Watch

The Cleaning and Regeneration of Reversed-Phase HPLC Columns

This month's "Column Watch" looks at practical ways to return a contaminated column to — or close to — its original state. Ron Majors also discusses cleaning procedures for bonded-silica and other types of reversed-phase columns.

Reversed-phase chromatography is by far the most widely used technique in high performance liquid chromatography (HPLC) (1). It is popular because it is applicable to most nonpolar analytes and to many ionizable and ionic compounds. Most of the stationary phases used in reversed-phase chromatography are hydrophobic in nature; therefore, analytes are separated by their degree of hydrophobic interaction with the stationary phase and matrices with hydrophobic components also can be retained in a similar manner.

Table I lists the most popular stationary phases usually bonded to silica gel (1). Phase subspecies — such as mixed phases (for example, phenyl–hexyl), endcapped and nonendcapped varieties, and polar-embedded phases — also exist within these bonded silicas. Various other packing materials have been used in reversed-phase chromatography, including polymers, polymer-coated silicas and aluminas, inorganic–organic hybrids, coated zirconia, and graphitized carbon. Each type of phase has its own advantages and disadvantages.

Reversed-phase chromatography columns are used in various applications with a wide variety of mobile phases and additives. Some of these techniques use additives that can change or modify the surfaces of the packing material. Sometimes, these additives themselves may contaminate the surface or bonded phase.

As with hydrophobic bonded phases, the surface of silica-gel packings has other chemical features. Residual silanols are present on the surface of all silica-gel bonded-phase packings. Figure 1 depicts the different types of silanols that can be present (2). Being weakly acidic in character, these silanols can interact with certain analytes and matrix components, particularly with basic compounds. Because the pK_a of the silanol is roughly 4.5, ionization can occur at intermediate pH values, and thus the possibility of electrostatic interactions with cationic species exists. The older Type A silicas can contain high concentrations of metal ions (sometimes 100 ppm or more) that impart even greater acidity to the silica

Table I: Relative use of stationary phases in HPLC*

Phase	Relative Usage (%)
C18 (octadecylsilane)	39
C8 (octyl)	26
Cyanopropyl†	14.5
Phenyl	12
C4 (butyl)	3.7
Hydrophobic interaction	1.8
C2 (ethyl)	1.1
C1 (methyl)	0.8
Other	0.8
Polymers	0.5

*Adapted from reference 1 and normalized.
†Includes normal-phase usage, because reversed-phase versus normal-phase chromatography use was not queried.

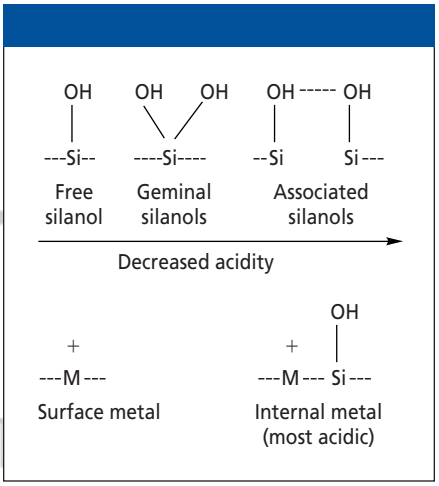

Figure 1: Types of silanols on the surface of silica gel (2).

Ronald E. Majors
Column Watch Editor

surface and also can interact with metal chelating or scavenging compounds (3). Residual silanols are more bothersome on nonendcapped bonded silicas and on short-chain-bonded phases such as C2 or C4 phases.

Users must be aware of the surface characteristics of their particular stationary phases and of possible analyte–surface interactions, so they can take into account possible matrix interactions when they are developing and using reversed-phase methods. For example, very hydrophobic sample matrices such as corn oil, highly aromatic materials, and waxes can stick to reversed-phase packing surfaces and change their characteristics. Biological fluids containing proteinaceous materials can adsorb on packing surfaces. Despite analysts' best attempts to protect HPLC columns from foreign substances, eventually certain analyte–matrix combinations can affect stationary phases detrimentally.

After a column is contaminated, its chromatographic performance can be different from that of an uncontaminated column. Contaminated columns can exhibit backpressure problems. A contaminated reversed-phase column must be cleaned and regenerated to return it to its original operating condition. This installment of "Column Watch" will discuss practical ways to return a column to, or nearly to, its original state. Because bonded-silica columns are the most popular, I will focus on them. At the end, I will discuss cleaning procedures for other types of reversed-phase columns.

What Causes Contaminant Buildup in Reversed-Phase Columns?

Usually, sample matrices contain compounds that are of no interest to analysts. Salts, lipids, fatty compounds, humic acids, hydrophobic proteins, and other biological compounds are a few of the possible substances that can come in contact with an HPLC column during its use. These materials can have lesser or greater retention than the analytes of interest. Those compounds that have lesser retention, such as salts, usually will be eluted from the column at the void volume. These undesired interferences might be observed by a detector and appear as chromatographic peaks, blobs, baseline upsets, or even negative peaks. If sample matrix components are retained strongly on the column and if the mobile-phase solvent composition itself never becomes strong enough to elute them, these adsorbed or absorbed compounds will accumulate, usually at the head of the column, after many injections. This behavior often is observed with isocratic conditions. Sample compounds that are of intermediate retention can be eluted slowly and appear as wide peaks, baseline disturbances, or baseline drift.

Sometimes the sorbed sample components build up to levels high enough that they begin to act as a new stationary phase. Analytes can interact with these impurities that contribute to the separation mechanism. Retention times can shift, and tailing can occur. If sufficient contamination occurs, the column backpressure can build to intolerably high levels, which overpressures the pump and can cause a column to settle and create a void, depending upon where the blockage occurs.

Table II: Column volumes of analytical columns

Column Size (mm × mm)	Void Volume (mL)
250 × 4.6	2.5
150 × 4.6	1.5
150 × 3.0	0.64
150 × 2.1	0.28
50 × 4.6	0.50
30 × 4.6	0.30
15 × 4.6	0.15

Washing Bonded-Silica Columns

The keys to rejuvenating a contaminated HPLC column are knowing the nature of the contaminants and finding an appropriate solvent that will remove them. When contamination results from the accumulation of strongly retained substances from repeated injections, a simple washing process to strip these contaminants often can restore column performance. Sometimes, after isocratic operation, flushing a column with 20 column volumes of 90–100% solvent B (the stronger solvent in a binary reversed-phase system) can remove the contaminants. (Table II lists the column volumes for various sizes of HPLC columns, so readers can easily determine a flush volume for a particular column.) For example, lipids can be removed by washing a column with nonaqueous solvents such as methanol, acetonitrile, or tetrahydrofuran. If you are using a buffered aqueous mobile phase, do not jump immediately to the strong solvent. An abrupt change to high organic solvent content could result in buffer precipitation in the HPLC flow system, which could cause even bigger problems such as plugged frits, plugged connecting tubing, pump seal failure, a scratched piston, or injection valve rotor failure. Instead, flush the column with a buffer-free mobile phase (that is, replace the buffer with water). After flushing with 5–10 column volumes, the stronger solvent then can be passed through the column.

Some additives that can change or modify the surfaces of the packing material may contaminate the surface or bonded phase.

Occasionally, the strong solvent component of a mobile phase is insufficient to remove the column contaminants. A stronger solvent or series of solvents will be necessary to clean the column. If the contaminants are nonbiological, then users can pass one or more additional organic solvents through the column to remove the undesired compounds. The solvents and solvent combinations that can be used are numerous. Visit one or more column manufacturers' web sites to see various recommended solvent systems.

Generally, all washing approaches follow a similar pattern. The wash solvents used are increased in their solvent strength, often ending with a solvent that could be very nonpolar (for example, ethyl acetate or even a hydrocarbon), which helps to solubilize nonpolar substances such as lipids and oils. It is important to ensure that each solvent in the series is miscible with the next solvent. At the conclusion of the wash cycle, go backwards through an intermediately miscible solvent before returning to the original mobile-phase system. For example, isopropanol is an excellent solvent for this intermediate step because it is miscible with organic solvents such as hexane or methylene chloride and also is miscible with aqueous solutions. Because isopropanol is quite viscous, make sure that the flow rate is not high enough to cause pump overpressure. Also, if using a UV detector, avoid using

solvents that absorb in the ultraviolet region of the spectrum because it may require a great deal of washing to remove all of the absorbing solvent to get a stable baseline.

A recommended column washing system for a typical bonded-silica column and a mobile phase without buffer salts is to use

- 100% methanol,
- 100% acetonitrile,
- 75% acetonitrile–25% isopropanol,
- 100% isopropanol,
- 100% methylene chloride, and
- 100% hexane.

When using methylene chloride or hexane, the column must be flushed with isopropanol before returning to an aqueous mobile phase because of solvent immiscibility. A minimum of 10 column volumes of each wash solvent should be passed through a column. For 250 mm × 4.6 mm analytical columns, analysts can use a typical 1–2 mL/min HPLC flow rate. To return to the original mobile phase, chromatographers usually can skip going through the entire series in reverse order. Using isopropanol as an intermediate solvent is recommended, followed by mobile phase without buffer, then finally with the starting mobile-phase composition. Tetrahydrofuran is another popular solvent that can be used for cleaning contaminated columns. If users suspect severe fouling, they can mix dimethyl sulfoxide (DMSO) or dimethylformamide mixed 50:50 with water and pass them at flow rates less than 0.5 mL/min. The successful regeneration of a reversed-phase column can be a time-consuming process, and solvent washings can be programmed into a gradient system for overnight operation.

A question arises as to whether to reverse the HPLC column during the washing procedure. Because most of the strongly held contaminants usually are at the head of the column, reversing the column can shorten the migration distance that the solubilized contaminants must travel to exit the column. As far as the packed-bed stability is concerned, most modern HPLC columns have been packed at a considerably higher pressure than the normal operating pressure; therefore, their beds should not be disturbed by the reversed flow. However, if a top frit is of a higher porosity than a bottom frit, this type of reversal could be detrimental. For example, if the bottom frit is of 2-µm porosity, it usually is sufficient to contain column packing with an average particle size of 5 µm (with a ± 2-µm particle size distribution). However, a manufacturer sometimes will put a larger-porosity frit at the top of the column to prevent plugging with sample or mobile-phase particulates. If the porosity of this frit is larger than that of the smallest particles in the particle-size distribution curve, some of the packing material conceivably could pass through the frit and be removed from the column, thereby creating a void. If a column has an arrow to recommend the direction of flow, I would consult the manual or instruction sheet, the manufacturer's web site, or the technical support group before reversing the column to make sure that it is a safe practice. Whether you reverse the column or not, it is a good practice to disconnect the column from the HPLC detector so that contaminants or particulates lodged on the frit are not swept into the detector cell, where they can cause contamination.

The frequency of cleaning fouled reversed-phase columns depends upon how much unretained material has been injected onto the column. Because reversed-phase columns sometimes can withstand a great deal of contamination before resolution loss or elution of extraneous compounds, users tend to wait until they observe some unusual behavior. However, an increased buildup of contaminants will make it more difficult to clean the column. For this reason, if you know that you are subjecting your reversed-phase columns to dirty sample matrices, I recommend cleaning your columns on a regular basis. The more frequent the cleaning, the less rigorous cleaning conditions you will need.

Table III: Wash solvents for removing proteinaceous material from HPLC reversed-phase columns*

Solvent	Composition
Acetic acid	1% in water
Trifluoroacetic acid	1% in water
0.1% Trifluoroacetic acid–propanol	40:60 (v/v) (viscous; use reduced flow rate)
TEA–propanol	40:60 (v/v) (adjust 0.25 N phosphoric acid to pH 2.5 with triethylamine before mixing)
Aqueous urea or guanidine	5–8 M (adjusted to pH 6–8)
Aqueous sodium chloride, sodium phosphate, or sodium sulfate	0.5–1.0 M (sodium phosphate pH 7.0)
DMSO–water or dimethylformamide–water	50:50 (v/v)

*Adapted from reference 6.

Cleaning Protein Residues from Bonded-Silica Reversed-Phase Columns

If biological materials such as plasma or serum build up on a reversed-phase column, chromatographers must use a somewhat different cleaning process. In most cases, neat organic solvents such as acetonitrile or methanol do not dissolve peptides and proteins and are ineffective for cleaning reversed-phase columns. However, mixtures of organic solvents with buffer, acids, and sometimes, ion-pairing reagents can be effective. Initially, flushing a column with mobile phase that has a somewhat higher percentage of the stronger solvent (solvent B) should be attempted.

Freiser and co-workers (4) found that repeated up-and-down gradients between aqueous trifluoroacetic acid and trifluoroacetic acid–propanol can regenerate contaminated reversed-phase columns. Bhadwaj and Day (5) suggested that a plug injection of 100 µL of trifluoroethanol in a 250 mm × 4.6 mm column could work. If these procedures fail, the strong eluents or solubilizing agents recommended by Cunico and colleagues (6) can be used to strip proteins (see Table III). Before flushing columns with these solvents, however, consult the column manual or the manufacturer to ensure that these solvents are compatible with the packing material. Silica-based columns usually are compatible but organic polymer–based columns can swell or shrink with certain solvent combinations, and the performance could be affected.

As with the previous solvent series, ensure that the sets of solvents in Table III used in series are miscible. Propanol is a good intermediate flush solvent. A minimum of 20 column volumes should be

used for each solvent system. Because some of the solvent systems are quite viscous, the flushing flow rates should be adjusted accordingly to ensure that no overpressuring occurs. After cleaning a column with the guanidine or urea reagents, use a minimum of 40–50 column volumes of HPLC-grade water to flush the column.

For reversed-phase columns, it has been inadvisable to use detergents such as sodium dodecyl sulfate (SDS) and Triton, because these compounds apparently are adsorbed strongly on bonded-silica packing and are difficult to remove. Using detergents can affect the surface of the packing and change its characteristics. However, a study by the Separations Group found that a column contaminated by a protecting group and scavenger products from a peptide synthesis could be cleaned by injecting 500 µL of 1% SDS solution into the mobile phase flowing at 1 mL/min (7). If followed by a gradient from 5% to 95% acetonitrile with 0.1% (v/v) trifluoroacetic acid and equilibration at the starting conditions, the polypeptide separation was restored.

The keys to rejuvenating a contaminated HPLC column are knowing the nature of the contaminants and finding an appropriate solvent that will remove them.

Special Techniques for Cleaning Bonded-Silica Reversed-Phase Columns

Sometimes, washing with organic solvents can fail to remove the column contaminants. This situation is particularly true if metallic ions are sorbed to the silica or bonded phase. A chelating reagent such as 0.05 M ethylenediaminetetraacetic acid (EDTA) can be flushed through a column. The EDTA complexes with many metallic species and solubilizes them. After treatment with an EDTA solution, analysts can wash the column thoroughly with water. If the sample matrix contains ionizable compounds, a change of pH could put them into an un-ionized form, and they could be flushed from the column with water–organic solvent mixtures. For example, a strongly basic matrix component sometimes can be removed by adjusting the pH to less than 3, at which point the protonated amine becomes more water soluble. Acidic matrix components can be removed by adjusting the pH to a higher value — greater than the pK_a — of approximately pH 8 or 9, at which the acids are in their ionized form. However, be cautious with silica-based columns because they can be damaged by long-term exposure to high pH levels (8).

To control bacterial growth that could be present in a buffer system or in columns left unattended in aqueous buffer, chromatographers can use common household bleach diluted 1:10 or 1:20. Run at least 50 column volumes followed by another 50 column volumes of HPLC-grade water. Do not run the bleach through the detector, because it could attack the flow cell. To prevent bacterial growth in the solvent reservoir, use just enough buffer for the day's use and store unused buffer in the refrigerator, use 0.1% sodium azide in the buffer, and don't let the column sit in buffer solution for long periods of time without any flow.

Chromatographers frequently have discussed the effect of the ion-pairing reagents on the stationary phase for columns used for ion-pairing chromatography. Apparently, ion-pairing reagents such as octanesulfonic acid (used for cations) and tetraalkylammonium bromide (used for anions) strongly sorb on the surfaces of bonded-silica columns at certain concentrations of organic modifier. The columns become contaminated and cannot be regenerated to their original state, and the story goes that any column used for ion-pairing work should be dedicated to that technique and never used again for regular reversed-phase chromatography.

Bidlingmeyer (9) disagrees with this generality and feels that the aggressive pH values used for the ion-pairing coupling actually can change the nature of some columns by either hydrolysis of the bonded phase or endcapping silane under acidic conditions (pH 1–3) or by silica dissolution at higher pH values (pH 7–8). To remove sulfonic acid ion-pairing reagents, he recommends first washing the column (minimum of 20 column volumes) with the same mobile phase without the ion-pairing reagent and then washing with mobile phase without the buffer (methanol might be a better organic solvent than acetonitrile in this wash step; for very long–chain ion-pairing reagents, use tetrahydrofuran). Apparently, sulfonic acid ion-pairing reagents and amine ion-pairing reagents exhibit different behaviors. Bidlingmeyer and co-workers (10) demonstrated that when using a C18 column with mobile-phase concentrations greater than 70% methanol, SDS, which is a long-chain anion-pairing agent, is not adsorbed onto the stationary phase. This finding agrees with the Separations Group work (7).

Bonded-silica monolith columns such as Chromolith columns (Merck KGaA, Darmstadt, Germany) should be treated as any other silica-based columns.

Regeneration for Polymeric Columns

Polymeric columns used to separate biological molecules also can become contaminated or require sanitization. The chemical stability of polymeric materials generally is considered one of their strengths. In fact, many manufacturers recommend washing their columns with 1.0 M nitric acid or 1.0 M sodium hydroxide. Certain reversed-phase polymeric columns such as those packed with poly(styrene–divinylbenzene) (PS–DVB) beads and polymeric monoliths such as CIM RP-SDVB disks (BIA Separations, Ljublijana, Slovenia) and Swift columns (Isco, Lincoln, Nebraska, USA) can withstand a wide range of pH values (usually pH 1–13 or sometimes pH 0–14), but users should exercise some care when washing these columns with harsh organic solvents. Depending upon their degree of cross-linking, swelling or shrinking can occur when the columns are exposed to some organic solvents. Highly cross-linked polymers with greater than 8–10% cross-linking usually have good mechanical stability with minimal shrinking in aqueous solvents and minimal swelling in organic solvents. Before washing a polymer column with a series of solvents, however, it is a good practice to consult the column's manual or contact the technical support group of the column's manufacturer.

According to BIA Separations (11), users can regenerate a polymer-based monolithic

column made of PS–DVB by

- washing the column with 10 column volumes of 0.1% trifluoroacetic acid in 2-propanol at one-half of the working flow rate,
- washing the column with at least 5 column volumes of 100% mobile phase B at one-half of the working flow rate, and
- re-equilibrating the column with at least 10 column volumes of 100% mobile phase A at the working flow rate.

If a methacrylate-based monolith with butyl or ethyl chemistry is cleaned, precipitated protein can be removed by flushing the column in a reversed direction with 10 column volumes each of 1.0 M sodium hydroxide, water, 20% ethanol solution, and the working buffer (12). For more hydrophobic proteins, users should insert a wash step of isopropanol (30% v/v) or 70% ethanol (v/v) after the water wash.

For sanitization or the inactivation of microbial populations, a PS–DVB monolithic column should be thoroughly washed with 0.5–1.0 M sodium hydroxide. The packed monolith column should be exposed to sodium hydroxide for at least 1 h at room temperature.

Columns with traditional polymer-based packing materials used to separate difficult proteins such as membrane proteins, structural proteins, and viral-coat proteins that are very insoluble require harsh cleaning conditions. For example, a solvent such as 50% isopropanol with 3 M guanidine hydrochloride at 60 °C might be necessary to elute such difficult proteins (13).

The cleavage of synthetic peptides from solid-phase resins generates reactive carbonium ions that are scavenged by anisole and thioanisole. The scavenger-carbonium reactions yield large, aromatic molecules that can foul reversed-phase columns during peptide purification. These contaminants are highly retained on C18 columns and cannot be removed with 100% acetonitrile or methanol. To clean these columns, reverse them and wash them with three to five column volumes of 100% isopropanol, three to five column volumes of methylene chloride, three to five volumes of isopropanol, and then the original solvent system (14). The elution of aromatic impurities can be verified with a UV detector at 260 nm.

Regeneration of Zirconia-Based HPLC Columns

ZirChrom Separations, Inc. (Anoka, Minnesota, USA), has manufactured a series of zirconia-based columns. The product line has several reversed-phase columns, including polybutadiene, polystyrene, and graphitized carbon versions. Zirconia is more pH resistant than silica gel, so coated zirconia should be more rugged and able to withstand harsher conditions such as higher pH and operating temperatures. However, due to the special surface characteristics, analysts must maintain certain experimental conditions to use these columns successfully with various analytes. Carboxylic acids, fluoride, and phosphate ions all adsorb strongly to zirconia-based columns. To remove them from a column, flush the column with 50 column volumes of a mixture of 20% acetonitrile–0.1 M sodium hydroxide or 0.1 M tetramethylammonium hydroxide, 10 column volumes of water, 50 column volumes of 20% acetonitrile–0.1 M nitric acid, 10 column volumes of water again, and 20 column volumes of 100% of organic solvent. For the polybutadiene and polystyrene columns, chromatographers can use methanol, acetonitrile, isopropanol, or tetrahydrofuran. The graphitized carbon column will require the same solvents with at least 20% tetrahydrofuran (15).

Conclusions

Reversed-phase HPLC columns can become contaminated by the repeated injection of samples that contain strongly retained substances in their matrices, especially compounds that are of high molecular weight or are very hydrophobic in nature, biological fluid components such as proteins, and strongly basic compounds that can adsorb on silanol groups. In addition, certain mobile-phase additives such as ion-pairing reagents and surfactants can sorb onto packing surfaces and change their nature. Contaminated columns can generate poor peak shapes, nonreproducible retention, high back pressures, and baseline artifacts. Often, washing with organic solvents and reagents that can break the strong interactions with the bonded phase or underlying silica surface can clean these columns.

Chromatographers should exercise some caution because using reagents that are too harsh can damage the bonded phase itself. Furthermore, it is good practice to prevent column contamination by using sample preparation procedures that will minimize column exposure to undesirable substances. For all applications, I highly recommend using guard columns to prevent contamination and possible damage to the analytical column.

References

(1) R.E. Majors, *LCGC* **15**(11), 1008–1015 (1997).

(2) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997), p. 180.

(3) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997), pp. 181–182.

(4) H.H. Freiser, M.P. Nowlan, M.N. Schmuck, D.L. Gooding, and K.M. Gooding, "The Use of Stabilized Silica Support with a Short Alkyl Chain for Reversed-Phase Preparative Chromatography of Proteins," product bulletin number 10, Micra Scientific (Northbrook, Illinois, USA, 1988).

(5) S. Bhardwaj and R.A. Day, *LCGC* **17**(4), 354–356 (1997).

(6) R.L. Cunico, K.M. Gooding, and T. Wehr, "Basic HPLC and CE of Biomolecules," Bay Bioanalytical Laboratory (Richmond, California, USA, 1998), p. 254.

(7) "Reversed-Phase Column Cleaning: SDS is OK," *Vydac Advances,* Grace/Vydac (The Separations Group) (Hesperia, California, USA, Winter 1998).

(8) R.K. Iler, *The Chemistry of Silica* (John Wiley & Son, New York, 1979), p. 639.

(9) B.A. Bidlingmeyer, *J. Chromatogr. Sci.* **38,** 226 (2000).

(10) B.A. Bidlingmeyer, F.V. Warren Jr., and R.A. Grohs, *Anal. Chem.* **63,** 384–390 (1991).

(11) "Product Specific Information Sheet for CIM RP-SDVB Disk," publication number PSIS-RPSD-0502, BIA Separations (Ljubljana, Slovenia, 2002).

(12) "Product Specific Information Sheet for CIM C4 Disk," publication number PSIS-C4D-0702, BIA Separations (Ljubljana, Slovenia, 2002).

(13) "A New Chemically Resistant and Heat-Stable Reversed-Phase Column for Protein and Peptide Separations," Vydac application note number 9702, Grace Vydac (Hesperia, California, USA, February 1997).

(14) "Cleaning of Reversed-Phase Columns Used for Synthetic Peptides," Vydac application note number 9603, Grace Vydac (Hesperia, California, USA, March 1996).

(15) "Recommendations for Use, Cleaning and Storage of Zirconia-Based HPLC Columns," ZirChrom Separations, Inc. (Anoka, Minnesota, USA, 2002).