Application Note B14.0

Reversed-Phase Chromatography: General Introduction for Improved Method Development

SiliaBond®

Today, more than 80% of all analytical chromatographic separations are performed using reversed-phase sorbents which have shown higher versatility compared to normal-phase chromatography sorbents. Reversed-phase sorbents have found their use in a wide range of applications such as process purification, isolation of active biomolecules, analytical separation of drugs and metabolites as well as extraction of various contaminants in environmental samples.

However, reversed-phase chromatography includes a large number of different phases that differ significantly in both chemical and physical properties which will have a significant impact on their chromatographic behaviours. Thus, one chromatographer will have to consider all of the following stationary phase properties while gathering information on the sample to be analyzed or purified:

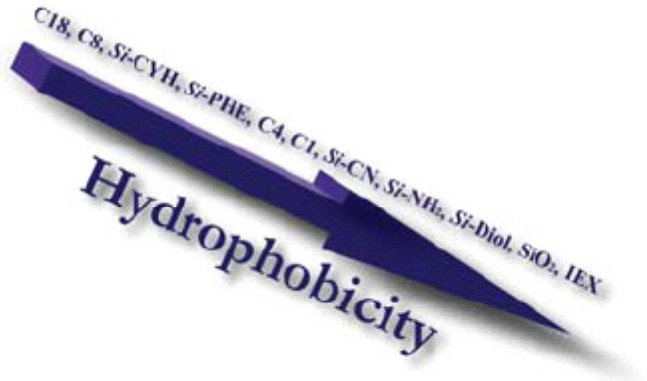
Stationary Phase and Analyte Properties to Consider Prior to Method Development

Stationary Phase Properties	Analyte Properties
Hydrophobicity	Hydrophobicity
Particle and Pore Diameter	pKa (charged?)
Bonding Chemistry (Surface Coverage, Polymeric vs Monomeric)	Molecular Weight
Particle Geometry (Irregular vs Spherical)	Sample Matrix (pH, salts, concentration, interferents)
Silica Purity	

OPTIMIZATION OF SEPARATION CONDITIONS

Choice of Stationary Phase:

1) **Stationary Phase hydrophobicity:** In reversed-phase chromatography, the packing material is always non-polar (hydrophobic) while the mobile phase is polar to non-polar. An important parameter that will affect the chromatographic efficiency is the hydrophobicity of the sorbent. As a general rule, the stationary phase hydrophobicity increases with the alkyl chain length:



SILICYCLE Inc. UltraPure SILICA GELS

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2) **Particle size (d_p):** The average particle size of a packing material has a dual effect on the separation. In order to increase a separation efficiency (plate numbers - N), a common strategy is to diminish the chromatographic material particle size.

$$N \propto 1/d_p^2$$

However, the use of smaller chromatographic particle also causes an increase in a column backpressure (ΔP) thus a diminution in the flow rate at identical pressure. A straightforward approach used to compensate the resulting decrease in an analyte migration time, the separation flow rate is usually increased.

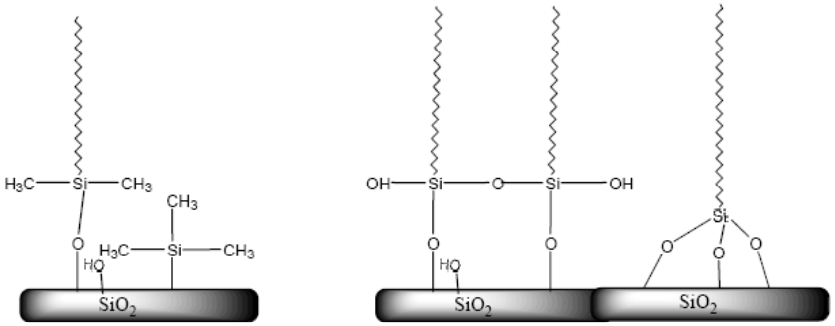
$$\Delta P \propto h/d_p^2$$

Another important factor often forgotten by chromatographers is the quality of the stationary phase particle size distribution. It has been shown that a narrow particle size distribution around the mean allows a great improvement in separation efficiency caused by a diminished number of different preferential flows that an analyte molecule can follow during its travel along the column. To accomplish this, SiliCycle has developed a proprietary technique for the manufacturing of all silica gels which results in a narrower particle size distribution than similar silica from other manufacturers.

3) **Pore Diameter:** About 50 to 80% of a column surface area accessible for chromatography is situated within the pores of the particle constituting the chromatographic bed. To optimize the chromatographic retention, the pore size diameter of the particle has to be wide enough to allow the analyte molecules diffusion within the pores. In general for molecules larger than 2,000 Da (g/mol), a pore size larger than 120Å is highly recommended.

4) **Bonding Chemistry:** All alkyl-functionalized reversed-phase sorbents (C1 to C18) possess variable surface density of Carbon (%C). As the percentage of Carbon increases, the hydrophobicity of the stationary phase also increases. This additional characteristic factor can also be used, for example, to improve efficiency or to tune the chromatographic separation duration.

Alternatively the type of surface functionalization also affects the chromatographic properties of the sorbents. There are two types of functionalization:



Monomeric functionalization

Polymeric functionalization

Monomeric functionalization (Mono)

By grafting a monochlorodimethylsilane, only one bond can be formed with the silica surface. This type of grafting is called monomeric. The dimethyl groups help to protect the surface by steric hindrance which also prevent from reaching the highest silane density possible. The residual silanol groups are inhibited by the grafting of a small molecule, trimethylsilane $Si(CH_3)_3$. This small reagent is called a capping agent, and this technique is called endcapping. Most of our modified silicas are available endcapped or non-endcapped. Even after endcapping, a small portion of the initial silanols is still present, unable to react due to steric hindrance and hence isolated from the mobile phase and analytes present. This product presents a very high stability, batch-to-batch reproducibility and good hydrophobic properties.

Polymeric functionalization (Poly)

By grafting a trichlorosilane, it is possible to form multiple bonds in three space directions with the surface and also between silane molecules. This grafting method is called polymeric functionalization. The silica surface is more hydrophobic, has greater stability in strong acidic condition (pH 2, 3) and has a longer lifetime.

5) **Particle Geometry:** Chromatographic sorbents are manufactured through different processes which results in either spherical or irregular shaped particles. While the chromatographic efficiency is independent of particle shape, the packing of chromatographic columns/cartridges differs from spherical to irregular particles as well as the resulting efficiency. Due to their regular and homogenous shape, spherical particles enable the fabrication of more homogeneous and more tightly packed chromatographic bed. In contrast, irregular material will be more susceptible to gap formation within the chromatographic bed if not properly packed. For this reason, spherical particles are used for analytical or preparative columns requiring high efficiency while irregular particles are commonly used in extraction, sample pre-treatment, purification or process chromatography.

6) **Silica Purity:** Depending on the quality of the manufacturing process, different levels of metal impurities are commonly found in chromatographic silica. High concentration of metal atoms can result in significant chromatographic peak tailing. For this reason, SiliCycle offers only high-purity acid-washed silica enabling higher separation efficiency.

7) **Other Properties:** Other sorbent properties such as the sorbent pH and residual silanol activity can affect the selectivity or efficiency through non-specific interaction.

CHOICE OF MOBILE PHASE: ORGANIC SOLVENT, BUFFERS (PH) AND ION-PAIRING AGENTS

Chromatographic differentiation is based on the difference in analyte's kinetic equilibrium between a stationary phase (sorbent) and a moving mobile phase. The nature and concentration of the mobile phase is selected either to increase the difference between different analyte molecules or to selectively isolate a single analyte for improved purification efficiency. There are few rules to obey when selecting a good mobile phase.

1) **Mobile Phase Hydrophobicity:** First, the mobile phase strength (hydrophobicity) is always kept lower than the stationary phase to prevent the analyte molecules from moving along the solvent front. The sample loading solvent should also obey this rule while providing sufficient analyte's solubility. In order to properly tune the mobile phase strength, solvent mixtures are commonly used. Since the lower the polarity (or higher hydrophobicity) of the mobile phase, the higher the elution power of the mobile phase, organic solvents are usually mixed with water or low-pH buffers to obtain intermediate solvent strength.

2) **UV Cut-Off and Viscosity (η)**: In order to decrease the method's limit of detection, the mobile phase UV cut-off should always be lower than the wavelength used for detection to minimize spectral interference. Another important characteristic often forgotten by chromatographer is the mobile phase viscosity (η) which has a direct influence on the separation efficiency (mass transfer kinetic) as well as on the resulting backpressure.

3) **Boiling Point:** The boiling point of the mobile phase is especially important in sample extraction or concentration because solvents with lower boiling points are easier to evaporate.

Organic Solvents and Properties

Solvent	Polarity	UV Cut-Off (nm)	Viscosity (cP at 20°C)	Boiling Point (°C)
Water	10.2	190	1.00	100.0
Acetonitrile	5.8	190	0.37	81.6
Ethanol	4.6	210	1.20	78.4
Methanol	5.1	205	0.60	64.7
n-Propanol	4.0	210	2.30	97.2
Iso-Propanol	3.9	205	2.50	82.3
Acetone	5.1	330	0.36	56.3
Chloroform	4.1	245	0.57	61.2

4) **Salts/Buffers/Ion-Pairing Agents:** To accomplish the efficient chromatographic separation of charged molecules such as basic compounds with reversed-phase sorbents, different strategies have been developed to increase the analyte's hydrophobicity in separation conditions. The most common strategy is to adjust the mobile phase pH close to the analyte pKa resulting in a net increase of the analyte hydrophobicity.

Buffers and Properties

Buffer	pH range	UV Cut-Off (nm)
Trifluoroacetic Acid (TFA)	1.5-2.5	210
Acetate	3.8-5.8	220
Formic Acid (FA)	2.5-4.5	220
Phosphate	2.1-3.1 & 6.2-8.2 & 11.3-13.3	195
Citrate	2.1-4.1 & 3.7-5.7 & 4.4-6.4	230
Formate	2.8-4.8	220
Carbonate	5.1-7.1 & 9.3-11.3	200
Tris(hydroxymethyl)aminomethane (TRIS)	7.3-9.3	210
Borate	8.2-10.2	195
Diethylamine	9.5-11.5	220

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In general, the use of buffers allows a clear improvement in separation/extraction reproducibility due to the influence of an analyte net charge on its hydrophobicity. For highly basic molecules or molecules possessing a permanent charge, positive and negative ion-pairing agents are commonly used. Examples include: Pentafluoropropionic acid (PFPA), Heptafluorobutyric acid (HFBA) and Tetramethylammonium chloride. In the case of reversed-phase sorbents presenting higher concentration of residual silanols, amine modifiers (ex: Triethyl- and Trimethylamine) are often used to diminish the non-specific interactions with strongly basic molecules. The nature of the buffers counter-ions can also become important in certain cases such as in mass spectrometry detection where more volatile counter-ions diminish ion-suppression (ex: ammonium salts).

Isocratic vs Gradient Elution:
Even after optimization of the stationary phase properties and tuning of the mobile phase, a separation might require additional selectivity in order to resolve two compounds. In such cases, a gradient elution is commonly used in analytical chromatography as well as in preparative chromatography.

What to aim for in Analytical Separation, Extraction and Purification:
Analytical: efficiency, reproducibility, speed, robustness, selectivity
Purification: loadability, speed
Extraction: selectivity, loadability

Scaling-Up in Reversed-Phase Chromatography:
Instead of optimizing separation conditions on large scale column, an experimented chromatographer will always perform prior optimization of separation conditions on a smaller scale separation format. This strategy permits great time, efforts and cost savings.

From Thin Layer Chromatography to Flash Chromatography:
A common technique used prior to Flash Chromatography is to first determine the experimental conditions using thin layer chromatography (TLC). Alternatively, method development becomes faster without using great amount of both compounds and solvents.

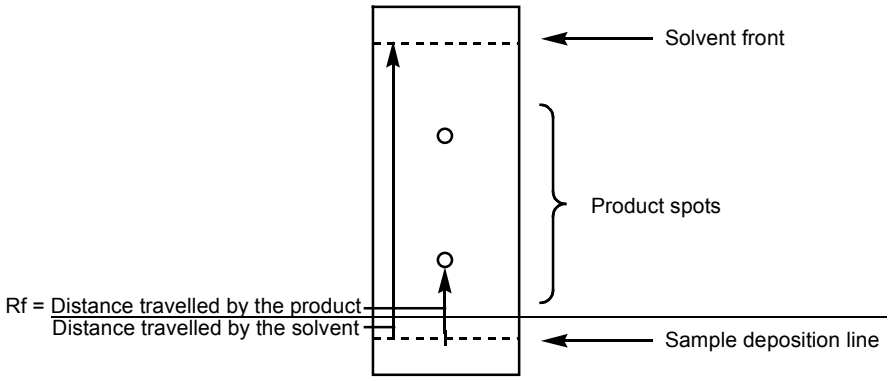
Initially, TLC allows a first approximation to be drawn on the number of products present within the sample as well as their relative polarity.

In order to obtain the best reproducibility in each steps of the scaling-up process, SiliCycle TLC plates and Flash Cartridges are made with the same UltraPure silica gel.

The working principle behind TLC is the same as for every other chromatographic technique where compound differentiation is based on their specific affinity for a stationary phase and a mobile phase. Due to chemical and structural distinctions, all products will bind to the stationary phase at different extent which results in a divergence between each compound retention time (chromatographic differentiation).

In practice, TLC is one of the most straightforward chromatographic techniques. The crude reaction mixture is initially spotted at the bottom of the TLC plate. Then, chromatographic elution is performed by placing the plate into a beaker that contains a small quantity of solvent (called eluent). To prevent the sample spot from diffusing out of the TLC plate, the sample spot should be kept above the solvent. Compared to classic chromatographic techniques where pumps are used to drive the eluent through a chromatographic channel, the solvent movement is achieved solely through capillary action. The solvent movement is allowed to proceed until the solvent front (observed as grey front due to silica wetting) reaches about 1 cm from the top of the TLC plate. Figure 1 shows the easiest way of how to use any TLC plates.

The calculation of chromatographic parameters (R_f, α) is performed upon revelation of the TLC using either direct UV light or a chemical dye. The R_f refers to the distance travelled by individual product compared to the solvent front as shown below.



The optimal condition will correspond to Rf values above 0.25 for the products of interest while the other compounds Rf values should differ of at least 0.2 unit from the main product.

From Analytical to Preparative/Process Chromatography:
The major difference between analytical and preparative chromatography is the amount of sorbents used for chromatographic separation which has an impact on amount of samples that can be separated/purified on a single chromatographic run.

Important considerations are to be respected when scaling-up to process chromatography. It has been demonstrated that the amount of sample loaded and separated in a chromatographic run has a severe impact on the quality of the separation and on the life-time of the chromatographic bed. In common separation conditions, samples should be loaded with a total concentration of products between 0.1 to 2% over the chromatographic sorbent weight used within the column, for example 0.1g of samples in a 100g flash cartridge. However, initial sample concentration of 0.1% is usually recommended to avoid any potential peak distortion due to unwanted column overloading.

Upon optimization of the chromatographic separation using a smaller-bore chromatographic column/cartridge, the optimized method can be implemented to bigger/larger columns using a scale-up factor (F) based on column volume increase. The scale-up factor is used to determine the new volumetric flow rate as well as the amount of sample to be injected:

$$F = \frac{d_{c,j}^2}{d_{c,i}^2}\frac{L_j}{L_i}$$

where d_c = Inner diameter of the smaller (i) and larger (j) column
L = Length of the smaller (i) and larger (j) column

SPECIAL ATTENTION AND COLUMN/CARTRIDGE CARE

Column Equilibration Between Injections:
In the same way as for initial column equilibration, 3 to 5 column volumes are passed on the column between runs to ensure that the column/cartridge properties are identical to the previous chromatographic runs. This procedure allows the chromatographic surface to gain back its initial activity and surface properties prior to the next chromatographic separation.

Column Cleaning and Storage:
In the gradient elution operations, the column should be washed after the last completed runs with 2 to 3 column volumes of 95% of the more polar solvent and the same volume of the initial solvent. For isocratic elution, 2 to 3 column volumes of the initial solvent should be used after each separation for column reconditioning. Finally, the column is stored in a solution containing at least 80% organics. It is imperative that once wetted any column/cartridge are prevented from drying.

SILICYCLE AVAILABLE REVERSED-PHASE PRODUCTS

Bulk Sorbents – SiliaBond® and Spherical Alternatives

Product Description	Product Number	Also available*	
		SiliaSphere™	SiliaSphere™ PC
SiliaBond C18 ne (23%)	R30130B	X	
SiliaBond C18 (23%;17%;11%)	R300230B;R30230B;R30430B	X	X
SiliaBond C12 (Adamantyl)	R53030B		
SiliaBond C8	R31030B	X	
SiliaBond C8 ne	R31130B		
SiliaBond C4	R32030B	X	X
SiliaBond C4 ne	R32130B		
SiliaBond C1	R33030B	X	
SiliaBond Phenyl	R34030B	X	
SiliaBond Phenyl ne	R34130B		
SiliaBond Cyclohexyl	R61530B		
SiliaBond Cyano	R38030B	X	
SiliaBond Bromophenyl	R55030B		
SiliaBond Pentafluorophenyl	R67530B		

* If not listed, all SiliaBond chemistries are available on SiliaSphere™ and SiliaSphere PC on a custom basis as well as on other supports (particle and pore size). Ask for information on our SiliaBond Reversed-Phase Kit (K32530B).

TLC Plates – SiliaPlate

Product Description	Plate Size (cm^2)	Thickness (µm)	Plates per box
SiliaPlate C18	10x10	150	25

Cartridges

SiliCycle offers most of these Reversed-Phase chemistries in a wide selection of cartridges (ISCO™, Flashmaster™ and Biotage™ compatible), SPE, 96 Well Plates. Mixed-mode cartridges are also available.

• SiliCycle ISCO™ Compatible Cartridges:

SiliaSep™ 4.3g (20/box)
13 g (20/box)
43 g (15/box)
90 g (12/box)
130 g (10/box)
360 g (4/box)

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• SiliCycle ISCO™ Compatible Cartridges:

Silia*Sep*	4.3g (20/box) 13 g (20/box) 43 g (15/box) 90 g (12/box) 130 g (10/box) 360 g (4/box)

• SiliCycle Flashmaster™ Compatible Cartridges:

Silia*Sep* OT	Open-Top 10, 15, 20, 25, 50 & 70 g

• SiliCycle Biotage™ Compatible Cartridges:

Silia*Sep* BT	12S & 12M 40S, 40M & 40L 65

• SiliCycle SPE Cartridges:

0.05 g/1 mL, 0.10 g/1 mL, 0.20 g/3 mL,
0.50 g/3 mL, 0.50 g/6 mL, 1.00 g/6 mL,
2.00 g/6 mL, 2.00 g/12 mL,
5.00 g/25 mL

• SiliCycle 96 Well Plate

0.05 g/2 mL, 0.10 g/2 mL, 0.20 g/2 mL

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