SELECTING HPLC COLUMNS:

GOING UNDER THE HOOD

HPLC Column Selection Guide

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Introduction

With hundreds of stationary phases and columns commercially available in the market, it can be difficult to select the right HPLC column for your application. We are here to help. This guide provides direction on column selection and helps assist you through the development of your methods.

At the end of this article you will find a concrete and useful application of theoretical and scientific work transformed into a practical tool enabling chromatographers to find the right HPLC column easily and in just a few seconds.
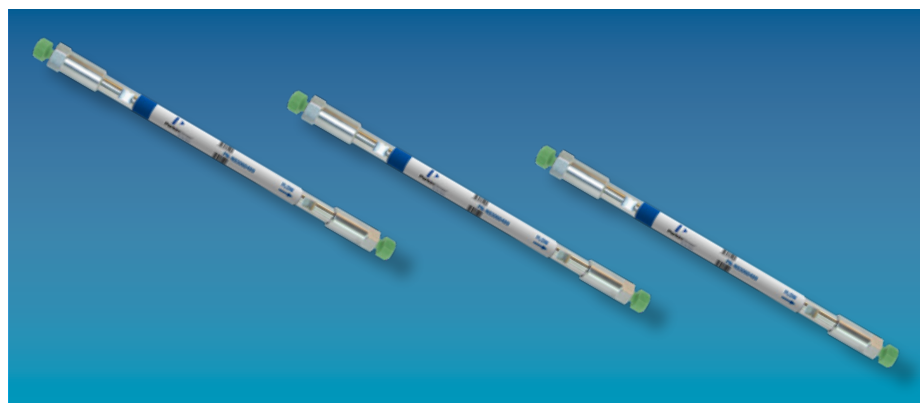
Chromatography Fundamentals

Retention Factor

The retention factor (k) described in Equation 1 and in Figure 1 is a thermodynamic parameter, independent of some key variable factors including flow rate and column dimensions. Because of this, it is a useful parameter when comparing the retention of various solutes obtained using different HPLC systems in isocratic mode.

$$k_A = \frac{t_{R_A} - t_o}{t_o}$$

Equation 1. With t_{R_A} the retention time of the analyte A, and t_o the dead time.
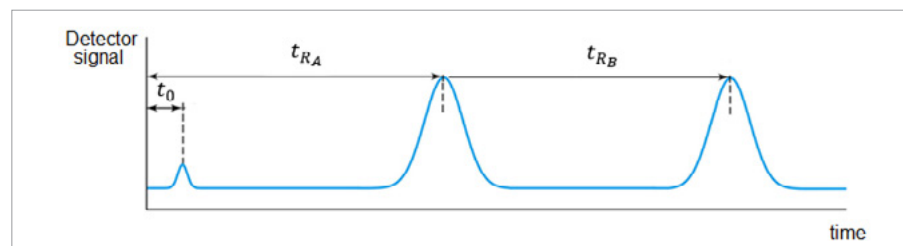

Figure 1. Retention in chromatography.

Chromatographers like keeping retention factor values between 2 and 10 for optimized separations. The most effective and convenient way to alter the retention factor is to adjust the solvent strength of the mobile phase, typically modifying the amount of organic solvent in the mobile phase mixture.

Selectivity Factor

The selectivity factor (a) described in Equation 2 is another thermodynamic parameter, and corresponds to the ability of the chromatographic system to distinguish two successive eluted components.

$$a_{A,B} = \frac{kB}{kA}$$

Equation 2.

The selectivity starts at one for co-eluted compounds, while high values indicate better separating power. This factor depends on the system analyte, mobile phase, and stationary phase for a given analysis temperature; and all these parameters may be altered tooptimize or change the HPLC separation. Altering the system selectivity is a good strategy to optimize the resolution, since small changes in selectivity can lead to large changes in resolution.

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For a specific application with a specific column, changes in the mobile phase can be applied to change the selectivity:

- Water content to change the solvent strength
- Organic modifiers to change the type of interactions
- pH value, which alters the degree of ionization of some analytes affecting their hydrophobicity
- Solvent additives

Also, the temperature has to be considered as it drastically alters the selectivity for some pairs of analytes.

Last, but not least, one of the most efficient ways to change the selectivity is to change the stationary phase to another chemistry or material "molecular" structure.

Efficiency

The efficiency of a peak is a kinetic parameter measuring the dispersion of the analyte band during the path through the HPLC system and column. This dispersion depends on the system analyte, mobile phase, and stationary phase for a given analysis temperature. The chromatographic parameter reflecting the column performance is the number of plates (N) and is described on the Equation 3:

$$N = 5.54 \times \left(\frac{t_R}{W_{1/2}} \right)^2 = \frac{L}{H}$$

Equation 2. Plate number. With W ½ the half-height peak width, H the height equivalent to a theoretical plate, and L the column length.

The model proposed in "Partition Chromatography" by J.P. Martin and Richard L.M. Synge relies on the separation of the column into plates. Each plate represents the distance over which the analyte achieves one equilibration between the stationary phase and the mobile phase. Consequently, the more plates available, the more equilibria possible, and the better the quality of the separation.

Many factors contribute to the peak broadening, but considering an optimized system with negligible extra-column volume, the biggest contribution comes from the column itself. The quality of the column packing, as well as the length of the column, but also both the particle size dispersion and dimension, play an important role in the verall efficiency.

The diffusion of analytes through the column is represented by the Van Deemter curve. The effect of the mobile phase velocity on the height equivalent to a theoretical plate (H) is linked to the efficiency and the column length by the equation in Equation 4 and Figure 2:

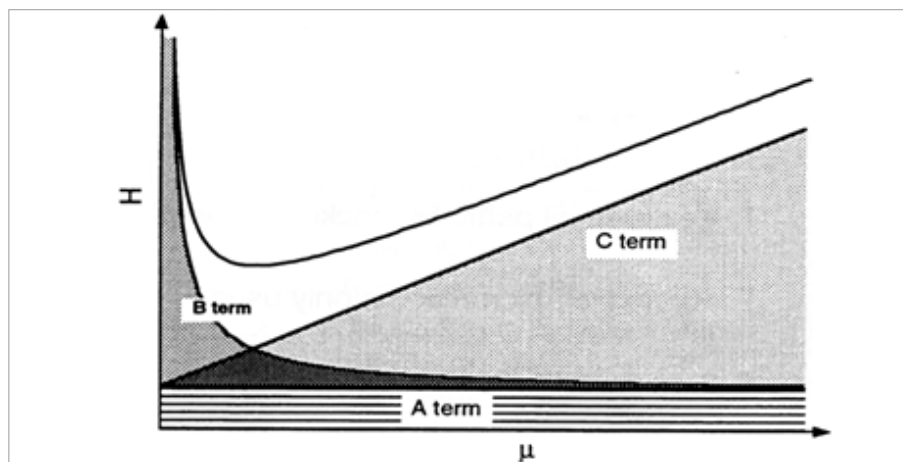$$H = A + \frac{B}{\mu} + C \times \mu$$

Equation 4.

Figure 2. Van Deemter curve.

A-factor is the convective dispersion or streamline-splitting (eddy-diffusion) parameter, related to channeling through a non-ideal packing. It can be minimized by packing columns homogeneously with silica particles of controlled granulometry.

Also, the nature and the size of the particles is very important for column efficiency. Smaller particles lead to higher efficiency and does so employing superficially porous particles (SPP) instead of totally porous particles.

B-factor is the longitudinal molecular diffusion coefficient of the eluting analytes in the longitudinal direction. It can be minimized by working at higher flow rates, by using lower inner-diameter tubing and connections, and using a organic modifier of lower viscosity.

C-factor is the resistance to the mass transfer of the analyte in the immobilized mobile phase and in the stationary phase. It can be minimized by using particles of a smaller diameter, superficially porous instead of totally porous particles, working with a lower flow rate, and higher temperatures.

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Resolution

Obtaining the optimal resolution in the least amount of time is a challenge for chromatographers. A value of 1.5 or more between two peaks of approximately the same magnitude ensures a convenient baseline separation. As demonstrated by the fundamental in Equation 5, the resolution (R_s) is affected by three important parameters: selectivity, efficiency, and retention.

$$R_{S_{A,B}} = \frac{\sqrt{N_B}}{4} \times \frac{a_{A,B} - 1}{a_{A,B}} \times \frac{k_B}{k_{B+1}}$$

Equation 5.

Peak Asymmetry

Ideally, all chromatographic peaks should be symmetrical (Gaussian). However, due to the effects of instrument extra-volumes, absorptive effects of the stationary phase, and the quality of the column packing, peaks are often distorted. Asymmetrical peaks lead to a loss of resolution, making quantification tricky. The asymmetry factor is estimated at 10% of the peak height. The tailing factor is estimated at 5% of the peak height. These two parameters are calculated according to Equation 5 and Figure 3.

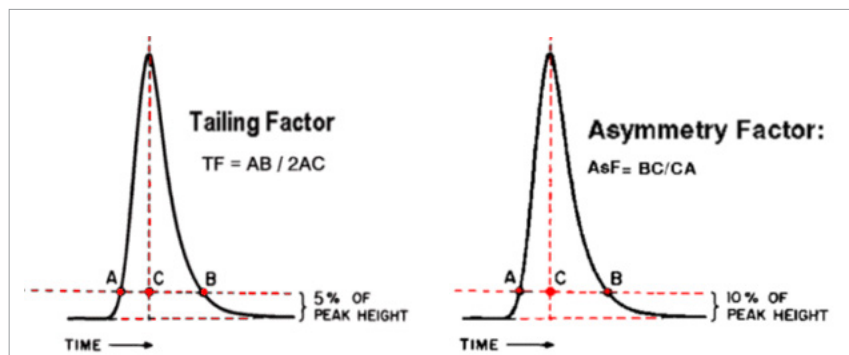$$As = \frac{BC}{AC} \quad and \quad TF = \frac{AB}{2AC}$$

Equation 6.

Figure 3. Tailing peak and asymmetry factor.

Standard limits are set for peak asymmetry. An asymmetry value between 1-1.05 (1<TF<1.033) is considered excellent, and up to 1.5 (corresponding to TF = 1.33) is considered acceptable.

Columns and Mobile Phases...Where Chemistry Meets Silica

Pore Size Dimensions

The choice of pore size is of crucial interest when developing a method for a class of compounds. Selecting a column based on silica particles with small or standard pore sizes (around 60 to 120 Å) to analyze large molecules (over 3000 g.mol-1) will result in size exclusion. Columns packed with 300 Å pore size particles are recommended for biomolecule analysis.

Particle Size Dimensions

The typical particle size for HPLC columns is 3 µm or 5 µm, but smaller diameters have grown in popularity. If high-speed or higher resolution analyses are required, columns containing 1.8 µm or 2-3 µm particles can be used. Shorter columns with these particles can produce faster separations, but longer columns are required for higher efficiency. Longer columns packed with smaller particles highly improve the global efficiency, but also dramatically increase back-pressure, making this procedure only possible with UHPLC instrumentation.

Column Dimensions

Choosing the dimension of a column is also essential and has dramatically changed in the past few years. While 4.6 mm id was largely used in method development, smaller 3.0 mm id or 2.1 mm id columns tend have become more common, due to the lower solvent use and the better MS detector compatibility.

The choice of the length of the column depends on the resolution needed, therefore, on the efficiency: shorter 50, 75 or 100 mm columns ensure fast analyses with a low solvent consumption. Longer columns (150 or 250 mm) are used when higher resolution is needed or when 3 and 5 µm particles are used. It is necessary to find the best match between efficiency and instrument performance capabilities. The columns packed with smaller particles are much more efficient, but cause higher back-pressure. Table 1 helps to find which combination of parameters can be used to obtain an equivalent efficiency.

Table 1. Effect of column dimensions and totally porous particles on efficiency.

Length (mm)	Column Efficiency for Different Particle Size (µm)			Analysis Time Saving
	5.0	3.5	1.8	
150	12,500	21,000	35,000	-
100	8,500	14,000	23,250	33%
75	6,000	10,500	17,500	50%
50	4,200	7,000	12,000	67%

pH and Mobile Phase: Solvent-Strength Selectivity and Solvent-Type Selectivity

Once the column has been selected, the mobile phase choice drives the separation, it is therefore, important to control the nature and the amount of the organic modifier.

Acetonitrile and methanol are the most commonly used organic modifiers. However, selectivity differences and sample retention will vary significantly among mobile phases containing acetonitrile, methanol, and tetrahydrofuran. The choice is determined also by the solubility of the sample and the need for UV detection at certain wavelengths (e.g., methanol is not suitable at 200 nm).

The pH and ionic strength of the aqueous portion of the mobile phase are important parameters to control in order to develop robust methods that are not sensitive to small variations in conditions. This is particularly the case with ionic compounds for which the retention is highly affected by pH. A pH between 2 and 4 is generally a great starting choice to stabilize the retention and the resolution of typical weak acids. For basic solutes a pH value between 7 and 8 is often selected, but requires using a buffered aqueous solution.

Stationary Phase Chemistries

Most of the time, the base material is high purity silica (Type-B) with totally porous particles. The content of the metal impurities has to be as low as possible to avoid strong interactions caused by metal in the silica matrix or metals adjacent to the surface. From a chromatographic point of view, the strongly interacting sites considerably broaden the chromatographic band, since they interact much more than "normal" adsorption centers.[1]

The quality of the silica plays a key role in column performance. Minimizing or eliminating contaminants great improves results. One of the main contaminants is metal ions and these ionic groups form great ion-exchange sites for ionized acids, results in badly tailing peaks for acidic solutes. Moreover, if the metals are adjacent to silanol groups, they withdraw electrons from the adjacent silanol and enhance its ionization, especially at high pH. These activated silanols act as cation-exchange sites for ionized bases, leading to strong retention and tailing peaks for basic solutes. These low-purity silicas correspond to **Type-A**, known today as acidic silicas.

Tremendous efforts have been made to enhance the purity of the silica material and the homogeneity of the particles. Today, the most commonly used is the **Type-B** silica, based on high purity-low metal content, spherical shape with narrow pore size distribution and high mechanical strength. As for the Type-A silica, the silanol groups can be functionalized, providing a wide range of column chemistries (e.g. C18, C8, CN…).

The **Type-C** silica support is structurally different as the surface is populated with silicon-hydride groups (-Si-H groups) which are very stable and relatively non-polar. It still has all the advantages of Type-B silica, but also other advantages. It can be used for three different modes of HPLC and occasionally two at the same time, improving the selectivity power. When using Type-C columns with the right choice of mobile phase, polar and non-polar compounds can be separated in the same isocratic run.

Reversed Phase Chemistries

The presence of silanol groups on the silica surface allows for the functionalization, and various chemistries have been developed offering a wide range of columns. Polymer materials present a wide range of pH stability and unique separation performances; but monomeric phases are more extensively used as it ensures a better reproducibility of the functionalized silica batch products.

In a great many cases method development begins with octyl (C8) or more frequently octadecyl (C18) stationary phases. Very often the recommended starting column choice is C18 RP, as it presents high hydrophobic properties and low amount of residual silanols thanks to an effective end-capping step. Therefore, it provides excellent peak shape and can be used over the pH range 2-9, accommodating most typical LC and LC/MS mobile phases.

If the solutes are more polar or need to be analyzed with water as a mobile phase, the C18 AQ is a good alternative as the polar groups incorporated in the stationary phase structure allow the analysis in 100% water without the collapse of the bonded chains.

If the separation is not optimal on these columns, CN and Phenyl columns may offer significant differences in selectivity from straight-chain alkyl phases to affect the separation.

Generally, the separation of larger solutes such as proteins is more efficient on short-chain reversed-phase columns (C3, C4, CN, C8); while peptides and small molecules are better separated on longer-chain columns (C18). However, there are many cases where this conventional method does not apply. It is better to begin with a phase of median hydrophobicity (e.g., C8), and change to a more hydrophobic phase or a more hydrophilic phase depending on the results obtained, and the solubility of the sample.

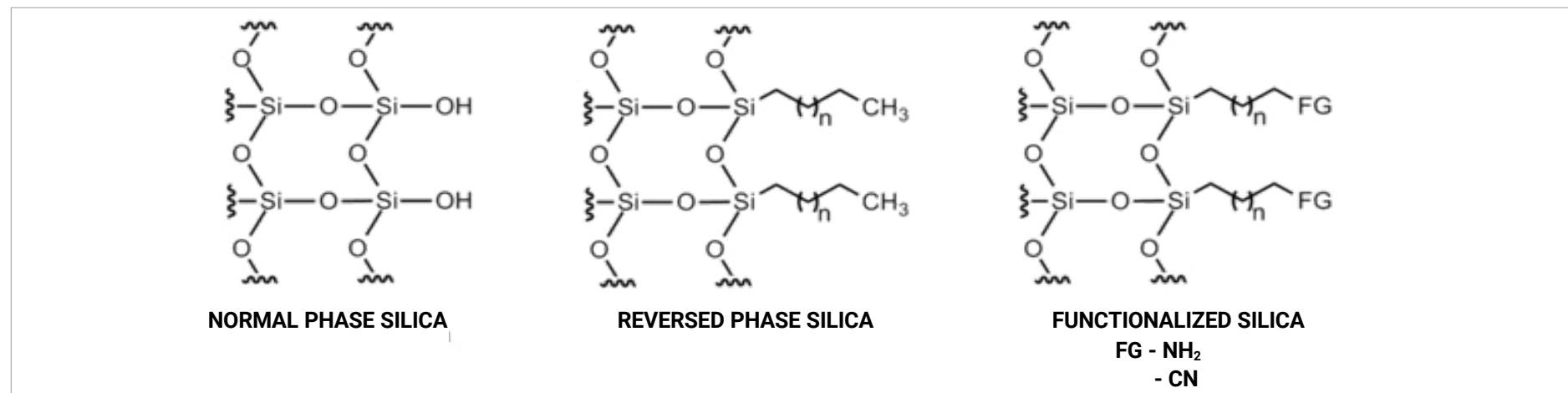| NORMAL PHASE SILICA | REVERSED PHASE SILICA | FUNCTIONALIZED SILICA
FG - NH$_2$
- CN |

Figure 4. Structural representation of some stationary phase chemistries.[2]

Characterization Of C18 Phases-Chromatographic Tests

Column manufacturers are usually willing to publish chemical and physical properties, like the bonding technology, or the composition of their column chemistries.

Some basic data such as the specific surface area (in $m^2.g^{-1}$), the particle diameter (in µm), the content of metal impurities and the carbon load (in %) may be specified. From these data, the bonding density can be calculated (in $mol.m^{-2}$) with the Equation 7:

$$\tau\ (\mu\,mol.m^{-2}) = \frac{10^6 \times P_c}{10^2 \times M_c \times n_c - P_c \times (M_w - 1)] \times s_{BET}}$$

Equation 7.

Where p_c is the carbon percentage per weight of the bonded material, M_c the atomic weight of carbon, M_w the molecular weight of the grafted molecule, n_c the total number of carbon atoms in the bonded organic group, and S_{BET} the specific surface area (m².g-1 of silica). Given that the carbon percentage includes both the bonded chains and the silanol end-capping groups, this value is more or less indicative.

Nevertheless, these parameters do not provide a full picture on phase properties and, therefore, replacing a C18 column with another C18, purely based on chemical and physical similarities, can be very challenging.

Chromatographic measurements can be achieved to complete these data, and various tests have been developed. Generally, the evaluation relies on the determination of the hydrophobicity (also called hydrophobic selectivity), the shape selectivity (also called steric selectivity), the hydrogen bond capacity (also called silanol activity), the ion exchange

capacity, and finally the metal content impurities. Chromatographic data based on the chromatographic peaks are also obtained assessing the asymmetry and the number of theoretical plates. To summarize, standard chromatographic values of interest are mainly the retention factor (k), the selectivity factor between appropriate solutes, and the peak asymmetry factor (AsF).

Even if different chromatographic tests have been developed to characterize LC stationary phases, none of these tests provides an exhaustive view of the potential power of separation of a stationary phase relative to all compound families to analyze.

Multiple goals are involved in the characterization of columns. First, obtaining a quantitative understanding of the specific characteristics of a column that impact the selectivity of a separation. This is helpful to rationalize the selection of a suitable stationary phase for a particular analysis, or for the selection of an alternative column when the first one did not result in a successful separation. Moreover, having a better knowledge of what differentiates one stationary phase from another is useful for screening in method development; when it is beneficial to maximize the differences between the columns and the mobile phases. Finally, one can be interested in understanding the interactions between the solutes, the mobile phase, and the stationary phase driving the separation.

Several reviews such as the one proposed by Neue[3] focus on stationary phase characterization. Significant work was performed by Snyder *et al.*, who characterized a huge number of columns and demonstrated the usefulness of a ranking scale.

The Tanaka test is a widely used test for characterization in reversed-phase liquid chromatography, especially C18. Analytes are widely commercially available, and not costly. Even if it does not properly assess all the properties of stationary phases, it remains a widely used comparative technique. It allows for a minimal evaluation of separation of ionisable compounds.

The Tchapla test based on the analysis of polymethylphenols, partially evaluates the hydrophobic selectivity. The Sander and Wise test furnishes a partial and complementary evaluation of the steric selectivity.

The Engelhardt *et al.* test partially evaluates the polar selectivity, while the Lesellier and Tchapla classification are interested in polar and steric selectivity.

Finally, a more specific test for evaluating stationary phases for analysis of basic compound has been proposed by Stella, Rudaz and Veuthey.

In 2002, Visky *et al.* reported 36 different tests evaluating these properties, but other tests have been elaborated by Neue and Layne.

Silica bonded with hydrophobic chains as C18 or C8 can establish dispersion interactions with analytes. The hydrophobicity depends on the chain length, the bonding density and the specific surface area of silica. For high-density bonded silica, the pore diameter may be an additional parameter.

The hydrophobic selectivity consists in determining the retention variation caused by the addition of a methylene group in the analyte structure.

The shape selectivity evaluates the ability of the stationary phase to differentiate analytes having the same elemental composition, but a different three-dimensional structure or configuration, as is the case for isomers. When the planarity, or the stretched configuration, of the molecule is affected, the penetration in the stationary phase may differ. The true structure of the bonded chain impacts the steric selectivity, especially the nature of stationary phase (monomeric or polymeric), the length of the alkyl chain (C8 to C30), and the nature of the organic modifier and the temperature. Also, the higher the bonding density, the more probable to have size exclusion.

The silanol activity includes the hydrogen bonding between acidic and basic solutes and neutral silanol groups, and ionic interactions between ionized basic solutes and ionized silanol groups. This parameter is directly linked to the amount and to the nature of available silanols, especially their acidic character, which is determining for the pH range of ionization. Indeed, different types of silanol exist onto the silica surface (single, geminal, and vicinal), showing different activities. It should be noted that the purity of silica is also of interest, as the presence of metallic impurities tends to increase the silanol acidity, and consequently, the secondary interactions are not suitable for the peak symmetry.

Finally, to compare the stationary phases, different parameters can be used, and different representations are proposed. If one property is taken into account, a histogram is enough; if two properties are considered, a two-dimensional plot is used; and finally five or six properties can be displayed in a histogram or radar plot.

Chemometrics

Chemometric tools can be used to classify and produce comparisons between columns. Among the different approaches, Principal Component Analysis (PCA) is used to assess the chromatographic similarity/dissimilarity of a range of columns. The stationary phases initially spreading in a multi-dimensional space (due to the number of parameters used for the comparison) are displayed in a two-or three-dimensional space providing differentiation of the groups as shown in Figure 5.

Such a method may introduce a loss of information when reducing the data from a multidimensional space to a limited number of significant axes. However, selecting the relevant criteria is not a simple task. More complex models involving thermodynamic approaches are also useful such as the linear solvation energy relationship (LSER), the Abraham solvation descriptors Poole and Poole, and the model of Snyder et al. These models can produce more precise information to understand the types and the relative strength of the chemical interactions that control retention and selectivity. However, the results of such models are sometimes difficult to interpret, especially when the analytes and the analytical conditions used are different.
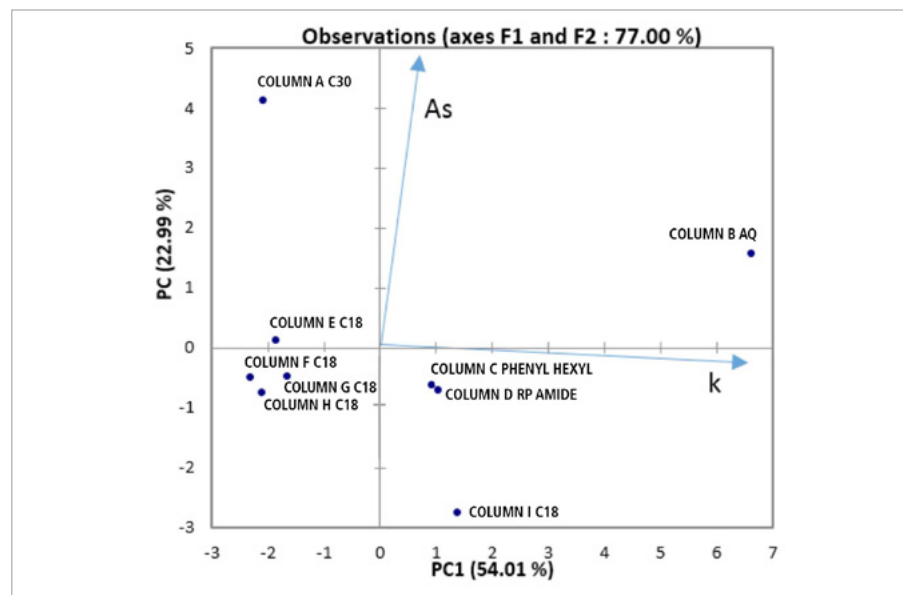

Figure 5. Score plots of some core-shell columns for the Veuthey test at pH 7. The solutes are numbered from 1 to 7. In blue the vectors corresponding to the parameters taken into account for the PCA (retention factor k, asymmetry factor As).

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Principal Component Analysis

With this new representation, columns close to each other (on the score plots) present similar chromatographic characteristics given the parameters considered (here retention and asymmetry factors). Conversely, the one located on another portion of the scores plot exhibit different properties.

A great example is presented in Figure 5. The variables taken into account are the retention factor (k) and the asymmetry factor (As) for solutes numbered from 1 to 7. The conditions of the analysis, as well as the solutes chosen correspond to those of the Veuthey test, particularly useful to characterize the chromatographic properties of a stationary phase toward basic solutes.

The calculation of the principal components, which are a linear combination of the initial values (and retains 77% of the information) ensures an accurate interpretation with minimal loss of information.

Here, most of the C18's are clustered together except the Column I C18 (Figure 5). They are located at the opposite direction of the vectors of retention factors (Figure 5), meaning that the solutes analyzed in these conditions do not develop important interactions with the stationary phases as they do not present high k values.

Column B AQ is particularly suitable for the analysis of basic solutes as those used for the Veuthey test. Indeed, it is located in the direction of the retention factor vectors, and far from the asymmetry vectors (Figure 5). It means that this column presents interesting retention properties, without too much distortion of the peak symmetry.

Column A C30 is also isolated and located in the vector direction of the asymmetry factor. The column is not suitable for the analysis of these types of solutes as it does not present interesting retention properties and high peak asymmetry.

References

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Authors

Mélanie Mignot, Pascal Cardinaël

Valérie Peulon - AgasseNormandie Université,

Laboratoire SMS- EA3233
Université de Rouen 76821, Mont Saint Aignan France

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