Supelco®

Chromolith® WP 300 Epoxy HPLC columns

General information and guidelines for care and use

All Chromolith® Widepore columns have been extensively tested and inspected to ensure highest quality. Please examine your column for any possible damage caused in transit. If damage has occurred, immediately notify your local Merck or MilliporeSigma representative and the delivery carrier.

Column information

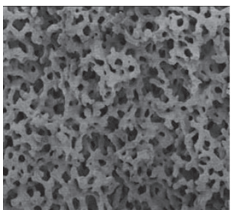
The label attached to the column indicates catalogue number, packing type, column dimensions and column number. Keep this important information with the column. If you have a problem, the column number allows us to trace the manufacturing history of your column.

Monolithic silica

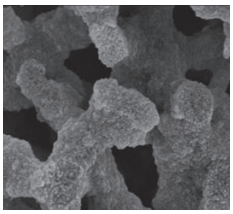
Chromolith® Widepore columns are made from a single piece of high-purity polymeric silica gel and are not packed with small silica particles. This new technology achieves a high separation performance along with a large reduction in operating pressure.

Chromolith® Widepore HPLC columns are made from highly porous monolithic rods of silica with a revolutionary bimodal pore structure providing a unique combination of macropores and mesopores. The **Macropores** allow a rapid flow of the mobile phase at low pressure.

The **Mesopores** form the fine porous structure and create the large uniform surface area on which adsorption takes place, thereby enabling high performance chromatographic separations.



a)



b)

Figure 1

Electron-microscope photographs a) Macropores; b) Mesopores

Chromolith® WP Epoxy columns are specially designed for user-specific immobilization of ligands and their later application in HPLC. The unique bimodal pore structure of silica monoliths allows for efficient coupling of molecules (ligands) to the silica surface without size-exclusion restrictions. The wider mesopores also enables the use of proteins and antibodies as ligands immobilized on the column and as analytes separated by an immobilized column.

Specifications

Table 1
Product specifications for Chromolith® Epoxy

Component	Description
Silica type	High purity, Type B
Particle size	Monolithic
Macropore size	2 µm
Mesopore size	30 nm (300 Å)
Pore volume	1 mL
Total porosity	>80 %
Surface area	~ 120 m^2/g
Epoxide concentration	~3.2 mol/m^2
Shipping solution	100 % isopropanol
Pressure limit	200 bar
pH stability immobilization	1.5 - 7.5 1.5 - 8.0 (up to 24 hours)
Storage temperature unimmobilized immobilized	 Room temperature 2 - 8 °C
Operating temperature	2 - 60 °C

Connection of Chromolith® columns to HPLC systems

Chromolith® columns are cladded with a mechanically stable and chemically robust polymer (PEEK - Poly Ether Ether Ketone). The end fittings are made of the same material. Do not remove the end fittings from the column.

The end-fittings of Chromolith® columns are connected with standard 1/16″ fittings to fit all standard HPLC, UHPLC and UPLC® systems. Short capillary tubing is recommended to minimize extra-column volumes. Install the column in the correct direction as shown on the column label.

We strongly recommend using adjustable plastic ferrules in order to avoid a possible damage to the plastic end-fittings of the Chromolith® column. The use of stainless steel ferrules is not recommended because they can damage the column end-fittings. Before connecting the column outlet to the detector, flush the column with mobile phase to remove any air.

We also recommend the use of wider capillaries during the immobilization process with ID > 0.5 mm to prevent blocking.

Preparing the column

The Chromolith® WP Epoxy column is shipped in 100 % 2-Propanol. The column has to be washed with 20 CV HPLC grade water before immobilization.

Immobilization via epoxide functions

The reaction mechanism for a direct immobilization using the epoxide group is shown in Figure 2.

The epoxy ring system enables a nucleophilic attack through a ring opening process leading to a covalent bond between the nucleophile function and the primary carbon atom. At the adjacent carbon atom, a hydroxyl group is formed. Epoxides could react with carboxyl, thiol, amine and hydroxyl functions depending on the pH of the medium. Very common is the reaction between epoxides and amines to form a secondary amine bond between the support and ligand. The use of lyotropic salts in the reaction media enhances the coupling yield. The use of lyotropic salts drives the soluble ligand toward the surface of the support by a salting out effect, enhancing the covalent reaction of epoxide and amine groups at moderate pH.

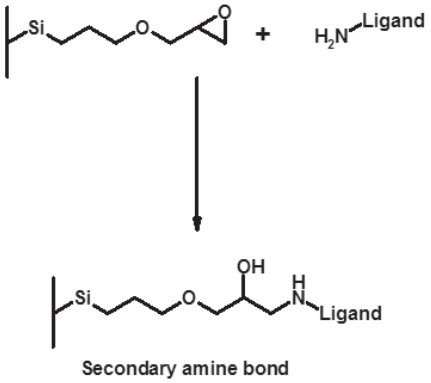


Figure 2

Reaction between epoxide functions on monolithic surface with amines forming a secondary amine bond

Additionally, other parameters influence the coupling yield of the epoxide reaction such as the ligand size and concentration, reaction time and temperature. Generally, a higher reaction time will lead to a higher surface coverage and coupling yield. Smaller molecules need higher concentrations than larger molecules (e.g. proteins) to achieve the same degree of surface coverage.

After coupling of the ligand, residual epoxide groups on the monolithic surface have to be quenched to avoid undesired backbone interactions with the analytes. Suitable reagents for quenching are 1 M glycine or 1 M urea. If the used ligand is stable at low pH, it is possible to use 150 mM phosphoric acid to hydrolyze remaining epoxide groups. All quenching reactions are finished after at least 30 minutes.

See below for an example immobilization protocol using epoxide functions for direct immobilization.

1. Connect the column to an HPLC pump (stand-alone-system is recommended) and equilibrate the column with 30 column volumes (CV) of 50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0 using a flow rate of 2.0 mL/min. The column end is connected directly to the waste. The equilibration step is performed at room temperature.
2. Dissolve the desired amount (1 - 10 mg/mL) of ligand in 25 mL of 50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0 buffer and recheck the pH value of the ligand solution. Subsequently, connect the solution to the HPLC pump.
3. Connect the column end also to the ligand solution and immobilize the ligand to the column in cycles for a maximum of 24 hours at a flow rate of 0.2 mL/min and room temperature.

4. Quench the remaining epoxide functions with 1 M glycine for 2 hours at a flow rate of 1.0 mL/min and at room temperature. The column end is directly connected to the waste.
5. Finally, equilibrate the column with starting mobile phase.

Immobilization via Schiff base mechanism

The immobilization via Schiff base mechanism requires reaction of the epoxide group to form an aldehyde. The aldehydes react with amines forming a Schiff base linkage, which is enhanced under alkaline conditions. The Schiff base linkage is susceptible to hydrolysis and can return back to the carbonyl and an amine, so the linkage can be stabilized by reduction to a secondary amine bond. As mild reductant, sodium cyanoborohydride can drive the immobilization to completion at neutral pH. Furthermore, it can be used at acidic conditions to quench the residual carbonyl groups at the monolithic surface.

The Schiff base mechanism is much more reactive in comparison to the epoxy reaction previously described. Nevertheless, the reaction is affected by several parameters like reaction time, temperature, ligand size and concentration.

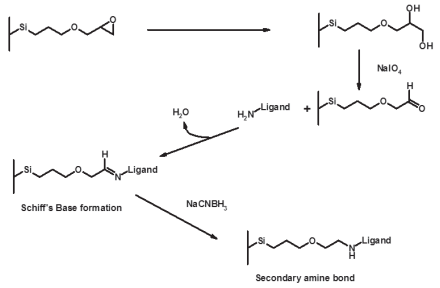


Figure 3

Scheme of immobilization via Schiff base mechanism

See below for an example immobilization protocol using Schiff base mechanism for immobilization of amines.

1. Connect the column to an HPLC pump (stand-alone-system at the best) and hydrolyze the epoxide functions with 70 CV of 2 % sulfuric acid using a flow rate of 2.0 mL/min at room temperature. The column end is connected directly to the waste.
2. Wash the column with at least 15 CV HPLC grade water at a flow rate of 2.0 mL/min. The column end is still connected to the waste.
3. The diol functions are oxidized by 100 CV 100 mM sodium periodate in water/methanol 4:1 (v/v) to carbonyl groups at 2.0 mL/min and room temperature. The column end is still connected to the waste.
4. Again, wash the column with at least 15 CV HPLC grade water at a flow rate of 2.0 mL/min. The column end is still connected to the waste.
5. Dissolve the desired amount (1 - 10 mg/mL) of ligand and 5 mM sodium cyanoborohydride in 25 mL 50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0 buffer and connect the solution to the HPLC pump.
6. Connect the column end also to the ligand solution and immobilize the ligand to the column and circulate for a maximum of 24 hours at a flow rate of 0.2 mL/min at room temperature.
7. Reduce the remaining carbonyl functions with 20 mM sodium cyanoborohydride, dissolved in 50 mM sodium phosphate pH 3.0, for 60 CV at 2.0 mL/min and room temperature. The column end is directly connected to the waste.
8. Finally, equilibrate the column with the starting mobile phase.

Generally, it is possible to perform all activation or immobilization steps at lower temperatures. However, please be aware that lower temperatures prolong the immobilization time.

Use as HPLC column

After immobilization, the column is ready to use for the desired purpose of the immobilized ligand. The type of required solvent or buffers depends on the type of ligand used.

Chromolith® WP Epoxy columns can be used with all commonly used HPLC grade organic solvents, with the following restrictions. The mobile phase should NOT contain more than 50 % Tetrahydrofurane (THF), 5 % Chlorinated solvent (e.g. Dichloromethane) or 5 % Dimethylsulfoxide (DMSO). However pure DMSO can be used as solvent for samples. Buffers, organic modifiers and ion pair reagents present no problems as long as the appropriate pH range is not exceeded. Nevertheless, be careful not to expose the column to conditions, which could cause denaturation of your ligand.

Do not exceed the **pH range from 1.5 to 7.5** with Chromolith® Widepore columns during analysis. Higher pHs will dissolve the silica, creating voids in the column. Lower pHs can eventually strip away some of the bonded phase. These defects will cause changes in retention times and loss of resolution.

Column lifetime is highly dependent on the sample and conditions, and cannot be generalized.

For samples with large quantities of contaminants, we recommend to apply one or more sample preparation methods prior to separation (e.g. solid phase extraction, filtration, centrifugation, etc.). Make sure that your samples and the mobile phases are clean and particulate free by using HPLC grade solvents and reagents.

If buffers or other salts are used, a final filtration of the mobile phase should be done with a membrane filter.

Regeneration

The robustness of monolithic columns is superior to particulate columns due to the fact that they consist of one piece of silica . The silica skeleton provides a higher stability and the specially designed bimodal pore system reduces the risk of column blockage.

To extend the lifetime of the column, "wash" the column after use and before storage to remove traces of samples and buffers from the column.

With continuous use of the column, it is necessary to monitor the column backpressure and to check the column performance with a control sample periodically.

Reverse the flow periodically to prevent particles and non-eluting sample components from accumulating on the column. When reversing the flow, flush the column before connecting it to the detector. In most cases, it is sufficient to use the standard solvent or buffer for the reverse flow washing procedure. For solvent-stable ligands, it is possible to clean the column with 100 % acetonitrile or methanol. For ligands, which are not solvent-stable, it is possible to use buffers with higher salt concentrations to clean the column.

Storage

Chromolith® WP Epoxy, which are not yet immobilized, could be stored at room temperature.

The optimum storage conditions for immobilized columns depends on the immobilized ligand.

For short-term storage, it is possible to use the initial buffer compositions as long as the column endcaps are in place and the column is stored at room temperature.

For long-term storage, it is necessary to include a bacteriostatic agent and to store the column in the refrigerator at 2 - 8 °C to prevent column fouling. It is necessary to use the endcaps to prevent drying of the column. Drying will damage the column and decrease the column performance and lifetime.

Example immobilization

Immobilization of concanavalin A

Background

Concanavalin A is a carbohydrate-binding protein which specifically binds to terminal α-D-mannosyl and α-D-glucosyl groups. It is used for the separation of glycoproteins from non-glycosylated proteins.

Materials

Concanavalin A	L7647
Sodium phosphate	137036
Ammonium sulfate	101217
Calcium chloride	102465
Sodium chloride	106444
Sodium acetate	106268
Glacial acetic acid	100063
Glycine	816013
Sodium hydroxide 50 %	158793
Methyl-α-D-mannopyranoside	M6882
Horseradish peroxidase	P8250
Ribonuclease A	55674

Immobilization according to Epoxy method

1. The Chromolith® WP Epoxy 100-4.6 mm column was connected to a HPLC pump and was equilibrated with 30 CV of 50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0 using a flow rate of 2.0 mL/min. The tubing from the column end was connected directly to the waste. The equilibration step was performed at room temperature.
2. 50 mg Concanavalin A from *Jack bean* were dissolved in 25 mL of 50 mM sodium phosphate, 1 mM calcium chloride + 1.9 M ammonium sulfate pH 8.0 buffer, and connected to the HPLC pump.
3. The tubing from the column end was also put into the ligand solution and the ligand was immobilized to the column in cycles for 4 hours at a flow rate of 0.2 mL/min at room temperature.
4. The remaining epoxide functions were quenched with 0.5 M glycine solution for 2 hours at a flow rate of 1.0 mL/min and at room temperature. The column end tubing was again directly connected to the waste.
5. The immobilized column was washed with 20 CV of 100 mM sodium phosphate puffer at pH 7.4.

HPLC application

Eluent A:	50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride pH 5.3
Eluent B:	Eluent A + 100 mM Methyl-α-D-mannopyranoside
Flow rate:	2.0 mL/min
Detection:	214 nm
Temperature:	25 °C
Injection volume:	5 µl
Gradient:	0.00 - 1.00 min 0 % B; 1.00 - 1.25 min 0 - 100 % B; 1.25 - 3.50 min 100 % B; 3.50 - 3.60 min 100 - 0 % B

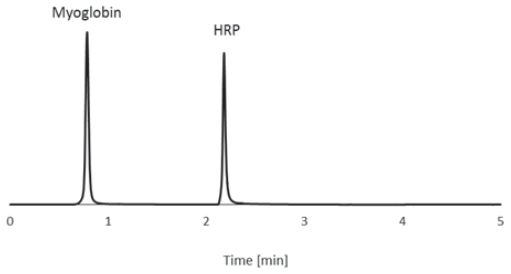


Figure 4

Separation of glycosylated HRP from non-glycosylated myoglobin with immobilized concanavalin A on Chromolith® WP Epoxy

Immobilization of human serum albumin (HSA)

Background

HSA is one of the most evaluated proteins used in chiral separations. It is also used for evaluation of drug-protein interactions. HSA is known for its ability to bind a variety of drugs and separate those in their respective enantiomers. HSA consists of three structurally similar domains I-III where each can be divided into two subdomains A and B. Binding site studies have shown that the main interactions for the enantiomeric separation takes place at two primary binding sites located at domain IIA and IIIA. Those binding sites are also known as Sudlow site I and Sudlow site II.

Materials

Sulfuric acid	112080
Sodium periodate	311448
Sodium phosphate	137036
Ammonium sulfate	101217
Ortho-phosphoric acid 85 %	100573
Sodium hydroxide 50 %	158793
Human serum albumin	A1653
Sodium cyanoborohydride	156159
Methanol	106018
Warfarin	A2250
Acetonitrile	100030

Immobilization according to Schiff base method

1. Chromolith® WP Epoxy column was connect to a HPLC pump and the epoxides were hydrolyzed to diol groups with 70 CV of 2 % sulfuric acid using a flow rate of 2.0 mL/min at room temperature. The column end tubing was connected directly to the waste.
2. Subsequently, the column was washed with at least 20 CV HPLC grade water at a flow rate of 2.0 mL/min.
3. The diol functions were oxidized to carbonyl functions by 100 CV of 100 mM sodium periodate in water/methanol 4:1 (v/v) at 2.0 mL/min and room temperature.
4. The aldehyde-activated column was again washed with 20 CV HPLC grade water at a flow rate of 2.0 mL/min at room temperature.
5. 250 mg HSA and 8 mg sodium cyanoborohydride were dissolved in 25 mL of 50 mM sodium phosphate + 1.9 M ammonium sulfate pH 8.0 buffer and connected to the HPLC pump.
6. The column end tubing was also put into the ligand solution and the column was immobilized in cycles for 24 hours at a flow rate of 0.2 mL/min and room temperature.
7. After immobilization, the remaining carbonyl groups were reduced by 20 mM sodium cyanoborohydride solution, dissolved in 50 mM sodium phosphate pH 3.0. In total, 60 CV at 2.0 mL/min were applied to the column at room temperature. The column end tubing was directly connected to the waste.
8. Finally, the immobilized HSA column was equilibrated with 100 mM sodium phosphate pH 7.4.

HPLC application

Eluent:	10 mM sodium phosphate pH 6.0/Acetonitrile 70/30
Flow rate:	1.0 mL/min
Detection:	214 nm
Temperature:	25 °C
Injection volume:	1 µl 1 mM racemic warfarin dissolved in acetonitrile

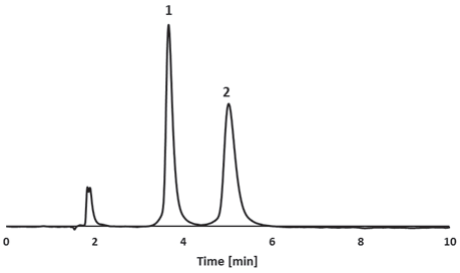


Figure 5

Separation of warfarin enantiomers by immobilized HSA on Chromolith® WP Epoxy

Peak No.	Name	k' value	Symmetry [USP]	Plates [N/m]	Resolution
1	Warfarin enantiomer 1	1.44	1.46	27460	3.61
2	Warfarin enantiomer 2	2.35	1.39	18020	

Ordering information for Chromolith® WP 300 products

Column dimension							
Length (mm)		ID (mm)	RP-18	RP-8	RP-4	Protein A	Epoxy
Chromolith® WP 300 HPLC Column [1 unit]							
25	x	4.6				1.52258.0001	1.52252.0001
25	x	2				1.52358.0001	1.52352.0001
50	x	4.6	1.52271.0001	1.52266.0001	1.52261.0001		1.52251.0001
50	x	2	1.52371.0001		1.52361.0001		1.52351.0001
100	x	4.6	1.52270.0001	1.52265.0001	1.52260.0001		1.52250.0001
100	x	2	1.52370.0001		1.52360.0001		1.52350.0001
Chromolith® Guard cartridges [3 units]							
5	x	4.6	1.52273.0001	1.52268.0001	1.52263.0001		1.52254.0001
5	x	2	1.52372.0001		1.52362.0001		1.52353.0001
10	x	4.6	1.52272.0001	1.52267.0001	1.52262.0001		1.52253.0001
Chromolith® Guard cartidge Holder							
5	x	4.6	1.52032.0001				
10	x	4.6	1.52033.0001				
Chromolith® Guard cartridge Holder							
for dimension			**Material**	**Item No.**			
5	x	2	Bioinert	1.52355.0001			
5	x	4.6	Bioinert	1.52255.0001			
10	x	4.6	Bioinert	1.52256.0001			

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