Phenyl Sepharose High Performance
Butyl Sepharose High Performance

Phenyl Sepharose™ High Performance and Butyl Sepharose High Performance are members of the GE Healthcare range of hydrophobic interaction chromatography (HIC) media for intermediate purification and polishing of proteins in packed bed. These media are particularly well suited for polishing step purification giving high resolution due to the small particle size.

Phenyl and Butyl Sepharose High Performance, are based on rigid, highly cross-linked, beaded agarose with a mean particle diameter of 34 µm. The functional groups are attached to the matrix via uncharged, chemically stable ether linkages resulting in a hydrophobic medium with minimized ionic properties. Some characteristics of Phenyl and Butyl Sepharose High Performance are listed in Table 1.

With their high physical and chemical stability, high batch-to-batch reproducibility and Regulatory Support File back-up, Phenyl and Butyl Sepharose High Performance are ideal for all stages of an industrial scale operation—from research and process development through scale-up and into production.

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1. Bioprocess

BioProcess™ media are developed and supported for production scale chromatography. All BioProcess media are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of media for production-scale.

Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities. BioProcess media cover all purification steps from capture to polishing.

2. Characteristics

The Sepharose High Performance base matrix is a highly cross-linked agarose derivative with good flow properties and high resolution, making it ideal for process scale applications, particularly the polishing stages of a separation when high resolution is required. The high physical and chemical stabilities of the matrix prevent bed compression and the formation of fines, and allow efficient maintenance procedures for increased life-length.

Figure 1 shows a pressure/flow curve for Phenyl Sepharose High Performance in a BPG 100/500 column recorded in an open bed mode to a final bed height of 10 cm, using water as packing solution. Due to the wall support offered by columns with smaller diameters, e.g. BPG100, the flow rate has to be lowered if columns with larger diameters are to be used to keep the pressure constant. In a large scale column, a flow rate of 100 cm/h at a 20 cm bed height is recommended.

Table 1 lists the main characteristics for Phenyl and Butyl Sepharose High Performance.

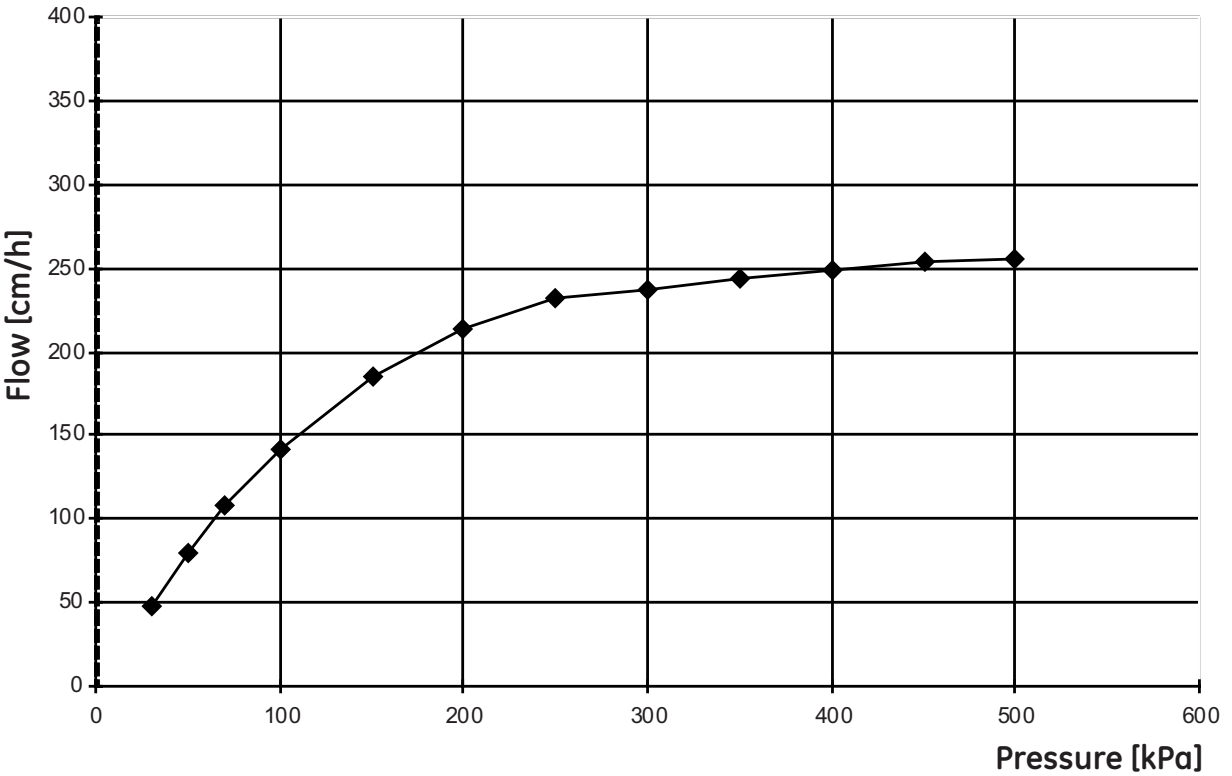

Fig 1. Pressure/flow curve for a Sepharose High Performance matrix.

Table 1. Characteristics of Phenyl and Butyl Sepharose High Performance.

	Phenyl Sepharose High Performance	**Butyl Sepharose High Performance**
Matrix	Highly cross-linked agarose 6%	Highly cross-linked agarose 6%
Degree of substitution	25 µmol phenyl groups/ml medium	40 µmol butyl groups/ml medium
Mean bead size	34 µm	34 µm
Bead size range	24–44 µm	24–44 µm
Exclusion limit M_r (globular proteins)	4×10^6	4×10^6
Max. back pressure	0.5 MPa (5 bar, 73 psi)	0.5 MPa (5 bar, 73 psi)
Recomm. linear flow rate*	Up to 100 cm/h	Up to 100 cm/h
Chemical stability	all commonly used buffers	all commonly used buffers
	1 M acetic acid	1 M acetic acid
	8 M urea	8 M urea
	6 M guanidine hydrochloride	6 M guanidine hydrochloride
	30% acetonitrile	30% acetonitrile
	30% isopropanol	30% isopropanol
	70% ethanol	70% ethanol
	2% SDS	2% SDS
pH stability	working range: 3–13	working range: 3–13
	cleaning-in-place: 2–14	cleaning-in-place: 2–14
Autoclavable.	At 120°C, pH 7 for 30 min.	At 120°C, pH 7 for 30 min.
Storage	0.01 M NaOH or 20% EtOH	0.01 M NaOH or 20% EtOH

3. Method design and optimization

The aim of designing and optimizing a method for the separation of biomolecules is to identify conditions that promote binding of the highest amount of target molecule, in the shortest possible time with highest possible product recovery.

The binding of proteins to a HIC medium is often promoted by the addition of salt. Thus, to get a high binding capacity, a high concentration of salt should be added to the feed stock and binding buffer. Since the promotion of binding by the salt is different between proteins it is possible to enrich the target molecule by choosing the most favorable concentration and type of salt. Some examples of salt type and concentration are ammonium sulphate 0.4-1.7 M, sodium sulphate 0.4-1.5 M and sodium chloride1.0-3.5 M. Other types of salt could also be used. Solubility of the target protein sets the upper limit of the salt concentration.

Low recovery can be remedied by adding some organic modifier, such as 30% iso-propanol, in the elution buffer

Design of Experiment (DoE) is an effective tool for investigation of the effect of several parameters on protein purity and recovery in order to establish the optimal elution protocol.

4. Suggested purification protocol

- Add the salt dissolved in a neutral buffer to the feed stock until the predetermined concentration is reached. The exact salt concentration has to be determined for each target molecule.
- Equilibrate the column with start buffer of the same salt concentration as in the feed.
- Apply the sample to the column.
- Wash out unbound sample using start buffer.
- Elute the target protein by applying a gradient of descending concentration of salt. Typically the gradient is 20 column volumes (CV).
- After identifying the elution volume for the target protein, the slope of the gradient can be leveled out in order to increase the resolution. It is also possible to employ a step-wise gradient.

5. Optimization of throughput

Balancing product recovery against throughput is the major consideration when optimizing a method. The dynamic binding capacity for the target protein should be determined by frontal analysis using real process feedstock. Since the dynamic binding capacity is a function of the flow velocity applied during sample application, the breakthrough capacity must be defined over a range of different residence times (flow velocities) to show the optimum level of throughput.

6. Scaling up

After optimizing the method at laboratory scale, the process can be scaled-up.

1. Select the bed volume according to required binding capacity.
2. Select a column diameter to obtain a bed height of 10-25 cm.
3. Scale-up is typically done by keeping bed height and flow velocity constant, while increasing bed diameter and volumetric flow rate.

However, since optimization is preferentially done with small column volumes, in order to save sample and buffer, some parameters like the dynamic binding capacity may be optimized using shorter bed height than those being used in the final scale. As long as the residence time is constant, the binding capacity for the target molecule remains the same.

Other factors, like clearance of critical impurities, may change when column bed height is changed and should be validated using the final bed height. The residence time is approximated as the bed height (cm) divided by the flow velocity (cm/h) applied during sample loading.

7. Column packing

General guidelines

Columns with large diameter may be difficult to pack if the bed height is too low. Bed heights of at least 10 cm are therefore recommended. Scaling up is preferentially done by keeping the bed height constant while increasing the diameter and volumetric flow-rate.

Recommended columns

Laboratory-scale columns

Column	Size	I.d. [mm]	Bed volumes up to [ml]	Bed heights up to [cm]
Tricorn™	5/50	5	1.1	5.8
Tricorn	5/100	5	2.1	10.8
Tricorn	5/150	5	3.1	15.8
Tricorn	10/50	10	4.6	5.8
Tricorn	10/100	10	8.5	10.8
Tricorn	10/150	10	12.4	15.8
XK	16/20	16	30	15
XK	26/20	26	80	15
XK	50/20	50	275	15

Large-scale columns

- BPG variable bed glass columns. Inner diameters from 100–450 mm, bed volumes up to 130 liters, bed heights up to 58 cm
- INdEX variable bed columns. Inner diameters from 70–200 mm; bed volumes up to 25 liters, bed heights up to 61 cm.
- FineLINE™variable bed columns. Inner diameters from 50-300 mm.
- Chromaflow™ variable and fixed bed columns. Inner diameters from 400–2000 mm.

Packing laboratory-scale columns

For instructions in good packing techniques look at Column Packing–The Movie that can be ordered from your GE sales office.

Packing Phenyl or Butyl Sepharose High Performance in Tricorn 10/100 column

To obtain a 10 cm bed height, pour 12 ml of a 75% medium slurry in 20% ethanol into the column to which a packing tube has been mounted. Add 20% ethanol to the top of the packing tube and mount the adaptor without trapping any air bubbles. Proceed with the packing as follows:

1. Pack the column at 2 ml/min (150 cm/h) until the bed is stabilized (~2.3 min).
2. Increase the flow to 5.2 ml/min (400 cm/h) and pack for 5 min.
3. Stop the flow and remove the packing tube and connector. If needed, remove the excess of medium. Mount and adjust the adaptor down to the surface of the medium.
4. Start the flow at 5.2 ml/min and pack for 1 min. Mark the bed height without turning the flow off.
5. Stop the flow and adjust the adaptor 2 mm below the mark.

Packing Phenyl or Butyl Sepharose High Performance in XK 16/20 column

To obtain a 10 cm bed height, fill the column tube with a 75% medium slurry. Mount the adaptor directly on the column as close to the upper edge as possible. The packing requires five steps:

1. Pack the column at 1 ml/min (30 cm/h) until the bed is stabilized (~20 min).
2. Increase the flow until a constant pressure of 5 bar is reached, and pack for 5 min or until the bed height is stable.
3. Stop the flow and adjust the adaptor down to the surface of the medium.

4. Pack for another 1-2 min at the same flow rate and mark the bed height without turning the flow off.
5. Stop the flow, adjust the adaptor 10 mm below the mark, and pack at 10 ml/min for 10 column volumes.

Packing large-scale columns

For packing instructions, refer to *www.gehealthcare.com/purification_techsupport*.

Evaluation of column packing

Test column efficiency to check the quality of the packing. Tests should be made directly after packing and at regular intervals during the working life of the column plus when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (As). These values are easily determined by applying a sample such as 2.0 M NaCl in water with 0.5 M NaCl in water as eluent. A solution of acetone (1%) in water can also be used as a test substance, but may interact with the hydrophobic medium.

Note: The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.
For optimal results, the sample volume should be at maximum 2.5% of the column volume and the flow velocity of 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use. Method for measuring HETP and As

Conditions	
Sample volume:	1% of the bed volume
Sample conc.:	2% v/v acetone in water
Fluid velocity:	30 cm/h
UV:	280 nm

Calculate HETP and As from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

- HETP = L/N
- $N = 5.54 (V_e / W_h)^2$

where

L = Bed height (cm)

N = Number of theoretical plates

V_e = Peak elution distance

W_h = Peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparison of column performance, the concept of reduced plate height is often used. Reduced plate height is calculated as:

- h = HETP/d50v

where d50v is the mean diameter of the bead, using the same unit as for HETP.

As a guideline, a value of $h<3$ is normally acceptable at the optimal test conditions presented above.

The peak should be symmetrical with an asymmetry factor as close as possible to 1 (values between 0.8 and 1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor is calculated as:

- As = b/a

where a = 1st half peak width at 10% of peak height

- b = 2nd half peak width at 10% of peak height.

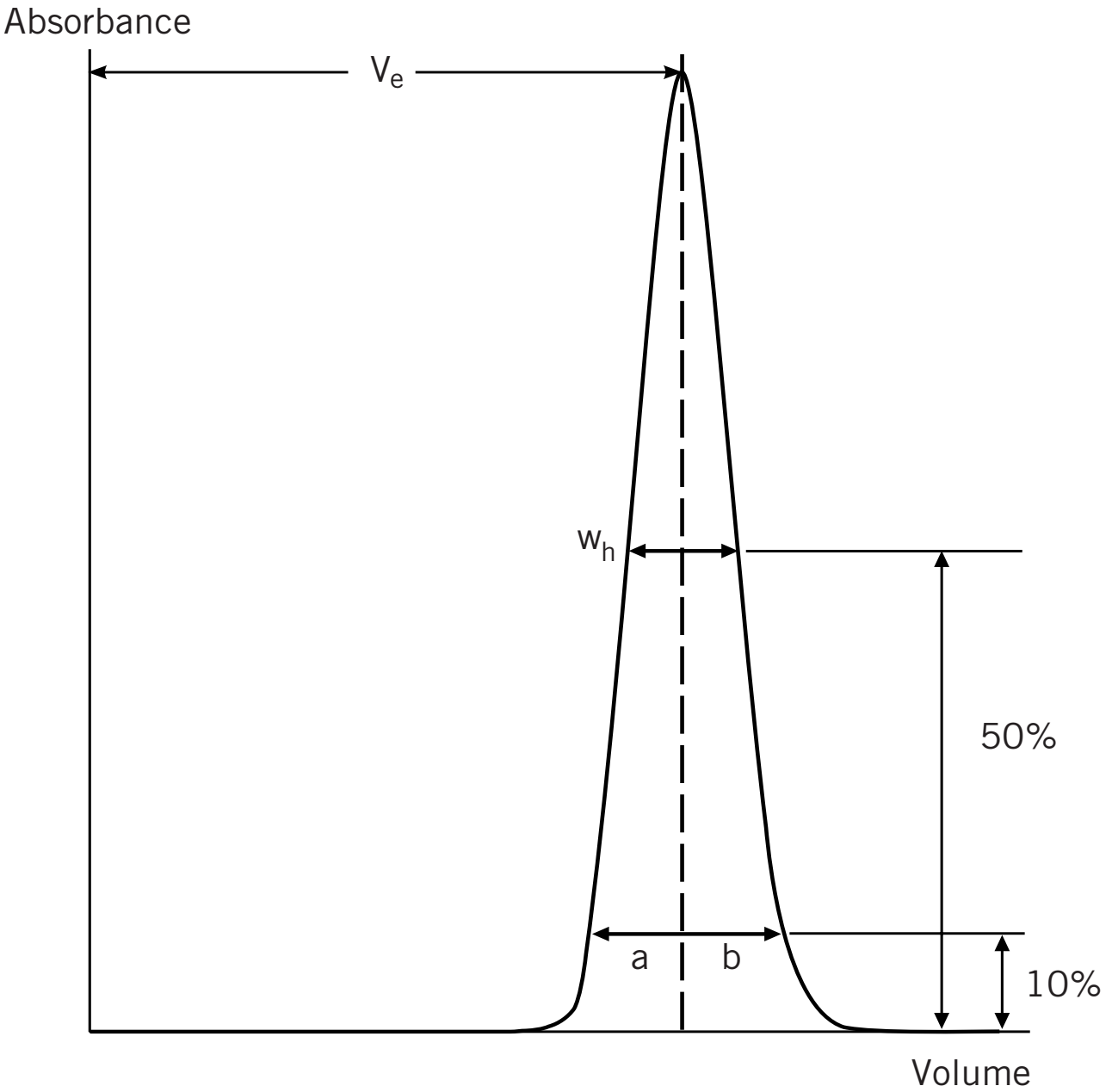

Fig. 2 A UV trace for acetone in a typical test

8. Media and column maintenance

Regeneration

For best performance from the medium, wash bound substances from the column after each chromatographic cycle.

Wash with 2 bed volumes of water, followed by 2–3 bed volumes of starting buffer.

To prevent a slow build up of contaminants on the column over time, you may have to apply more rigorous cleaning protocols on a regular basis.

Cleaning-in-place (CIP)

CIP removes very tightly bound, precipitated or denatured substances generated during previous production runs. In some applications, substances such as lipids or denatured proteins may remain in the column bed and not be eluted by regeneration. You should therefore develop

CIP protocols for the types of contaminants known to be present in the feed. Recommended procedures for removing specific contaminants are described below. CIP procedures can normally be carried out for hundreds of cycles without affecting column performance.

Suggested protocol to remove precipitated proteins

- Wash the column with 4 bed volumes of 0.5–1.0 M NaOH at 40 cm/h, followed by 2–3 bed volumes of water.

Suggested protocol to remove tightly bound hydrophobic proteins, lipoproteins and lipids

- Wash the column with 4–10 bed volumes of up to 70% ethanol or 30% isopropanol followed by 3–4 bed volumes of water. (Apply gradients to avoid air bubbles forming when using high concentrations of organic solvents.)
- Alternatively, wash the column with detergent in a basic or acidic solution, e.g. 0.5% non-ionic detergent in 1 M acetic acid. Wash at a flow rate of 40 cm/h. Remove residual detergent with 5 bed volumes of 70% ethanol followed by 3–4 bed volumes of water.

Caution: Specific regulations may apply when using 70% ethanol since it can require the use of explosion-proof areas and equipment. Consult your local safety regulations for more information.

To remove other contaminants, the following method is suggested

- Wash the column with 4 bed volumes of 0.5–1.0 M NaOH at 40 cm/h, followed by 2–3 bed volumes of water.

The CIP protocols given above should be used as guidelines when formulating a cleaning protocol specific for the raw material used. The frequency of CIP will depend on the raw material applied to the column, but we recommended using a CIP procedure at least every 5 cycles during normal use.

Depending on the nature of the contaminants, different protocols may have to be used in combination. If fouling is severe, the protocols may have to be further optimized. During CIP, reverse the flow direction through the column.

Sanitization

Sanitization is the reduction of microbial contamination in the column and related equipment to an acceptable minimum. A specific sanitization protocol should be designed for each process according to the type of contaminants present. The following is a recommended protocol.

Wash the column with 0.5–1.0 M NaOH at a flow rate of approximately 40 cm/h, contact time 30–60 minutes.

Storage

Store Phenyl Sepharose High Performance and Butyl Sepharose High Performance in 20% ethanol at 4 to 30 °C to avoid microbiological growth.

9. Ordering information

Product	Pack size	Code No
Phenyl Sepharose High Performance	75 ml	17-1082-01
	1 l	17-1082-03
	5 l	17-1082-04
Butyl Sepharose High Performance	25 ml	17-5432-01
	200 ml	17-5432-02
	1 l	17-5432-03
	5 l	17-5432-04

Related product	Quantity	Code No
HiTrap™ HIC Selection Kit	7 x 1 ml	28-4110-07
HiTrap Butyl HP	5 x 1 ml	28-4110-01
HiTrap Butyl HP	5 x 5 ml	28-4110-05
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
HiTrap Phenyl HP	5 x 5 ml	17-5195-01
HiLoad™ 16/10 Phenyl Sepharose HP	1 (20 ml)	17-1085-01
HiLoad 16/10 Phenyl Sepharose HP	1 (53 ml)	17-1086-01
Tricorn 5/100 column	1	18-1163-10
Tricorn 10/100 column	1	18-1163-15
Packing Equipment 10/100	1	18-1153-25
XK 16/20 column	1	18-8773-01
BPG 100/500 column	1	18-1103-01

Related literature	Quantity	Code No
Column Packing–The Movie	1	18-1165-33
Hydrophobic Interaction and Reversed Phase Chromatography Handbook	1	11-0012-69

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