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Focused Gradient Purification of Peptides with a Water/Acetonitrile (0.1% TFA) Mobile Phase using the ACCQ*Prep*® HP150 System

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ACCQ*Prep*, Focus Gradient Generator, HPLC, Peptides

Application Overview

Peptides are becoming increasingly important as tools for life science research and as new medicines in a range of disease areas. Despite many advances in peptide synthesis technologies, the 'bottle-neck' in their production is often at the purification stage. If each individual amino acid coupling step is 99% efficient, and assuming complete deprotection and cleavage from resin, then a sequence consisting of 30 amino acids would yield only 74% of the desired peptide. The remaining 26% will consist mostly of incomplete sequences due to failed amino acid couplings, which may have very similar chromatographic properties to the desired peptide. Very high purity is normally required for further applications, such as *in vitro* and *in vivo* studies. Therefore, purification of the crude mixture presents significant opportunities for new technological advances.

Purification of peptides is normally achieved using reversed phase preparative HPLC to obtain high purity. The trial-and-error process of optimising the chromatographic separation method to give the best purification is often time consuming, expensive, and wasteful (peptide material and solvents). Several parameters can be modified for improving the separation of compounds such as choice of mobile phase, method gradient, sample loading and stationary phase particle size. The physicochemical properties of the desired peptide may be similar to unwanted by-products. This can cause challenges, as the unwanted products will be eluted together with the desired product.

The Focused Gradient feature in ACCQPrep HP150 generates an optimised gradient based on an initial 'scout run' that separates the desired compound with high purity and resolution when using water (50 mM ammonium formate, pH 3.5) and methanol. In this application note, the built-in Focus Gradient Generator is adjusted to accommodate the use of the common solvent composition of water and acetonitrile (MeCN) (0.1% TFA), with the aim of separating a desired peptide from the unwanted side-products. To establish how successful the Focus Gradient Generator is for this application, we attempted the purification of several model peptides.

Experimental Details

Crude peptide mixtures were dissolved at a concentration of ~20 mg/mL in a solvent dictated by their solubility. More water-soluble peptides were fully dissolved in water or water/MeCN (1:1), whereas the more hydrophobic peptides required the addition of DMSO to fully dissolve them. Note: higher %DMSO generally afforded a loss of peak resolution; however, in some cases peptide precipitation was observed upon the addition of water. Therefore, some peptides were injected as a solution in 100% DMSO..

The generation of a Focused Gradient required an initial small injection (0.5 mL) of crude sample using the scout run method, and the retention time of the eluted peak of interest determined the %mobile phase composition for the Focused Gradient.

A new Focused Gradient was generated first by using the scout gradient method, and then by incrementally adjusting the starting %MeCN composition of the mobile phase (maintaining a 12 min, 10% gradient), for a series of peptides with different hydrophobicities. Chromatographic separations were performed according to the parameters in Table 1.

Instrumentation	Teledyne ISCO ACCQ*Prep* HP150 with AS 2x2 AutoSampler	
Column	RediSep Prep C18, 100Å, 5 µM	
Wavelengths	214 nm (red) 254 nm (purple)	
Mobile Phases	Solvent A: Water (0.1% TFA) Solvent B: MeCN (0.1% TFA)	
Flow Rate	18.9 mL/min	
Equilibration Volume	153.2 mL	
Scout Gradient	Solvent B %	Time (min)
	10	Initial
	100	6
	100	5

Table 1. Fixed parameters using in preparate HPLC.

Method Development and Purification

Crude peptide **1** (Table 2) was injected onto the column and separated using the built-in scout run (Figure 1B). The injection artefact, consisting of the solvent used to dissolve the peptide mixture, was consistently eluted at a retention time of 2 minutes. The large peak at ~4 minutes contained the group of compounds from the analytical HPLC chromatogram. (Figure 1A).

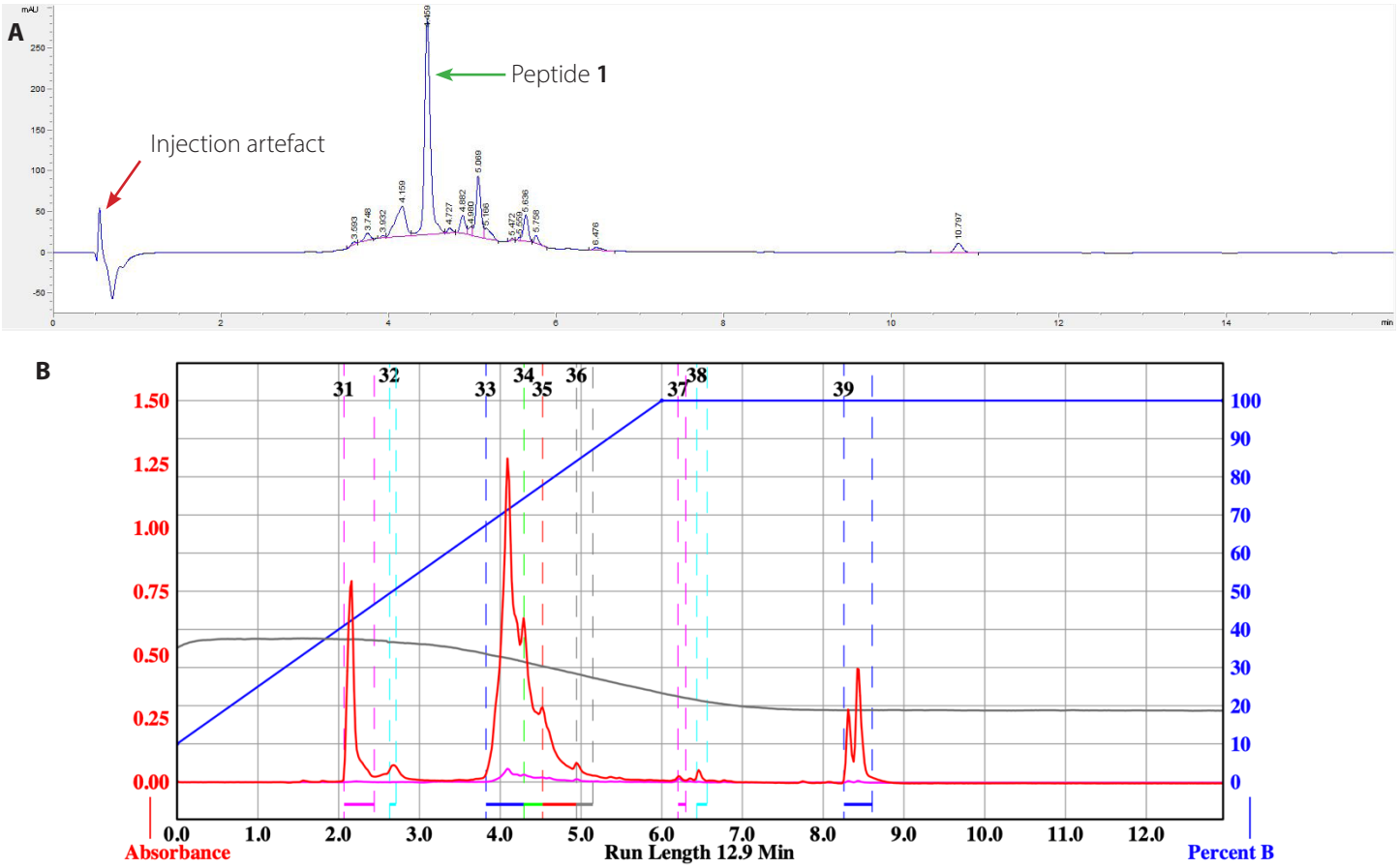

Figure 1. A) Analytical HPLC chromatogram of crude peptide **1**. B) Scout run chromatogram of crude peptide **1** using the ACCQ*Prep* HP150.

Using the built-in Focused Gradient feature and selecting the peak at 4.1 minute from the scout run resulted in a generated gradient that caused the peaks to be eluted later in the wash step (Figure 2A). Therefore, the Focused Gradient was adjusted from 17% to 30% starting MeCN composition, which eluted the desired peptide in the middle of the gradient (Figure 2B). Mass spectrometry identified the desired peptide in fraction 50 (Figure 2B) and afforded 98.9% HPLC purity (Figure 2C).

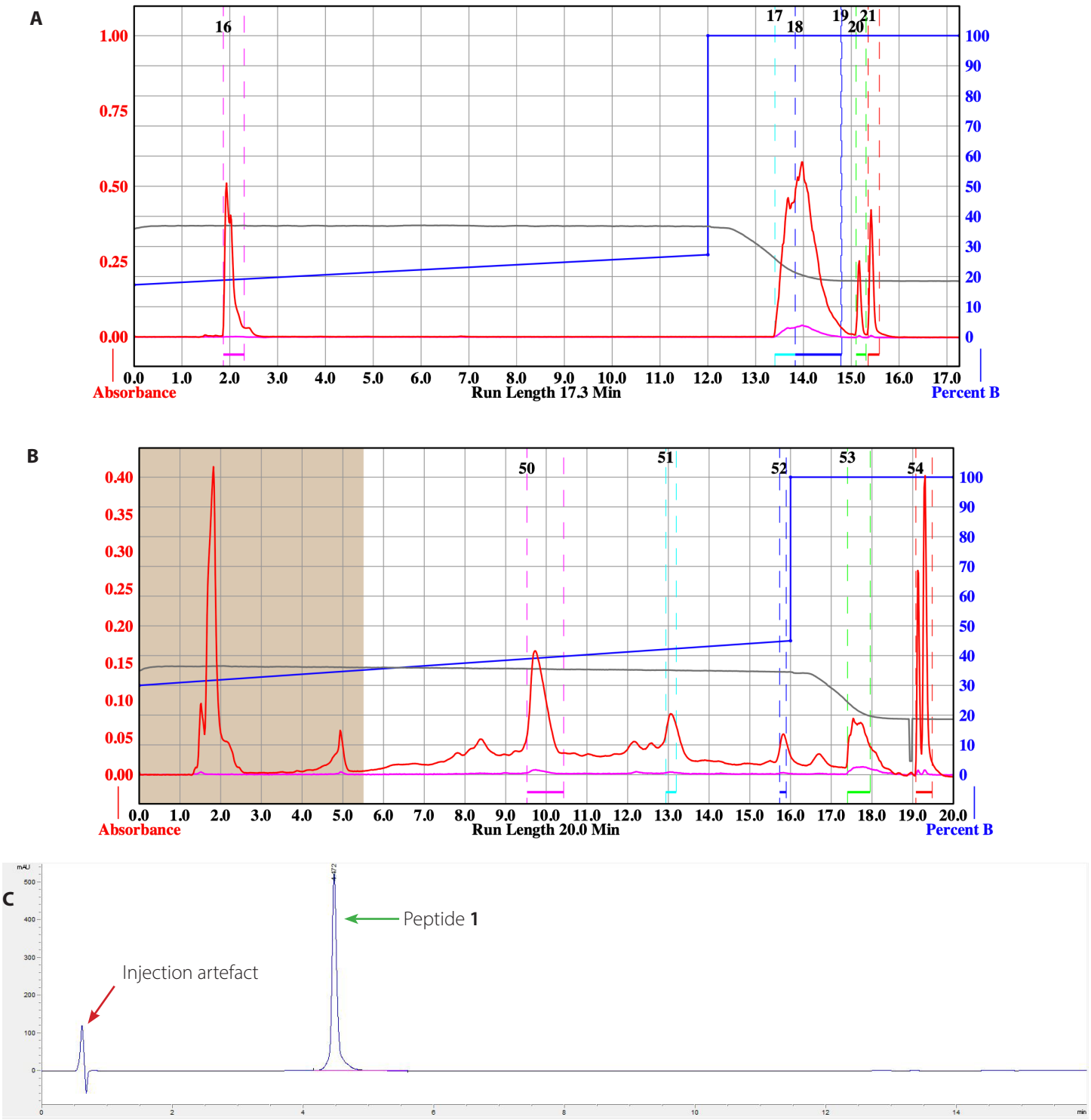

Figure 2. A) Built-in Focused Gradient run of peptide **1**. Desired peptide is eluted in the washing stage. B) Focused Gradient adjusted to a higher polarity. This results in the desired product to be eluted during the gradient and before 12 minutes. Initial waste window (brown area) was used for the first 5.5 minutes. C) Analytical HPLC chromatogram of purified peptide **1** using the ACCQ*Prep* HP150.

To establish a relationship between the peak retention time in the scout run method and the starting %MeCN composition in the optimised Focused Gradient, a series of peptides with different retention times (Table 2) were examined. The built-in Focused Gradient starting %MeCN was incrementally adjusted until the desired compound was eluted between 5-12 minutes. The dataset used to train the new Focused Gradient is shown in Table 2.

Focused Gradient for Peptides - Training Dataset			
Peptide	Scout Retention Time (min)	Scout % MeCN	Focused Gradient % MeCN at 6 min
1	4.10	71.5	37
2	3.65	64.8	27
3	3.75	66.3	31
4	4.30	74.5	38
5	5.15	87.3	50
6	3.90	68.5	32
7	4.25	73.8	36

Table 2. Dataset used for generating the Focused Gradient that uses water (0.1% TFA) and MeCN (0.1% TFA) mobile phases.

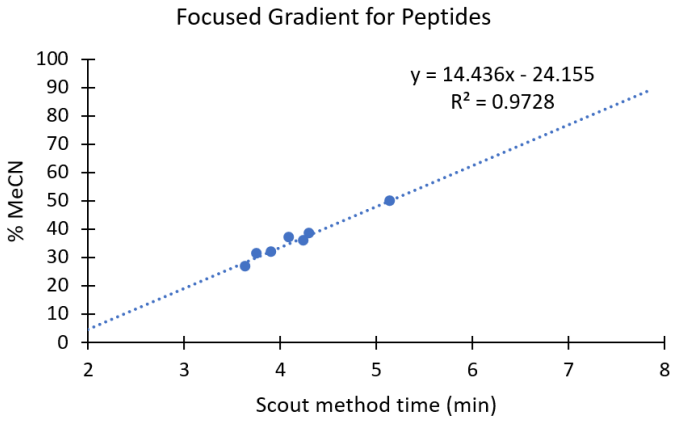

Figure 3. Linear regression analysis of the dataset shown in Table 2.

A correction factor was applied in order to shift the initial %MeCN down by 2%, whilst retaining the 10% gradient over 12 minutes. This made the desired compound elute after 5 minutes, which allowed a 5-minute initial waste window to be used, and so the injected solvent was not collected. Therefore, the straight-line equation used for generating the new Focused Gradient is:

$$y = 14.436x - 26.155$$

Where x is the retention time of the expected compound peak from the scout run. The output value (y) is the %MeCN of the gradient at 6 minutes, i.e., the middle of the gradient. Therefore, the initial %MeCN is -5% and the end %MeCN is +5% of the output value (y). The general method of the Focused Gradient is a 10% gradient over 12 minutes. After 12 minutes, the %MeCN increases to 100% for 5 minutes, i.e., the washing step (Figure 4B).

This calculator was used on a variety of different peptides, from short to long, linear to helical peptides, and different hydrophilicities. All test peptides using this Focused Gradient calculator afforded purities >95%. An example of this calculator being applied to peptide **8**: a peptide-based antagonist of the CGRP receptor is shown (Figure 4), affording a purity of peptide **8** of 98%.

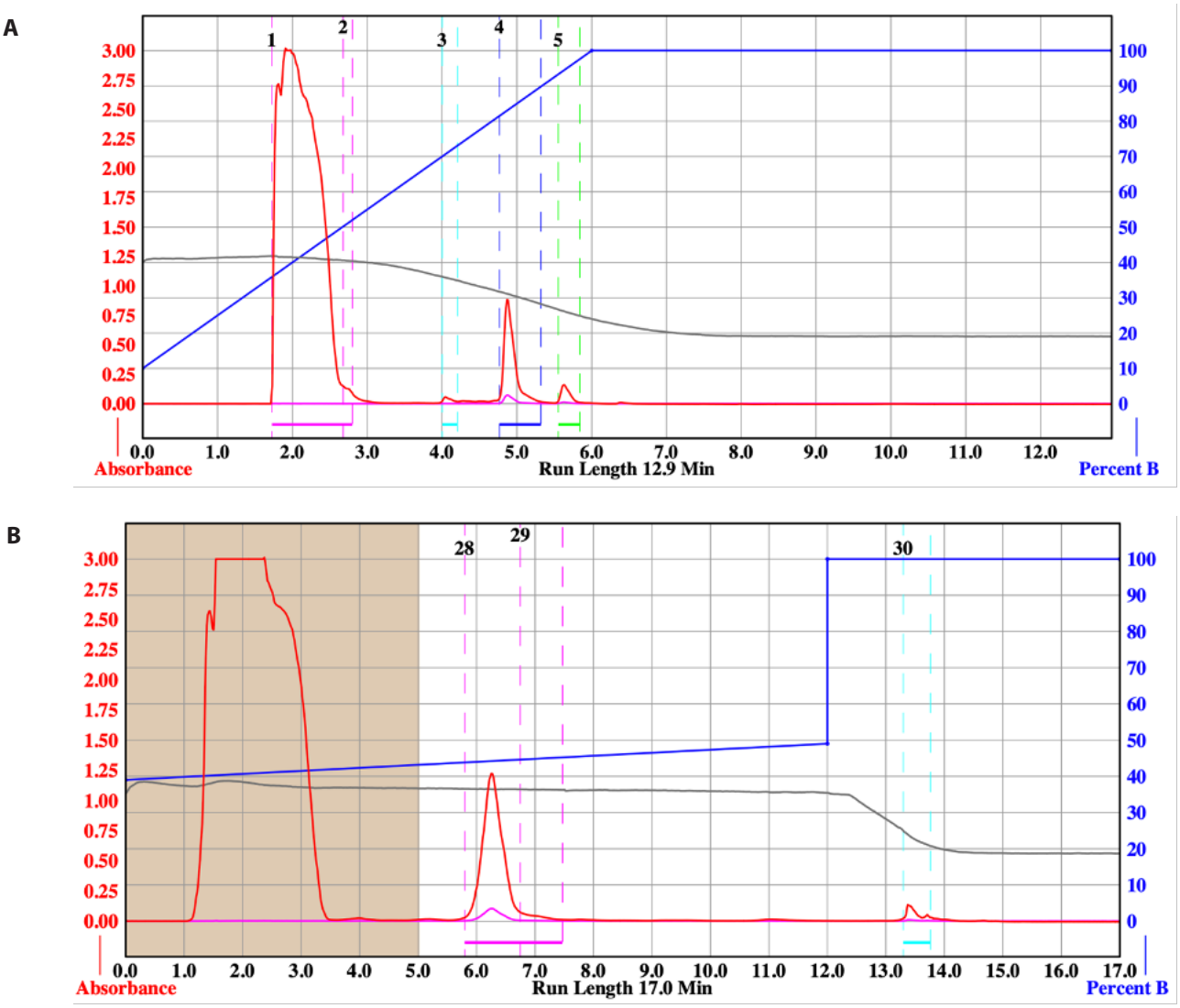

Figure 4. A) Scout run of crude peptide 8 dissolved in 100% DMSO. B) Focused Gradient of peptide 8 using the Focused Gradient calculator.

Conclusion

The purification of peptides can be achieved using reversed-phase preparative HPLC and can result in a high purity % that is necessary for further applications. The ACCQPrep HP150 Focus Gradient Generator is an excellent feature that generates an optimised gradient for quick and easy purification. The built-in feature was designed based on the use of water (50 mM ammonium formate, pH 3.5) and methanol. In this work, purification of the desired peptide was successfully achieved through the use of a Focused Gradient calculator that uses the common solvent choice of water (0.1% TFA) and MeCN (0.1% TFA). In total, 9 different peptides were purified with greater than 95% using this Focused Gradient calculator. A Microsoft Excel spreadsheet containing the Focused Gradient calculator is available on request.*

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Addendum

This technique and corrections are useful to avoid changing from a water/acetonitrile/trifluoroacetic acid gradient method.

However, also see Application Notes *Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds 2: Peptides*[a] and *Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds*,[b] which explain how ionizable compounds cause deviations from the calculations of the Focus Gradient Generator and how proper pH control and buffering can cause these compounds to elute within the Generator's calculations.

The Focus Gradient Generator is based on the Time-on-Target algorithm described by Silver.[c] The algorithm is the same as that implemented in Teledyne ISCO chromatography systems and used a non-polar compound for calibration to avoid any dependance on pH.

References

a. Application Note 141 *Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds 2: Peptides*. www.teledynelabs.com

b. Application Note 114 *Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds*. www.teledynelabs.com

c. Silver, J. Overview of Analytical-To-Preparative Liquid Chromatography Method Development. *ACS Combinatorial Science* **2019**, *21* (9), 609–613; https://doi.org/10.1021/acscombsci.8b00187