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Application Note 166

Getting Started with RP-HPLC for Peptides

Reversed-Phase (RP-HPLC) is an extremely useful tool for analytical biochemists. However, unlike small molecule HPLC, separations of proteins and peptides are nearly always performed under gradient conditions. There are other differences that one needs to be aware of in order to develop RP-HPLC separations of proteins and peptides as efficiently as possible. The general guidelines given in this short article may help reduce your method development time.

Key Words:
- proteins • peptides • RP-HPLC • LC/MS
- gradient elution • method development

Reversed-phase high performance liquid chromatography (RP-HPLC) of complex peptide or protein mixtures remains a general method of choice because of the resolution it provides. Unlike small organic molecules whose chromatographic behavior is described by a finite partitioning equilibrium between the stationary phase and the mobile phase, proteins and peptides typically do not exhibit such an effect. Instead, they exhibit an adsorption phenomenon in which the polypeptide is adsorbed onto the stationary phase and elutes only when the solvent strength of the mobile phase is sufficient to compete with the hydrophobic forces keeping it there. For this reason, elution of peptides or proteins from reversed-phase supports is by gradients of increasing solvent strength. When run under isocratic conditions, peaks for proteins and peptides are typically much broader than their small molecule counterparts.

Getting Started

Column: A good starting point for separating peptide or polypeptide mixtures is to start with a C18-bonded silica column designed for these applications. The Discovery BIO Wide Pore C18 is an ideal choice. Begin with 5µm particles packed into 15cm L x 2.1mm ID columns with a mobile phase flow rate of 0.2mL/min. The 2.1mm ID or "narrowbore" column configuration is a good balance between sensitivity (with 4.8-times the sensitivity of a 4.6mm ID column) and analysis time. If the dwell volume* of your HPLC system is greater than 500µL, a 4.6mm ID column run at 1.0mL/min is likely to be a better dimension to begin with.

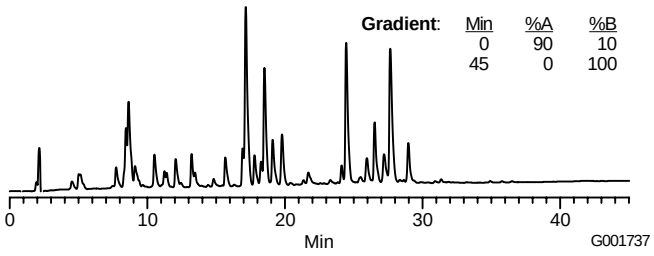
Mobile phase: Choice of mobile phase will in part be dictated by the means of detection. If detection is by mass spectrometry (MS), then the options are more limited and also generally don't provide optimal chromatography. Commonly used MS-compatible ionic mobile phase additives are acetate, formate, and carbonate and their corresponding ammonium salts. The organic component is most often acetonitrile (CH_3CN).

If detection is by traditional UV absorption, then mobile phases can be selected which provide for superior chromatography, which is largely conferred by including ion pairing reagents like TFA (trifluoroacetic acid) and HFBA (heptafluorobutyric acid). Typical concentrations are 0.05 – 0.1% *v/v.* A good starting point is to prepare mobile phase A to be 0.1% TFA in water and mobile phase B 0.1% TFA in a mixture of 50:50 water:acetonitrile. (Alternatively, having some acetonitrile in both A and B minimizes the refractive index changes that occur upon mixing and can provide more stable baselines.)

An initial run should begin with a gradient of 10 to 100%B in 45 minutes. An example of this starting gradient for peptides derived from a protein digest is shown in Figure A. While this separation does not provide significant resolution of component peptides, there are means that can be employed to improve the resolution for virtually all parts of the chromatogram.

Figure A. Initial Chromatographic Run of Complex Peptide Sample

Column:	**Discovery BIO Wide Pore C18, 15cm x 2.1mm, 5µm**
Cat. No.:	**568202-U**
Mobile Phase A:	0.1% TFA (v/v) in water
Mobile Phase B:	50:50, (0.1% TFA, v/v in water):(0.1% TFA, v/v in CH_3CN)
Flow Rate:	0.208mL/min
Temp.:	35°C
Det.:	215nm
Inj.:	10µL
Sample:	Carboxymethylated β-Lactoglobulin B tryptic digest



Optimizing the Method

Changing gradient profile: A first strategy for improving the resolution is to reduce the gradient slope. Gradient slope is the change in organic modifier concentration over time. There are limits to the practicality of this strategy because a reduction in gradient slope usually means longer run time. However, it is a strategy that is usually employed early in method optimization. The initial run (Figure A) has a slope of 1% CH_3CN/minute. The first step is to reduce the gradient slope by half. Concurrently, any wasted space in the chromatogram should be minimized. In the example shown in Figure A, assume nothing of significance elutes after 30 minutes. At 30 minutes, the percent of mobile phase B is 70%. Therefore in a second run both factors can be combined; a shallower gradient and reduction of the final %B to 70%.

The effect of these changes is shown in Figure B where the gradient profile was changed from 10 to 70%B in 60 minutes. The shallower gradient provided some improvements in resolution in the first half of the gradient. However, just as significant is the differences in selectivity in various groups of peaks. This is a fundamental effect due to the differential responses of the sample components to changes in the gradient slope. Also note that there is still wasted space at the end of chromatogram that can be reduced.

To see if a lower initial concentration of acetonitrile improves resolution of early eluting peaks, a third run is needed. This run has the same slope as previous runs, but in order to maximize the resolution of early-eluting peaks, the gradient starts at 100% aqueous. Under these conditions, the bonded phase is better able to distinguish between small differences in the hydrophobicity of the peptides. Although this may come at a cost of longer run time and may result in some wasted space in the initial part of the chromatogram, the improved resolution afforded may offset these consequences. The chromatogram in Figure C shows that this change had the desired effect. Peaks eluting before 25 minutes were better resolved than in Figure B.

Another option to improve resolution is to increase the run time. Although this reduces through-put, it may in some cases be the best way to obtain the required resolution. In Figure D the run time is increased to 130 minutes: 0 to 65%B in 130 min (0.25% CH_3CN/minute) as compared to Figure B where the slope is 0.5% CH_3CN/minute.

One last option not to overlook but not illustrated here is column length. It may be that increased column length may provide the desired improvements in resolution. The separations in this article were all run on 15cm columns, but very often 25cm columns are used.

In conclusion, the gradient profile (rate of change in % CH_3CN and the starting and ending %CH_3CN) has enormous power to increase resolution and decrease analysis time. By combining gradient methodology with a highly-efficient RP-HPLC material, like Discovery BIO Wide Pore C18, one has all the tools needed to maximize the separation of peptides and protein digests.

Ordering Information

(Other dimensions available. Please call or visit our web site.)

Description	Cat. No.
Discovery BIO Wide Pore C18 HPLC Columns, 15cm x 2.1mm, 5µm	**568202-U**
Discovery BIO Wide Pore C18 Guard Columns	
2cm x 2.1mm, 5µm, Kit	**568271-U**
2cm x 2.1mm, 5µm, 2 cartridges	**568270-U**

The following literature is available upon request, or by downloading from our website **sigma-aldrich.com/supelco**

Discovery BIO Wide Pore Brochure	T402038

Conditions for Figures B-D

Column:	**Discovery BIO Wide Pore C18, 15cm x 2.1mm, 5µm**
Cat. No.:	**568202-U**
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	50:50, (0.1% TFA in water):(0.1% TFA in CH_3CN)
Flow Rate:	0.208mL/min
Temp.:	35°C
Det.:	215nm
Inj.:	10µL
Sample:	Carboxymethylated β-Lactoglobulin B tryptic digest

Figure B. Change Gradient Profile

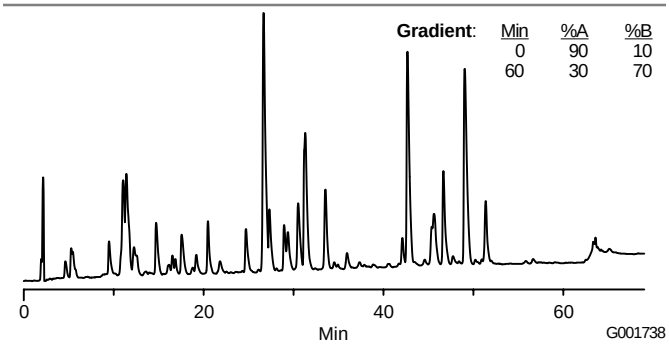


Figure C. Enhanced Resolution Early in the Gradient

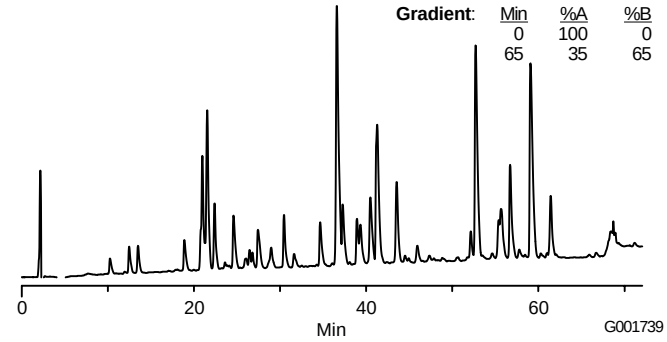
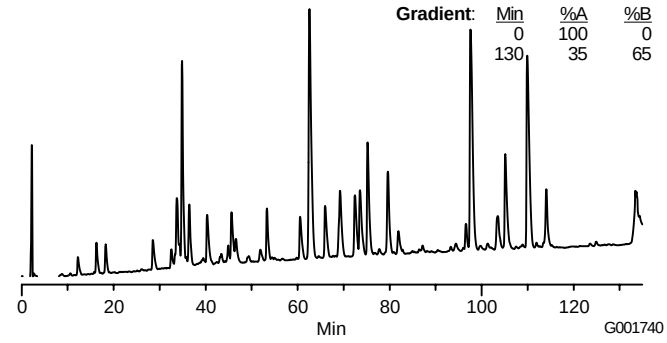


Figure D. Further Refine Gradient Slope



Reference

Geng, X. and Regnier, F.E. 1984. Retention Model for Proteins in Reversed-Phase Liquid Chromatography. *J. Chrom* 296, 15.

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*Dwell volume is the volume from and including the mixer to the column inlet. Low pressure mixing systems generally have larger dwell volumes than high pressure mixing systems.

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