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Bulletin 935

Enhanced Stability of Discovery BIO Wide Pore C5 Compared to Other Short Alkyl Chain Phases

Key Words:
● HPLC ● reversed-phase ● peptides ● proteins ● C4 ● C5
● column stability ● TFA ● phase hydrolysis

The most common reversed-phase HPLC (RP-HPLC) phase for protein separations is C4-modified porous silica. However, this popular phase is susceptible to hydrolysis in the acidic, TFA-containing mobile phases these separations employ. Phase hydrolysis causes run-to-run irreproducibility and reduced column operating lifetime with the expense of column replacement and downtime. Supelco's Discovery BIO Wide Pore C5 addresses the limitation of C4 phases directly. It is less susceptible to hydrolysis and provides long-term use under conditions where other silica-based reversed-phase columns show loss of performance. In this report, the benefits of the Discovery BIO Wide Pore C5 compared to a C4 will be demonstrated in terms of its superior chemical stability, and superior resolution with comparable selectivity.

Hydrolytic Conditions of RP-HPLC of Proteins and Peptides

Reversed-phase HPLC (RP-HPLC) of proteins and peptides typically employs mobile phases that contain trifluoroacetic acid (TFA) in concentrations from 0.05 to 0.1%, depending on the method. TFA has characteristics that make it beneficial to HPLC of proteins: its volatility, protein solubility, low UV absorbance, and ability to improve the peak shape of basic proteins. However, a 0.1% v/v aqueous TFA solution has a pH of around 2. These acidic conditions can damage alkyl-modified silica HPLC stationary phases, especially those with short alkyl chains.

RP-HPLC bonded phases that comprise alkyl chains with less than eight carbon units bonded to porous silica particles are commonly used for protein and hydrophobic peptide separations. Compared to longer alkyl chain phases, like the classic C18, the short alkyl chain phases have distinct advantages. Their lower hydrophobicity reduces the likelihood of protein denaturation and allows mobile phases with lower percentages of organic modifiers to be used. Also, they can provide subtle selectivity differences for peptide separations.

However, short alkyl chain phases are more susceptible to stationary phase hydrolysis. The low pH TFA mobile phases used in RP-HPLC of proteins provides ideal conditions for the hydrolysis to occur. The improved stability of longer alkyl chain phases is consistent with computational modeling of hydrogen bonding and hydrophobicity of alkanols as a function of a number of carbons (1,2).

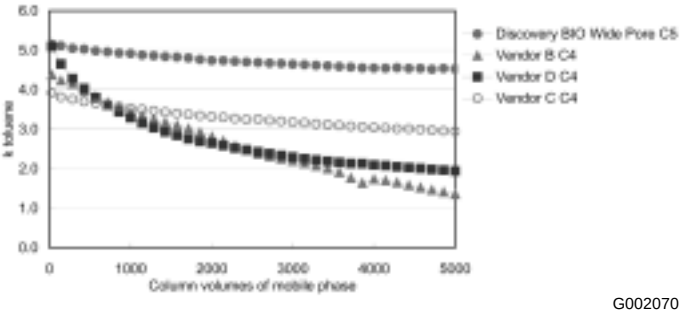
Unique Chemistry of C5 Combines Stability with Benefits of Short Alkyl Chain RP-HPLC

The stability of Discovery BIO Wide Pore C5 and several competitive, market-leading C4 columns was measured under very harsh conditions of low pH and high temperature. These conditions were designed to accelerate acid-catalyzed phase hydrolysis and are much more aggressive than one would typically employ for protein or polypeptide separations. Phase hydrolysis was measured indirectly as a loss in retention of toluene (a purely hydrophobic probe) as a function of mobile phase volume passed through the column.

The results comparing the Discovery BIO Wide Pore C5 and the C4 columns appear in Figure A. Clearly, the Discovery column was significantly more stable than market-leading C4 columns, all of which showed a marked decrease in retention of toluene upon exposure to the low pH mobile phase.

Figure A. Stability of Short Alkyl Chain Reversed-Phases at Low pH and High Temperature

Column:	**Discovery BIO Wide Pore C5, 5cm x 4.6mm ID, 5µm particles** (other columns, same dimensions)
Cat. No.:	**568420-U**
Mobile Phase:	(81:19) (1% TFA in H_2O):(1% TFA in CH_3CN)
Flow Rate:	1mL/min
Temp.:	60°C
Det.:	UV, 254nm
Inj.:	5µL



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Enhanced Stability under Normal Operating Conditions

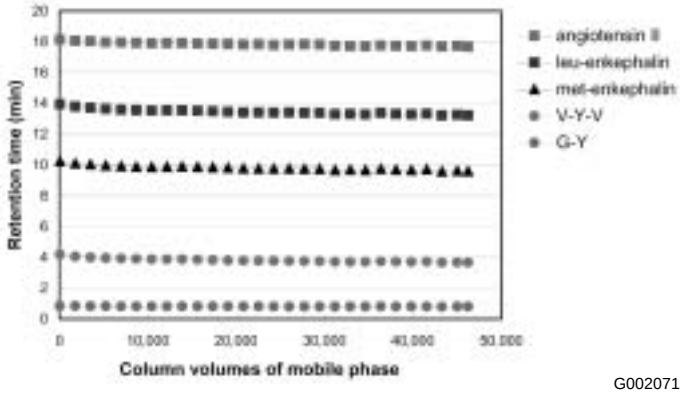
Under mobile phase and temperature conditions typically used in RP-HPLC protein and peptide separations, the Discovery BIO Wide Pore C5 is very stable. Figure B shows retention of a mixture of five peptides as a function of mobile phase volume. The retention shows no sign of change even after 45,000 column volumes of mobile phase.

Figure B. Stability of Discovery BIO Wide Pore C5 Under Typical Conditions

Column: **Discovery BIO Wide Pore C5, 5cm x 4.6mm ID, 5µm particles**
Cat. No.: **568420-U**
Mobile Phase: (A) (95:5) (0.1% TFA in H_2O):(0.1% TFA in CH_3CN)
(B) (25:75) (0.1% TFA in H_2O):(0.1% TFA in CH_3CN)
Flow Rate: 2mL/min
Temp.: 35°C
Det.: UV, 220nm
Inj.: 5µL
Sample: Sigma Peptide Mix, Cat. No. H2016, 0.125mg/mL of Gyl-Tyr and 0.5mg/mL of the other peptides in mobile phase A

Gradient Table:

Time (Min)	%A	%B
0	100	0
22	78	22



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C5 Has Better Stability, but Comparable Selectivity to C3 and C4 Phases

Despite the problems with phase hydrolysis and column stability, many protein and polypeptide methods have been developed on C4 or, less frequently, C3 phases. When choosing a phase that gives enhanced stability, it is desirable to have similar selectivity (retention time, elution order, peak spacing, etc.) to a C4 or C3 so that the existing methods can be easily transferred with minimum changes.

The Discovery BIO Wide Pore C5, although it has one additional carbon unit, will give the same selectivity as most C4 or C3 phases under the same conditions. To demonstrate this, a separation of six proteins that vary in molecular weight from 12.4 to 67kDA was run on Discovery BIO Wide Pore C5 and three market-leading C4 or C3 phases. The results are shown in Figure C. Note the similar selectivity of the C5 to the popular C4 or C3 phases.

Figure C. Protein Separation on Short-Chain Reversed-Phases

Column: **Discovery BIO Wide Pore C5, 15cm x 4.6mm ID, 5µm particles** (other columns, same dimensions)
Cat. No.: **568422-U**
Mobile Phase: (A) (75:25) (0.1%TFA in H_2O):(0.1%TFA in CH_3CN);
(B) (25:75) (0.1%TFA in H_2O):(0.1%TFA in CH_3CN)
Flow Rate: 1mL/min
Temp.: 30°C
Det.: UV, 220nm
Inj.: 10µL
Sample: each protein, 1mg/mL in mobile phase A, except BSA (2.5mg/mL) and ovalbumin (3.5mg/mL)

Gradient Table:

Time (Min)	%A	%B
0	100	0
25	0	100

1. RNAse (13.7kDA)
2. Cytochrome c (12.4kDA)
3. Lysozyme (14.3kDA)
4. BSA (67kDA)
5. Myoglobin (18.8kDA)
6. Ovalbumin (45.3kDA)

Figure C1. Discovery BIO Wide Pore C5

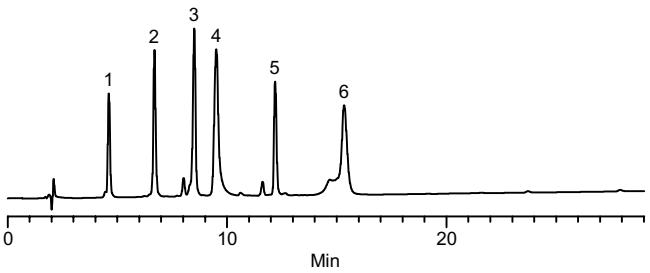

Figure C2. Vendor A C3

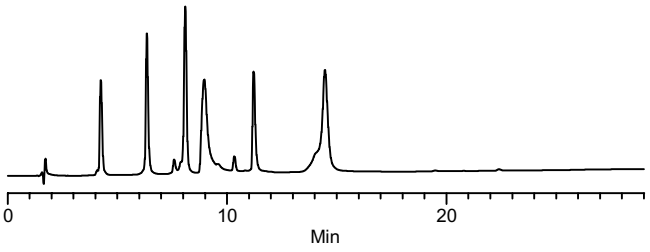

Figure C3. Vendor B C4

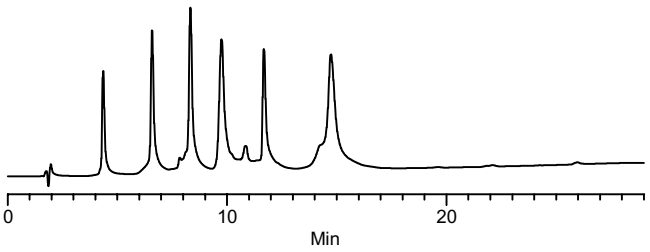

Figure C4. Vendor C C4

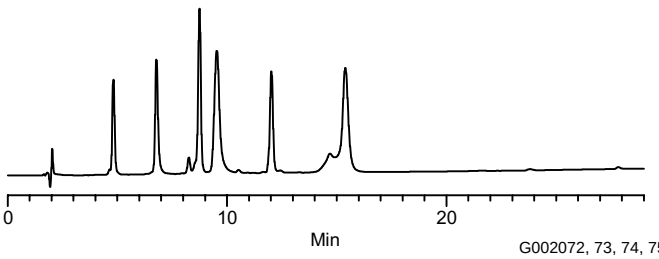

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Figure D. Separation of Cytochrome C Variants on Short-Chain Reversed-Phases

Column:	**Discovery BIO Wide Pore C5, 15cm x 4.6mm ID, 5µm particles** (other columns, same dimensions)
Cat. No.:	**568422-U**
Mobile Phase:	(A) (70:30) (0.1%TFA in H_2O):(0.1%TFA in CH_3CN); (B) (64:36) (0.1%TFA in H_2O):(0.1%TFA in CH_3CN)
Flow Rate:	1mL/min
Temp.:	30°C
Det.:	UV, 220nm
Inj.:	12µL, 4mg/mL
Sample:	10µg each variant

Gradient Table:

Time (Min)	**%A**	**%B**
0	100	0
30	0	100

1. Horse cytochrome c
2. Rabbit cytochrome c
3. Cow cytochrome c
4. Pigeon cytochrome c
5. Chicken cytochrome c
6. Dog cytochrome c

Figure D1. Discovery BIO Wide Pore C5

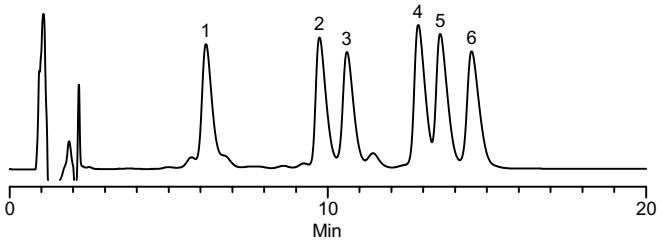


Figure D2. Vendor A C3

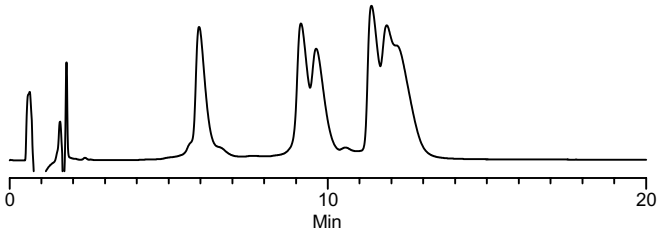


Figure D3. Vendor B C4

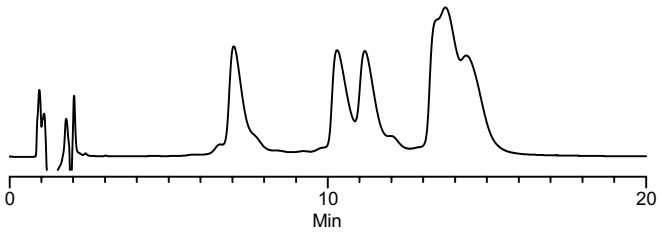
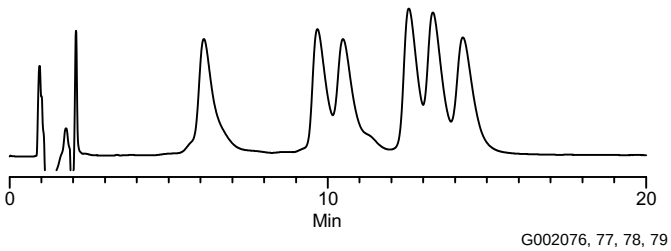


Figure D4. Vendor C C4



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C5 Typically Has Higher Efficiency and Better Resolution than C4 Phases

Stability and selectivity are important, but so is efficiency. Efficient columns resolve more compounds per unit time and allow detection of lower concentrations. The Discovery BIO Wide Pore C5 gives higher efficiency separations than C4 or C3 phases under normal conditions. To demonstrate, a high-resolution separation of cytochrome c from various sources is shown on Discovery BIO Wide Pore C5 and three market-leading C4 or C3 columns in Figure D. First, this figure demonstrates the power of RP-HPLC as a separation mode. All six cytochrome c variants were resolved even though they have 88% peptide sequence identity. Second, the figure shows that the Discovery BIO Wide Pore C5 gave higher efficiency but similar selectivity of the cytochrome c variants compared to the C4 or C3 columns.

Conclusion

Discovery BIO Wide Pore C5 is less susceptible to phase hydrolysis and exhibits longer lifetime than C4 or C3 phases. It does exhibit similar selectivity to C4 and C3, which permits easy transfer of methods from C4 or C3 to C5. Discovery BIO Wide Pore C5 was shown to be a leading performer in the market of short alkyl chain phases.

References

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2. Hanai, T. et al. "Molecular Recognition in Chromatography Aided by Computational Chemistry." Supramolecular Chemistry, 3:243-247, (1994).

Ordering Information

Other dimensions, phases, and separation modes of Discovery BIO are available. Please call or visit our web site.

Description	Cat. No.
Discovery BIO Wide Pore C5, 5µm particles	
5cm x 4.6mm ID	**568420-U**
15cm x 4.6mm ID	**568422-U**
2cm x 4mm ID Supelguard kit*	**568473-U**
2cm x 4mm ID Supelguard cartridges, pk. of 2	**568472-U**

*Kit includes a holder and one replaceable cartridge.

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