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Sepax C18 Column User Manual

1. Introduction

One of the most critical parts of an HPLC instrument is the HPLC column. Both the life of the column and the reliability of the analysis therefore depend on the proper use of the HPLC column and the instrument. Appropriate cleaning of the instrument, especially after running an aqueous salt-buffer, before installing the C18 column is extremely important. Make sure all parts of the HPLC system have been cleaned, including, but not limited to sample loops, buffer inlet lines, precolumn filter, column attachment lines, and injection ports. This critical step helps ensure that residual particles and impurities from previous runs are effectively removed, preventing system clogging and pressure issues, while also extending column lifetime. For additional guidance, refer to the cleaning protocols provided by the instrument manufacturer.

After the system has been cleaned, start the instrument at a low flow rate and confirm that the column inlet line has started to drip mobile phase, then stop the flow. Remove both column endcaps and attach the column to the instrument, following the directional orientation noted on the column label.

Sepax C18 columns are shipped in an acetonitrile/water mixture, which is generally compatible with most reversed-phase mobile phases. Direct switching to the target mobile phase is possible; however, we recommend first flushing the column with the strong solvent component of your mobile phase (e.g., acetonitrile or methanol) before introducing the full mobile phase composition. For new columns, begin with a reduced flow rate (see Table 3) and maintain this flow until at least 2–10 column volumes (CVs) have passed, and the baseline becomes stable. Once the system pressure has stabilized, increase the flow rate incrementally using a ramp rate of 0.1 mL/min² until the desired running flow rate is reached. Continue flushing the column at the running flow rate for up to 10 CVs. As a best practice, always ramp up the flow rate gradually to minimize pressure surges and preserve column integrity. Continuously monitor system pressure, and ensure it remains stable for several minutes before increasing to the next flow rate increment.

If the mobile phase consists of only organic solvent and water or buffer, a new column can be equilibrated by running 10-20 CVs of the mobile phase through the column. However, if the mobile phase contains an ion-pairing agent, 20-50 CVs may be needed to equilibrate the column.

Take note of the system pressure without the column. Record start & end pressures once the desired flow rate and stabilized baseline have been achieved. This will allow you to gauge when the column should be cleaned after running the target sample(s). All sample compositions are different and proper column cleaning will greatly prolong the column lifetime.

2. Product Information

Sepax C18 bonded phase is manufactured by the surface modification of pure and mechanically stable spherical silica particles. The C18 bonded phases have been innovatively and specially designed to ensure maximum coverage that results in very reliable column-to-column reproducibility. The columns are packed with a proprietary slurry technique to achieve uniform and stable packing bed density for maximum column efficiency. Since the packing material is silica-based, never run caustic or any solvent mixture above pH 8.5 which will irreversibly damage the resin surface. A representative

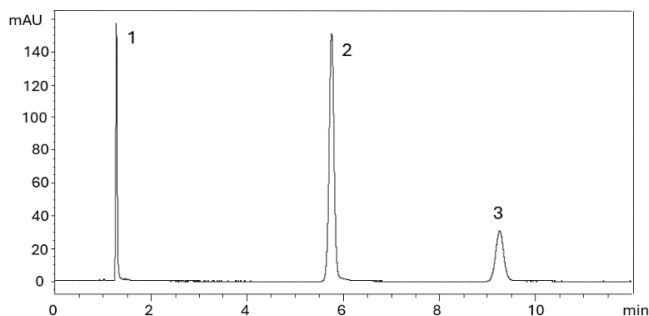


Figure 1. Separation profile of a QC standard mixture by Sepax GP-C18 (5 μ m, 120 Å) 4.6 $\times$ 150 mm column.

Mobile phase: acetonitrile: water (55% : 45%) mixture;
Flow rate: 1 mL/min; Detector: UV 214 nm;
Column temperature: 25 °C;
Samples: 1) uracil, 2) anisole, 3) toluene.

QC chromatogram of Sepax GP-C18, 4.6 x 150 mm column is shown in Figure 1.

3. Technical Specifications

Table 1. Technical specifications of Sepax C18 columns

Property	Description
Material	C18 Bonded silica
Particle size	1.8 - 15 μm
Pore size	60 – 300 Å
pH Stability	2.0 - 7.5
Recommended operating temperature	4 - 40 °C
Mobile phase compatibility	Aqueous and organic

4. Column Installation

When a column is shipped or not in use, it is always capped at both ends. When installing the column to the system, first remove the end caps. Make the flow direction as marked on the column. Unless the user has a special purpose of reversing the flow direction (for example: to clean inlet contamination or clogging), follow the flow direction as labeled. Column connections are an integral part of the chromatographic process. If ferrules are over-tightened, not set properly, or are not specific for the fitting, leakage can occur.

Set the ferrules for column installation to the HPLC system as follows:

- 1) Place the male nut and ferrule, in order, onto a 1/16" o.d. piece of tubing. Be certain that the wider end of the ferrule is against the nut.
- 2) Press the tubing firmly into the column end fitting. Slide the nut and ferrule forward, engage the threads, and finger-tighten the nut.
- 3) While continuing to press the tube firmly into the end fitting, use a 1/4" wrench to further tighten if necessary.
- 4) Repeat this coupling procedure for the other end of the column.

5. Column Protection/ Safety Precautions

Sepax C18 columns are pre-packed under high pressure in stainless steel hardware.

- Sepax does not recommend opening the column hardware and cannot guarantee the performance of a column that has been tampered with.
- Sepax cannot guarantee the performance of a column that has experienced inappropriate physical force or impact.

6. Sample and Mobile Phase

- To avoid clogging the column, all samples and solvents including buffers should be filtered through 0.45 μm or 0.2 μm filters before use.
- To avoid bubbling during measurements, always degas the mobile phase. A simple way for degassing is to sonicate it for 5 minutes under a pumped vacuum.

Sepax-C18 bonded stationary phase is compatible with both aqueous and organic mobile phases. The columns can tolerate a maximum aqueous phase proportion of up to 95%. The choice for the mobile phase is broad, including organic solvents, aqueous buffer, or a mixture of organic and water, such as methanol or acetonitrile and water. Gradient elution methods for Sepax-C18 columns often begin with 5% methanol or acetonitrile as the initial mobile phase.

7. Column Equilibration

New Sepax C18 columns are shipped in a mixture of methanol or acetonitrile and water. Prior to sample injection, ensure compatibility of the mobile phase with the column's shipping solvent and it's crucial to adequately equilibrate the column with the intended mobile phase for the stable separation. For more details, please refer to the following.

- To activate the column prior to its initial use, ensure to flush it with 5-10 CVs of a pure organic solvent, such as methanol or acetonitrile, continuing this process until a stable and flat baseline is achieved.
- When using a mobile phase that does not include buffers or additives, equilibrate the column and system with the mobile phase to be used for 10-20 CVs or until a stable and flat baseline is achieved. When changing to the mobile phase of the designed system, flush the column with the mobile phase while gradually increasing the flow rate from 0.1 mL/min² to the operation condition, until the baseline is stable.
- When using a mobile phase that includes buffers or additives, start by equilibrating the column and system with a diluted water/organic solvent mixture at a low concentration ratio. This should be done for at least 10 CVs. Maintaining the same water-to-organic solvent ratio, subsequently, continue the equilibration with the organic/buffered mobile phase for an additional 10 CVs. When using a mobile phase composed of a ternary mixture or complex systems such as ion-pairing reagents, it's crucial to equilibrate the column thoroughly. Continue equilibration until

the baseline stabilizes to minimize any drift in retention times.

- Salt precipitation can occur during buffer exchange. To prevent this, ensure to use an appropriate aqueous/organic solvent mixture to remove any salts before transitioning to the final buffer solution.
- If the column backpressure and baseline fluctuate, this might be due to the air bubbles trapped inside the column. Flush the column with a higher flow rate for 2-5 minutes, for example, 2 mL/min for a 4.6×150 mm column.

In general, flushing a column with 20 CVs of the mobile phase will equilibrate the column. The equilibration time for typical column dimensions is given in Table 2. When equilibrating a column, the volume of the solvent, not the time, is most important. The equilibration time needs to be adjusted according to the flow rate.

Table 2. Equilibration time for typical column dimensions at given flow rate.

Column id (mm)	Empty Column Volume (mL)	Flow Rate (mL/min)	Equilibration Time (min)
2.1 × 30	0.1	0.2	10
2.1 × 50	0.17	0.2	17
2.1 × 100	0.35	0.2	35
2.1 × 150	0.52	0.2	52
2.1 × 250	0.87	0.2	87
4.6 × 50	0.83	1	17
4.6 × 100	1.66	1	33
4.6 × 150	2.49	1	50
4.6 × 250	4.15	1	83
7.8 × 250	11.95	2	120
10.0 × 250	19.63	4	98
21.2 × 50	17.65	21	17
21.2 × 150	52.95	21	50
21.2 × 250	88.25	21	84

8. Column Cleaning

Contamination of the resin bed can deteriorate the column performance. The impurities can be removed by washing the column with a sequence of solvents outlined below. Note that this is a general guideline, and the washing steps should be tailored to the nature of the impurities. Sometimes the column can be backflushed for cleaning to allow contaminants to flow out of the column. The minimum volume for the solvent used to clean the analytical column is 20 CVs. Before any cleaning procedure is done, make sure the solvents are compatible and supported by the HPLC system. Always

adjust the flow rate to maintain the back pressure within the allowed limit.

- 1) Remove any used/fouled guard column prior to cleaning.
- 2) Disconnect the column from the detector and allow the cleaning solvent to flow into the collection vessel.
- 3) If using a buffer mixture, wash the column first with a mobile phase without buffer salts (water/organic phase).
- 4) Wash with 100% organic phase (methanol or acetonitrile).
- 5) If more polar samples were injected, the following cleaning sequence can be used: water → methanol → THF → methanol → water → mobile phase.
- 6) Use the following sequence if more nonpolar samples were injected: 2-propanol/water (1:1) mixture → 2-propanol → THF → dichloromethane → heptane → 2-propanol → mobile phase.

If ion-pairing reagents are used, complete removal of them might not be achievable. Given that these reagents can affect the selectivity of the column, it is highly recommended to allocate specific columns exclusively for ion-pairing methods, thus ensuring consistent reproducibility and avoiding cross-contamination issues.

9. Column Regeneration

It is recommended to rejuvenate the HPLC column periodically to extend its life by washing the column with different mobile phases. Eluent type and volume may be tailored to specific needs, with a general recommendation of cleaning 5-10 CVs for each mobile phase. During the process of mobile phase exchange and reactivation, it's important to monitor column pressure and ensure compatibility between solvents. The recommended sequences are designed to be gentle, safeguarding the column against potential damage from abrupt solvent changes. These regeneration steps are optional, which can be implemented individually or in combination, tailored to address the specific contaminants and situational requirements as well as to ensure the phase's durability. For example, a larger column size may not be practical for processing 5-10 CVs of each solvent. In such cases, reducing the column volume may be more efficient, provided that this adjustment still allows the baseline to stabilize. Following is a typical washing sequence that helps to rejuvenate the column.

- 1) Wash with the mobile phase without buffer salts.
- 2) Wash with 10% acetonitrile, then 50% acetonitrile followed by 100% acetonitrile.
- 3) Then wash with 50% acetonitrile/ 2-propanol, then change to 100% 2-propanol and then back to 50% acetonitrile/ 2-propanol.
- 4) Wash with 50% acetonitrile/ water and then with the mobile phase.

It is worth noting that after using a mobile phase containing ion-pairing reagents, it is recommended to regenerate or reactivate the column. In this case cleaning the column with solvents such as isopropanol or tetrahydrofuran mixed with water will be helpful.

10. Column Maintenance

Routine maintenance of the column and proper usage helps to extend the lifetime as well as the performance of the column. Following are some recommendations:

- Periodically check for any physical damage or leak from the end-fittings.
- Filter the mobile phase and sample solution to remove particulates and contaminants from entering the column that could potentially harm the column.
- Use guard columns to protect the analytical column from contamination.
- Avoid extreme conditions such as pH, temperature, sudden pressure change, and drastic change in the mobile phase composition.
- Never use strong acidic or basic solvents to clean the column. This will result in irreversible damage to the column. Recommended pH range of any silica based HPLC column is 2.0-7.5. pH closer to neutral is always beneficial to the stationary phase.

11. Column Storage

When not in use for an extended time, do not allow water or aqueous buffer to remain in the column. The following are the recommended storage conditions for Sepax C18 columns:

- Short term storage (few hours to overnight): same mobile phase used in the last analysis.
- Medium interval storage (two days or over the weekend): wash the column with 100% water and store in water.
- Long term storage (more than two days): use an aprotic solvent without salt buffer. Water content should not be higher than 50%.

Acetonitrile: water mixture (1:1) is one of the best solvents for the long-term storage of the column.

Each column is shipped with two removable end plugs. To prevent the drying of the column bed, seal both ends of the column with the end plugs provided. If abnormal peak shapes occur when using the column after a long period of storage, the column can be reactivated by overnight washing with the storage solvent at a low flow rate.

12. Additional Information

- Upon receipt, first evaluate the column using the manufacturer's quality control procedures. The theoretical plate number and tailing factor should match the QC report. Due to differences in instruments and experimental conditions, the actual test results may deviate from the QC report.
- Please ensure that the column is used within the range of column temperature, pressure, and pH values specified in the manual. Any conditions that exceed these ranges may lead to irreparable damage to the column.
- It is recommended to dissolve the sample in the mobile phase to avoid the occurrence of solvent effects. Additionally, it is important to ensure that the sample to be tested is highly soluble in the mobile phase to prevent the sample from precipitating in the mobile phase, which can lead to increased column pressure and system contamination. If this happens, the column can be backflushed at a low flow rate to remove impurities blocking the column head. Filter the buffer and sample solution before the further usage of the column.

Table 3. Standard and recommended flow rates of typical C18 columns.

Column* [i.d. × length]	Empty Column Volume (mL)	Standard Flow Rate mL/min	Recommended Cleaning Flow Rate** mL/min
2.1 × 30 mm	0.1	0.2	0.1
2.1 × 50 mm	0.17	0.2	0.1
2.1 × 100 mm	0.35	0.2	0.1
2.1 × 150 mm	0.52	0.2	0.1
2.1 × 250 mm	0.87	0.2	0.1
4.6 × 50 mm	0.83	1	0.5
4.6 × 100 mm	1.66	1	0.5
4.6 × 150 mm	2.49	1	0.5
4.6 × 250 mm	4.15	1	0.5

7.8 × 250 mm	11.95	2	1
10.0 × 250 mm	19.63	4	2
21.2 × 50 mm	17.65	21	10
21.2 × 150 mm	52.95	21	10
21.2 × 250 mm	88.25	21	10

* Different package sizes are also available.

**The standard flow rate is determined by the inner diameter (I.D.) but may vary depending on the specific instrumental setup. The operating pressure must remain below the maximum allowed limit. Depending on the situation, the recommended flow rate should be either 1/3 or 1/2 of the standard flow rate.

13. Product Ordering Information

Pore Size	Product	Part Number
60Å	GP-C18 (5 µm, 60 Å)	101175-0000
	GP-C18 (10 µm, 60 Å)	101179-0000
100Å	HP-C18(2) (2.2 µm, 100 Å)	143182-0000
	HP-C18(2) 3 µm, 100 Å)	143183-0000
	HP-C18(2) (5 µm, 100 Å)	143185-0000
	HP-C18(2) (10 µm, 100 Å)	143189-0000
120Å	GP-C18 (1.8 µm, 120 Å)	101181-0000
	BR-C18 (1.8 µm, 120 Å)	102181-0000
	GP-C18 (2.2 µm, 120 Å)	101182-0000
	GP-C18 (3 µm, 120 Å)	101183-0000
	BR-C18 (3 µm, 120 Å)	102183-0000
	HP-C18 (3 µm, 120 Å)	103183-0000
	GP-C18 (5 µm, 120 Å)	101185-0000
	BR-C18 (5 µm, 120 Å)	102185-0000
	HP-C18 (5 µm, 120 Å)	103185-0000
	GP-C18 (10 µm, 120 Å)	101189-0000
	HP-C18 (10 µm, 120 Å)	103189-0000
	GP-C18 (15 µm, 120 Å)	161815-0000
200Å	Bio-C18 (3 µm, 200 Å)	105183-0000
	Bio-C18 (5 µm, 200 Å)	105185-0000
	Bio-C18 (10 µm, 200 Å)	105189-0000
300Å	Bio-C18 (3 µm, 300 Å)	106183-0000
	Bio-C18 (5 µm, 300 Å)	106185-0000
	Bio-C18 (10 µm, 300 Å)	106189-0000

Most common column dimensions are I.D. (mm): 2.1, 4.6, 7.8, 10.0, 21.2, 30, 50 and length (mm): 30, 50, 100, 150, 250. If you need additional information about the product, please visit our website (www.sepax-tech.com), or contact your area representative, or technical support team (techsupport@sepax-tech.com), or call (302) 366-1101 (toll free: 1-877-SEPAX-US).