GE Healthcare

Instructions 71-5002-39 AC **Hydrophobic Interaction Chromatography**

Phenyl Sepharose® 6 Fast Flow (low sub)
Phenyl Sepharose 6 Fast Flow (high sub)

Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) are part of the GE Healthcare media range for hydrophobic interaction chromatography (HIC). They are also part of the Fast Flow media range developed for preparative separations. They are based on rigid, 90 µm (45–165 µm) diameter highly cross-linked agarose beads, designed to meet industrial demands on reliability and scalability.

These instructions contain information about media characteristics, column packing and maintenance. To ensure best performance and trouble-free operation, please read these instructions before using Phenyl Sepharose 6 Fast Flow (low sub) or Phenyl Sepharose 6 Fast Flow (high sub).

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1. Characteristics

The base matrix, Sepharose 6 Fast Flow, is a highly cross-linked, 6% agarose derivative with excellent kinetics, making them ideal for process scale applications, particularly during initial capture and intermediate stages of a separation process, when high flow rates are required.

The high physical and chemical stability of the matrix prevents bed compression and formation of fines, and allows efficient maintenance procedures for increased media life time. Typical pressure flow rate curves are shown in Fig 1.

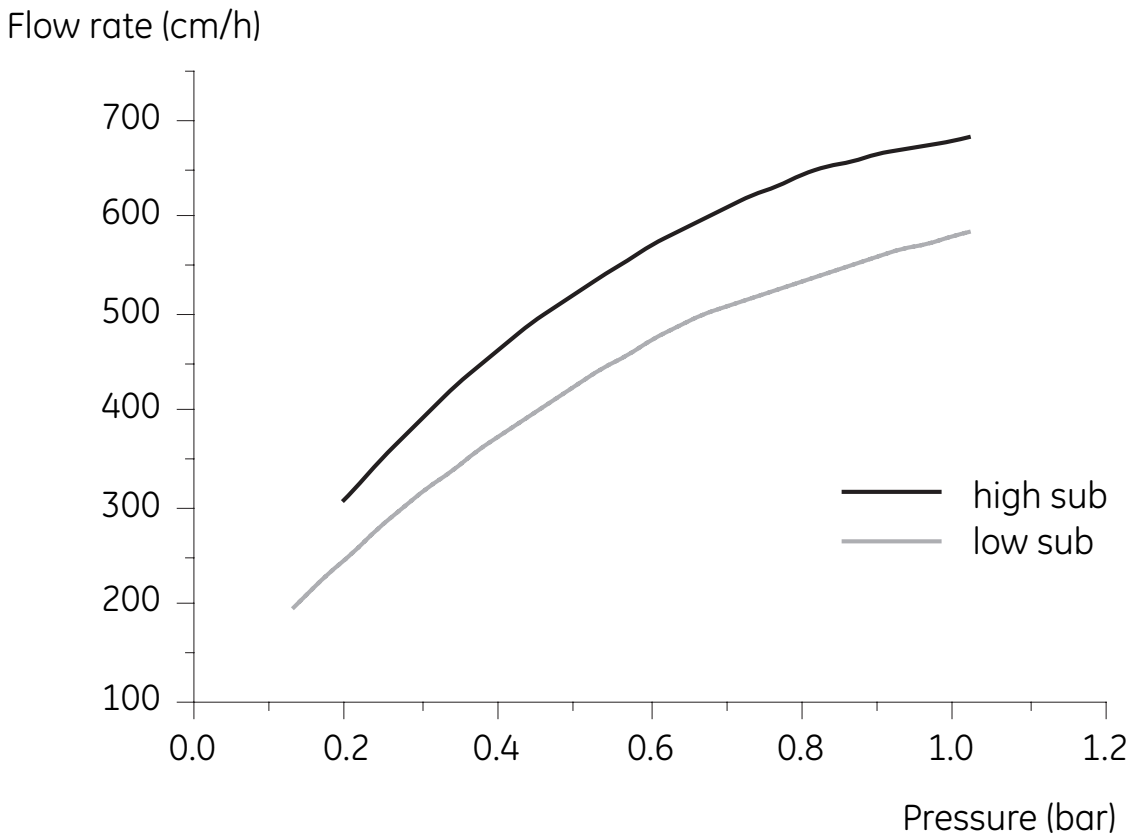


Fig 1. Typical pressure/flow rate curves for Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) in an XK 50/30 column, bed height 15 cm; mobile phase 0.1 M NaCl.

Method design and optimisation

The main purpose of optimising a chromatographic step is to reach the pre-defined purity level with highest possible product recovery by choosing the most suitable combination of the critical chromatographic parameters. In process chromatography, in contrast to analytical or small scale preparative chromatography, this has to be accomplished as quickly and economically as possible, i.e. finding the conditions that give the highest possible productivity and process economy.

Recommendations for optimising the critical operational parameters which affect the maximum utilisation of a HIC step can be found in our handbook: Hydrophobic Interaction Chromatography: Principles and Methods, Code number 18-1020-90, available from your local GE Healthcare office.

Media screening

To help with screening and selection of media, a HiTrap HIC Test Kit is available. It consists of 6 × 1 ml HiTrap columns packed with Butyl Sepharose 4 Fast Flow, Butyl-S Sepharose 6 Fast Flow, Octyl Sepharose 4 Fast Flow, Phenyl Sepharose 6 Fast Flow (high and low substitution) and Phenyl Sepharose High Performance, code number 11-0034-53.

Table 1. Characteristics of Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub).

Matrix	Highly cross linked agarose 6%
Type of ligand	Phenyl: R-O-CH_2-CH(OH)-CH_2-O-C_6H_5
Bead form	Rigid, spherical, macro porous
Mean particle size	90 µm diameter
Particle size distribution	45–165 µm
Degree of substitution	Low sub. – approx. 20 µmol phenyl/ml medium High sub. – approx. 40 µmol phenyl/ml medium
pH stability:	
working range	3–13
cleaning range	2–14
Chemical stability	Stable in commonly used aqueous buffers - 1 M NaOH, 3 M Ammonium sulphate, 30% isopropanol, 70% ethanol, 10% ethylene glycol, 0.5% SDS, 6 M guanidine-hydrochloride, 8 M Urea
Autoclavable	20 min at 121 °C
Linear flow rate at 25°C	≥ 400 cm/h at 100 kPa (1 bar, 14.5 psi) XK 50/30, 15 cm bed height
Operating temperature	4–40°C
Delivery conditions	20% ethanol

* The actual loading capacity in a real working situation will depend on the nature and concentration of contaminants in the sample, and the degree of resolution required.

2. Column packing guidelines

General

Purifying biological macromolecules by HIC is a typical high selectivity technique where the difference in retention for the molecules to be separated can be substantial at any specific ionic strength.

Therefore, relatively short columns can be used if the selectivity of the adsorbent is exploited in an optimal way. Recommended bed heights range from 3 to 15 cm, which will minimise back pressure and allow high throughput.

Recommended process scale columns

- BPG™, variable bed, glass columns: inner diameters from 100–450 mm, bed volumes up to 130 litres, bed heights max 58 cm.
- BioProcess™ Stainless Steel (BPSS) fixed bed columns: inner diameters from 400–1400 mm; fixed bed volumes from 19–230 litres; fixed bed height 15 cm.
- INdEX™ variable bed columns: inner diameters from 70–200 mm; bed volumes up to 25 litres; bed heights max 61 cm.
- CHROMAFLOW™ variable and fixed bed columns. Inner diameters from 280–2000 mm.

Packing process scale columns

General packing procedures

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the relevant column instruction manual carefully. Sepharose 6 Fast Flow based media are easy to pack since their rigidity allows the use of high flow rates, see Fig 1.

Four types of packing methods are described:

- Pressure packing (for columns with adaptors)
- Suction packing (for large columns with fixed bed heights)
- Hydraulic pressure packing
- CHROMAFLOW packing

How well the column is packed will have a major effect on the result of the separation. It is therefore very important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow rate. Guidelines are given below for determining the optimal packing flow rates for columns with adaptors and fixed bed heights.

Determining optimal packing flow rates

The optimal packing flow rate is dependent on column size and type, medium volume, packing solution, and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system.

To determine the optimal packing flow rate, proceed as follows:

1. Calculate the amount of medium needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of medium required per litre packed volume is approximately 1.15 litres sedimented medium.
2. Prepare the column exactly as for column packing.
3. Begin packing the medium at a low flow rate (30 cm/h).
4. Increase the pressure in increments and record the flow rate when the pressure has stabilised. Do not exceed the maximum pressure of the column, or the maximum flow rate for the medium.
5. The maximum flow rate is reached when the pressure/flow curve levels off or the maximum pressure of the column is reached. Stop the packing and do not exceed this flow rate. The optimal packing flow rate/pressure is 70–100% of the maximum flow rate/pressure.
6. Plot the pressure/flow rate curve as in Fig 1 and determine the optimal packing flow rate.

The operational flow rate/pressure should be <70% of the packing flow rate/pressure.

Note: For BPSS columns, first pack the column by suction packing at a low flow rate then determine the flow/pressure characteristics as above by pumping with downward flow through the column.

Packing methods

Pressure packing

BPG columns

BPG glass columns are supplied with a movable adaptor. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at a constant flow rate (or back pressure).

1. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 cm of liquid in the column.
2. Mix the packing buffer with the medium to form a 50–70% slurry. (Sedimented bed volume/slurry volume = 0.5–0.7). Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped under the adaptor. Secure the adaptor in place.
3. Seal the adaptor O-ring and lower the adaptor a little further into the slurry to fill the adaptor inlet with packing solution.
4. Connect a pump and a pressure gauge, then start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or medium.
5. When the bed has stabilised, mark the bed height on the column tube, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adaptor to 0.5–1 cm from the bed surface.
6. Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a maximum of 1 cm between bed surface and adaptor when the bed has stabilised.
7. Close the bottom valve, stop the pump, disconnect the column inlet and push the adaptor down to approximately 3 mm below the mark on the column tube without loosening the adaptor O-ring. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Suction packing

BioProcess Stainless Steel (BPSS) columns

BioProcess Stainless Steel Columns are supplied with fixed end-pieces. They are packed by sucking packing solution through the chromatographic bed at a constant flow rate.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave 2–3 cm of liquid in the column.
3. Mix the packing solution with the medium to form a 50% slurry (sedimented bed volume/slurry = 0.5). Pour the slurry into the column. Stir gently to give a homogeneous slurry.
4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow rate, see Fig 2. Keep the flow rate constant during packing.
5. When the bed has stabilised, the top of the bed surface should be just below the junction between the column and the packing device. If, when stabilised, the level of the bed is incorrect, add or remove slurry. Always stir the slurry thoroughly before packing.
6. Just before the last of the solution has entered the packed bed (before the surface starts to dry), close the valve on the column outlet, stop the pump, quickly remove the packing device and replace it with the final lid.

This final operation should be completed as quickly as possible because the bed will start to expand as soon as flow through it stops.

7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

Hydraulic packing

INdEX columns

INdEX columns are supplied with a hydraulic function which allows an extremely simple, rapid and reproducible packing procedure to be used. The medium is packed at the same time as the adaptor is lowered into position at the correct pressure.

The adaptor is pushed down by a constant hydraulic pressure, forcing water through the slurry and compressing it so that a packed bed is gradually built up. The hydraulic pressure can be generated using a pump and a pressure relief valve.

When the adaptor reaches the surface of the settled medium, it continues downwards under hydraulic pressure compressing the medium. The extent to which the medium is compressed depends upon the pressure from the adaptor and the elasticity of the medium. The quantity of medium required when packing Sepharose 6 Fast Flow based media by hydraulic pressure is approximately 1.2 litres sedimented medium per litre packed bed.

1. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 cm of liquid in the column.
2. Pour the 75% slurry carefully into the column. Fill the column with buffer solution up to the edge of the glass tube. Mix the slurry and buffer solution. Allow the medium to settle to below the bevel (G) on the glass tube, see Fig 2.
3. Rest the adaptor against the bevel (G) on the glass tube. Tilt the lid slightly to avoid trapping air bubbles under the net when mounting it on the column. Lower and secure it in place.
4. Connect a pump to the inlet of the hydraulic chamber (A) in-line with a manometer and a pressure relief valve, between the pump and the hydraulic chamber. The manometer should be placed after the valve in the direction of the flow.
5. Open the hydraulic inlet (A) and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual medium.
6. Close (C) and open the elution inlet/outlet (B) to expel trapped air in the adaptor net.

A. Hydraulic inlet
B. Elution inlet/outlet
C. Hydraulic outlet
D. Elution inlet/outlet
E. Hydraulic chamber
F. Slurry/Packed bed
G. Bevel on glass tube

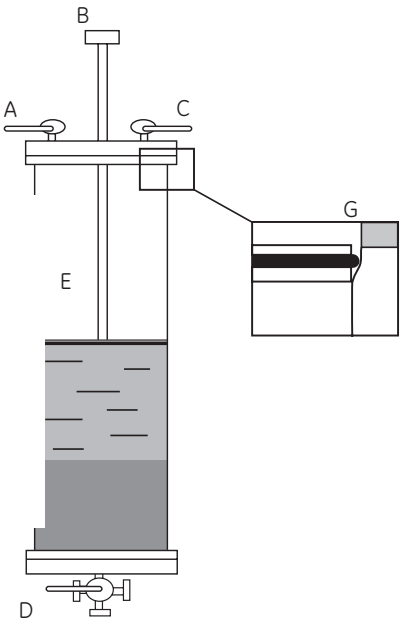


Fig 2. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

7. Close (B) and open the elution inlet/outlet (D) to start packing. Apply a pre-defined constant hydraulic packing pressure. When packing Sepharose 6 Fast flow based media in an INdEX column to a bed height of 15 cm, the recommended hydraulic packing pressure is 1.5 bar for INdEX 100 and 0.8 bar for INdEX 200.
8. When the bed has finally settled (no flow at the column outlet), stop the packing procedure by closing (A) and (D). The adaptor stops moving when the hydraulic force, acting downwards is equal to the mechanical force of the bed, expressed upwards.
9. To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adaptor to rise from the bed surface.

Note: The hydraulic pressure used for packing is not comparable to the back-pressure generated when packing with a pump or pressure vessel. When using hydraulic pressure packing, the bed is mechanically compressed during the last part of the procedure. As a result, the flow properties of the packed bed will be limited by this mechanical compression.

At any flow rate, the pressure drop over the bed under running conditions is higher than expected from the hydraulic pressure applied during packing.

It is therefore important to carefully optimize the hydraulic packing pressure in order to achieve the same flow properties as for columns packed with conventional techniques using a pump.

Packing CHROMAFLOW columns

Procedure

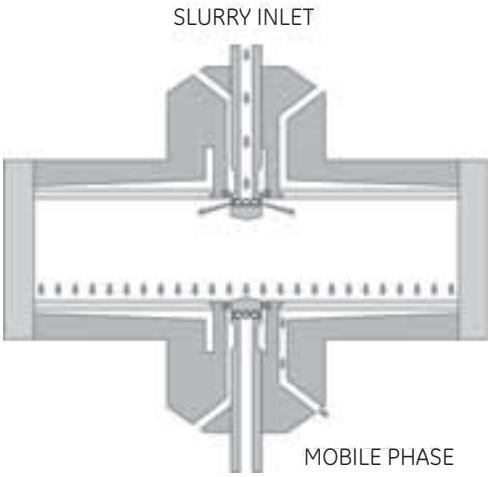
Prepare the column for packing as described in the User Manual.

Packing from the top

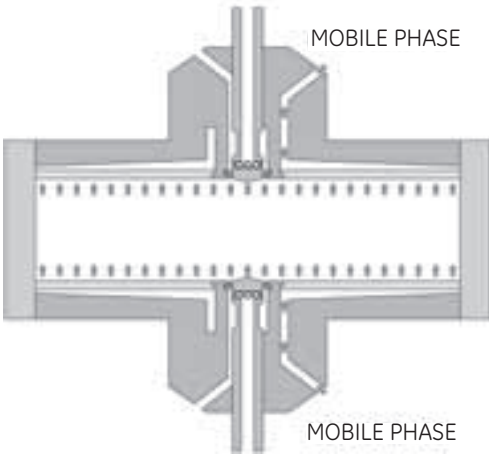
1. Set the top nozzle to the pack position (mid-position).
2. Fully retract the bottom nozzle (run position).
3. Ensure that the top mobile phase is closed.
4. Open the bottom mobile phase.
5. Open Inlet C and start the packing pump. Adjust the flow to achieve the required packing conditions for the selected medium. Monitor column pressure and the outlet flow rate in order to record column packing parameters. (Remember to stir the medium slurry during packing to prevent it from settling.)
6. Continue pumping until the column is fully packed and the pump stalls due to build-up of medium in its pipelines. Turn off the packing pump.
7. Fully retract the top nozzle to its run position. Close Outlet (C). Open Inlet (B) from the water/buffer tank and open Outlet (D). The pump should now be restarted to rinse the top slurry lines. (If the nozzle is full of liquid when in the packing position, make sure that the waste slurry outlet is open before retracting the nozzle.)
8. To clean-in-place, exchange the buffer tank for wash/buffer tank containing cleaning solution.

Packing from below

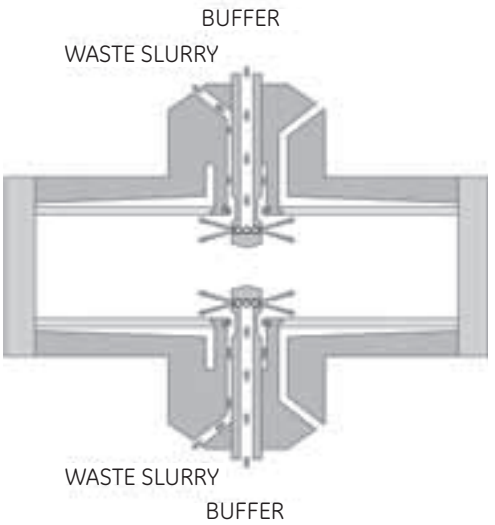
To pack from the bottom, carry out the same procedure for the connections and flow path via the bottom nozzle. The column is now ready to equilibrate and test.



Packing position
The top nozzle is extended part of the way (mid position) into the column. The bottom nozzle is fully retracted. Slurry enters the column via the top nozzle and excess liquid exits via the bottom mobile phase outlet. After packing, the slurry lines are isolated from the mobile phase and can be cleaned independently from the rest of the column.



Running position
The bottom and top nozzles are retracted. Mobile phase enters the column directly into an annulus, immediately behind the bed support. The annulus is cut through at an angle to ensure that linear flow rate is kept constant during distribution of the mobile phase across the bed.



Unpacking position
In this position, both bottom and top nozzles are fully extended into the column, thereby exposing a third passage through which medium leaves the column.

Cleaning solution can be pumped through the nozzles and sprayed into the column. In this way the column is easily and effectively cleaned without exposing the interior or the medium to the environment, or without dismantling the column.

Fig 3. Principle of operation – CHROMAFLOW columns.

Note: It is also possible to use a slightly different packing method where the amount of medium is predetermined. In this case the complete alount of medium is packed into the column causing compression of the bed. When all medium has entered the column the pump is stopped, the top nozzle is retracted, the bottom mobile phase valve closed and the medium is allowed to decompress within the column.

3. Evaluation of column packing

To check the quality of the packing and to monitor it during the working life of the column, column efficiency should be tested directly after packing, at regular intervals afterwards, and when separation performance is seen to deteriorate.

The recommended 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 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent. Sometimes a concentrated buffer solution, e.g. 10-fold, is preferred.

It is of utmost importance to realise that the calculated plate number will vary depending on the test conditions and it should therefore be used as a reference value only. It is also important that conditions and equipment are kept constant so that results are comparable. Changes in solute, solvent, eluent, sample volume, flow rate, 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 rate between 15 and 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use.

Method for measuring HETP and A_s

To avoid dilution of the sample, apply it as close to the column inlet as possible.

Conditions	
Sample volume:	1.0% of bed volume
Sample conc.:	1.0% (v/v) acetone in water, 2.0 M NaCl or 10x buffer
Eluent:	0.5 M NaCl in water, or dilute buffer
Flow rate:	30 cm/h
Detection:	
Acetone:	UV 280 nm;
NaCl, buffer:	Conductivity

Calculate HETP and the number of theoretical plates as follows:

$HETP = L/N$

and

$N = 5.54 (Ve/W_h)^2$

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.

The reduced plate height is calculated as

$$HETP/d$$

where d is the mean diameter of the beads. As a guide line, a value of <3 is normally acceptable.

The peak should be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8–1.5 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 calculation:

$$A_s = b/a$$

where

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

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

Figure 4 shows a UV trace for acetone in a typical test chromatogram in which the HETP and As values are calculated.

Column: BPG 300
Media: Sepharose 6 Fast Flow
Bed height: 57.5 cm
Bed volume: 40.6 litres
Eluent: Distilled water
Sample: 1.05 litres (1% acetone)
Flow rate: 19 cm/h
V_e =18.7
W_h =0.9
HEPT=0.024 cm
a: 0.90
b: 0.85
A_s: 0.94

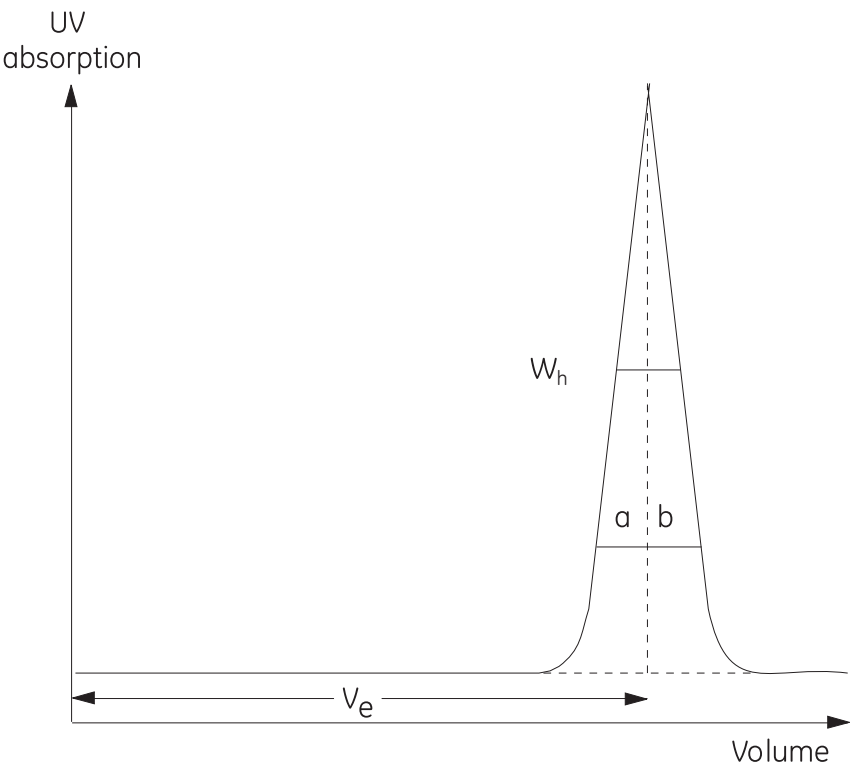


Fig 4. UV trace for acetone in a typical test chromatogram showing the HETP and A_s value calculations.

4. Media and column maintenance

Regeneration

For best performance from the media, bound substances must be washed 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, more rigorous cleaning protocols may have to be applied on a regular basis.

Cleaning-in-place (CIP)

Cleaning-in-place (CIP) is the removal of very tightly bound, precipitated or denatured substances from the purification system generated in previous purification cycles. If such contaminants accumulate on the column, they may affect the chromatographic properties of the column. If the fouling is severe, it may also block the column, increasing back-pressure and reducing flow rate.

The following are suggested methods to remove strongly 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 bubble formation when using high concentrations of organic solvents.
- Alternatively, wash the column with 1–2 bed volumes of 0.5% non-ionic detergent (e.g. in 1 M acetic acid), followed by 5 bed volumes of 70% ethanol to remove the detergent, and 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 it is recommended to use 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 optimised. During CIP flow direction through the column should be reversed.

Sanitisation

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

Wash the column thoroughly with running buffer after sanitization.

Sterilisation

To sterilise Phenyl Sepharose 6 Fast Flow (high sub or low sub), dismantle the column and autoclave the medium for 20 minutes at 120 °C.

Storage

Store Phenyl Sepharose 6 Fast Flow (high sub or low sub) in 20% ethanol at +4–+30 °C, to avoid microbiological growth.

5. Further information

Please read these instructions carefully before using Phenyl Sepharose 6 Fast Flow media. For further information visit www.gehealthcare.com or contact your local GE Healthcare representative.

6. Ordering information

Product	Pack size	Code No
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
	200 ml	17-0965-05
	1 litre	17-0965-03
	5 litres	17-0965-04
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
	200 ml	17-0973-05
	1 litre	17-0973-03
	5 litres	17-0973-04
	10 litres	17-0973-06
HiTrap HIC Test Kit	6 × 1 ml	11-0034-53

Handbook	
Hydrophobic Interaction Chromatography: Principles and Methods	18-1020-90
HiPrep 16/10 Phenyl (low sub) 20 ml	17-5094-01
HiPrep 16/10 Phenyl (high sub) 20 ml	17-5095-01

The complete range of Sepharose Fast Flow media includes other HIC media as well as media for ion exchange and affinity chromatography. Further information is available upon request.

Columns

For information about process scale columns, please ask for the following Data Files.

Data File	Code No
BPG 100, 140, 200, 300, 450	18-1115-23
INdEX	18-1115-61
BioProcess Stainless Steel	18-1121-08
CHROMAFLOW columns	18-1138-92

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