Reversed-phase HPLC separation of peptides is often accomplished by subtle interactions of peptides with the reversed-phase surface. Various aspects of the reversed-phase surface can affect peptide separations in small, but important ways. In particular, some peptide separations are very sensitive to the density and uniformity of the hydrophobic phase bonded to the silica matrix.

**Sensitive Test of Chromatographic Carbon Load**

A very sensitive test for monitoring the carbon load on some types of C\textsubscript{18} reversed phase columns has evolved from a test developed at the U.S. NIST (National Institute for Standards and Technology) for evaluation of C\textsubscript{18} phase types\textsuperscript{48}. Based on studies of the influence of shape selectivity on the reversed-phase HPLC separation of polynuclear aromatic hydrocarbons, this test consists of the isocratic separation of three polyaromatic hydrocarbons - phenanthro(3,4c)phenanthrene, tetrabenzenophthalene and benzo(a)pyrene, and the calculation of a resolution factor - $\alpha$ - between tetrabenzenophthalene and benzo(a)pyrene (Figure 41). The original NIST studies relate the $\alpha$ factor to the phase type: $\alpha$ values greater than 1.7 result from monomeric bonding of the reversed-phase and $\alpha$ values less than 1.0 result from polymeric bonding.

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**Figure 41**

NIST-Derived C\textsubscript{18} RP-HPLC Chromatographic Carbon Load Test

The resolution between polynuclear aromatic hydrocarbons constitute a sensitive test of chromatographic carbon load.

**Conditions:**
- **Column:** Vydac 218TP54 (C\textsubscript{18}, 5 \textmu m, 4.6 x 150 mm)
- **Eluent:** 85:15 acetonitrile:water
- **Sample:** phenanthro(3,4c)phenanthrene, tetrabenzenophthalene and benzo(a)pyrene

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Micro-combustion carbon analysis of C\textsubscript{18} reversed-phase is an imprecise measure of carbon load, whereas the NIST alpha value measured on polymerically bonded C\textsubscript{18} reversed-phase columns is very sensitive to minute variations in the carbon load. Use of the NIST-derived test has significantly improved the carbon load reproducibility of polymerically bonded C\textsubscript{18} columns (Figure 42).

The precise control of C\textsubscript{18} carbon load resulting from the NIST-derived carbon load test led to an observation by scientists at Genetics Institute that a pair of peptide fragments in an Asp-N protein digest were barely separated on standard C\textsubscript{18} columns but were well resolved on a C\textsubscript{18} column with a slightly lower carbon load (Figure 43). Nine of ten peptide fragments eluted at the same time on both columns, however one peptide fragment, with a pK of 7.3, eluted earlier on the lower carbon load column, increasing the resolution between it and an adjacent fragment with a pK of 4.3. This illustrates the subtle affect that C\textsubscript{18} reversed-phase carbon load can have on peptide separations.

**Figure 43**

The subtle effect of carbon load on peptide separations

**Low carbon load C\textsubscript{18} RP-HPLC column (B) separated two peptides that were only partially resolved on a standard carbon load column (A).**

**Conditions:**
- **Columns:**
  - A. Vydac 218TP52 - standard C\textsubscript{18}, 5 \textmu m, 2.1 x 250 mm
  - B. Vydac 218LTP52 - low carbon load - C\textsubscript{18}, 5 \textmu m, 2.1 x 250 mm
- **Eluent:** 6 mM TFA/4 mM HFBA, 11 - 95% ACN in 75 min at 0.25 mL/min
- **Sample:** Asp - N protein digest

Data courtesy of Herminia Catipon and Thomas Salati, Genetics Institute, 1 Burtt Rd., Andover MA
Reversed-phase HPLC peptide separations are sensitive to the shape of the gradient and hence, to the characteristics of the system hardware being used. Pumps and gradient formers can affect peptide separations in subtle ways, especially at low flow rates.

**Evaluation of gradient systems and response delay time**

To experimentally examine the actual gradient produced by an HPLC system, replace the column with a short length of small diameter tubing and run a 30 minute gradient at 1.0 mL/min from water to 0.3% acetone (for absorbance) in water and monitor at 254 nm. The UV profile represents the gradient actually generated by the system hardware (Figure 44). The gradient UV profile can be used to:

- Check on system reproducibility;
- Determine system performance at the extremes of the gradient;
- Calculate the gradient response delay - the time from when the controller or computer signals a change in the gradient to when this change actually reaches the column. In the example (Figure 44) the gradient delay is about 3 minutes (3 mL at 1 mL/min) calculated from when the run begins to where the profile begins to rise. Hardware systems that differ in gradient response delay times will produce different gradient shapes, which may result in apparent differences in peptide selectivity.

Figure 45 shows the effect that the gradient response delay has on narrowbore columns run at low flow rates. The peptide separation on a narrowbore HPLC column at 0.20 mL/min (Fig. 45B) is compared with the separation on an analytical column at 1.0 mL/min (Fig. 45A) using the same HPLC system and programmed gradient. The 10 minute gradient response delay distorts the peptide separation (Fig. 45B). Delaying sample injection and data collection ten minutes after starting the gradient cancels the effect of the gradient response delay and the resulting narrowbore separation (Fig. 45C) is similar to the analytical separation (Fig. 45A).

**Calculation of Desorbing Solvent Concentration**

Because of internal volume in the flow system - tubing, mixing chamber, column void volume, etc. - the solvent concentration given by the system when the polypeptide elutes is higher than the actual solvent concentration that desorbs and elutes the polypeptide. To calculate the solvent concentration that desorbs the polypeptide ($C_D$):

1. Enter the retention time of the peak
2. Subtract the retention time of the injection peak
3. Subtract the gradient response delay time
4. And subtract any initial gradient hold time
5. Equals corrected elution time ($T_{corr}$)

The solvent concentration ($C_D$) at the corrected elution time is:

$$C_D = C_S + \left(ET_{corr}/T_g\right)(C_{E} - C_S);$$

where:
- $C_S$ = solvent concentration at start of gradient
- $C_E$ = solvent concentration at end of gradient
- $T_g$ = time duration of gradient

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**Figure 44**

Gradient hardware system evaluation

The gradient generated by the system hardware is visualized by the profile of a gradient increasing in acetone.

**Conditions:**
- **Column:** Replaced by low-volume tubing
- **Gradient:** 0 - 0.3% acetone in water over 30 min at 1.0 mL/min
- **Detection:** UV at 254 nm

**Figure 45**

Effect of system hardware on gradient shape in narrowbore HPLC

The system hardware gradient delay distorts the gradient shape at low flow rates and affects the peptide separation (B). Delaying sample injection to adjust for the gradient delay produces similar separation results (C) as obtained with an analytical column (A).

**Conditions:**
- **Column:** A. Vydac 218TP54 ($C_{18}$, 5 µm, 4.6 x 250 mm)
  - B and C. Vydac 218TP52 ($C_{18}$, 5 µm, 2.1 x 250 mm)
- **Eluent:** 15 - 30% ACN in 30 min with 0.1% TFA
- **Flow rate:** A. 1.0 mL/min     B and C. 0.20 mL/min
- **Peptides:** 1. bradykinin 2. oxytocin 3. angiotensin II
  - 4. neurotensin 5. angiotensin I

**Note:** In C, sample injection and data collection were delayed 10 min after initiating the gradient.
Appendix C: Guide to maintaining and restoring RP-HPLC column performance

Reversed-phase HPLC columns, if properly cared for, give good performance for hundreds to over a thousand injections. Although the following ideas are specifically applicable to Vydac RP-HPLC columns, they also apply to many other RP-HPLC columns.

Column Protection
Column lifetime can be extended by filtering all solvents and samples and using an eluent filter and a guard column. *We recommend using an eluent filter* between the solvent delivery system and the injector to trap debris from the solvents, pumps or mixing chamber. *We also recommend using a guard column* between the injector and the column if samples contain insoluble components or compounds that strongly adsorb to the material.

Column Conditioning
Because of the nature of the reversed-phase surface, column performance (resolution, retention) may change slightly during the first few injections of proteins larger than 5-10,000 MW. A column can be conditioned by repeated injections of the protein until the column characteristics remain constant (requires injection of about 100 µg of protein) or by injection of 100 µg of a commonly available protein, such as ribonuclease, followed by running an acetonitrile/0.1% TFA gradient.

Column storage
RP-HPLC columns can be stored in organic solvent and water. For long term storage the ion-pairing agent or buffer should be rinsed from the column and the organic content should be at least 50%.

Chemical Stability
Reversed-phase HPLC columns are stable in all common organic solvents including acetonitrile, ethanol, isopropanol and dichloromethane. When switching solvents it is important to only use mutually miscible solvents in sequence. Silica-based RP-HPLC columns are stable up to pH 6.5 to 7 and are not harmed by common protein detergents such as sodium dodecylsulfate (SDS).

Pressure and Temperature Limits
RP-HPLC columns are generally stable to 60 degrees C. and up to 5000 psi (335 bar) back-pressure. Typical back-pressures for RP-HPLC columns are shown in Table 6.

RP-HPLC column trouble-shooting
The performance of RP-HPLC columns may deteriorate for a number of reasons including use of improper eluents, such as high pH, contamination by strongly adsorbed sample constituents, insoluble materials from the solvent or sample or simply age or extensive use. Here are some suggestions to restore the performance of a RP-HPLC column.

- **High back-pressure.** Disconnect the column from the injector and run the pumps to ensure that the back-pressure is due to the column and not the HPLC system. If the column back-pressure is high, most HPLC columns can be reversed and rinsed to try to flush contaminants from the inlet frit. Begin the reverse rinse at a low flow rate - 10 to 20% of normal - for 10-15 minutes and then increase to the normal flow rate.

- **Contaminated column.** Wash the column either with 10-20 column volumes of a strong eluent or run 2-3 'blank' gradients (without sample injection) to remove less strongly adsorbed contaminants.

<table>
<thead>
<tr>
<th>Column Size (mm)</th>
<th>Flow rate (mL / min)</th>
<th>Typical Back-pressure (with 50:50 ACN:Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 x 250</td>
<td>0.20</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 250</td>
<td>1.0</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 150</td>
<td>1.0</td>
<td>600 - 1200 psi</td>
</tr>
<tr>
<td>10 x 250</td>
<td>5.0</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 250</td>
<td>1.0</td>
<td>500 - 1000 psi</td>
</tr>
<tr>
<td>10 x 250</td>
<td>5.0</td>
<td>500 - 1000 psi</td>
</tr>
<tr>
<td>22 x 250</td>
<td>25</td>
<td>500 - 1000 psi</td>
</tr>
</tbody>
</table>
**Protein contamination.** If the loss in column performance appears to be due to adsorbed protein we recommend rinsing the column with a mixture of one part 0.1 N nitric acid and four parts isopropanol. Rinsing at a low flow rate - 20% of normal - overnight is most effective.

**Lipids or other very hydrophobic contaminants.** If lipids or very hydrophobic small molecules are causing the change in column performance, we recommend rinsing the column with several column volumes of dichloromethane or chloroform. When changing from water to chloroform or dichloromethane or back again it is important to rinse the column with a mutually miscible, intermediate solvent such as isopropanol or acetone between the two less miscible solvents.

**Spurious - "ghost" - peaks.** Unexpected peaks sometimes appear in HPLC chromatograms. These are usually caused by contaminants in the solvents used. Hydrophobic contaminants in Solvent A - contaminants may be present in the water or the ion-pairing agent or buffer - accumulate on the column during equilibration and at low solvent concentrations and elute as "ghost" peaks during the gradient. This can be easily diagnosed by making two gradient runs, the first with a relatively long equilibration time - 30 minutes - and the second with a short equilibration time - 10 minutes (example, Figure 46). The short equilibration will have smaller peaks than the long equilibration if the "ghost peaks" are due to contaminants in the 'A' solvent because less contaminants will adsorb onto the column with the short equilibration. To correct the problem use higher purity or fresh water or ion-pairing agent or buffer.

**Figure 46**
Evidence of solvent contaminants as source of ghost peaks

| A. 10 min. equilibration - small "ghost peaks" |
| B. 30 min. equilibration - larger "ghost peaks" |

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**Basic Principles and Conditions**

2. **Occurrence of Methionine Sulfoxide During Production of Recombinant Human Insulin-like Growth Factor (IGF-I),** M. Hartmanis and A. Engstrom, *Second Symposium of the Protein Society, 1988, Abstract Number 502*