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SUPELCO

Technical Report

Peptide Separations on a Stable, Aqueous Compatible C18 Column

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Because of their omnipresence in biological systems, peptides have diverse molecular structures. This diversity makes it difficult for one type of HPLC column to selectively retain and resolve the components of an entire peptide map. Retention on reversed-phase columns is due to hydrophobic (non-polar) interactions. Retention problems arise with polar peptides that do not interact strongly with the hydrophobic stationary phase, e.g. C18 chains, resulting in little or no retention. This can be overcome by using a C18 HPLC column that can be used with very little organic modifier in the mobile phase.

Key Words
● HPLC ● reversed-phase ● aqueous mobile phases
● peptides ● peptide mapping ● phase collapse
● proteomics ● tryptic digest

The Importance of C18 Columns in Peptide HPLC

Bio-analysts performing peptide separations often encounter polar or very hydrophilic peptides that require highly aqueous mobile phases to achieve necessary reversed-phase retention. A C18 column is commonly employed. However, most C18 columns are not designed for use in mobile phases that contain less than 5% organic. Under these conditions, the C18 phases exhibit a "phase collapse" phenomenon where the retention decreases over time[1,2]. This is generally thought to be the result of the stationary phase folding down on the silica surface with concomitant loss in hydrophobic surface area. Fortunately for the bio-analyst, there are C18 columns that are stable and compatible with highly aqueous mobile phases. In this article, we describe the Discovery BIO Wide Pore C18 column that does not undergo phase collapse and is therefore ideally suited for use in 100% aqueous mobile phases.

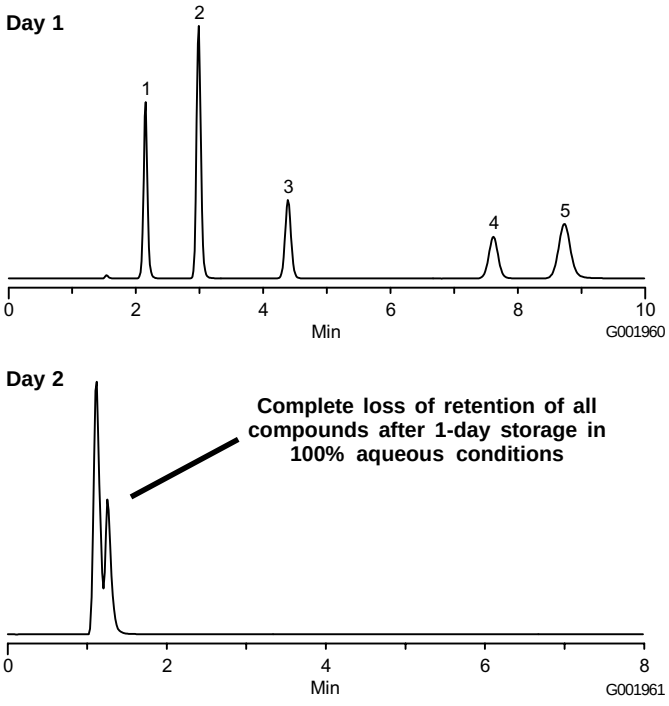
The C18 Phase Collapse Phenomenon

An example of C18 aqueous phase collapse is shown in Figure A. To demonstrate the effect, we first tested a commercially available C18 column with five nucleotide bases in phosphate buffer, pH 7. The column was then stored in the buffer component of the mobile phase overnight and retested under the same conditions the following day. The results are shown in Figure A. The retention and resolution of the five components have changed dramatically from Day 1 to Day 2. Although the column gave initially excellent retention and resolution, after overnight storage in 100% aqueous conditions, all retention and resolution is lost and the five peaks co-elute at the void volume.

Figure A. Phase Collapse on Conventional C18 Column After Aqueous Storage

Column:	**Standard silica-based C18, 15cm x 4.6mm ID, 5µm particles**
Mobile Phase:	20mM Potassium Phosphate, pH 7.0
Flow Rate:	1.5mL/min
Det.:	UV 254nm
Temp.:	35°C
Inj.:	10µL, each compound 10µg/mL

1. Cytosine
2. Uracil
3. Guanine
4. Thymine
5. Adenine



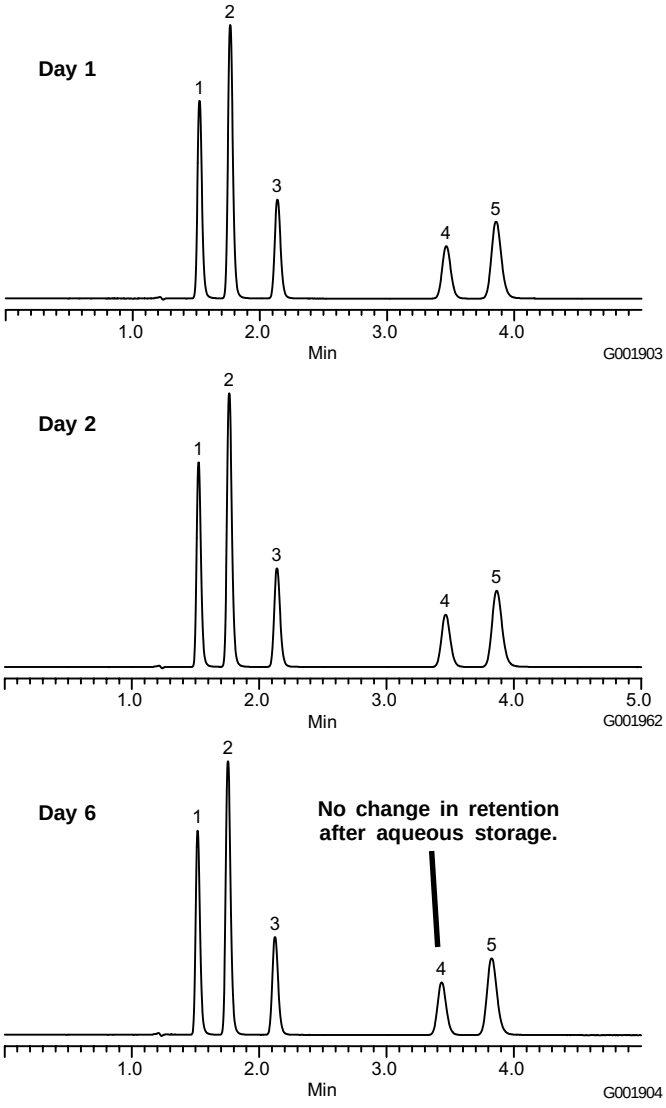
Discovery BIO Wide Pore C18 — Aqueous Compatible C18 Column

Figure B presents the same test on a Discovery BIO Wide Pore C18 column that is compatible with aqueous run and storage conditions. The column was tested in the same potassium phosphate buffer as described in Figure A each day and stored in the buffer overnight. As the chromatograms demonstrate, the retention time of each peak and the resolution of the peaks do not change over a period of 6 days. This indicates that the column is compatible with 100% of aqueous buffer and does not undergo phase collapse.

Figure B. Aqueous Compatibility Test on an Aqueous Compatible C18 Column

Column: **Discovery BIO Wide Pore C18, 15cm x 4.6mm ID, 5µm particles**
Cat. No.: **568222-U**
Mobile Phase: 20mM Phosphate Buffer, pH 7.0
Flow Rate: 1.5mL/min
Det.: UV, 254nm
Temp.: 35ºC
Inj.: 10µL, each compound 10µg/mL

1. Cytosine
2. Uracil
3. Guanine
4. Thymine
5. Adenine



Applicability to Peptide Separations

Most peptide separations are performed under gradient conditions. In order to retain early-eluting polar peptides, the initial gradient conditions should be 100% aqueous. However, as demonstrated in Figure A, most C18 columns do not perform reliably under these high aqueous conditions. The Discovery BIO Wide Pore C18 phase is not susceptible to phase collapse and can therefore be run under conditions that will retain polar peptides. In the example show in Figure C, a gradient of 0 – 75% acetonitrile at constant 0.1% TFA was used. Note that starting conditions were 100% aqueous. The five peptides were well resolved with excellent peak shape and efficiency initially and after repeated gradient cycles. Ultimately, the column gave stable results after 600 injections following 600 gradient cycles over a two-week period.

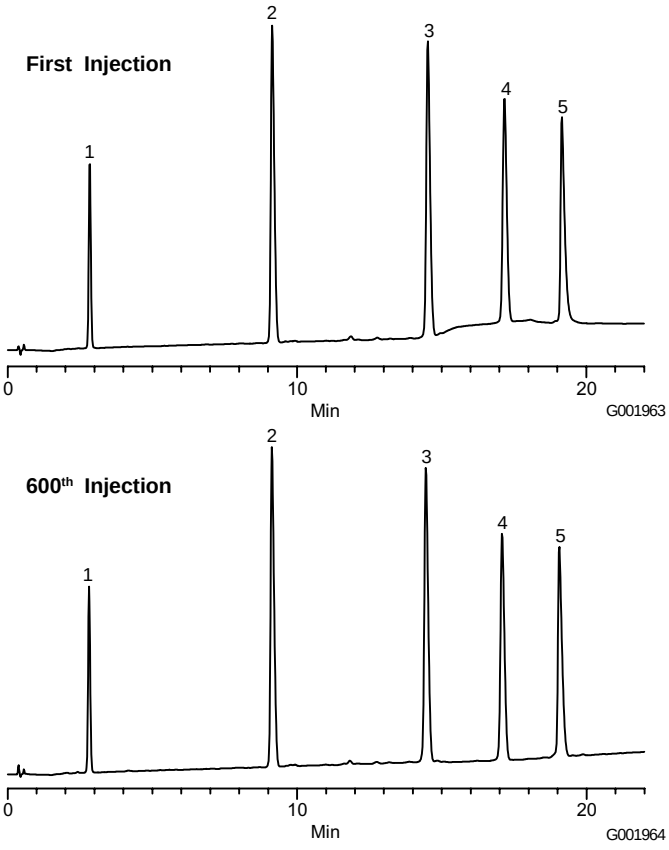
Figure C. Test of Peptide Mix Under 100% Aqueous Starting Gradient Conditions

Column: **Discovery BIO Wide Pore C18, 5cm x 4.6mm ID, 5µm particles**
Cat. No.: **568220-U**
Mobile Phase: A: 0.1%TFA in Water; B: 25:75, 0.1%TFA in Water:CH_3CN
Det.: UV, 220nm
Temp.: 35°C
Inj.: 5µL, Peptide Standard (Sigma H2016) in mobile phase

Gradient:

Min.	%A	%B	Flow, mL/min
0.00	100	0	2
22.00	70	30	2
22.10	100	0	0
24.00	100	0	0
24.10	100	0	2
32.00	100	0	2

1. Gly-Tyr
2. Val-Tyr-Val
3. Met-enkephalin
4. Leu-enkephalin
5. Angiotensin II

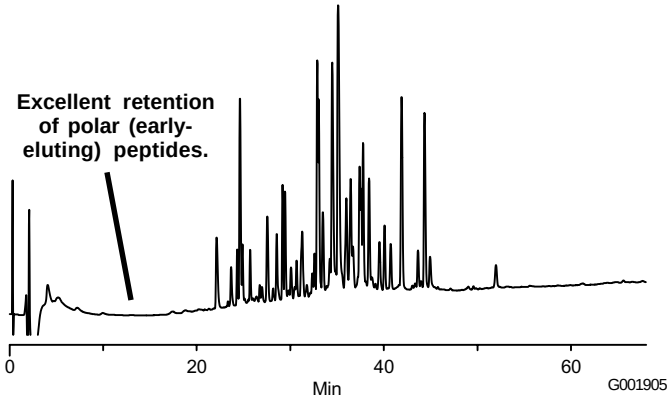


Utility for Peptide Mapping

Peptide mapping is another important application where an aqueous compatible C18 column is necessary. In peptide mapping, proteins are enzymatically digested to produce peptide fragments with a wide range of polarities. The peptide fragments are then subjected to reversed-phase HPLC or LC/MS analysis on a C18 column. Under reversed-phase conditions, polar peptides elute first. However, because of their low affinity for the C18 stationary phase, polar peptides have very low retention and often elute too close to the void volume for reliable identification. By running under extremely high aqueous mobile phase conditions, the polar peptides have a greater likelihood of being retained and resolved. To do this however, one needs a C18 column that is compatible with highly aqueous mobile phases, like the Discovery BIO Wide Pore C18. Figure D shows the peptide map of a tryptic digest of carboxymethylated hemoglobin on a 10cm x 0.5mm ID column packed with 3µm Discovery BIO Wide Pore C18. Here, a small diameter column is used because it consumes less sample and produces higher sensitivity than a regular (4.6mm ID) column for the smaller sample size.[3] The mobile phase is a gradient of acetonitrile in water, both containing 0.1% TFA. The flow was controlled at 12.2µL/min with a capillary LC system. After the column is equilibrated in the 100% aqueous starting conditions and the sample is loaded, elution occurs by a linear gradient up to 60% acetonitrile in 60 minutes. As the chromatogram in Figure D demonstrates, most of the peaks are well resolved and the first major peptide peak is eluted well away from the void volume, indicating adequate retention. Retention is definitely sufficient to prevent the co-elution of peptides at the void volume. Additionally, some small peaks can be seen at the region between void volume and the first major peaks. These minor peptides could provide important information on the parent protein structure in proteomic study. If the conditions did not start at 100% aqueous, these peptides could co-elute at the solvent front or be buried under the major peaks.

Figure D. Peptide Map of Carboxymethylated Hemoglobin Tryptic Digest

Column:	**Discovery BIO Wide Pore C18, 10cm x 0.5mm ID, 3µm particles**
Cat. No.:	**65518-U**
Mobile Phase:	A: 0.1% TFA in Water; B: 40:60, 0.1%TFA in Water:CH_3CN
Flow Rate:	12.2µL/min
Temp.:	ambient
Det.:	UV 215nm
Inj.:	1.25µL
Sample:	carboxymethylated hemoglobin tryptic digest
Gradient:	0 to 100%B in 60minutes



Conclusion

Most C18 columns are not stable under 100% aqueous conditions and undergo a phase collapse phenomenon and loss of retention. However, Discovery BIO Wide Pore C18 columns have exceptional stability and compatibility with 100% aqueous mobile phases. This feature makes Discovery BIO Wide Pore C18 ideally suited for use in peptide mapping, and other applications where the retention of polar peptides is important for reliable characterization of protein or peptide samples.

References

1. R. G. Wolcott and J. W. Dolan, "Lessons in Column Washing," LCGC 17(4) (April 1999).
2. Ryan D. Morrison and John W. Dolan, "Reversed-Phase LC in 100% Water" LCGC Europe, October, 2000.
3. "Increase Sensitivity and Decrease Sample Consumption Using HPLC Microbore and Capillary Column Dimensions" (Supelco Application Note 167, T302167, FCB).

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 particles	568222-U
5cm x 4.6mm ID, 5µm particles	568220-U
10cm x 0.5mm ID, 3µm particles	65518-U

Technical Literature

The following literature can be obtained by calling us at 800-359-0682, or visiting our web site.

Description	Lit. Code
Discovery BIO HPLC Columns Brochure (T402038)	ERI
Getting Started with RP-HPLC for Peptides (Application Note 166 - T302166)	FCA
Increase Sensitivity and Decrease Sample Consumption Using HPLC Microbore and Capillary Column Dimensions (Application Note 167 - T302167)	FCB
Eliminate TFA and Improve Sensitivity of Peptide Analyses by LC/MS (Application Note 168 - T302168)	FCC
Effect of Stationary Phase on Selectivity of Reversed-Phase HPLC Separations of Polypeptides (Application Note 169 - T302169)	FCD

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