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Application Note 169

Effect of Stationary Phase on Selectivity of Reversed-Phase HPLC Separations of Polypeptides

RP-HPLC separations of peptides and polypeptides is influenced by the chemistry of the bonded phase. The objective of most peptide separations is to obtain as much information about the sample as possible, especially when working with peptide maps. Therefore, it is to the researcher's advantage to run the sample on different stationary phases, like a C18, C8, and C5.

Key Words:
- selectivity • polypeptides • peptides
- peptide maps • RP-HPLC

What is Meant by Selectivity?

The resolution equation:

$$R_S = (1/4)\, N^{1/2} \{(\alpha - 1)/\alpha\} \{k/(1 + k)\}$$

tells us that retention (k), efficiency (N), and selectivity (α) each play a role in a chromatographic separation. There are few improvements that can be made to column efficiency if one is working with small particles and modern packing materials. Retention also gives limited options because of the need to keep analysis times as short as possible. However, selectivity wields great power to increase resolution. Selectivity can be thought of as peak spacing. The further the peaks are spaced from one another, the better the selectivity. Selectivity, or separation factor, between peaks 1 and 2 is measured by the equation:

$$\alpha = k_2 / k_1$$

where $k = (t_R - t_0) / t_0$

Of course, improvements in selectivity beyond allowing for complete baseline resolution of all sample components is of no additional benefit. Application Note 166 (T302166) showed that improvements in selectivity (and thus resolution) for a complex peptide sample can be achieved by altering the gradient slope and start conditions for the run. These are the most common strategies for optimizing selectivity with polypeptide samples, but there are other tools as well. In this short article, we will discuss the effect of stationary phase chemistry on the selectivity of peptide separations.

How Does the Stationary Phase Effect Selectivity?

Unlike the partitioning mechanism exhibited by small molecules, retention of polypeptide analytes on a reversed-phase matrix is by differential adsorption to the stationary phase, primarily due to differences in their hydrophobicity. More hydrophobic peptides are retained longer by the bonded phase, and *vice versa*. By reducing the alkyl chain length of the bonded phase, not only is the hydrophobicity reduced, but also the total surface area that is in contact with the peptide analytes. For small molecule separations, a C18 and C8 will usually give the same selectivity, although different retention. However because of different retention mechanisms for peptide and polypeptide separations, the differences in selectivity between a C18, C8, and C5 can be dramatic. The same sample run on C18, C8, and C5 phases will yield different information about the sample, an important consideration for peptide mapping.

There are other factors which affect adsorption to the matrix as well, even when comparing only linear aliphatic alkyl bonded phases. These other factors involve polar or H-bonding interactions with the silica surface itself, or the indirect effects of the silica surface chemistry on the conformation of the bonded phase. Thus, not only differences in the hydrophobicity of the bonded phase can influence selectivity, but also secondary effects impacted by the bonding chemistry and surface silanols: bonding density, extent of endcapping of silanols, and type of bonding (mono-, di-, or trifunctional).

An example of selectivity differences conferred by bonded phase chemistry is shown in Figures A and B (on reverse). Here, a proteolytic digest of apohemoglobin is chromatographed on the three Discovery BIO Wide Pore reversed-phases C18, C8, and C5. The chromatograms displayed only represent a portion of the entire run to better illustrate the subtle, but significant, differences in selectivity conferred by each phase. Each of the phases displays better selectivity in different parts of the chromatogram. If the goal is purification of a specific peptide, then this has particular utility. If the goal is the best overall resolution of the entire sample, then a decision process should be applied which evaluates the performance of each phase with its optimized method.

In conclusion, different bonded phase chemistries give subtle yet significant differences in selectivity toward peptides and polypeptides. Running the sample on each of the three Discovery BIO Wide Pore reversed-phase chemistries will yield different, useful information about the sample.

Figure A. Proteolytic Digest Discovery BIO Wide Pore C18, C8, and C5 (0-16 minute window)

Mobile Phase A: 95:5, (0.1% TFA (*v/v*) in water):(0.1% TFA (*v/v*) in CH_3CN)
Mobile Phase B: 50:50, (0.1% TFA (*v/v*) in water):(0.1% TFA (*v/v*) in CH_3CN)
Column: **Discovery BIO Wide Pore, 15cm x 4.6mm, 5µm**
Flow Rate: 1.0mL/min
Temp.: 30°C
Det.: UV, 215nm
Inj.: 50µL
Sample: tryptic digest of carboxymethylated apohemoglobin

Gradient:

Min	%A	%B
0	100	0
65	0	100

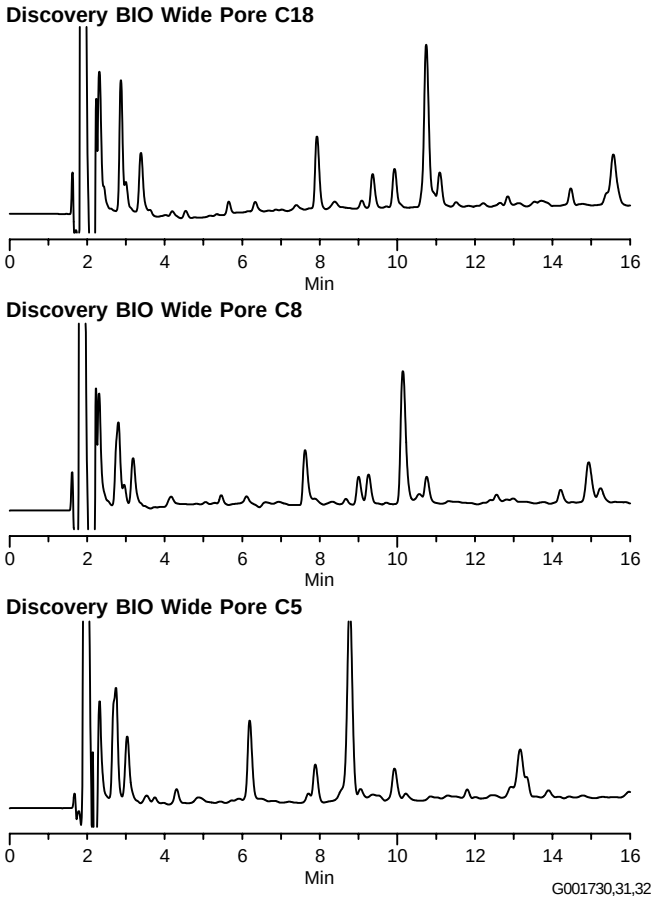

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Figure B. Proteolytic Digest Discovery BIO Wide Pore C18, C8, and C5 (28-38 minute window)

Mobile Phase A: 95:5, (0.1% TFA (*v/v*) in water):(0.1% TFA (*v/v*) in CH_3CN)
Mobile Phase B: 50:50, (0.1% TFA (*v/v*) in water):(0.1% TFA (*v/v*) in CH_3CN)
Column: **Discovery BIO Wide Pore, 15cm x 4.6mm, 5µm**
Flow Rate: 1.0mL/min
Temp.: 30°C
Det.: UV, 215nm
Inj.: 50µL
Sample: tryptic digest of carboxymethylated apohemoglobin

Gradient:

Min	%A	%B
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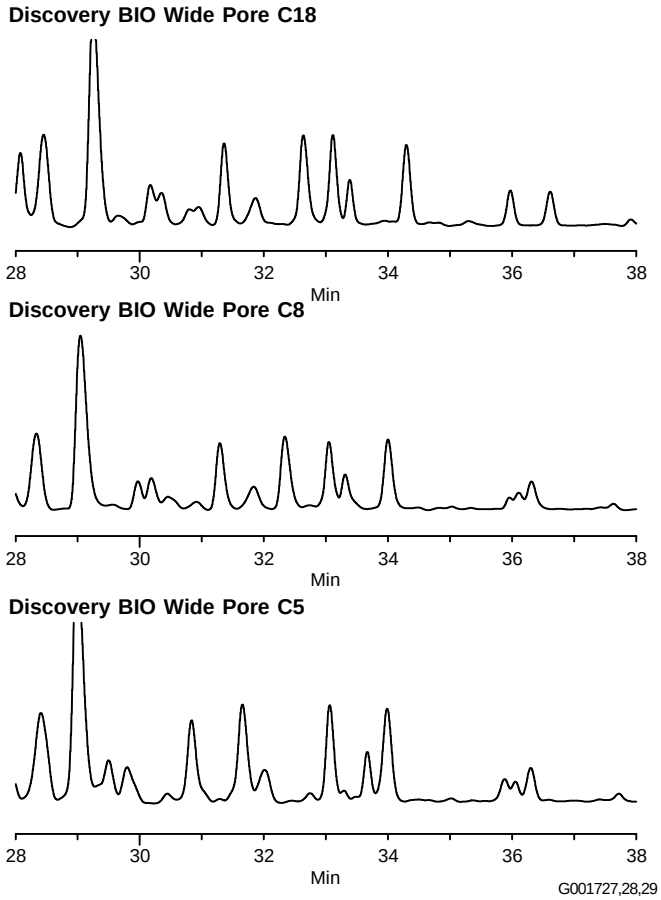
65	0	100



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Ordering Information

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

Description	Cat. No.
Discovery BIO Wide Pore C18, 15cm x 4.6mm ID, 5µm	**568222-U**
Discovery BIO Wide Pore C8, 15cm x 4.6mm ID, 5µm	**568322-U**
Discovery BIO Wide Pore C5, 15cm x 4.6mm ID, 5µm	**568422-U**

Description	Cat. No.
Guard Column Kits (holder plus one cartridge)	
Supelguard Discovery BIO Wide Pore C18, 2cm x 4mm ID, 5µm	**568273-U**
Supelguard Discovery BIO Wide Pore C8, 2cm x 4mm ID, 5µm	**568373-U**
Supelguard Discovery BIO Wide Pore C5, 2cm x 4mm ID, 5µm	**568473-U**

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