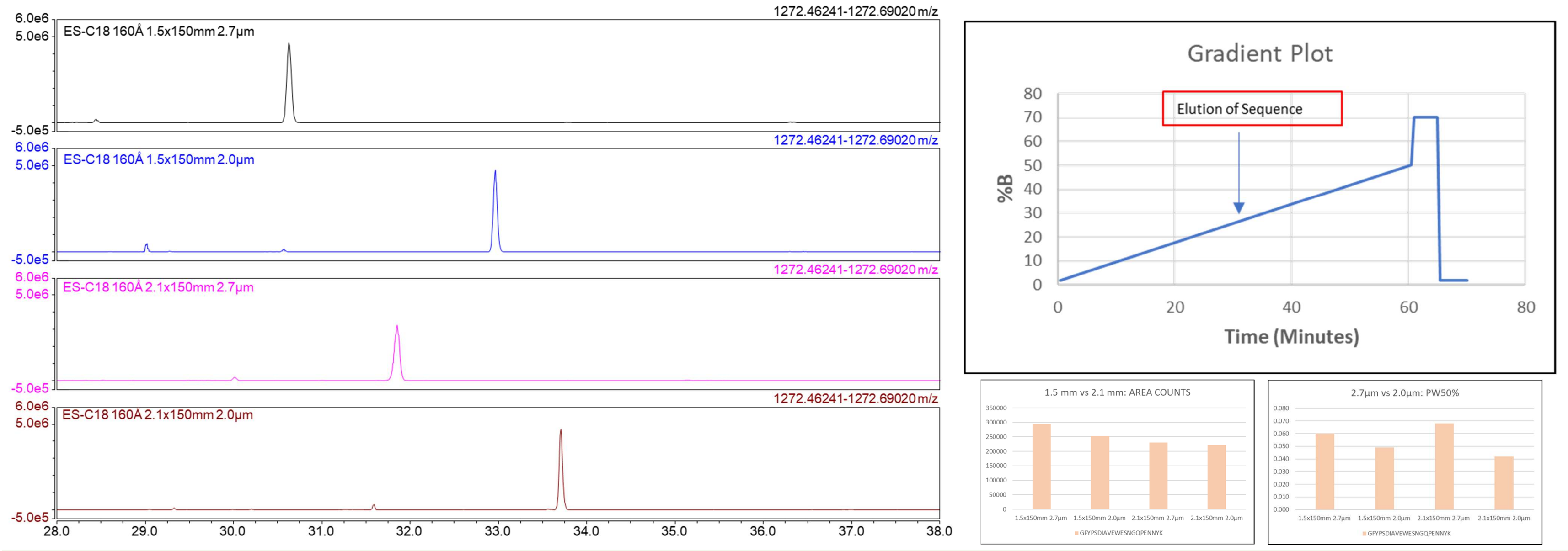
Extracted Ion Chromatograms: Column Evaluation

Peptide mapping can make evaluating column performance complicated. By choosing three different known sequences from the tryptic digest of Trastuzumab, an evaluation can be made on the performance of the column used for the separation. The three sequences chosen, with the help of the computer software BioPharma Finder (Thermo Scientific), were YADSVKGR (M+2), GFYPSDIAVEWESNGQPENNYK (M+2), and WGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSK (M+3). Each sequence was chosen based off of two requirements. The first requirement was the confidence of the sequence. This means that the computer program that was used to determine the sequence, based on its MS data, had matched the MS data taken from this experiment to the online library. The second requirement was retention time of the sequence. In order to gain an understanding of the column effects, a sequence from the beginning, middle, and end of the gradient were chosen.

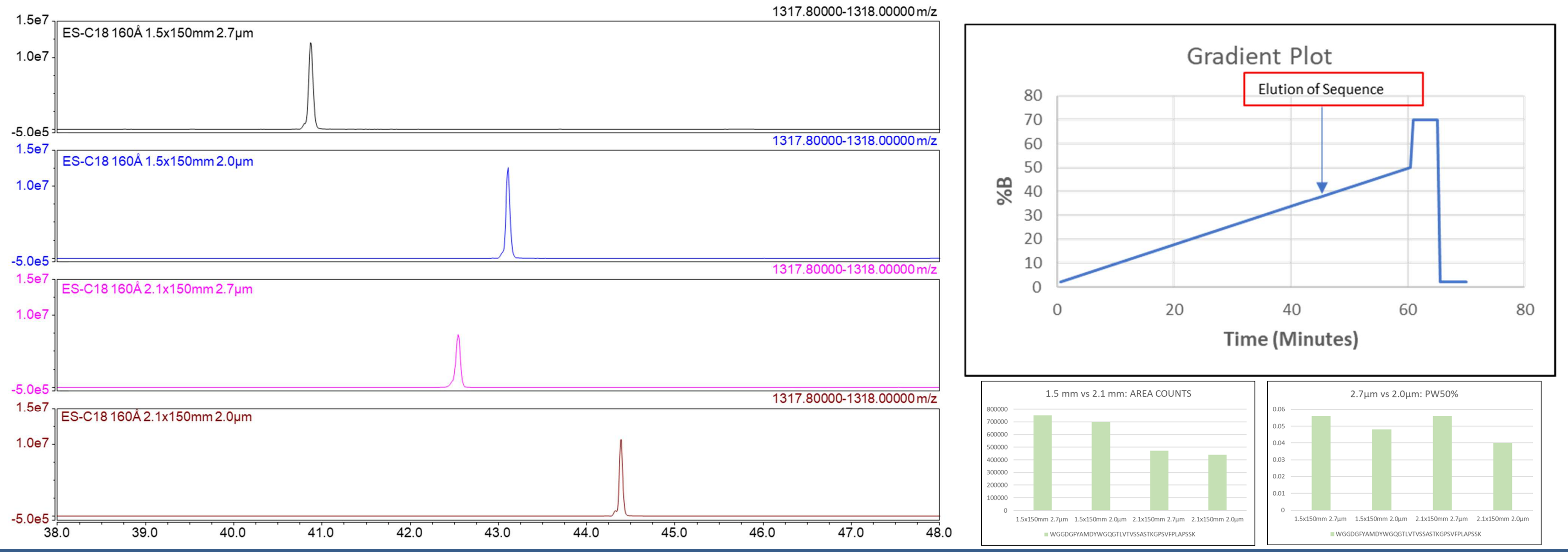
YADSVKGR Sequence



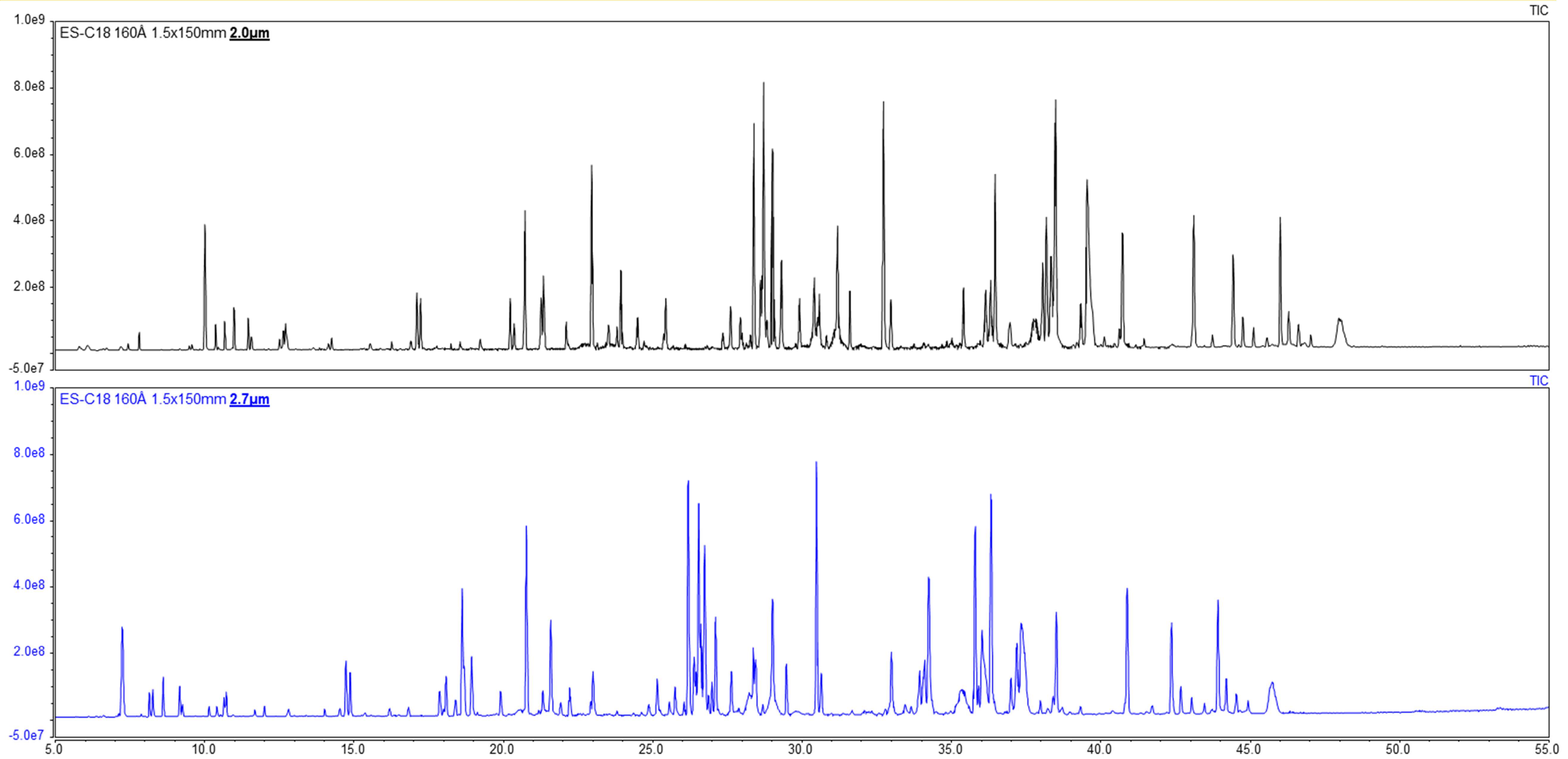
GFYPSDIAVEWESNGQPENNYK Sequence

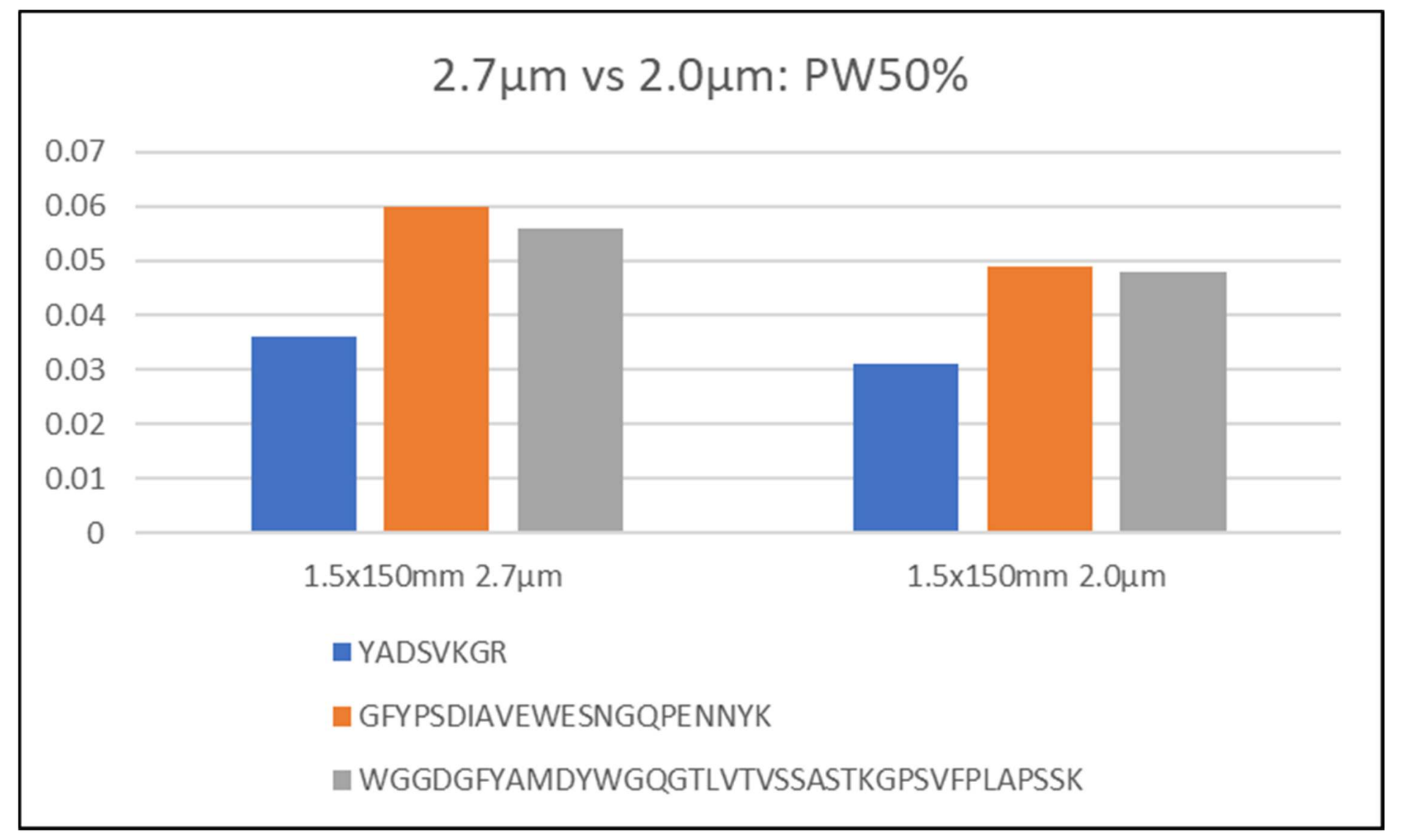


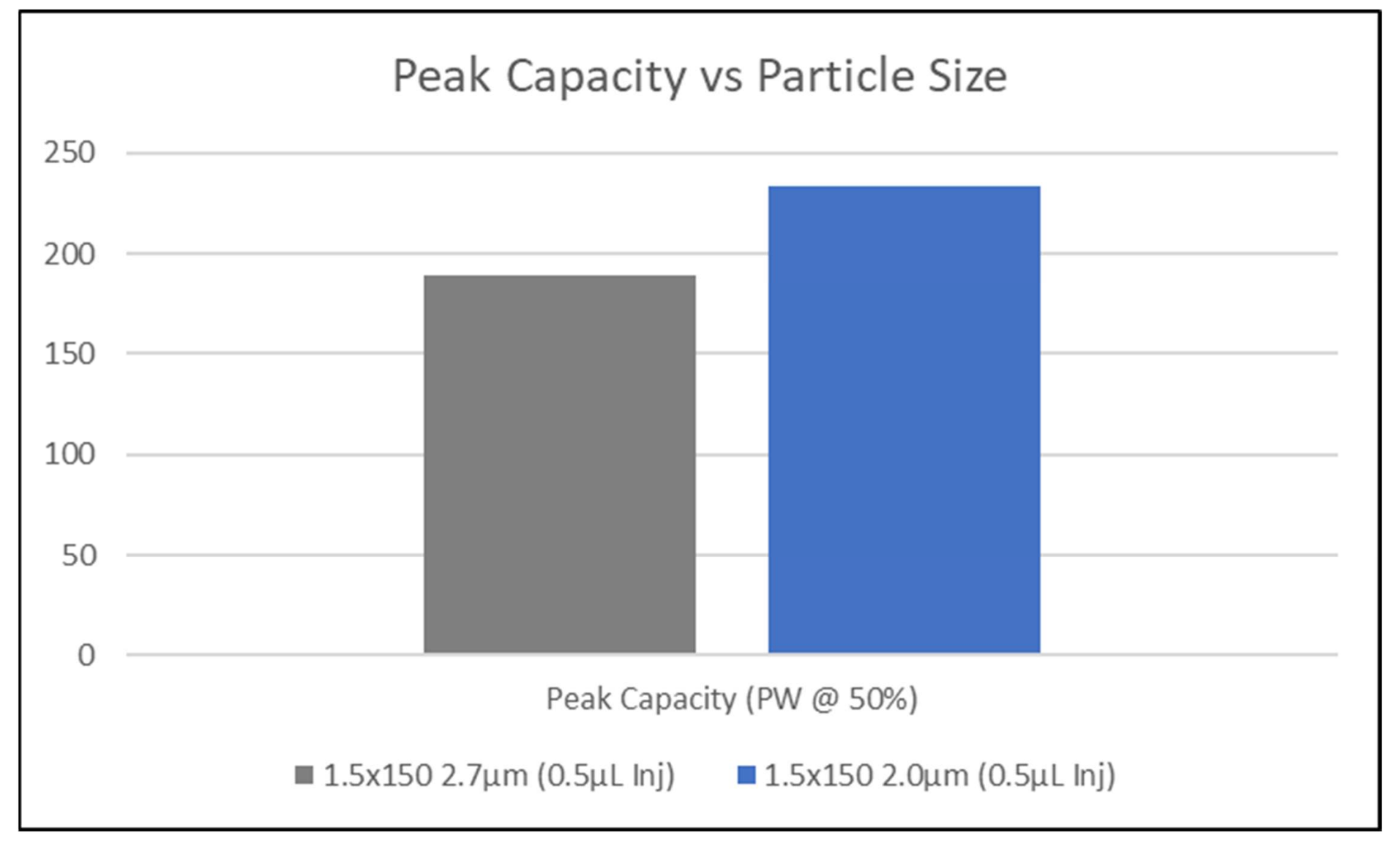
WGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSK Sequence



1.5 mm ID Particle Size Comparison







The full MS scan above shows of a sample of Trastuzumab that underwent trypsin digestion to produce peptide fragments of varying length. A comparison between the two different particle sizes (2 and 2.7 µm) of the ES-C18 stationary phase show an increase in peak capacity when switching from a 1.5 mm column packed with 2.7µm particles to a 1.5 mm column packed with 2µm particles. Using Chromeleon software (Thermo Scientific), peak width data was obtained for multiple sequences. The data above gives a closer look at the benefits of a 2µm 1.5 mm ID peptide column. Samples with similar complexity require the increased efficiency from a smaller particle size and long shallow gradients. By switching from a 2.1 mm ID to a 1.5 mm ID not only can solvent usage be cut in half, but sensitivity can also be increased.

Conclusions

The chromatographic and MS separation demands of new biotherapeutic drugs has become more challenging which requires improved column technology. By decreasing particle size, column efficiency and resolution can be increased to enable better peak identification. Higher peak capacity can be obtained using advanced column technologies along with increases in sensitivity and decreases in peak widths. With the drive to perform these separations with higher efficiency and a smaller environmental footprint, the use of smaller particle sizes along with reduced column ID's can help chromatographers achieve these goals.