

Solute Functionality Provides Clues to Effective Choice of Polar Reversed Phase HPLC Columns

Abstract

Understanding specific polar functional group interactions between solutes and reversed phases is important for effective column selection. Retention and selectivity are compared for three different reversed phases: C18, F5, and PEG. Interesting differences among the phases are illustrated. This knowledge can improve column selection and enable quicker, better HPLC method development.

Reversed Phase Retention and Selectivity

Retention and selectivity in reversed phase HPLC result from complex combinations of hydrophobic and polar interactions among the solute, mobile phase, and stationary phase. Stationary phases have evolved from simple alkyl hydrocarbon chains to alkyl hydrocarbon chains in combination with other moieties, typically polar functional groups. This changes retention and selectivity, particularly for solutes that have specific interactions with the polar portion of the stationary phase. So-called polar reversed phases always provide different retention and selectivity compared to C18 and frequently provide valuable improvements in separation.

Retention and Selectivity with Polar Reversed Phases

A polyethyleneglycol (PEG) phase has ether groups that can attract other polar analytes. PEG provides a very different separation of phenols compared to C18 (Figure A). There are three interesting differences: 1. Selectivity differences as illustrated by elution order changes, 2. Greater retention of some phenols (peaks #2 and #3) that are poorly retained on C18, 3. Faster analysis including the elution of peak #9 that did not elute on C18. The very polar compound, 1,3,5-

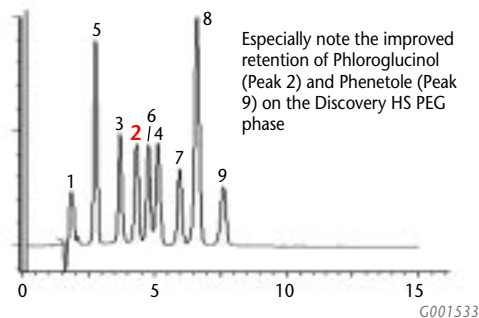
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Figure A. Resolution of Phenolic Compounds on Discovery® HS PEG Compared to Standard C18

Column: 15cm x 4.6mm, 5µm; Mobile Phase: 85% 10mM ammonium acetate, pH 6.8 : 15% MeCN; Flow Rate: 1.0mL/min; Temp: 20°C; Detection: UV/Photodiode Array; Injection: 10µL (50µg/mL for each analyte)

Discovery HS PEG

Note the selectivity differences of the phenolic compounds between the conventional C18 and the Discovery HS PEG phase.



Conventional C18 Column

Phenetole (9) is not eluted under these conditions on C18

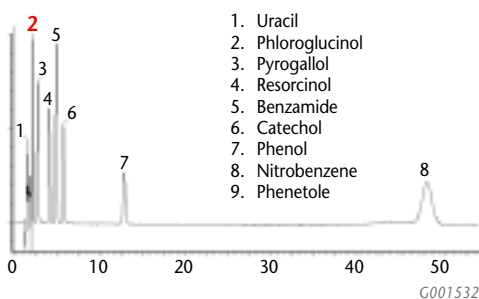
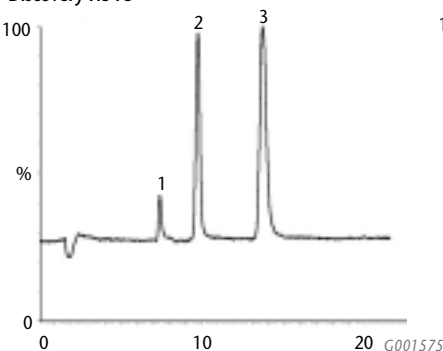


Figure B. Superior Retention of Some Basic Compounds on Discovery HS F5 Compared to a Standard C18 Phase

Column: 15cm x 2.1mm, 3µm; Mobile Phase: 30% 10mM ammonium acetate, pH 6.8: 70% MeCN; Flow Rate: 200µL/min; Temp: 35°C; Inj.: 2µL; Sample: 25µg/mL each in mobile phase, ephedrine and methcathinone; Det.: LC/MS, esi (+) mode

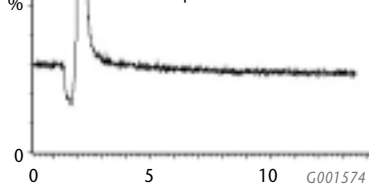
Discovery HS F5



Discovery HS C18

Peaks elute at void as determined by LC/MS

1. Impurity from Ephedrine
2. Methcathinone
3. Ephedrine



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NEW PRODUCTS

NEW Discovery BIO and HS Columns

Supelco introduces six new phase chemistries into the Discovery family of HPLC columns. In order to better service the diverse needs of today's researcher, each phase chemistry is available in three different particle sizes. We are now offering Discovery BIO for biomolecule analysis and purification. These wide pore (300Å) columns are available in three different phase chemistries: C18, C8, and C5. In addition to BIO, the HS (120Å) line of columns has grown to include two new unique phases in addition to time tested C18. The entire HS line is now available in the following bonded phase chemistries: C18, PEG, and F5.

Discovery BIO WidePore C18 (3µm, 5µm & 10µm)

- Excellent resolution for synthetic peptide analysis and peptide mapping
- Highly stable in alkaline pH mobile phase and constant column selectivity over time
- Scalable from analytical to prep
- Excellent lot to lot column reproducibility for robust assays of synthetic peptides and peptide mapping
- Ideal for LC/MS applications - No detectable bleed

☞ For more information, request T401097, or visit sigma-aldrich.com/discovery-bioc18

Discovery BIO WidePore C8 (3µm, 5µm & 10µm)

- Intermediate hydrophobicity (less than C18; greater than C4/C5 phases) offers an excellent starting point for method development
- Ideal for the analysis and purification of peptides, polypeptides and smaller proteins
- Scalable from analytical to prep
- Excellent column stability at alkaline pH and constant column selectivity over time
- Well suited for LC/MS applications

☞ For more information, request T401098, or visit sigma-aldrich.com/discovery-bioc8

Discovery BIO WidePore C5 (3µm, 5µm & 10µm)

- Excellent performance for large polypeptide and protein analysis and purification
- Faster separations for hydrophobic peptides compared to C18
- Increased column stability in acidic and alkaline mobile phase compared to conventional C4
- Scalable from analytical to prep
- Excellent lot to lot column reproducibility for robust assays of biotechnology products

☞ For more information, request T401099, or visit sigma-aldrich.com/discovery-bioc5

Discovery HS C18 (3µm, 5µm & 10µm)

- Specifically developed for pharmaceutical analysis and purification
- Ideal for LC/MS applications - No detectable bleed as independently tested
- Highly stable to ensure excellent run-to-run and lot-to-lot reproducibility and long column life
- Scalable from analytical to prep

☞ For more information, request T401095, or visit sigma-aldrich.com/discovery-hsc18

Discovery HS PEG (3µm, 5µm & 10µm)

- A unique polyethylene glycol reversed phase specifically developed for pharmaceutical analysis and purification
- Unique selectivity and faster separations of polar compounds compared to C18 phases
- Outstanding performance for phenolic compounds offers unique retention and selectivity and faster analysis compared to C18
- Scalable from analytical to prep
- Highly stable to ensure excellent run-to-run and lot-to-lot reproducibility and long column life

☞ For more information, request T401100, or visit sigma-aldrich.com/discovery-hspeg

Discovery HS F5 (3µm, 5µm & 10µm)

- A unique pentafluorophenylpropyl terminated reversed phase column specifically developed for pharmaceutical analysis and purification
- Unique retention and selectivity compared to C18 phases
- Scalable from analytical to prep

☞ For more information, request T401096, or visit sigma-aldrich.com/discovery-hsf5

For more information on all the NEW Discovery BIO and HS Columns, visit sigma-aldrich.com/supelco-discovery



NEW APPLICATIONS

PEG Column Yields Faster Analysis for Flavones

Flavones are a group of multi-ring, hydroxyl-containing compounds that are being studied widely for their nutritional value and their use in preventive health care measures. These compounds are found in products as diverse as Ginkgo Biloba, orange juice, and in garden herbs such as dill, oregano and parsley. A standard C18 column may be used to separate many of these compounds, however, co-elution of some of the compounds can occur. In this applications report, both unique selectivity as well as faster separations are demonstrated on the Discovery HS PEG phase as compared to standard C18.

Figure E shows the structures of some common flavones. Note the large number of OH groups on the rings. The mixture was first run on a standard C18 column. Figure F shows a co-elution of the flavone and chrysin, and the separation

takes 50 minutes to complete! Running the same mixture on a PEG column (Figure 3) shows some reversals in elution order compared to C18, no co-elutions, and a run time of only 16 minutes! The separation on the PEG column is clearly superior and this separation is amenable to use with LC/MS in that the mobile phase contains 0.1% formic acid.

The Discovery HS PEG column provides a faster separation as well as unique selectivity for the flavones when compared to standard C18. In addition, good resolution of each component in the mixture is observed. If you are trying to separate compounds with many OH groups and are experiencing problems with standard C18 phases, consider the Discovery HS PEG column for better retention of these types of compounds.

For more information, request T401100, or visit sigma-aldrich.com/thereporter-applications

Figure E. Structures of Some Common Flavones

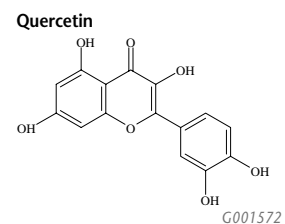
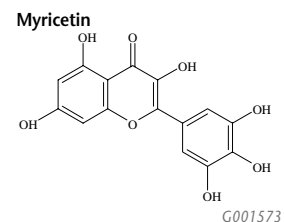
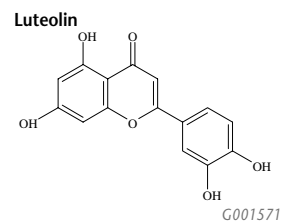
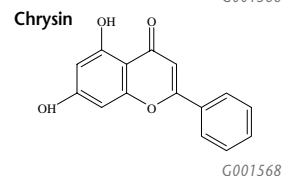
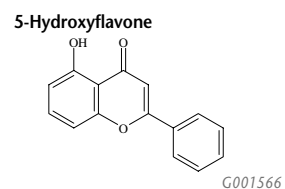
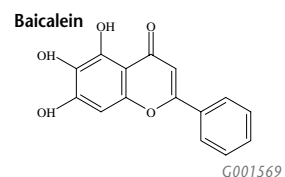
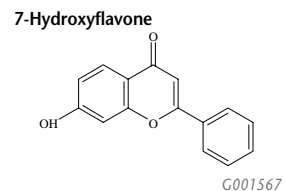
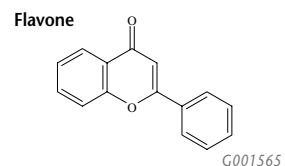
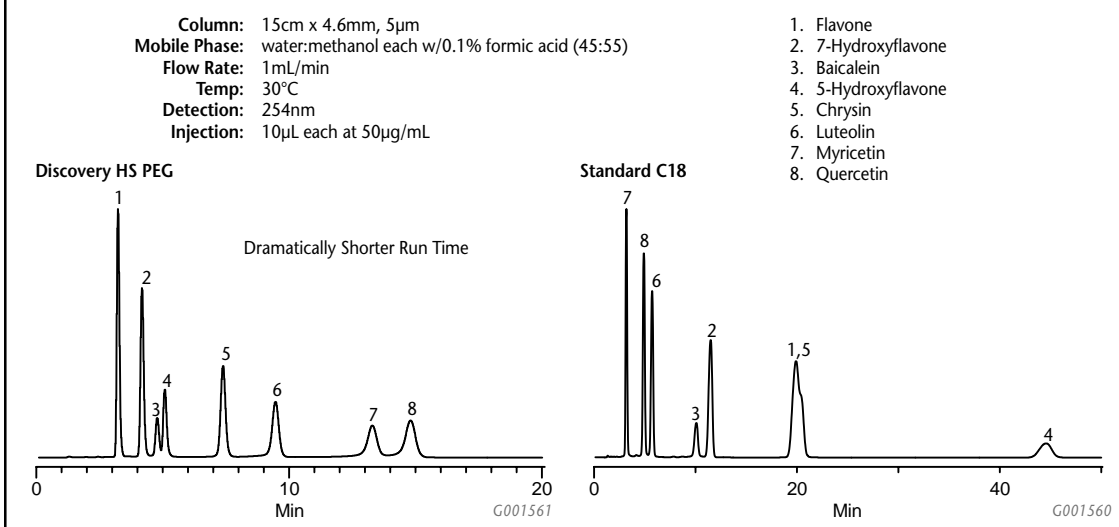


Figure F. Comparison of Flavones Analysis on Discovery HS PEG vs. Standard C18



LIQUID CHROMATOGRAPHY PERFORMANCE TIP

Solvent Compatibility: Sample, Mobile Phase and Wash Solvent

A chemist at Supelco observed very broad peaks in a chromatogram. After checking the system and testing the column on another system, attention was turned to the sample, sample solvent, and mobile phase. The sample dissolved in the mobile phase completely. In re-checking the system, it was found that the wash solvent for the autoinjector had a very high percentage of organic solvent (acetonitrile). The samples were not soluble in this solvent. A change was made to a high aqueous wash solvent (90% water/10% methanol), then the autosampler was purged several times to remove any of the previously used high organic solvent. The sample was then injected onto the column and the chromatography looked normal.

In general, the sample must be soluble in the mobile phase or another compatible solvent. Also, thought must be

given to the mobile phase solubility in terms of the aqueous (possible salt containing) and organic portions of the mobile phase. Another area of compatibility that needs our attention is wash solvent compatibility with sample and mobile phase. As discussed above, this can lead to poor chromatography.

Knowing how soluble samples are in mobile phase is standard practice in liquid chromatography. Sometimes overlooked is the compatibility of the wash solvent in the autoinjector. Be certain that this wash solvent is compatible with your samples and mobile phases as well.

For more information, request T100826.

For an expanded version of this Performance Tip, visit sigma-aldrich.com/thereporter-performance-tip

Solute Functionality Provides...

(continued from page 1)

trihydroxybenzene (phloroglucinol) shows the most retention on PEG relative to C18 clearly illustrating the relatively greater importance of polar retention mechanisms on the PEG phase.

The pentafluorophenyl phase (F5) also exhibits unique retention and selectivity compared to C18. It exhibits greater retention for amine-containing compounds like ephedrine and methcathinone (Figure B on page 1). These compounds are not retained on C18 using the same mobile phase. Perhaps this is explained by hydrogen bonding between the F5 and the amines since fluorine is the only halogen atom that can hydrogen bond.

Conclusion

Polar reversed phases can provide dramatically different separations compared to C18. The PEG phase produced excellent separations of phenolic solutes and should be considered for applications such as drug metabolite analysis. The F5 phase exhibits unique, interesting separations of amine-containing solutes and should be considered for applications such as basic pharmaceuticals. Both phases offer valuable alternatives to C18 when confronted with poor resolution on C18. Retention and selectivity of polar reversed phases is a continuing research and development focus at Supelco. Our aim is to develop unique, valuable phases and applications for HPLC.

For more information, request T401096 and T401100, or visit sigma-aldrich.com/thereporter-mainarticle

CASE STUDY

1

Stability at pH1.5 (0.5% TFA) and 70°C

Use of 0.1% trifluoroacetic acid (TFA) at pH2 is common practice in reversed phase separation of proteins and peptides. Sometimes higher concentrations of TFA or a lower pH is required to achieve the desired selectivity and to separate impurities from the main peak of interest. However, due to the instability of silica-based packing materials, most manufacturers recommend that the pH should not be lower than 2 and the temperature not higher than 50°C. Here we demonstrate a column that is stable at lower pH1.5 (0.5% TFA) and high column temperature (70°C).

The stability test was conducted on a Discovery BIO Wide Pore C18 column. Discovery BIO Wide Pore C18 is an octadecyl (ODS) bonded phase on 300Å pore size silica developed for the separation of peptides and small proteins. The test was conducted with 0.5% TFA at 70°C and the conditions are listed in Figure C. Figure C (a) is the chromatogram from the initial injection. Figure C (b) is the chromatogram from an injection after 40,000 column bed volumes passed through the column. The chromatograms show the resolution and peak shape did not change over the prolonged test period. Figure D presents the chart of retention times for the five peptides over the column bed volumes. The data clearly shows the column is very stable over 40,000 column bed volumes of mobile phase at low pH and high column temperature.

In conclusion, the Discovery BIO Wide Pore C18 phase can be used for low pH and high column temperatures. The next time you need to use low pH (to 1.5) and high column temperature (to 70°C) you may want to consider using the Discovery BIO Wide Pore C18 phase.

For more information regarding Discovery BIO Wide Pore products, request T401097, T401098 and T401099, or visit sigma-aldrich.com/thereporter-casestudy

Figure C. Comparison Between Initial Injection (a) and Injection after 40,000 Column Bed Volume (b) Passed Through the Column

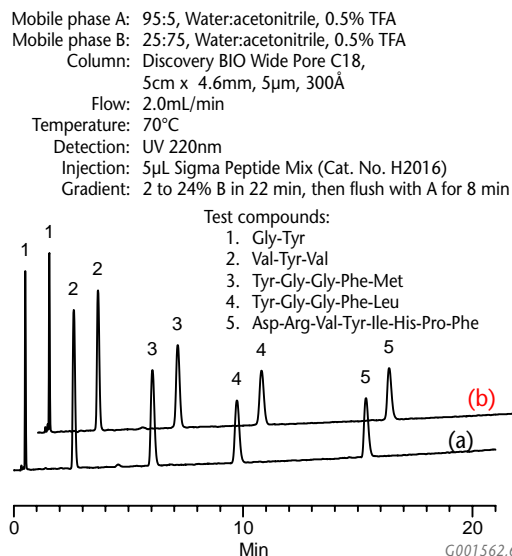
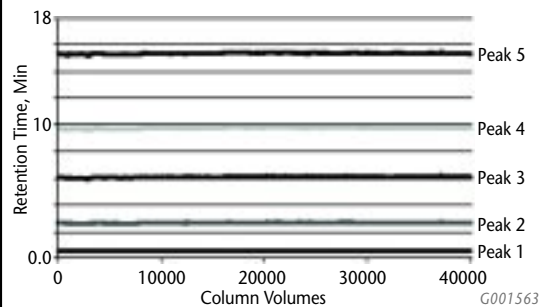


Figure D. Retention Time Over the Entire Test in 0.5% TFA at 70°C



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