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Zenix-C SEC-MALS User Manual

Column Information

Utilizing proprietary surface technologies and 3 µm particle size, Zenix-C SEC phases are made of uniform, hydrophilic, and neutral nanometer thick films chemically bonded on the high purity and enhanced mechanical stability silica. The proprietary surface technologies allow the chemistry of thin film formation to be well controlled, which results in high column-to-column reproducibility. The nature of the chemical bonding and the maximum bonding density of the thin film benefit Zenix-C SEC phases with high stability. The uniform surface coating enables high efficiency separation. The narrowly dispersed, spherical silica particles of the Zenix-C packings for SEC-100, SEC-150 and SEC-300 have nominal pore sizes at 100 Å, 150 Å, and 300 Å, respectively. With a small particle size of 3 µm and specially designed large pore volume (ca. 1.35 mL/g for Zenix-C SEC-150 and 300, and ca. 1.1 mL/g for Zenix SEC-100), Zenix-C phases have achieved unprecedented high separation efficiency and resolution. Zenix-C SEC columns are packed with a proprietary slurry technique to achieve uniform and stable packing bed density for maximum column efficiency.

Zenix-C SEC phases are designed to ensure highest resolution and maximum recovery for a very broad range of separation applications. These applications cover large biological molecules, such as proteins and nucleic acids; small biological molecules, such as peptides and oligonucleotides; natural polymers, such as polysaccharides; synthetic polymers; biological cells, such as bacteria and virus; and nanomaterials, such as nanoparticles. Typical applications for Zenix SEC columns are separation and detection in aqueous buffer mobile phases.

System cleaning for MALS Applications

MALS is very sensitive to particles and foreign objects entering the detector and the results can be complicated or confusing. Thorough cleaning of the HPLC system and particles-free mobile phase are critical to get reliable MALS results. Following is the recommended procedure to minimize any interference of foreign particles with the MALS data.

Full HPLC system clean once a week. This includes sonicating the in-bottle filter, precolumn filter, and precolumn housing in 50% aqueous ethanol for 10 minutes. After sonication, rinse everything in HPLC grade water. Reinstall the in-bottle filter but keep the precolumn filter off. Run 100% ethanol through the HPLC system for 20 minutes followed by running water for 20minutes. Reinstall the precolumn filter after both runs are complete. The system is now ready for buffer. Use freshly made buffer and filter through a 0.2 µm filter just before use.

Daily System clean every night. At the end of everyday operation, run water through the system for 20 minutes followed by running 20% aqueous ethanol for 20 minutes. Leave the system stored in 20% ethanol.

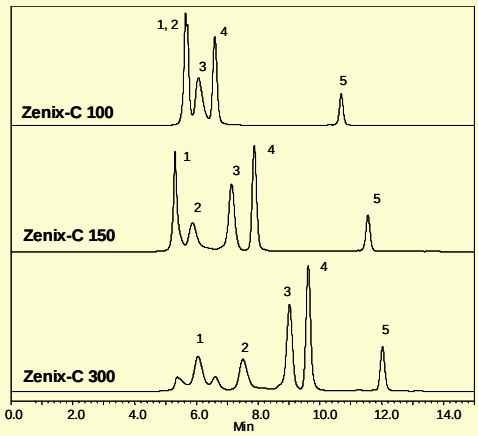
Always use a precolumn filter (0.5 µm stainless steel frit) to trap any residual particles. The flow rate should be within the recommended range of the MALS flow cell. Most of the cases the flow should not exceed 1mL/min.

Column Stability and Performance

Zenix-C SEC columns use full coverage bonded silica packing, which allows exceptionally high stability. They are compatible with most aqueous buffers, such as ammonium acetate, phosphate, tris, etc. When 150 mM phosphate buffer at pH 7.0 is used as the mobile phase to run Zenix-C SEC columns, 300 injections with protein standards of thyroglobulin, BSA, ribonuclease A and uracil, or 1 month of usage has negligible deterioration for Zenix-C SEC columns.

The neutral and hydrophilic Zenix-C stationary phases have negligible nonspecific interactions with biological molecules, such as proteins, DNA, RNA and peptides. Combined with their high capacity, Zenix-C SEC columns enable high efficiency and high recovery separations. Figure 1 shows an example of separating Biorad protein mixture using 7.8x300 mm Zenix-C SEC columns.

Figure 1. Separation of Biorad protein mixture by Zenix-C 100, 150 and 300 columns.



Columns: Zenix-C (3 µm, 7.8x300 mm); Mobile phase: 150 mM Sodium Phosphate, pH 7.0; Flow rate: 1.0 mL/min; Detection: UV214 nm; Injection: 10 µL

Sample: 1) Thyroglobulin, 670 kD; 2) γ-Globulin, 158 kD; 3) Ovalbumin, 44 kD; 4) Myoglobulin, 16.9 kD; 5) Vitamin B12, 1355 D.

Column Characteristics

Silica: *Spherical, high purity (<10 ppm metals)*

Particle size: *3 µm*

Pore sizes for protein separation:

100 Å, MW range 100 ~ 100,000
150 Å, MW range 500 ~ 150,000
300 Å, MW range 5,000 ~ 1,250,000

Safety Precaution

The columns are normally operated under moderate pressure. Loose connections will cause leaking of organic solvents and injected samples,

all of which should be considered as hazards. In the case of leaking, proper gloves should be worn while handling the columns. When opening the columns, proper protections should be used to avoid inhalation of the small silica particles.

Samples and Mobile Phases

To avoid clogging the column, all samples and solvents should be filtered through 0.45 μm or 0.2 μm filters before use. Zenix-C SEC columns are compatible with an aqueous mobile phase or a mixture of organic and water, such as methanol or acetonitrile and water. Always degas the mobile phase. A simple way for degassing is to sonicate it for 5 minutes under water pumped vacuum.

Column Protection

Frequent solvent exchange between organic and aqueous buffers can have effects on the resin properties and effect the column lifetime. As viscosity of the mobile phase can influence the backpressure across the column, please check the viscosity of solvents used and adjust the flow rate accordingly. This will ensure the column does not undergo pressure spikes or become over pressurized. When switching from aqueous to organic, run a minimum of 2-5 CV water in between to prevent salting out/precipitations which can damage the column. It is also recommended to minimize the time the column is in pure water to only 5 CV. Sepax does not recommend leaving the column long term in pure water. For proper equilibration, it is recommended to let the column sit overnight in the running buffer or at a slow flow rate before injection of sample.

Solvent	Viscosity at 20 °C
Acetone	0.32
Acetonitrile	0.37
Methanol	0.55
Water	1.00
Ethanol	1.20
2-Propanol	2.40

In addition to filtering the sample and the mobile phase, the best way to protect the column is to install a pre-column filter in front of it. In most cases a pre-column filter helps to remove the residual particulates that are in the sample, the mobile phase, or leached from the LC system, such as pump and injector seals. A Sepax pre-column filter does not contain resin, and as such will not change the retention time of the analyte and does not require equilibration. The filter contains a replaceable frit, and an increase in pressure indicates that the filter frit should be replaced.

Column Installation and Operation

The column should always be capped at both ends when it is not in use. When installing a column to the system, first ensure that the system is clean and primed with mobile phase. Before the column is connected, it is recommended to run the system at a high flow rate to ensure all bubbles are flushed from the system and a flat baseline is obtained. If the baseline is not flat, it is not recommended to connect the column until further system maintenance is performed. These steps will ensure that the column lifetime is maintained. Once the system is cleaned, primed with mobile phase, and has a flat baseline across the detector, stop the flow of the mobile phase, ensuring that mobile phase is still primed in the lines. Remove the end caps of the column and connect the column, ensuring that there are no air gaps in the connection. Unless a user has special purpose to reverse the flow direction, for example, removal of the inlet blockage, follow the flow direction as marked on the column. 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 LC system as follows:

(a) Place the male nut and ferrule, in order, onto a 1/16" outer diameter piece of tubing. Be certain that the wider end of the ferrule is against the nut.

(b) Press tubing firmly into the column end fitting. Slide the nut and ferrule forward, engage the threads, and finger-tighten the nut.

(c) Repeat this coupling procedure for the other end of the column.

Once the column is connected, start the system at a slow flow rate of mobile phase and monitor the pressure of the system and ensure that there are no leaks.

Column activation: It is recommended to activate or condition new columns and columns that have not been used for a long time. To activate, flush the column with 10-20 column volumes of the running mobile phase starting at a low flow rate and gradually increasing until desired operating flow rate is met. Below is a table for reference. (If you plan to run the column in organic solution over 20% organic, flush the column with 2-5 column volumes of water at slow flow rate before switching to desired organic running buffer to avoid precipitation issues.) Please keep in mind that some organic buffers, such as IPA and EtOH, will have a higher viscosity/backpressure and will need to be run at slower flow rates. For organic use over 20%, it is also recommended to equilibrate the resin at a slow flow or no flow in the running buffer overnight to allow for the resin to adjust. For aqueous use, flush the column with your mobile phase while gradually increasing the flow rate to your operating flow rate allowing the column to equilibrate the pressure until the baseline is stable at each step.

The table below offers recommended flow rates for startup. It is suggested to start the flow rate to be 1/5th of the normal running flow rate, ramping up with an increment of 1/5th of the flow rate every two or three minutes. Monitor the baseline and pressure as it should be stable for a few minutes before moving to the next increment. If using organic additives such as IPA, you may need to ramp up in smaller increments due to the higher pressure.

Column Dimension	Column Volume	New Column Flow Rate (mL/min)	Ramp up Increments (mL/min)	Running Flow Rate (mL/min)
2.1×300mm	1.04 mL	0.01	0.01	0.07
4.6×300mm	4.98 mL	0.05	0.05	0.35
7.8×300mm	14.3 mL	0.1	0.1	1.0
10×300mm	23.5 mL	0.3	0.1	1.65
21.2×300mm	106 mL	1.4	0.5	7.5
30×300mm	212 mL	2.8	1.2	15
50×300mm	589 mL	7.8	3.5	41

If using TRIS buffer as the HPLC running buffer: to prevent precipitation, it is recommended to remove the phosphate buffer from the column before switching to TRIS buffer. This can be achieved by flushing the column with 5 column volumes of 100 mM NaCl buffer, pH 7.0 before using TRIS buffer.

Flow rate range Normal operating flow rate can be found in the table above for use with phosphate buffer as the mobile phase when run at ambient temperature. Running the instrument in a cold room or addition of any solvent having viscosity higher than water may cause back pressure to increase. The flow rate should be adjusted to ensure that the back pressure does not exceed above the limit. Any change in the mobile phase composition should be introduced gradually such that column experiences a shallow pressure variation.

Shipping solvent New columns are shipped in 50 mM sodium phosphate buffer containing 0.02% (w/v) sodium azide (NaN_3).

pH For optimum performance and operation during the longest lifetime, columns should be maintained in a pH range between 2 and 7.6. A pH of up to 8.5 can temporally be used, but not recommended for extended use for optimal column lifetime. It is recommended to flush the column with neutral pH buffer after using the column at high or low pH.

Pressure These columns can operate at a pressure up to 3,500 PSI. The normal operating pressure is usually under 2,000 PSI. Continuous use at a high pressure may eventually damage the column. Sudden system/pump fluctuations in pressure can also lead to irreversible damage. Since the pressure is generated by the flow rate, the maximum flow rate is limited by the backpressure. It is expected that the backpressure might gradually increase with its service. A sudden increase in backpressure suggests that the column inlet frit might be blocked. In this case it is recommended that the column be flushed with reverse flow in an appropriate solvent.

Temperature The maximum operating temperature is 80ºC temporarily. The optimum operating temperature for the longest lifetime is 10 – 30 ºC. Continuous use of the column at a higher temperature (>80ºC) can damage the column, especially under high pH (>8).

Shutdown protocol At the end of workday and after the column is equilibrated into appropriate buffered storage solution, the flow rate should be gradually decreased. Once the flow rate and pressure have dropped to zero, the column can be taken off from the system. Each column is shipped with two removable end plugs. To prevent drying of the column bed, seal both ends of the column with the end plugs provided.

Storage When the column is not in use for several days, it should be stored in a low salt pH-neutral salt buffer, such as 50mM Phosphate buffer pH 7.0. (If you plan to store the column in organic solution, such as 10% EtOH, make sure to slowly flush the column with water first to prevent salting out, then switch to the storage solution at slower flow rate, such as half the running flow rate.) When the column is not in use for an extended time, Sepax recommends adding a bacteriostatic agent such as 0.02% sodium azide or 20% acetonitrile, or 10% ethanol.

- 50mM Phosphate buffer or TRIS, pH 7.0 with 0.02% sodium azide

  OR
- 50mM Phosphate buffer or TRIS, pH 7.0 with 20% acetonitrile or 10% ethanol.

Preventative daily care For preventative daily care, a single injection of 6M guanidine HCl under normal running condition and mobile phase can gently remove any residual components that may be stuck on the column before storage to increase the lifetime of the column and inhibit irreversible binding to the resin. Please see the table below for reference of injection volume of guanidine for your specific column dimension. After the guanidine injection, continue with the shutdown protocol and storage solution.

Column Dimension	Column Volume	Guanidine HCl Injection Volume
4.6 x 300 mm	4.98 mL	20-50 ul
7.8 x 300 mm	14.3 mL	100 ul
10 x 300 mm	23.5 mL	240 ul
21.2 x 300 mm	106 mL	1 mL
30 x 300mm	212 mL	2.2 mL
50 x 300 mm	589 mL	4.9 mL

Guard/precolumn filter: It is recommended to use a guard or pre-column filter to extend the column lifetime. If you notice an increase in pressure on the column, replace the guard/precolumn filter immediately.

PN	Description	Frit Size
102002-3UMKIT	Pre-Column Filter Kit for 3 µm Columns	0.5 µm PEEK

**Include column coupler.*

Cleaning Proper and regular system maintenance and cleaning are just as important as column care. From time to time, some samples could get adsorbed onto the inlet frit or the packing material. When the adsorption accumulates to a certain level, it is usually indicated by an increase in back pressure and a broader peak. If using a guard column, make sure to disconnect and clean independent of the analytical or prep column.

The general procedure for column cleaning is as follows:

1. Disconnect the column from the detector.
2. Clean the column in the reverse flow direction for columns packed with stainless steel tubing.
3. Gradually increase the flow rate and never exceed 50% of the maximum recommended flow rate. For higher viscosity cleaning agents, such as IPA and EtOH, it is recommended to use at least 1/3 the running flow rate. Monitor the backpressure. Make sure that the pressure reading is not over a 15 bar increase when switching buffer solutions. If you see the pressure difference is much higher than the normal operating conditions, you need to lower the flow rate to avoid a pressure spike causing more damage onto the column.
4. Typically, 5 column volumes of cleaning solution are enough. To prevent precipitation of the salt or immiscibility of different solvents, one may need to gently flush the column with 2-3 column volumes of distilled, deionized water between each exchange.

Cleaning solutions Low pH salt solutions help remove basic proteins. Organics are useful when removing hydrophobic proteins. Chaotropic agents help to remove strongly adsorbed materials (via hydrogen bonding). Only use chaotropic agents when neutral salts or organics have not improved resolution. All Silica SEC columns, including Sepax, are susceptible to corrosive damage; and solvents such as NaOH should not be used. Two separate cleaning solutions are recommended as a general starting point for cleaning.

- Concentrated neutral salt (e.g., 0.5 M Na_2SO_4) at low pH (e.g., pH 3.0). (This will work on the electrostatic interactions)

OR

- Water soluble organic (MeOH, ACN, EtOH, 10 %-20 %) in aqueous buffer (e.g., 50 mM sodium phosphate, pH 7.0) to remove any hydrophobic samples.

**Always run DI water in between cleaning solutions. Before and after.*

Technical Support: *If you have any other additional questions, please contact technical support @ techsupport@sepax-tech.com or call (302) 366-1101*