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HPLC COLUMNS AND PACKINGS

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ABSTRACT

In this chapter on HPLC columns, we are discussing both the surface chemistry of a packing as well as column design and performance. In the section that covers column chemistry, we cover modern options of base materials as well as the commonly used approaches towards the surface chemistry of a packing. Specific subsections are dedicated to the selectivity of reversed-phase packings, HILIC, monolithic structures, and the reproducibility of modern packings. In the section on speed and resolution, we familiarize the reader with the principles of how to choose a column. In the section on specialty columns, we cover briefly preparative chromatography and columns with a very small diameter.

I. INTRODUCTION

When we consider the role of HPLC column technology, we should remind ourselves that the birth hour of true high-performance LC was when it became possible for the first time to pack efficient columns with particles smaller than 30 µm.¹ This improved the separation performance of a standard column from a few hundred theoretical plates to a few thousand, and quite satisfactory analyses could be performed in a time frame of around 15 min to half an hour. This performance resulted in the rapid acceptance of HPLC in the analytical laboratories in the pharmaceutical industry.

An important element of column performance is the particle size of the packing. However, there is much more to the story than just the particle size. We will provide in this chapter the important aspects of column performance in chromatography. In Section II, we will familiarize the reader first with the different options available today with respect to the particle backbone or the structure of chromatographic columns. Then we will look into the very important subject of the surface chemistry of chromatographic packings. What creates the selectivity of the separation? How can this selectivity be characterized in a quantitative way? What are the characteristics of the commonly used types of chromatographic packings? How are they useful in the practice of HPLC? Finally, we will go back to the parameters that make HPLC what it is today: the speed and the resolving power in a separation. How can we assess both in a quantitative way? How can we judge the overall capability of a column? Is there a difference in the case of isocratic chromatography and gradient chromatography, and how can we evaluate column performance under gradient conditions?

In most of these discussions, we will focus on particle-based columns. However, today another option has become available: monolithic columns, both based on silica and on polymeric structures. We will briefly discuss the basic properties of these new structures as well. The most commonly used packings are silica-based packings. Polymeric packings have been an alternative for a long time, but their disadvantages outweighed the advantages, and they are much less frequently used for the HPLC analysis of small molecules. However, polymer-based packings are successfully used in sample preparation techniques. In modern times, other alternatives to silica have been developed, and these newer alternatives have gained a wider acceptance. These include hybrid packings and packings based on zirconia or titania.

In Section IV, we will briefly cover some aspects of unusual column dimensions. Is there any advantage to a column with a very small diameter? What are the aspects of column design and the choice of particle size that are important for preparative applications? This discussion will be brief only.

Of course, all these sections do not need to be read in sequence and stand on their own.

II. COLUMN CHEMISTRY

A. General Comments

All but few particles used in HPLC are fully porous packings. Fully porous packings have a large specific surface area, and this creates a larger retention and a larger loadability, i.e. fully porous packings are less prone to exhibit broad peaks with increased injection (with respect to both volume and mass). A typical specific surface area is in the range of 200–300 m²/g. This quite sizeable surface area resides in the pores of the packing. The pore size must be sufficiently large to allow access of the analytes to the surface of the packing. For the analysis of small molecules (molecular weight 100–500), a pore size of 10 nm is about right. Some packings have a slightly smaller pore size (around 8 nm), some have a bit larger pores (about 13 nm). One needs to be careful with some of these designations though, since the methods used for the pore size measurements are not consistent from manufacturer to manufacturer, and often only nominal values are given.

The 10 nm (or 100 Å) packings can be used for peptide analysis as well, but for larger molecules (proteins) a larger pore size, nominally 30 nm (or 300 Å), is needed. The larger pore size provides a smaller specific surface area, therefore these larger pore packings are less retentive, and they are not commonly used for small molecules.

The most important properties of a porous particle are the specific surface area and the specific pore volume. “Specific” means that these values are given per gram of packing. This approach is acceptable for a comparison of different silica-based packings, but such values can be misleading, if silica-based packings are compared to zirconia-based packings or polymer-based packings. Chromatographers are usually more interested in which packing would result in more retention or less retention. The retentivity of a particle can be estimated quickly by looking at the ratio of the specific surface area to the specific pore volume. This is called the particle phase ratio β_p :²

$$\beta_p = \frac{A_{sp}}{V_{sp}} \quad (1)$$

A_{sp} is the specific surface area (in m²/g) and V_{sp} is the specific pore volume (in mL/g). However, for a quantitative assessment, the ratio of the surface area of the packing in the column to the column void volume needs to be considered. This true phase ratio inside the column can be calculated as follows:²

$$\beta = (1 - \varepsilon_i) \frac{A_{sp}}{V_{sp} + (\varepsilon_i / \rho_p)} \quad (2)$$

ε_i is the interstitial fraction. Its value is typically 0.4 for most HPLC columns. The skeletal density ρ_p is 2.2 g/mL for silica. For polymeric packings, it is around 0.8–1 g/mL. This equation allows for a meaningful comparison of different packings, including non-porous packings.

The advantage of fully porous packings is clear: they have a much larger surface area than non-porous particles. Therefore, their retentivity and loadability is much larger. Equation (2) permits a direct comparison of the retentivity of all packings, whether they are non-porous, porous, superficially porous, or based on silica or another carrier.

It is important to get a true understanding of the basic properties of different particles to obtain a proper assessment of their value in chromatography. You will find that the true phase ratio gives you quite a different impression than the surface area alone. Let us compare two packings! One is a silica packing with a specific surface area of 350 m²/g and a specific pore volume of 1 mL/g; the other is also a silica packing, but now with a specific surface area of 200 m²/g and a specific pore volume of 0.5 mL/g. When we calculate the phase ratios, we find that both are practically identical: 178 m²/mL compared to 176 m²/mL. Thus, the retentivity and the preparative loadability of both packings should be equal, unless other particle properties affect these values. If we would have used only the specific surface area for judging the retention of these two packings, we would have believed that the one with the larger surface area would be nearly two times as retentive. This comparison points out the importance of Equation (2).

The strength of a particle depends on its specific pore volume. For normal HPLC applications, strength is of no issue, if the specific pore volume is 1 mL/g or less. For ultra-high-pressure applications, a slightly lower pore volume is needed.

B. The Particle Backbone

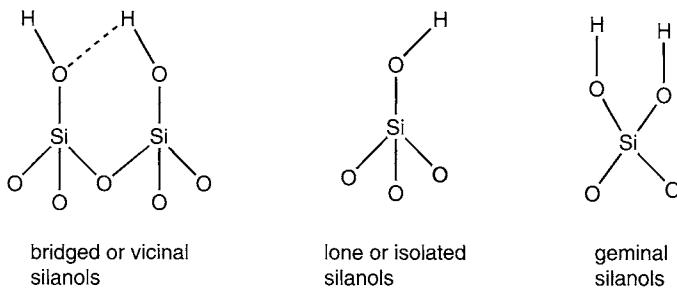
In HPLC, packings based on silica are predominantly utilized. The most important reason for the broad use of silica is its mechanical strength together with the availability of well-established surface modification techniques, especially for the creation of reversed-phase packings (see Section II.C). Another important feature of silica is the freedom with which surface area and pore size can be tailored to specific applications.

One needs to distinguish two types of silica. The classical type is of low purity, and contains a fair amount of other ions in its matrix and on its surface. Common impurities are alumina and iron oxide. Both stem from the raw materials, which are of inorganic origin (water glass). These contaminants are embedded in the matrix of the material, and create acidified surface silanols. Acidic surface silanols are a significant problem when reversed-phase packings based on a classical silica are used in applications common to the pharmaceutical industry, such as the

analysis of compounds containing basic functional groups (amino groups). The classical way to solve the problem is to work only under acidic mobile-phase conditions and/or with bases as competing additives to the mobile phase, or both.

This problem was reduced significantly when high-purity silicas became commercially available. They are synthesized from very pure organic silanes, such as tetraethoxysilane, and metal contamination can be kept out of a silica based on these raw materials by using carefully controlled manufacturing processes. There is a class difference in the peak shape quality for basic analytes between reversed-phase packings based on classical silicas and those based on high-purity silicas. Today, packings based on high-purity silicas are widely available, and there are no compromises that would prevent anyone from using these much higher quality packings based on high-purity silicas. Since method development with high-purity or other modern packings is so much easier, we recommend the use of modern packings for this purpose.

The surface of a silica packing (or a silica-hybrid packing, see below) is occupied by silanols. One distinguishes three types of silanol groups.



Lone silanols are more acidic than the other types of silanols, which exist on well-hydrated surfaces. The generally accepted average pK_a of silanols on a fully hydroxylated high-purity silica is around 7.³ On a low-purity silica, a significant portion of the silanols are more acidic,⁴ which is the cause of base tailing on the older silicas.

In recent years, a derivative technology of high-purity silica has been developed: hybrid organic/inorganic packings based on a co-condensation of tetraethoxysilane and other silanes containing silicon–carbon bonds. This technology provides a mixed organic/inorganic matrix. The primary advantage of this technology is an improved stability of the packing to an alkaline environment. Silica-based packings are commonly unstable above pH 8 even at room temperature. A dense coating with a C₁₈ layer can alleviate this problem somewhat, but the real solution to this weakness of silica is a modification of the matrix. The incorporation of methyl groups or ethyl bridges into the matrix significantly improves the pH stability of the packing. Methyl-hybrid packings have been reported to be stable above pH 11, while the stability of a newer version, the ethyl-bridged hybrid packing,⁴ has been demonstrated to reach pH 12.

A side benefit of at least one version of the hybrid silicas is a shift of the pK_a of the surface silanols into the alkaline pH range.⁵ Under the same conditions of measurement, the pK_a of a high-purity silica was measured to be around 7, while it was around 10 for the methyl hybrid packing.⁶ This means that the “silanol activity” of the methyl hybrid packing is much reduced compared to silica-based packings, resulting in generally improved peak shapes for basic analytes compared even to high-purity silica packings.

Zirconia-based packings are stable from pH 1 to 14, as well as at elevated temperatures. However, bonding procedures like the silanization of silica are not available for zirconia. For reversed-phase applications, packings are prepared by either a coating of the zirconia with polybutadiene or polystyrene, or by preparing a thin surface layer of carbon, with a subsequent derivatization of the carbon with a C₁₈ layer. The zirconia carrier has a large pore size, 30 nm, with an associated lower specific surface area (30 m²/g). The lower surface area means that commonly 10–30% less organic modifier is needed for getting equivalent retention to a standard silica-based C₁₈ column for a neutral analyte.

Due to the unique character of the zirconia surface, one needs to rethink the method development process. Phosphate, bicarbonate, or carboxylic acid buffers are preferred mobile phase additives, but they are also strongly adsorbed to the zirconia surface. The carbon-coated zirconia is more akin to activated carbon than to a C₁₈ packing, and it retains some of these characteristics after the C₁₈ coating.

Polymer-based packings are very rarely used in reversed-phase applications for small molecules. The reason for this lack of use is, on one hand, the commonly inferior mass transfer properties of polymeric packings (i.e., they give wider peaks), and on the other hand, the swelling and shrinking that goes along with an organic polymer. Polymeric packings are more frequently used in special applications such as size exclusion chromatography, hydrophilic interaction chromatography, large molecular weight biomolecule analysis, or in sample preparation techniques.

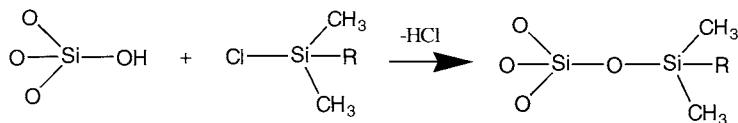
C. The Particle Surface

The particle surface is the most important part of the packing, since it is the interaction with this surface that creates the separation. We will once again focus on silica and silica-hybrid packings since they are most commonly used.

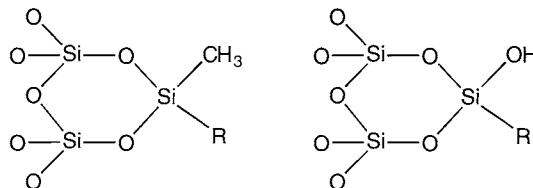
I. Surface Chemistry

The surface silanols of silica are derivatized with organosilanes to create the different types of packings.⁷ Typical reagents are chlorosilanes, although other silanes can be used as well. Monofunctional, difunctional, and trifunctional silanes can be equally employed in this surface reaction.

Since HCl is formed during the reaction, a base is used to scavenge the acid. The surface reaction of a monofunctional silane is shown below:

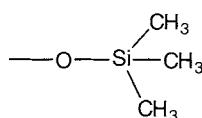


If a difunctional or trifunctional silane is used, more than one bond with the surface is created. This is shown schematically below:



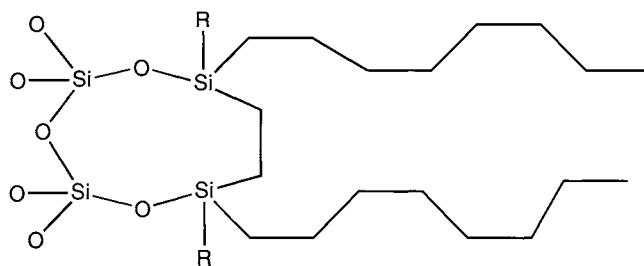
The desired predominant reaction product of a difunctional silane with the surface is the structure shown above. In this way, a maximum number of surface silanols can be removed. However, the details of the actual reaction conditions determine how close one can get to obtaining a large concentration of bidentate bonding. For many bonded molecules, only one link to the surface is formed, and the second silanol is left open.

Trifunctional silanes almost never form a triple link to the surface. Thus, the derivatization with a trifunctional silane creates at least as many new silanol groups as there are ligands attached to the surface. In order to remove these new silanols of multi-functional silanes and residual silanols that were not accessible to the larger monofunctional silanes, the packings are commonly endcapped with trimethylsilyl groups:



This procedure removes additional accessible silanols. However, we must keep in mind that there are about $8 \mu\text{mol}/\text{m}^2$ of silanols on a fully hydroxylated silica, but the surface reaction and the endcapping can only eliminate about $4 \mu\text{mol}/\text{m}^2$, leaving still plenty of silanols on the surface. These silanols tend to interact with basic analytes, and may produce peak tailing. This is one of the reasons why hybrid packings have been created, since they exhibit a smaller number of silanol groups on the surface to start with. Modern, high-quality, more sophisticated endcapping techniques can also remove a larger number of these “residual” silanol groups.

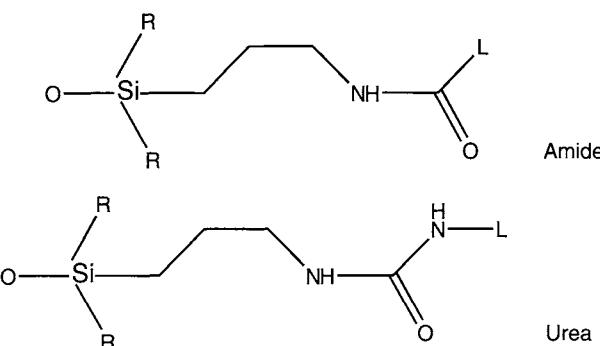
Another bonding option not yet mentioned is the use of bidentate ligands. Such ligands have the capability to attach to the surface in more than one spot. This results in an additional improvement in stability.

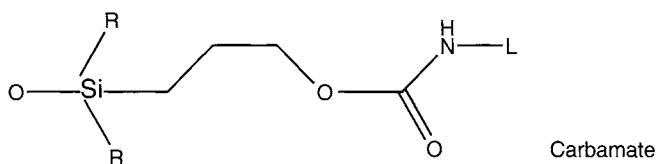


The common side group of monofunctional silanes is a methyl group. However, added stability under acidic conditions can be achieved with more bulky side chains, such as isopropyl or isobutyl groups. The disadvantage of this approach is that the bulkiness of these side chains reduces the surface coverage, and thus more silanols remain and accessible by analyte molecules.

The most common ligand in reversed-phase chromatography is the C₁₈ ligand, an 18-carbon hydrocarbon chain. This chain creates the hydrophobic retention of the reversed-phase packing. As an alternative, a C₈ ligand is sometimes used. Due to the shorter chain length, it provides a bit less retention than the C₁₈ ligand, but it is occasionally preferred for several reasons. Sometimes, a shorter retention is desired. Some C₈ packings give a slightly improved peak shape compared to a C₁₈ ligand, since the smaller ligand can result in a slightly better surface coverage. Or, a slightly different selectivity is desired. It should be pointed out that the selectivity differences between C₁₈ and C₈ packings based on the same substrate and with the same surface coverage are rather small, but they still can be useful on occasion.

If one is looking for an appreciable difference in selectivity, packings with an embedded polar group are recommended as the first choice. These packings have a polar group incorporated into the long-chain ligand, three carbons away from the silicon group that is used to attach the ligand to the surface.⁸ In order to be effective, the polar group is best selected from the group of amide, carbamate, or urea, which all provide excellent hydrogen bonding capability. The three commonly used examples are shown below.



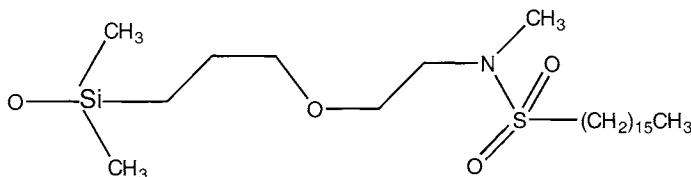


R represents the possible side groups. For a monofunctional ligand (such as the commercially available carbamate phase), R represents a methyl group. Most other packings are based on a trifunctional silane, which means that R is either an additional attachment to the surface or a free silanol.

The length of the ligand varies with the manufacturer. Some short ligands with eight carbons are available, other packings use longer chains, such as a C₁₄ ligand.

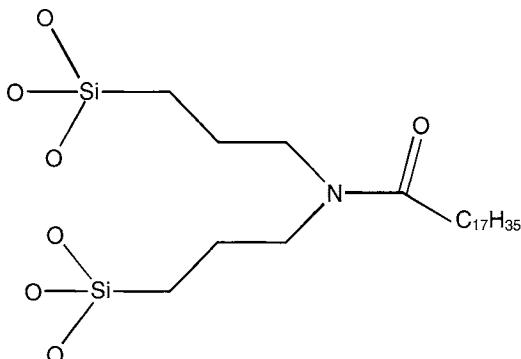
Many (but not all) of the commercially available amide phases are prepared in a two-step reaction, where the silica is first derivatized with an amino silane, and the amide is formed in the second step. This leaves residual amine on the surface. This gives these packings an additional anion exchange function, which can be as detrimental to the peak shapes of acids as silanols are for the peak shapes of bases.

Recently, another packing of the same class was introduced. It is based on a sulfonamide function as the embedded polar group.



This phase does not appear to have the hydrogen bonding ability of the phases mentioned above, but it improves the peak shape for difficult analytes as the other packings with embedded polar functions do.

Finally, a combination of the idea of the bidentate ligand with the idea of an embedded polar group has become commercially available as well. In this case, both attachments to the surface are based on a trifunctional silane, and the distance between “neighboring” attachment groups is rather large:



All packings with an embedded polar group share with each other the fact that they can be used in 100% aqueous mobile phases without dewetting,⁹ an improved peak shape for basic analytes, and significantly different selectivity patterns compared to standard C₁₈ phases. These differences in the selectivity compared to the classical C₁₈ phases can be attributed to the participation of the polar group in the interaction of analytes with the surface of the packing.

Another packing with an embedded polar group is based on a phenol ether. However, this group does not exhibit the characteristic selectivity effects typical of the amide, carbamate, and urea phases.

A comparison of the selectivity difference between different packings is shown in Figure 1. In this figure, the retention times of over 70 analytes measured in an acetonitrile or a methanol gradient at pH 3, 7, and 10 are shown. In Figure 1a, the retention data obtained on a C₈ packing are compared to the same data on a C₁₈ packing. In Figure 1b, the same comparison is made for a classical C₈ column and a packing with an embedded carbamate group of the same chain length. One can see that the scatter is much larger for the comparison of the classical column and the embedded-polar-group column (EPG column). This demonstrates the larger selectivity difference created by the embedded polar group.

Figure 2 depicts the details of the selectivity difference between a classical C₈ packing and a packing with an embedded polar group. For simplicity, only the data obtained in methanol are shown. The patterns observed in acetonitrile were similar, but with a smaller impact of the stationary phase selectivity. Generally, the retention is higher for both ionized acids and bases on the classical C₈ packing. However, since the XTerra MS packing is well deactivated, the patterns for the ionic compounds do not differ much from the patterns for general neutral compounds. Analytes with the ability to hydrogen bond to the embedded carbamate group are more retained on the XTerra RP₈ packing. These include phenols, sulfonamides, and non-ionized acids, with the strongest effect for the first two classes of compounds. It should be pointed out that the hydrogen bonding ability of phenols is lost when the phenol group becomes ionized. The same is true for the hydrogen bonding ability of carboxylic acids.

The selectivity differences between EPG packings and classical packings are exciting. However, they have a larger MS bleed than C₁₈ packings, which bothers some LC/MS users. The bleed is not due to a larger ligand loss compared to a standard C₁₈ packing, but rather due to the much greater ease of ionization of the ligand, which is caused by the embedded polar group. The user needs to decide, if the significant advantages of well-designed EPG packings outweigh this drawback.

Several different types of phenyl ligands are commercially available. They all share a phenyl group, but the attachment to the surface and the length of the chain between the surface and the ligand varies. The common linker is a three-carbon chain, but newer materials have a six-carbon

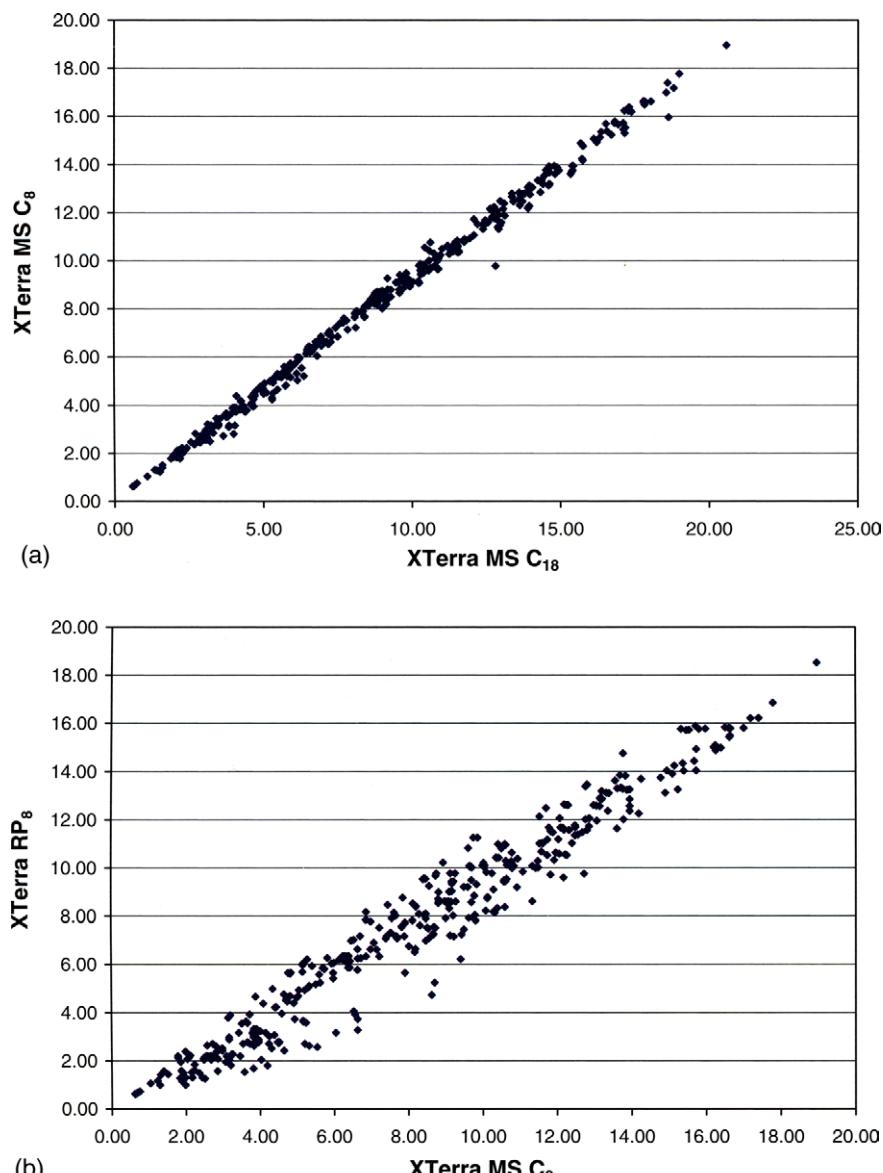


FIGURE 1 Plot of the retention times for over 70 analytes for methanol and acetonitrile gradients at pH 3, 7, and 10 for a change in ligand chain length (a) and a comparison of a packing with an embedded polar group (Xterra RP₈) with one without it (Xterra MS C₈) but with equal chain length (b). The larger scatter for (b) demonstrates the larger selectivity difference between the columns in (b) compared to the columns in (a). Data courtesy of Alberto Méndez, Waters Corporation.

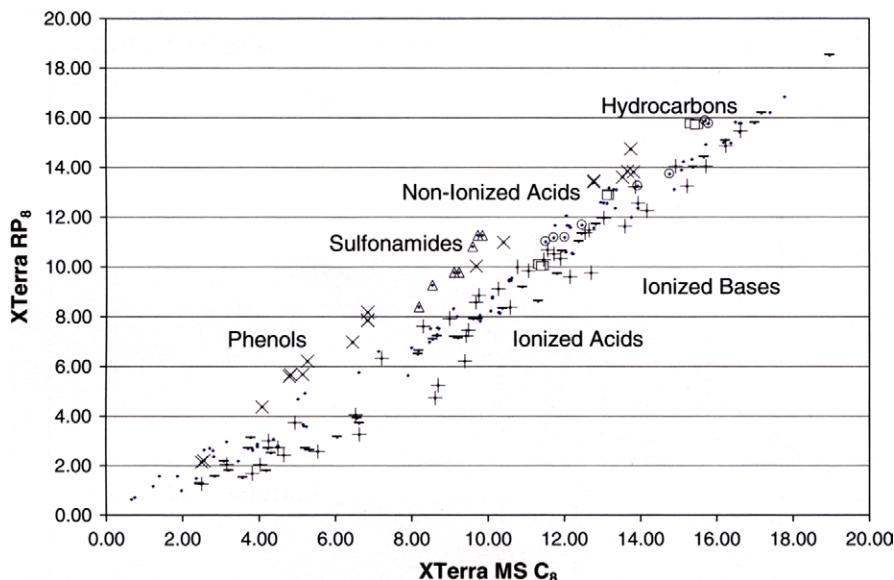


FIGURE 2 Detailed comparison of the retention pattern of different compound classes on a packing with a classical ligand and one with an embedded polar group. (+) Ionized bases, (−) ionized acids, (X) phenols, (O) non-ionized carboxylic acids, (Δ) sulfonamides. Data courtesy of Alberto Méndez, Waters Corporation.

spacer (phenylhexyl packings). The phenyl group shows $\pi-\pi$ interactions with suitable analytes. Most analytes of interest in the pharmaceutical industry contain aromatic rings and one often sees selectivity differences when switching from a C₈ or a C₁₈ packing to a phenyl packing. However, in many cases these selectivity differences are rather small, which actually may be more of a benefit than a detriment. In some special cases, the selectivity and retention differences between a phenylhexyl packing and a C₁₈ packing can be striking. Figure 3 shows this for the analysis of a set of explosives standards, carried out on 1.7-μm particles using ultra-high-pressure LC (see also Section III.C). Due to the $\pi-\pi$ bonding, the phenylhexyl column exhibits a larger retention and a significant selectivity difference compared to the C₁₈ packing.

There are two principal types of perfluorinated packings: one is the aliphatic perfluoro octyl packing and the other one is the aromatic pentafluoro phenyl (PFP) packing. Due to the larger steric hindrance around the more bulky fluorinated groups, the coating levels are usually lower than those for equivalent non-fluorinated packings. As a consequence, both packings exhibit a larger amount of silanol interactions than their non-fluorinated counterparts. Therefore, it is often difficult to determine which selectivity effects stem from the ligand, and which originate in the increased level of silanol interaction. For example, the retention of bases is enhanced on typical PFP packings, which cannot be

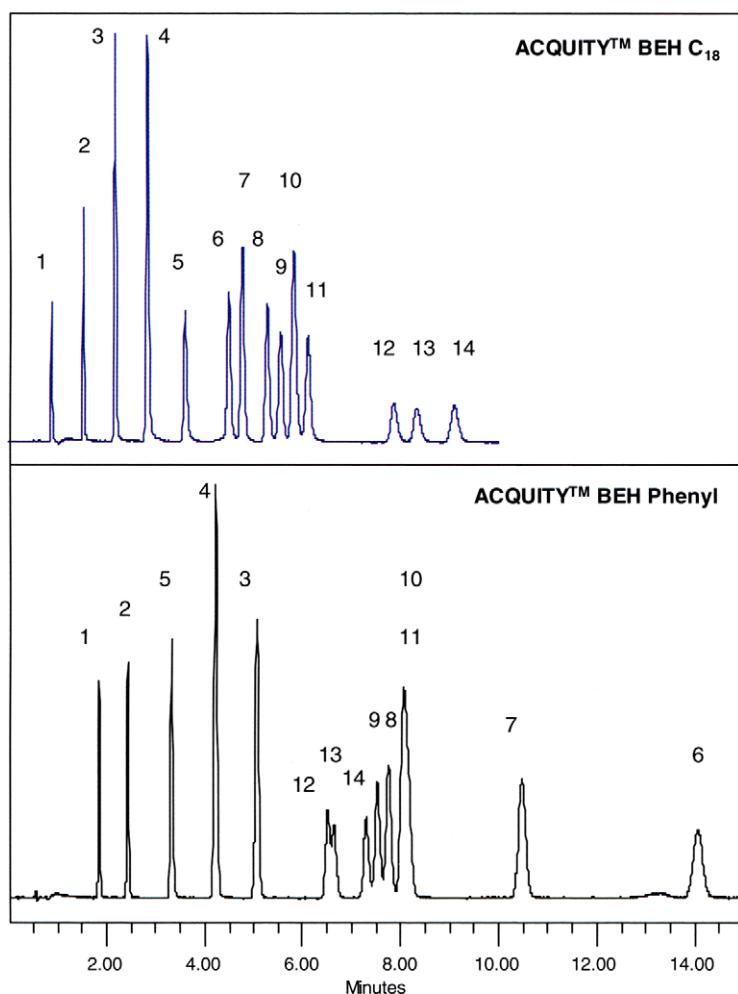


FIGURE 3 Comparison of the retention of explosives standards on 1.7- μm UPLC packings. Top: 100 mm \times 2.1 mm ACQUITY™ BEH C₁₈. Bottom: 100 mm \times 2.1 mm ACQUITY™ BEH Phenyl. Flow: 0.5 mL/min. Mobile phase: 28% methanol, 72% water. Instrument: ACQUITY™ UPLC with ACQUITY™ Photodiode Array Detector at 254 nm. Peak designations: (1) HMX, (2) RDX, (3) 1,3,5-trinitrobenzene, (4) 1,3-dinitrobenzene, (5) Nitrobenzene, (6) tetryl, (7) 2,4,6-trinitrotoluene, (8) 2-amino-4,6-dinitrotoluene, (9) 4-amino-2,6-dinitrotoluene, (10) 2,4-dinitrotoluene, (12) 2-nitrotoluene, (13) 4-nitrotoluene, (14) 3-nitrotoluene. Data courtesy of Eric S. Grumbach, Waters Corporation.

explained readily by a specific interaction with the ligand. Selectivity differences between a perfluoro octyl packing and a standard C₁₈ are rather small, except for those that could be attributed to surface silanols.

Cyano (CN) packings have been around since the beginning of HPLC, largely due to their success in GC. However, in LC, many users became

disenchanted with CN packings. This is largely due to multiple stability issues, but not due to a lack of selectivity difference compared to a C₁₈. The standard ligand is a propyl cyano ligand. The attachment to the surface can be done with the use of a monofunctional silane or a trifunctional silane. Due to the shorter chain length and the polar functional group, CN packings are significantly less retentive in reversed-phase HPLC compared to C₈ or C₁₈ packings. Due to the difficulties with existing packings, the use of CN packings is not recommended.

2. Selectivity

The general selectivity properties of commercial reversed-phase columns are of significant interest to the applied chromatographer. Several researchers have dedicated large resources to attempt to characterize the main features of commercially available columns.¹⁰⁻²⁸ Reference 28 (Chapter 4 in this book) describes the approach by one group of authors. The most erudite method and the most inclusive overview of different packings can be found in reference 26, with a recent refinement of the method in reference 27. However, with the exception of the judgment about the hydrophobicity of different packings, the agreement between the different methods is rather poor.¹⁴ The reasons for these discrepancies are not understood. One possibility is that the mobile phase plays a more significant role, and that the dismissal of its influence is the underlying cause for the differences between the characterization methods. From the standpoint of the practitioner, this simply means that one needs to look at the described column features as paintings that have been created with a broad brush, and that one can only extract a general character of a column from the measured values. However, this is still of significant value.

In the following, we will discuss the data collected by us using the method described in reference 13. We have measured the retention of a purely hydrophobic compound, acenaphthene, and that of a compound that shows a strong interaction with surface silanols, amitriptyline, in a methanol/buffer 65.0/35.0 (v/v) mobile phase. The buffer is a 20 mM pH 7.0 phosphate buffer. The logarithm of the retention factor of acenaphthene is the yardstick for the hydrophobicity of the packings. From the logarithm of the relative retention between acenaphthene and amitriptyline, a measure for the activity of the surface silanols is derived. This is demonstrated in Figure 4. In this figure, the silanol activity is plotted versus the hydrophobicity of the packings. C₁₈ packings are marked with a black square and C₈ packings with a gray square. Generally, C₁₈ packings are more hydrophobic than C₈ packings, but this is not always the case, since the retention for a purely hydrophobic compound depends also on the ligand density and the phase ratio of the parent silica. The circles mark phenyl packings and the diamonds are CN packings. Most phenyl packings are less hydrophobic than C₈ packings, and CN packings are the most polar packings. The stars represent fluorinated packings.

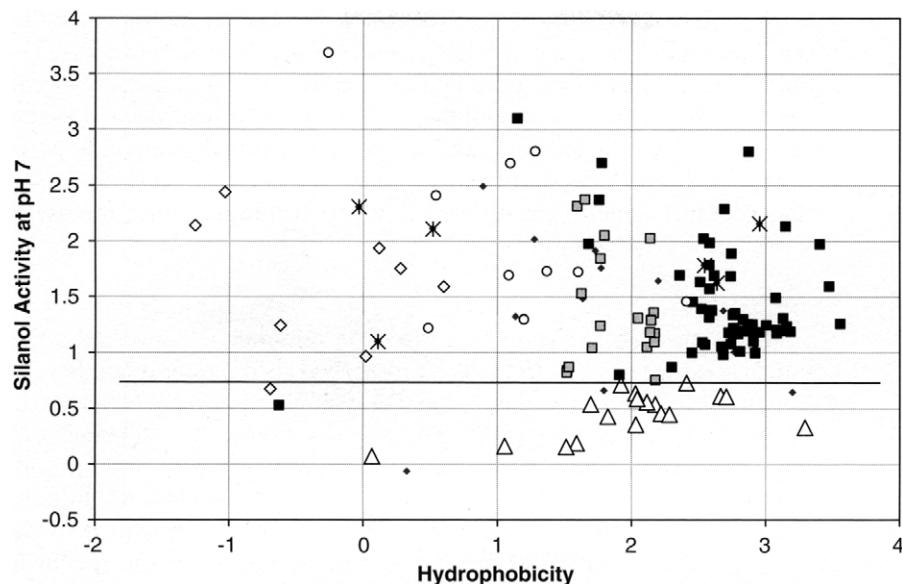


FIGURE 4 Selectivity chart summary. Designation of packings: black squares: C_{18} , gray squares: C_8 , circles: phenyl, diamonds: cyano, stars: fluorinated packings, triangles: packings with embedded polar groups, small back diamonds: uncategorized packings. Reprinted from reference 43.

The packings marked by a triangle are all packings with an embedded polar group. They clearly show a much lower silanol activity than the packings lacking this feature. This is a general advantage in method development. A line is drawn that shows the difference between packings with an embedded polar group and those packings that lack this feature.

In Figure 5, plots of the values of the silanol activity versus the hydrophobicity are shown for 148 packings. Figure 5a is drawn at the same scale as Figure 4, which was used to orient the reader in the chart. Figure 5b is a magnified view of the indicated section of (a), and (c) is a magnification of (b). The charts cover a broad selection of different packings, from older stationary phases based on low-purity silicas to newer packings based on high-purity silica, to packings based on hybrid particles. The packings were selected from commercial sources in different parts of the world. Occasionally, some packings were tested multiple times to check the reproducibility of the packings. Therefore, the chart contains more data points than packings.

Some packings with a very high silanol activity such as the Zorbax Classic or the Resolve C_{18} were not included in this chart due to the extremely high silanol activity. The same holds true for unencapsulated C_{30} packings such as the YMC C_{30} . Among the remaining packings, the highest silanol activity on the chart is found for Waters Spherisorb Phenyl (#4), followed by Platinum EPS (#18). Low silanol activity is

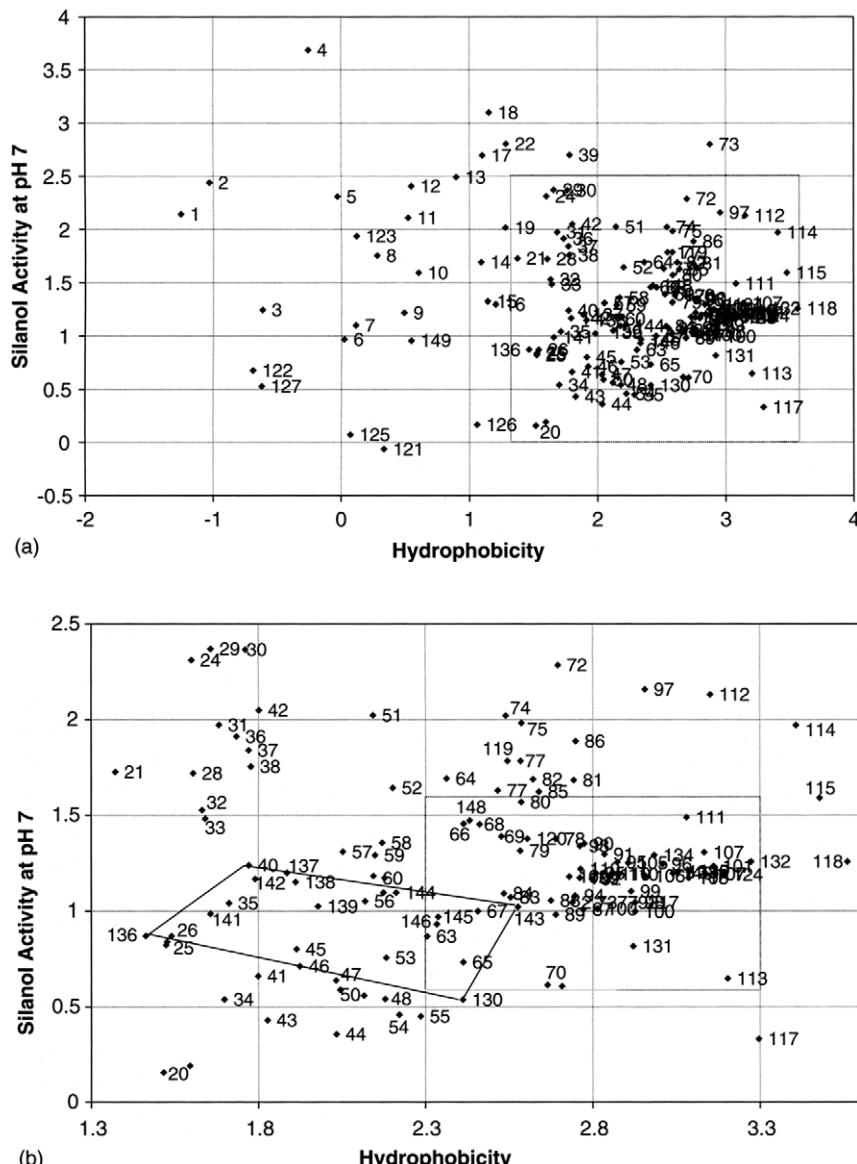
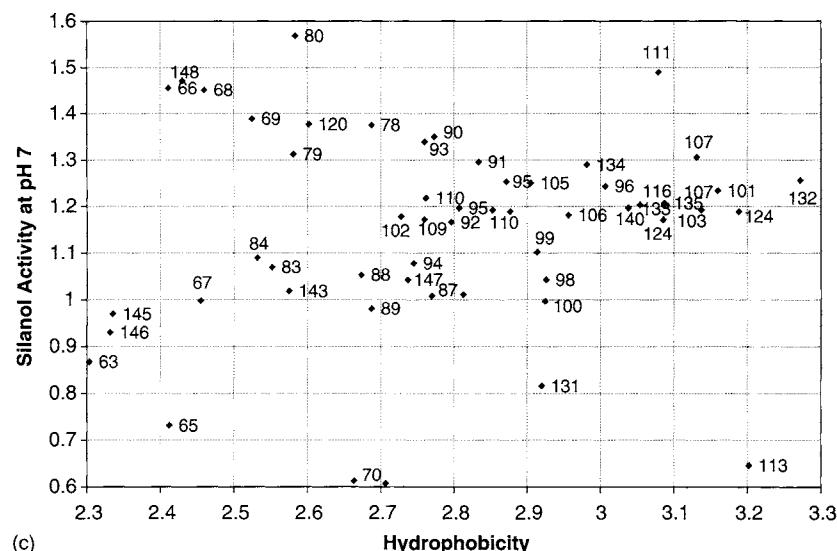


FIGURE 5 (a) Column selectivity chart. (b) An expansion of (a), as indicated, and (c) is an expansion of (b). The same column designations are used in (a)–(c). (1) Nova-Pak CN HP, (2) Waters Spherisorb CN RP, (3) Hypersil CPS CN, (4) Waters Spherisorb Phenyl, (5) Keystone Fluofix 120N, (6) YMC-Pack CN, (7) Ultra PFP, (8) Zorbax SB-CN, (9) Hypersil BDS Phenyl, (10) Inertsil 3 CN, (11) Fluophase RP, (12) Hypersil Phenyl, (13) Zorbax SB-Aq, (14) YMC-Pack Ph, (15) YMC Basic, (16) Ultra Phenyl, (17) Inertsil Ph3, (18) Platinum EPS C₁₈, (19) Syngeri Polar-RP, (20) XTerra RP₈, (21) Nova-Pak Phenyl, (22) Zorbax SB-Phenyl, (24) Zorbax Rx C₈, (25) XTerra MS C₈, (26) Prodigy C₈, (28) Zorbax Eclipse XBD Phenyl, (29) Zorbax SB C₈, (30) μBondapak C₁₈.

**FIGURE 5** Cont.

(31) YMC J'Sphere L80, (32) Supelcosil LC DB-C₁₈, (33) ZirChrom PBD, (34) Discovery RP Amide C₁₆, (35) Hypersil BDS C₈, (36) HydroBond AQ, (37) Lichrospher Select B, (38) Allure Ultra IBD, (39) Platinum C₁₈, (40) Nova-Pak C₈, (41) Capcell Pak C₁₈, (42) Alltima C₈, (43) Discovery RP AmideC₁₆, (44) Xterra RP₁₈, (45) Symmetry300 C₁₈, (46) Spectrum, (47) Zorbax Bonus RP, (48) Supelcosil LC-ABZ Plus, (50) SymmetryShield RP₈, (51) Lichrosorb Select B, (52) PolyEncap A, (53) Prism, (54) Supelcosil LC-ABZ+, (55) Supelcosil LC-ABZ, (56) Luna C₈ (2), (57) Inertsil C₈, (58) Kromasil C₈, (59) Zorbax Eclipse XDB C₈, (60) Symmetry C₈, (63) Hypersil HyPurity Elite C₁₈, (64) Hypersil ODS, (65) Polaris C₁₈-A, (66) Luna Phenyl-Hexyl, (67) Hypersil BDS C₁₈, (68) Supelcosil LC DB-C₁₈, (69) Aqua C₁₈, (70) SymmetryShield RP₁₈, (72) Nucleosil C₁₈, (73) Waters Spherisorb ODS-2, (74) Waters Spherisorb ODSB, (75) YMC J'Sphere M80, (77) Zorbax SB-C₁₈, (78) Synergi Max RP, (79) YMC Hydrosphere C₁₈, (80) Nova-Pak C₁₈, (81) PolyEncap C₁₈, (82) TSK-Gel 80T_s, (83) Ace C₁₈, (84) Xterra MS C₁₈, (85) Fluophase PFP, (86) Purospher RP₁₈, (87) Develosil C30 UG 5, (88) Develosil ODS UG 5, (89) Hypersil Elite C₁₈, (90) Zorbax Rx C₁₈, (91) Zorbax Eclipse XDB C₁₈, (92) L-Column ODS, (93) YMC ODSAQ, (94) Prodigy C₁₈, (95) Luna C₁₈ (2), (96) Kromasil C₁₈, (97) Allure PFP Propyl, (98) Discovery HS C₁₈, (99) Inertsil ODS-2, (100) Symmetry C₁₈, (101) L-column ODS, (102) Puresil C₁₈, (103) Cadenza CD-C₁₈, (105) Luna C₁₈, (106) Zorbax Extend C₁₈, (107) Inertsil ODS-3, (109) Zorbax Eclipse XDB C₁₈, (110) YMC Pack Pro C₁₈, (111) Purospher RP₁₈e, (112) Alltima C₁₈, (113) ODPerfect, (114) YMC J'Sphere H80, (115) Develosil ODS SR 5, (116) Nucleodur Gravity C₁₈, (117) Inertsil ODS-EP, (118) YMC-Pack Pro C₁₈ RS, (119) Discovery HS F5, (120) Atlantis dC₁₈, (121) Discovery HS PEG, (122) Discovery Cyano, (123) Luna CN, (124) Cadenza CD-C₁₈, (125) experimental carbamate CN packing, (126) experimental carbamate phenyl packing, (127) Imtakt Presto FT C₁₈, (128) Aquasil C₁₈, (129) Pursuit Diphenyl, (130) XBridge Shield RP₁₈, (131) Acclaim PA, (132) Inertsil ODS 3V, (133) Capcell Pak MGII 5 μm, (134) Capcell Pak MGII 3 μm, (135) Acclaim C₁₈, (136) XBridge C₈, (137) ACT Ace C₈, (138) ACT Ace C₁₈, (139) Hypersil Gold, (140) SunFire C₁₈, (141) Xterra Phenyl, (142) XBridge Phenyl, (143) XBridge C₁₈, (144) SunFire C₈, (145) Pursuit C₁₈ 3 μm, (146) Discovery C₁₈, (147) Gemini C₁₈, (148) Synergi Fusion RP. Data supplied by Bonnie Alden.

shown for all the types of packings with embedded polar groups. The polyethylene-glycol-derivatized Discovery HS PEG (#121) marks the bottom of the chart.

The most hydrophobically retentive packing on the chart is YMC-Pack Pro RS combined with a silanol activity typical of high-purity silicas (#118). Intermediate hydrophobicity for a C₁₈-type packing is exhibited by XTerra MS C₁₈ (#84). The most polar stationary phase on the chart is the Nova-Pak CN HP packing (#1). The reader is encouraged to examine the three selectivity charts in detail to find the positions of his or her favorite packings.

Today, many automated method development schemes use a parallel exploration of the selectivity of different columns. Based on the characteristics of the packings described here and in the previous paragraph, we have recommended to use a classical C₈ or C₁₈, a short-chain or long-chain EPG column, and a phenyl column together with the selectivity of the mobile phase to search for a quick start in method development.²⁹ A typical scheme would use one of each type of columns, methanol and acetonitrile as solvents, and, if the analytes are ionizable, pH as the primary tools in the exploration of separation selectivity. Subsequently, mobile phase composition and temperature can be used for a method fine-tuning. Such a scenario uses every one of these principal selectivity parameters with maximal effect.

These different selectivity characteristics can even be found within a given family of packings. The advantage of such an approach lies in other common factors of the packings. For example, the family of XBridge packings exhibits an excellent chemical stability at alkaline pH. At the same time, the character of the surfaces is measurably different. We can find the packings of this family on Figure 5b (connected lines): XBridge C₈ (#136) is found on the left side of the graph. The most hydrophobic packing in this family is XBridge C₁₈ (#143). The packing with the lowest silanol activity is the one with the incorporated polar group XBridge Shield RP₁₈ (#130). The XBridge Phenyl packing (#142) has an intermediate hydrophobicity, but also shows selective π–π interaction typical of the phenyl packings (see Section II.C.1). When combined with mobile phase selectivity, these different stationary phase characteristics maximize the chances that a suitable separation will be found rapidly.²⁹

3. Other Techniques: HILIC and Mixed-Mode Phases

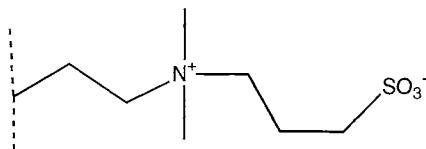
In the majority of this chapter, we are focusing on reversed-phase chromatography, since it is the most commonly used technique in the pharmaceutical industry. However, some alternative techniques may be of advantage under appropriate circumstances. One of these techniques is hydrophilic interaction chromatography, commonly abbreviated as HILIC. Like reversed-phase chromatography, aqueous–organic mobile phases are used. However, the stationary phases are very polar, and thus the overall retention patterns are opposite to what is found with

reversed-phase chromatography. The most common organic modifier is acetonitrile, and retention increases with an increase of acetonitrile in the mobile phase, and decreases with the addition of water. A good overview of the application of this technique for bioanalytical LC/MS can be found in reference 30.

The basic mechanism of HILIC is partitioning of the analytes from the acetonitrile-rich mobile phase to the water-rich surface of the packing.² In other words, more polar compounds such as sugars are well retained, while hydrophobic compounds such as toluene are unretained and can serve as void-volume markers. In addition, some of the commercial packings used for HILIC also have ion-exchange properties, which can be advantageous for improving the separation or altering retention. It should be mentioned here that HILIC methods have been demonstrated to yield superior sensitivity in applications where electrospray mass spectrometry is used as the detection technique.³⁰ The improvement in sensitivity is often one order of magnitude, and may occasionally reach much higher values.³¹

The classical HILIC application is the separation of sugars on silica-based propylamino columns.³² Due to stability issues with a standard amino column, modern “carbohydrate columns” are stabilized to overcome this difficulty. Also, polymer-based amino columns are available for this application. Due to the amine function, these columns can also be used in a combination of HILIC with an anion-exchange mechanism. Underivatized silica columns can also be used for HILIC applications. In this case, the polar surface of the silica is responsible for the attraction of the water from the mobile phase. In the case of silica, the HILIC retention mechanism is supported by cation-exchange with the surface silanols.³¹ It needs to be mentioned that in HILIC, silanols are not a detriment. The tailing observed with silanol-rich packings in reversed-phase chromatography is due to the fact that the silanols are few in number and buried under the hydrophobic C₁₈ layer. In HILIC, the silanols are on the surface and easily accessible. Therefore, they can interact freely and without steric hindrance with the analytes. Thus, tailing is observed only rarely.

A modern development in HILIC are zwitterionic stationary phases. The structure of the group responsible for the retention is shown below:



Phases with such groups can be prepared on silica and on organic polymers, and both forms of this packing are commercially available. Note that the opposite charges balance each other. The retention is created by the preferential binding of water by the charged groups. These packings work equally well for anionic, cationic, and neutral analytes.

Another recent development of interest is the use of mixed-mode stationary phases, where hydrophobic interaction is combined with an ion-exchange mechanism. In these stationary phases, ion-exchange groups are incorporated into a reversed-phase ligand. Several different combinations of this type are commercially available, combining strong and weak cationic and anionic functions on the surface with reversed-phase ligands. Details about the structure of the ligands are unfortunately not available.

Mixed-mode stationary phases on the basis of polymeric packings have been used extensively in sample preparation.³³ In this case, a packing prepared from divinylbenzene and N-vinyl pyrrolidone is partially derivatized with sulfonic acid groups, carboxylic acid groups, quaternary ammonium groups, or tertiary amino groups to create strong and weak cation and anion exchangers. Such packings interact both via the ion-exchange groups as well as via the hydrophobic matrix (divinylbenzene). Applications include the sample preparation of plasma and urine samples prior to HPLC analysis, often in combination with MS detection. Generally, it was found that techniques using the mixed-mode packings resulted in cleaner HPLC samples with significantly less ion-suppression in MS.

Occasionally, good sample preparation techniques can be combined in an optimal way with HILIC as the analytical technique prior to MS detection. Next, we will describe an interesting application that combines the sample cleanup with the column chemistry and the detection. The ionizable analyte in a (rat) plasma sample is cleaned up via solid-phase extraction using a mixed-mode ion-exchange technique. The elution solvent in this technique is methanol to eliminate hydrophobic interaction, together with an acid or a base to break the ion-exchange interaction.³³ This sample can subsequently be injected directly (i.e. without evaporation to dryness and reconstitution in another solvent) onto a HILIC column. Under HILIC conditions, methanol is a weak eluent and therefore a rather large injection volume is possible. Retention is further enhanced for basic compounds, if the HILIC column is a silica column, since the ion exchange with surface silanols assists the HILIC process. If the detection method is ESI-MS, one can achieve a higher sensitivity in mobile phases that are rich in organic solvent, as is typical for a HILIC method.³⁰ Such a strategy takes full advantage of the sample preparation, column chemistries, and detector sensitivity available.

D. Monolithic Structures

Since their inception in the mid-1990s, monolithic structures have inspired the imagination of chromatographers. However, the practice has remained far behind these early expectations.

What are monoliths? They are continuous structures of the stationary phase, with a continuous macropore structure that provides the channels for the flow of the mobile phase.³⁴ The stationary phase can be

porous or non-porous, with the same drawbacks of a non-porous structure as exhibited by non-porous beads in packed beds. In standard monoliths, the internal pore structure, also called micropores, provides the surface area necessary to achieve adequate retention in chromatography.

Monoliths are available based on both silica³⁵ and organic polymers.³⁶ Most of the following discussion will focus on silica monoliths.

With packed beds, the volume of the interstitial channels between the particles is typically 40% of the column volume (it is a bit higher for irregular-shaped particles, and it may also be somewhat lower for spherical particles, depending on the properties of those particles). The size of these interstitial channels determines the backpressure of the column, while the size of the particles determines the column performance. With a fixed interstitial volume fraction, the column performance and the column backpressure are simultaneously determined by the particle size in packed beds.

With monoliths, this link no longer exists. Thus, it is possible to create structures with a larger interstitial fraction combined with a smaller characteristic size for the stationary phase than would have been possible with a packed bed. Since the backpressure depends inversely to roughly the fifth power on the interstitial fraction (in the range of 40% interstitial fraction), one can gain significantly by creating a more open monolithic structure. On the other hand, new methods for achieving a high level of macroscopic structural uniformity could lead to further improvements.

The analysis of the performance of standard commercial monoliths³⁷ showed that the chromatographic performance (van Deemter A-term) of the devices examined was similar to a well-packed bed of 3.5–5-μm particles, while the backpressure was equivalent to that of a column packed with 8–10-μm particles. This gives a clear impression of the performance advantage of a standard monolith. At the same time, the pressure that can be applied to a commercial monolith is limited by the fact that the encasement of the column is made out of PEEK™, which limits the pressure that the column can be exposed to (and thus the speed of analysis that can be achieved). Commercial silica monoliths are still limited to a single internal diameter. Within these constraints, good performance can be achieved.

Organic monoliths are commercially available as well, but they are primarily used as media in the separation of large molecular weight compounds, mostly biomolecules. Typical applications include proteins, peptides, or DNA fragments. A study of the operational variables in such systems has been published.³⁸

E. Reproducibility

In the pharmaceutical industry, the reproducibility of a separation is of utmost importance. Once a separation has been developed,

it is expected that it can be transferred to other departments (e.g. QC department) or to other locations, often in other parts of the world, without any difficulties. The method developers also count on the stability of the method over long periods of time, i.e. decades. Both of these subjects have several implications.

First of all, after the completion of method development, it is very important to test the reproducibility of a method from instrument to instrument, and from column to column. With respect to the column reproducibility, it is also worthwhile to compare used and new columns, and columns containing different preparations (=batches) of the packing material. Major column manufacturers can supply such columns, and some have such column sets in their standard offering. The comparison of a used column and a brand-new column ensures that the method is indeed reproducible. The comparison between columns from the same batch of material should give essentially identical results. The comparison between different preparations of the packing material gives an indication of the long-term reproducibility of a method. Good results increase the comfort of the QC department that the method will be stable over long periods of time.

Second, the choice of columns used in method development needs to take into consideration the distribution capability of the column manufacturer. Many pharmaceutical companies have manufacturing sites in different parts of the world, and the same column needs to be readily available in different countries.

The third point is the capability of the column manufacturer to ensure a long-term supply of the packing. This is more likely, if the column supplier is an integrated manufacturer. This means that the column manufacturer synthesizes the raw silica *and* does the surface bonding and column manufacturing. Some column manufacturers do not have the capability to synthesize the raw silica. Thus, they depend on another supplier to provide them with the silica. This has consequences for the security of the supply, and potentially, also the reproducibility of the final product.

The quality of the batch QC-test of the column manufacturer together with reproducibility data of a packing material can also be taken into account in the selection of columns deemed for the development of QC procedures. Such data have, for example, been published in reference 39. Data on the long-term reproducibility of packings are also available from manufacturers upon request. An example of a dataset on the long-term reproducibility of one packing is shown in Figure 6. Relative retention values are plotted versus the batch numbers of all released batches of the product. The range of each retention window is $\pm 10\%$ around the mean value. Let us discuss briefly the different values measured and the meaning of these values. The simplest value is the hydrophobic selectivity measured by the relative retention between

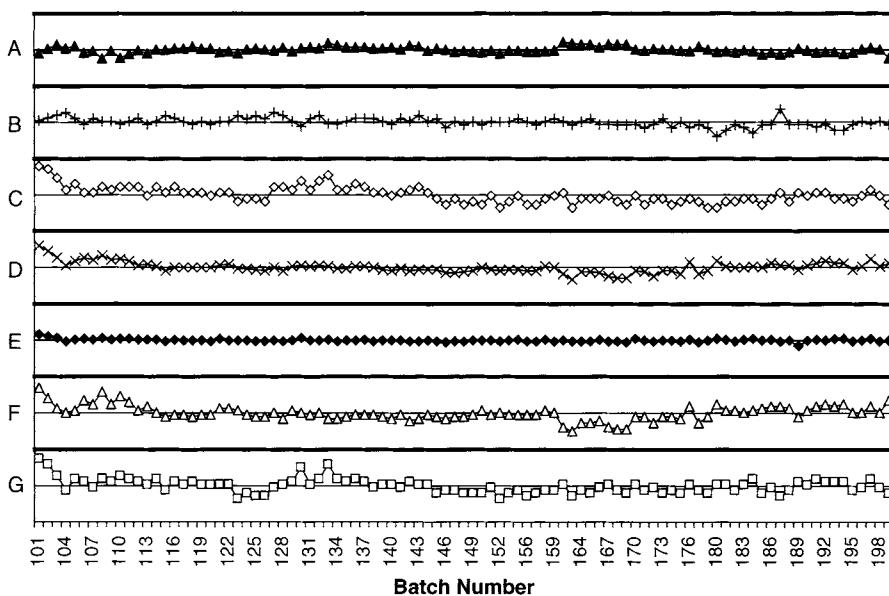


FIGURE 6 Batch-to-batch reproducibility of the Symmetry C₁₈ packing. Range of each value: $\pm 10\%$ around the mean. (A) ▲: α -dipropylphthalate/butylparaben, (B) +: α -amitriptyline/propranolol, (C) ◊: α -amitriptyline/acenaphthene, (D) x: α -dipropylphthalate/acenaphthene, (E) ●: α -naphthalene/acenaphthene, (F) Δ: α -butylparaben/acenaphthene, (G) □: α -propranolol/acenaphthene. The chart includes all data of the released product since the introduction of the packing. Data supplied by Ed Grover, Waters Corporation.

acenaphthene and naphthalene (Figure 6E). This value has a relative standard deviation of $<0.4\%$. As discussed above, the relative retention between a base and a neutral purely hydrophobic reference compound is a measure of the silanol group activity of a packing (Figure 6C and G). The test is carried out at pH 7.0, where the partial ionization of the surface silanols critically influences the retention of the positively charged base. The standard deviations of the relative retention of both bases to acenaphthene are around 2%. The relative retention between both bases (Figure 6B) is a measure of access to the surface silanols: one base is a secondary amine and the other a tertiary amine. The standard deviation of this value is 1.2%. The relative retention values between dipropylphthalate and acenaphthene (Figure 6D) and between butylparaben and acenaphthene (Figure 6F) reflect hydrogen bond acceptor and hydrogen bond donor activity of the surface. The first parameter has a standard deviation of 1.5% and the second one a value of 2.0%. The relative retention between the two polar compounds dipropylphthalate and butylparaben (Figure 6A) is a direct measure of the hydrogen bonding capability of the packing (without reference to hydrophobic interaction), and it exhibits a standard deviation of $<0.9\%$ for this packing. It should

also be mentioned that the retention factor of acenaphthene itself has a standard deviation of 2.7%. However, the reproducibility of the selectivity factors is a much better and more important indicator of the reproducibility of a packing than the reproducibility of the retention factors.

III. SPEED AND RESOLUTION

In this section, we will discuss the impact of column length and particle size on the performance of a separation. On first glance, it is already clear that it is not possible to run very fast separations on a long column packed with very small size particles. Conversely, we can also imagine that the separation capability of a short column packed with rather large particles is limited. In the following, we will attempt to shed some light on column performance capabilities in both isocratic and gradient chromatographies.

A. Column Performance in Isocratic Chromatography

The foundation of the views presented here is a series of publications by Martin, Eon, and Guiochon in the mid-1970s.^{40–42} Later publications^{2,43} cover the material in a similar fashion as described here.

In isocratic chromatography, the analysis of column performance is very straightforward, and we can visualize what is happening with little difficulty. Let us assume that we have a 15-cm 5-μm column with an internal diameter of 4.6 mm! We typically will operate this column at a flow rate of 1 mL/min. Now, let us take a standard separation, and let us increase the flow rate. The separation is happening faster and the peaks are narrower in time units, but we also will loose some resolution, since the peaks will be getting a bit wider compared to the distance between the peaks, as we increase the flow. If we do not have a tight pair of peaks in our chromatogram, we can still recognize that the peaks are getting wider with higher flow rate, since they are also getting shorter. At some point, we cannot increase the flow rate any further, because we would have reached the pressure limit of the instrument.

What will happen, if we do the same thing with a 25-cm column, or with a 5-cm column, packed with the same size particles (5 μm)? We will start off with a higher plate count on the 25-cm column, but we will also hit the instrument pressure limit at a lower flow rate than with the 15-cm column. Conversely, the 5-cm column will have a much lower plate count, but we will be able to operate it with three times the flow rate as the 15-cm column. Figure 7 puts these thoughts into a graphic format. Plotted is the square root of the plate count (which is proportional to the resolution of the separation) versus the analysis time for these three columns. We call the y-axis the “resolving power” of a separation. The

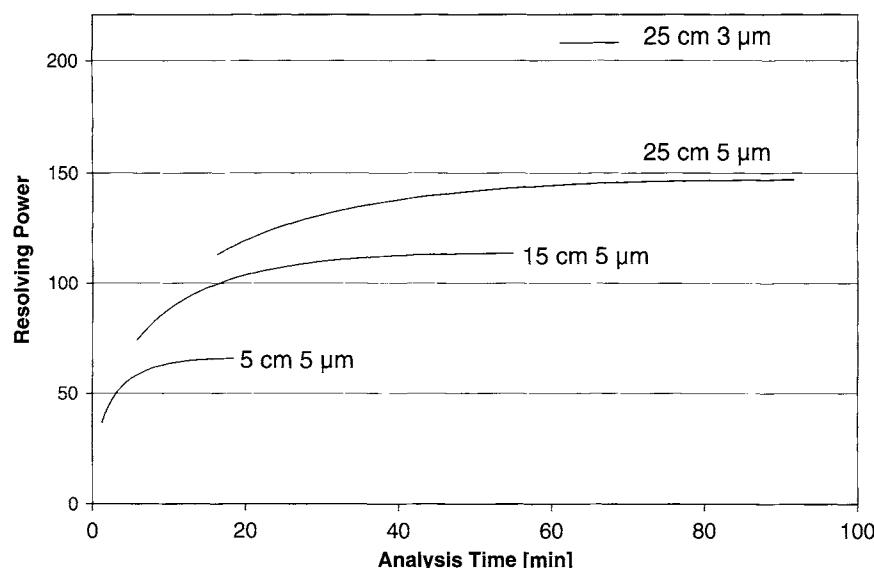


FIGURE 7 Influence of column length and particle size on the resolving power of a separation.

x-axis is the analysis time. We can clearly see the pattern of the change in column performance when we change the column length for the three 5- μm columns.

Also included in Figure 7 is the case, when we try to get even more resolving power out of a column by changing the particle size in the 25-cm column to 3 μm . Indeed, we do get what we had wanted, but the analysis time range over which this column can be used is very limited. If we run too slowly, we loose column performance (longitudinal diffusion takes over). We cannot run any faster, since we quickly reach the pressure limit with the 3- μm particles.

The results in Figure 7 are not unexpected. Longer columns have more resolving power, shorter columns provide a faster separation. Reducing the particle size gains resolving power (for a given column length), but it limits the speed of analysis that can be achieved.

There is another way though to think about column performance. This second way will gain speed and often also separation performance at the same time. Let us now change column length and particle size in proportion to each other! Let us compare the performance patterns of a 25-cm 5- μm , a 15-cm 3- μm , and a 10-cm 2- μm column to each other! In this case, the ratio of column length to particle size remains constant. The performance pattern for this set of columns is shown in Figure 8 up to a pressure of 4000 psi (266 bar). Now, each of these columns has the same maximum resolving power. However, as we decrease the particle size, this maximum

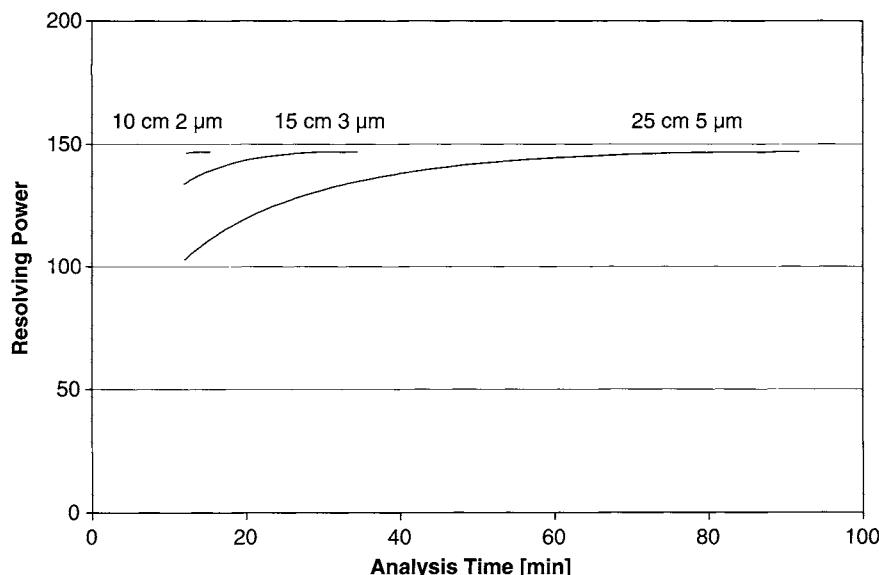


FIGURE 8 Resolving power as a function of analysis time for various columns with a constant ratio of column length to particle size.

resolving power is reached in a shorter analysis time. At the point of maximum column performance, the 3-μm column is about 2.8 times faster than the 5-μm column. In addition, we see that all three columns have the same limiting short analysis time. Given the pressure limit, the 10-cm 2-μm column cannot be operated any faster than the 25-cm 5-μm column, but it reaches the maximum column performance at a very short run time.

We have seen now that the maximum column performance remains constant when we change the column length and the particle size in the same proportion. We also have seen that the fastest analysis that can be performed on columns scaled this way remains constant. The advantage of the shorter column with the smaller particles is only that the resolving power at or close to the speed limit is higher for the smaller particles.

How do we get then to a faster analysis? We have to reduce the ratio of column length to particle size. Alternatively, if we want to maintain maximum column performance, we need to go to an instrument that can provide a higher pressure. We cover the latter aspects in Section III.C.

Let us see what happens when we choose another ratio of column length to particle size! In Figure 9, we examine a 5-cm 5-μm, a 3-cm 3-μm, and a 2-cm 2-μm column. Once again we see that all three columns have the same maximum plate count, all three columns can achieve the same shortest analysis time. The maximum resolving power is a little bit less than one half of what was achievable with the five times longer columns shown in Figure 8. But the speed is much higher: the fastest analysis at a

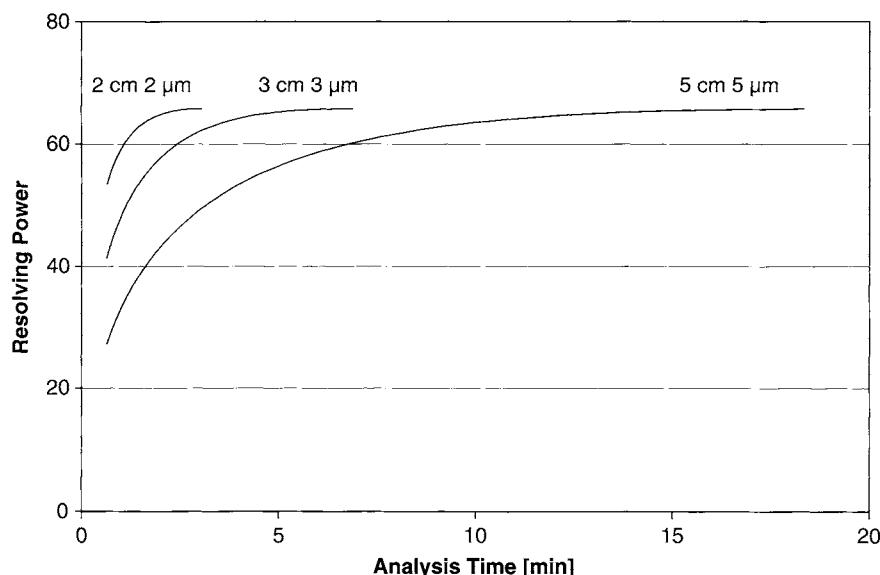


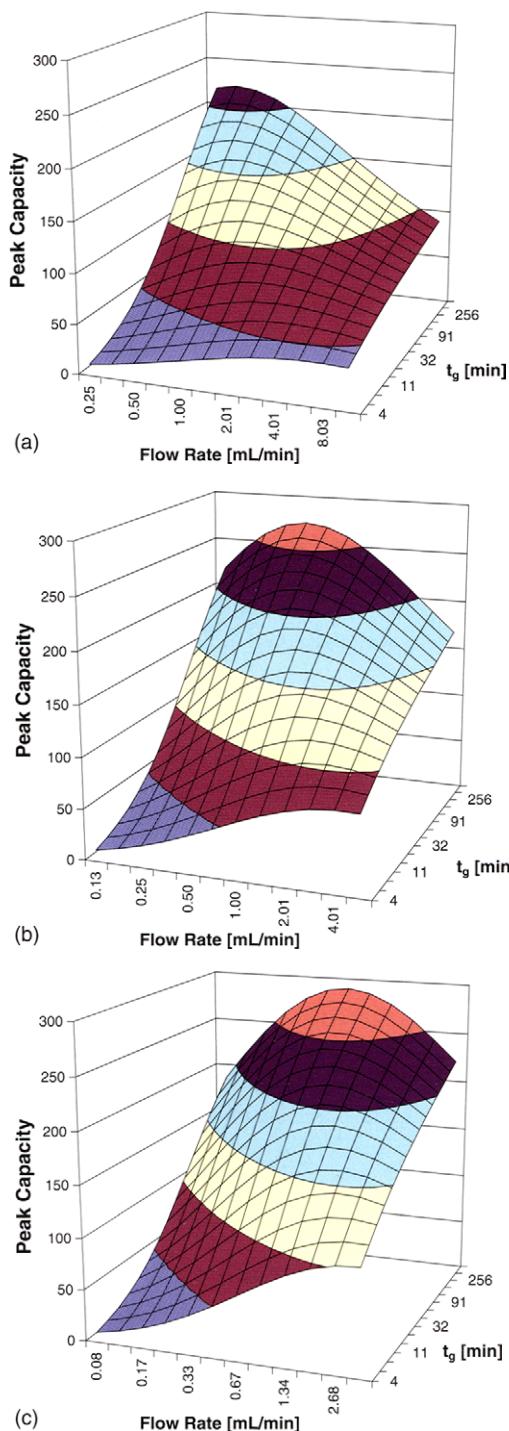
FIGURE 9 Resolving power as a function of analysis time for various short columns with a constant ratio of column length to particle size.

pressure of 3000 psi (200 bar) is just around 40 s for our standard analysis to a retention factor of 10. Of course, the smaller particle sizes always outperform the larger particles. Quite reasonable analyses can be performed in less than 2 min with either the 3-μm or the 2-μm columns. Obviously, the focus of the use of columns with a small ratio of column length to particle size is the speed of the analysis, not the maximum separation power.

Before we conclude this section, it should be mentioned that the exact speed values that can be achieved depend on the viscosity of the mobile phase. In our examples, we assumed a viscosity of 1 cP, the value for water. If one is using a methanol–water mobile phase, the speed will be a bit slower, whereas for acetonitrile-based mobile phases, it will be a bit higher. However, in these comparisons it was more important to understand the influence of the column parameters such as column length and particle size.

B. Column Performance in Gradient Chromatography

The same principles that we have just applied to isocratic chromatography also apply to gradients. Despite the fact that the use of gradients complicates the picture a bit, the overall rules are similar to what we have seen in isocratic chromatography. We should use a fixed ratio of column length to particle size for the column comparisons in the same



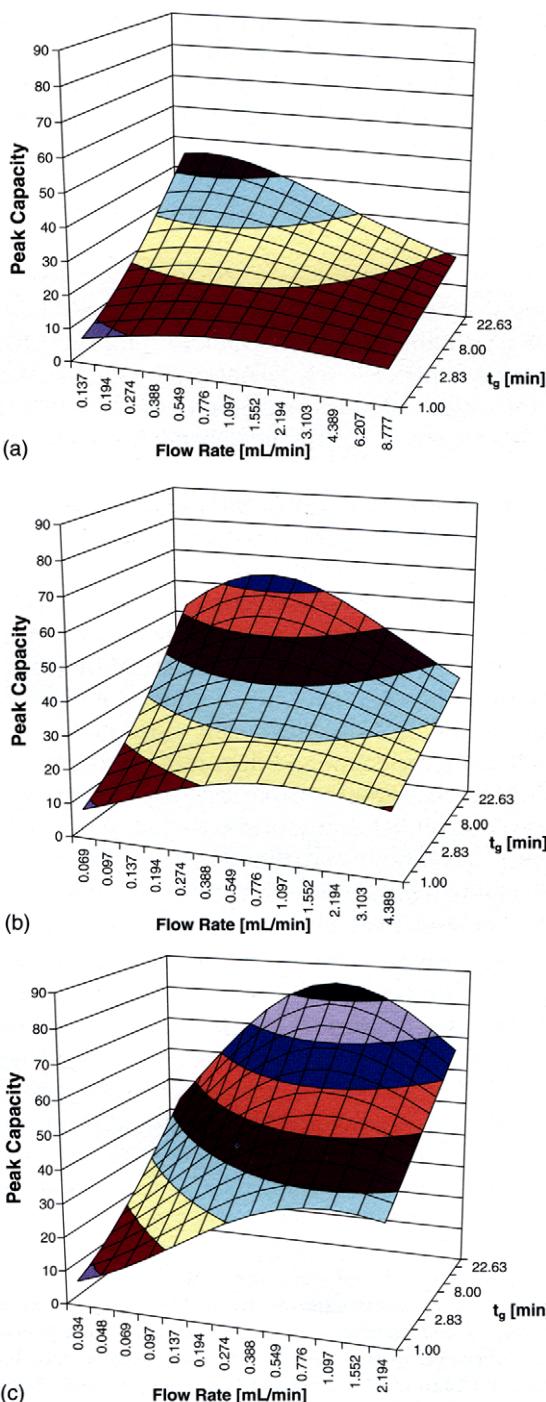
way as we have done in isocratic chromatography. We will assume a fixed gradient from 0% to 100% organic for all the comparisons. In order to measure the separation performance of the gradient, we use the peak capacity, defined as the gradient run time t_g divided by the average peak width w in the gradient:

$$P_c = 1 + \frac{t_g}{w} \quad (3)$$

This is simply a measure of how many peaks could be eluted with a nearly baseline separation in a given gradient run time. Other measures for judging the performance of a gradient can be used as well (e.g. reference 44), but the overall findings remain essentially unchanged.

In Figure 10, we have plotted the dependence of the peak capacity on the operational parameters flow rate and gradient run time for three different columns. Before discussing the details of the graphs, let us examine first the nature of the plot. Note that both the flow rate axis and the axis of the gradient duration are logarithmic. This permits a display of the column performance over a wide range of operating conditions. First, let us look at the conditions of a very long gradient run time, which was 512 min (=approximately 8 h) at the back end of the graphs. Under these circumstances, the peak capacity exhibits a maximum. The location of the maximum is at a slow flow rate, close to the optimum of the relationship between plate count and flow rate in isocratic chromatography. As we shorten the gradient run time, the maximum of the peak capacity moves to higher velocities. It also becomes lower. Generally, we obtain maximum resolution, and therefore the best peak capacity, at slow flow rates. However, an analysis time of 8 h or so is not practical. Most gradient analyses are carried out with run times less than 1 h, often even much faster than this. A 30-min analysis is pretty much in the center of the graphs. The flow rate at which the maximum of the peak capacity occurs for this gradient run time is around 1 mL/min for a column with an internal diameter of 3.9 mm. This is in good agreement with the standard operating practices in many labs: gradient run time about 30 min, flow rate about 1 mL/min. The graphs stop on the right at the flow rate where we reach the pressure limit of the instrumentation. In our calculations, this limit was assumed to be around 6000 psi (400 bar).

FIGURE 10 Peak capacity as a function of the flow rate and the gradient duration for three columns with an internal diameter of 3.9 mm. The logarithmic axis for the gradient duration t_g is same for all three graphs and ranges from 4 to 512 min. The logarithmic axis for the flow rate varies from graph to graph. (a) Column: 300 mm × 3.9 mm, 10 μm ; flow rate from 0.25 to 11.35 mL/min. (b) Column: 150 mm × 3.9 mm, 5 μm ; flow rate from 0.125 to 5.67 mL/min. (c) Column: 100 mm × 3.9 mm, 3.5 μm ; flow rate from 0.08 to 3.78 mL/min.



Now that we have oriented ourselves in the graphs, let us examine them in more detail. In all three graphs, the maximum of the peak capacity at a particular gradient run time moves to a higher flow rate as the gradient run time becomes shorter. The reason for this observation is simple. If we reduce the gradient run time at a particular flow rate, the gradient volume becomes smaller, and we lose resolution. If we want to compensate for this, we need to increase the flow rate. The increase in flow rate brings with it a reduction in column plate count. Therefore, a reduction in run time will always be accompanied by a reduction in peak capacity, but it is not that bad if we increase the flow rate at the same time.

Let us now compare the three graphs in Figure 10 to each other! Figure 10a shows the gradient performance of a 30-cm 10- μm column. Figure 10b represents a 15-cm 5- μm column, and Figure 10c a 10-cm 3.5- μm column. We see that the performance at very long run times improves substantially from the 10- μm column to the 5- μm column, but the increase in peak capacity at the longest run time is only small going from the 5- μm column to the 3- μm column. This is in agreement with the experience shown above for isocratic chromatography: there is a gain in speed, when we reduce the column length together with the particle size, but for the longer run times, the gain in performance is small. However, when we look at the front of the graph, at quick gradient times as fast as 4 min, the advantage of the shorter column packed with smaller particles is substantial (about 30% improvement in peak capacity). At the same time, we are operating close to the selected pressure limit. However, if we compare the gain in speed for equal performance, we can obtain the same peak capacity with the 3.5- μm column in about 4 min as was possible with the 10- μm column in about 30 min.

The smaller particles provide a gain in speed, even in gradient separations. This gain is more pronounced, if we select shorter columns. This is shown in Figure 11, where we compare the peak capacities for 2.1-mm columns of a shorter length. Here we compare 10-cm 10- μm , 5-cm 5- μm , and 2.5-cm 2.5- μm columns. The fastest analysis time considered is a 1-min analysis. The 10- μm column only delivers a peak capacity of around 13, completely inadequate for a real analysis. The 5- μm column reaches a peak capacity of 22. Only the 2.5- μm column reaches an adequate peak capacity, i.e. 37, for this fast analysis. The flow rate is also fairly high for such a short column: 1 mL/min, in agreement with data for fast gradients reported in the literature.^{45,46}

FIGURE 11 Peak capacity as a function of the flow rate and the gradient duration for three fast columns with an internal diameter of 2.1 mm. The logarithmic axis for the gradient duration t_g is same for all three graphs and ranges from 1 to 32 min. The logarithmic axis for the flow rate varies from graph to graph. (a) Column: 100 mm \times 2.1 mm, 10 μm ; flow rate from 0.14 to 8.78 mL/min. (b) Column: 50 mm \times 2.1 mm, 5 μm ; flow rate from 0.07 to 4.39 mL/min. (c) Column: 25 mm \times 2.1 mm, 2.5 μm ; flow rate from 0.03 to 2.19 mL/min.

C. The Need for Pressure

It is clear that smaller particles are of advantage if one wants to achieve a reasonably high performance at very short analyses times. At the same time, the pressure required to operate a column increases drastically. At a given flow rate, the pressure grows inversely to the square of the particle size. However, the flow rate needed to reach the optimum performance of columns packed with small particles also increases, as the particle size decreases. The consequence of both facts is that the pressure to operate a column under the best conditions increases with the inverse of the third power of the particle size. If we had been comfortable at 750 psi (50 bar) with 3.5- μm particles, we will need 6000 psi (400 bar) to operate the same column packed with 1.7- μm particles at the optimum flow. On the other hand, the gain in speed and performance is very much worth the effort. An example of an isocratic separation that takes advantage of the smaller particles at a constant column length is shown in Figure 12. The measured gain in resolution was 1.5-fold, while the separation was 2.6 times faster. The consequence was

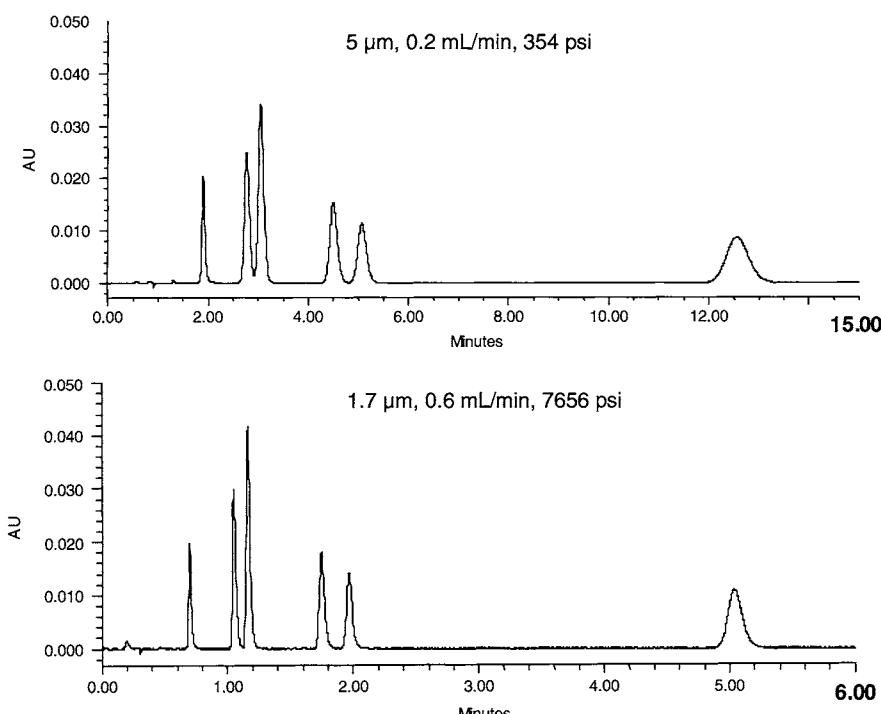


FIGURE 12 Improvement in a separation by reduction of the particle size at a constant column length of 5 cm. Columns: 50 mm \times 2.1 mm. Top: 5 μm . Bottom: 1.7 μm . Chromatogram courtesy of Eric S. Grumbach, Waters Corporation.

a roughly 22-fold increase in pressure. All of these figures are in close agreement with the theoretical expectations. There is no question of the gain in performance from the very small particles. There is also no question that the cost of this improvement is pressure.

Similar analyses can also be made if the column length is reduced with the particle size, as we have done above. We have shown there that under these circumstances, the performance optimum changes to shorter and shorter run times, as the particle size is reduced. If we continue to do this, we will at some point reach this performance maximum at the pressure limit of the instrument. The only way to further continue on this path towards increased performance is to increase the available pressure.

Small particles are also of advantage for separations that require a very large horsepower in a reasonable time. The first examples of the use of very high pressure by Jorgenson and co-workers focused on maximizing column plate count.⁴⁷ In a later publication, the benefit of very small particles was demonstrated also for gradient applications.⁴⁸ In more recent times, the instrumentation to execute separations that require a higher pressure and the columns needed for high-power separations have become commercially available.⁴⁹ At the time of this writing, a typical particle size for ultra-performance LC applications is 1.7 µm (as in Figure 12) with column lengths up to 15 cm.

An example of an analysis that requires a very high separation power is metabolite profiling in biofluids, i.e. the science of metabolomics.⁵⁰ Also, impurity profiling does benefit from the improvement in separation power. Finally, further reduction of interferences in the LC/MS analysis of drugs in biofluids is possible by increasing the peak capacity of the chromatographic separation. The improved separation power results in fewer coelutions for each peak. Fewer interferences mean less ion suppression, which was the surprising side benefit of the performance increase due to small particles when complex separations were performed with MS detection.

IV. SPECIALTY COLUMNS FROM NANOBORE TO PREPARATIVE CHROMATOGRAPHY

In this section, we will briefly touch the subject of columns with both a wider and a smaller internal diameter than commonly used in HPLC. The technology of column preparation has not stood still at these fringes of standard column technology. However, special equipment is required to use these columns. For the proper use of preparative columns, pumps with higher flow rates and injectors with a larger injection volume are required. For the use of columns with a very small diameter, the main technological difficulty is the design of sensitive detectors with very small extra-column band spreading.

A. Preparative Columns

Preparative chromatography can be carried out at many different levels. The preparation of milligram quantities of purified substances from crude synthesis product has become commonplace in the pharmaceutical industry. Often, open-access systems are available that allow an automated purification of the crude product with pre-established chromatographic methods.

The packings used in an open-access preparative system are identical to the ones used for chromatographic analysis. The commonly used particle size is 5 µm, and the common column length is 50 mm, but longer lengths are used as well. Common column diameters range from 7.8 to 30 mm.

The loadability of a column can be estimated by the rule of thumb that column overload will deteriorate a separation if more than 1 mg of sample per 1 g of packing is injected. Another good rule of thumb is that a column contains about 0.75 g of packing per milliliter of column volume. Table 1 gives an estimate of how much sample can be injected on columns of various dimensions before significant overload occurs. Of course, these values need to be taken as guidelines, since for example an overload of the mobile phase buffer or the influence of the sample solvent can result in peak distortion at much smaller injections. A special injection technique, at-column dilution, can be employed to get around early peak distortion due to the sample solvent.⁵¹ In this technique, the sample is injected onto the column in a solvent stream that is compatible with the sample, and this solvent stream is converted just in front of the column to the mobile phase composition required to get good retention and loadability in the preparative chromatography.

TABLE I Proposed Operating Conditions and Load for Various Column Dimensions

I.d. (cm)	L (cm)	Flow rate (mL/min)	Load (g)
0.46	5	0.5	0.5–1
0.46	15	2	0.5–1
0.78	5	2	1.5–3
1	5	3	2.5–5
1	10	6	2.5–5
1	15	9	2.5–5
2	5	12	10–20
2	10	25	10–20
2	15	35	10–20
3	5	25	25–45
3	10	55	25–45
3	15	80	25–45

Most preparative columns have some special hardware to stabilize the packed bed. Common techniques used for this purpose are various forms of axial or radial compression. Column stability is improved by increasing the packed bed density. Columns of this type have demonstrably a more predictable lifetime than columns without such features.

B. From Microbore to Nanobore

Columns with diameters of about 4 mm continue to be the most commonly used columns in HPLC. Due to the high linear velocities needed for fast analysis, 2.1-mm columns are often used for very rapid applications. Recently, 2.1- and 1-mm columns have been used for analyses requiring a higher pressure, since the heat generated from the pressure can be dissipated better with a smaller column diameter. Smaller volume columns also generate a higher detector response for equal sample amounts injected, which is of advantage in the analysis of small sample quantities. Thus, there are advantages in the use of smaller diameter columns.

There could be several reasons for the use of columns with a smaller internal diameter. Some MS detectors work best at flow rates lower than standard HPLC flow rates. Fast separations need to be carried out at a high linear velocity. This is best achieved by using a smaller column diameter. In this case, the diameter is best chosen to be around 2 mm. Solvent consumption in a high-throughput lab can be of concern. Under these circumstances, the use of a 3-mm column is often the best solution, since the standard HPLC equipment can be used with little modification. A modern low-dispersion system with a small gradient delay volume is needed for the successful implementation of 3-mm columns.

Another important reason for the use of smaller diameter columns is a limit in the available sample volume. However, as the column diameter is reduced, the peak volumes become smaller as well. The smaller peak volumes require a smaller detector volume (or better, a lower post-column band spreading). If this is not done, the extra-column band spreading can deteriorate the separation. At the same time, a smaller detector volume often goes along with a reduced sensitivity of the detector. Thus, one needs to carefully think which column volumes go along with which detector volumes to not deteriorate the separation power of the columns by too much or compromise detector sensitivity by too much. Part of this thought process should also be the consideration of a sample enrichment on the column by dissolving the sample in a solvent composition that is a weaker eluent than the mobile phase. This permits a larger injection volume, and thus a larger sensitivity, i.e. better limits of detection and quantitation.

There is a reasonable rule of thumb (for isocratic chromatography) that helps in the choice of the right column for the right detector or vice versa: the standard deviation of the system band spreading should not

exceed 1% of the retention volume of the first peak of interest in the chromatogram. For a 15 cm × 4.6 mm column with 10,000 plates, this means that the system band spreading should not exceed 16 µL if the unretained peak is important. Conversely, if only retained peaks are important, a 3-mm column with the same performance can be run on the same system. For a column with an internal diameter of 100 µm, a system band spreading not exceeding 25 nL is desirable. However, these are rough guidelines that should be checked against the performance of the actual column/detector combination.

V. SUMMARY

In this chapter, we have described HPLC columns and packings. The first part deals with the chemistry of the packings, covering both the surface chemistry as well as the solid support matrix. This is followed by a discussion of the physical properties of the columns, i.e. column dimensions and particle sizes. Finally, a section has been devoted to unusual column dimensions. In all cases, special attention has been paid to items that we feel are important for HPLC users in the pharmaceutical industry.

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