instructions

Butyl-S Sepharose™ 6 Fast Flow

11-0026-33 Edition AA

Amersham Biosciences

1. Introduction

Butyl-S Sepharose™ 6 Fast Flow forms part of the GE Healthcare range of media for hydrophobic interaction chromatography (HIC). It also belongs to the BioProcess™ Media family. BioProcess Media are separation media developed, made and supported for industrial use, especially the manufacture of biopharmacauticals. With their high physical and chemical stability, very high batch-to-batch reproducibility and Regulatory Support File back-up, BioProcess Media are ideal for all stages of an operation - from process development through scale-up and into production.

These instructions contain information about medium characteristics as well as column packing, testing and maintenance.

To ensure best performance and trouble-free operation, please read these instructions before using Butyl-S Sepharose 6 Fast Flow.

2. Characteristics

The base matrix, Sepharose 6 Fast Flow, is a highly cross-linked, 6% agarose derivative with excellent flow characteristics, making it ideal for process scale applications, particularly the initial capture and intermediate purification stages of a separation when high flow rates are 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 typical pressure/flow curve for Butyl-S Sepharose 6 Fast Flow. Table 1 lists its main characteristics.

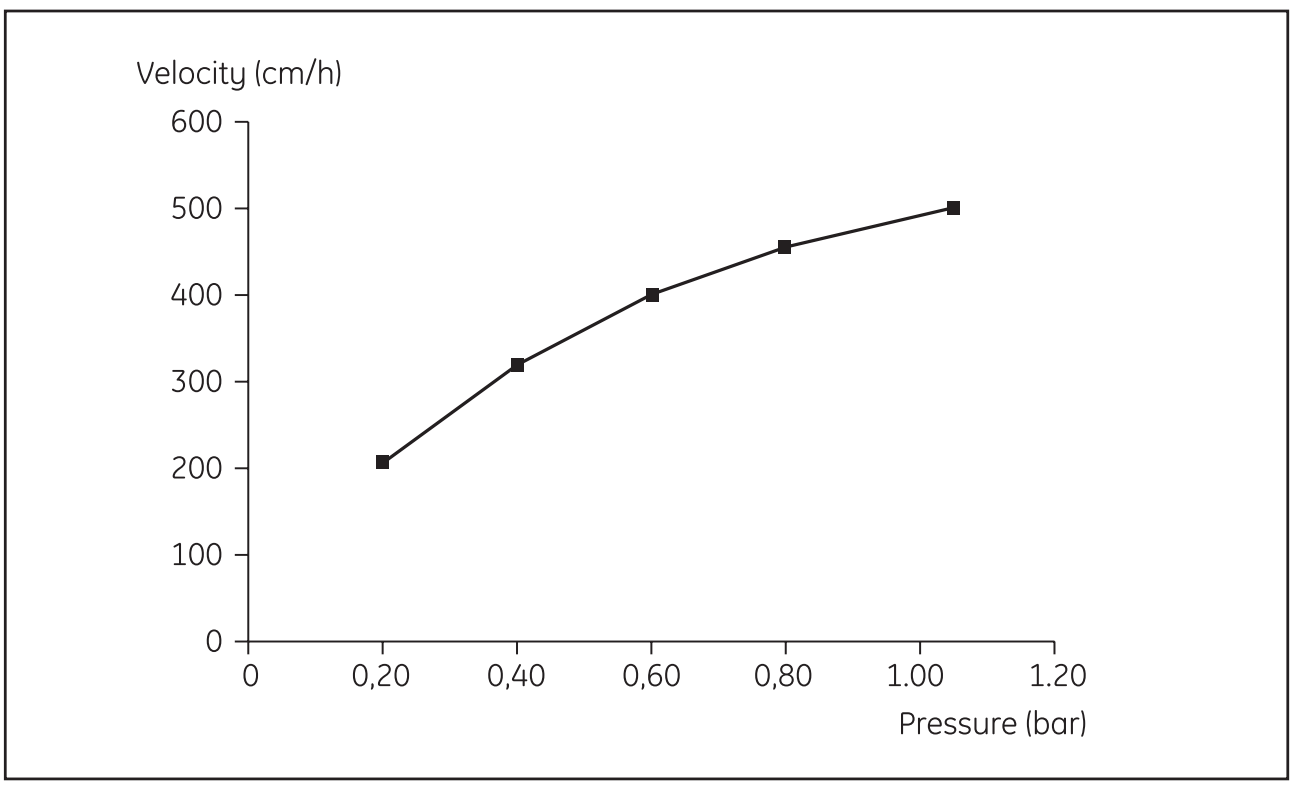


Fig. 1. Typical pressure/flow curve for Butyl-S Sepharose 6 Fast Flow in an XK 50/30 column, bed height 15 cm; mobile phase 0.1 M NaCl.

Table 1. Characteristics of Butyl-S Sepharose 6 Fast Flow.

Matrix	Highly cross-linked agarose 6%
Type of ligand	Butyl-S: -S-$(CH_2)_3$-CH_3 attached via a 12-atom spacer
Bead form	Rigid, spherical, macroporous
Mean particle size	90 µm diameter
Particle size distribution	45-165 µm
Degree of substitution	Approx. 10 µmoles ligand/ml medium
pH stability	
working range	3-13
cleaning range	2-14
Chemical stability	Stable in commonly used aqueous buffers: 1 mM HCl, 1 M NaOH, 30% isopropanol, 50% ethylene glycol, 70% ethanol, 6 M guanidine-hydrochloride, 8 M urea
Autoclavable	In 0.05 M sodium phosphate buffer pH 7.0 at 121 °C for 20 min.
Linear flow velocity 25 °C with 0.1 M NaCl as mobile phase	Approx. 400 cm/h at 100 kPa (1 bar, 14.5 psi)
Operating temperature	4-40 °C
Delivery conditions	20% ethanol

2.1 Method design and optimization

The main purpose of optimizing a chromatographic step is to reach the pre-defined purity level with the highest possible product recovery by choosing the most suitable combination of 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 optimizing the critical operational parameters that maximize the use of a HIC step can be found in our handbook Hydrophobic Interaction Chromatography and Reversed Phase Chromatography: Principles and Methods, code number 11-0012-69, or in related literature available from your local GE Healthcare office.

3. Column packing

General guidelines

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 also minimizes back-pressure and allows high throughput.

3.1 Recommended columns

Laboratory-scale columns

- Tricorn™ 5/50 (5 mm i.d.) for bed volumes up to 1.1 ml at bed heights up to 5.8 cm.

- Tricorn 5/100 (5 mm i.d.) for bed volumes up to 2.1 ml at bed heights up to 10.8 cm.
- Tricorn 5/150 (5 mm i.d.) for bed volumes up to 3.1 ml at bed heights up to 15.8 cm.
- Tricorn 10/50 (10 mm i.d.) for bed volumes up to 4.6 ml at bed heights up to 5.8 cm.
- Tricorn 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 10.8 cm.
- Tricorn 10/150 (10 mm i.d.) for bed volumes up to 12.4 ml at bed heights up to 15.8 cm.
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.
- XK 50/20 (50 mm i.d.) for bed volumes up to 275 ml at bed heights up to 15 cm.

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.
- Chromaflow™ variable and fixed bed columns. Inner diameters from 400-2000 mm.

3.2 Packing laboratory-scale columns

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adaptor with packing buffer. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with packing buffer.
3. Re-suspend medium stored in its container by shaking (do not stir sedimented medium). Mix the packing buffer with the medium to form a 50–70% slurry (sedimented bed volume/slurry volume = 0.5–0.7).
4. Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
5. If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Mount the adaptor (or lid of the packing reservoir) and connect the column to a pump. Avoid trapping air bubbles under the adaptor or in the inlet tubing.
6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow based media are packed at a constant pressure of approximately 1.5 bar (0.15 MPa). If the packing equipment does not include a

pressure gauge, use a packing flow velocity of approximately 500 cm/h (10 cm bed height, 25 °C, low viscosity buffer).

If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed.

Note: Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures using the same pump.

7. When the bed has stabilized, close the bottom outlet and stop the pump.
8. If using a packing reservoir, disconnect the reservoir and fit the adaptor to the column.
9. With the adaptor inlet disconnected, push the adaptor down approximately 2 mm into the bed. Allow packing solution to flush the adaptor inlet.
10. Connect the pump, open the bottom outlet and continue packing. The bed will compress further -and a space will form between the bed surface and the adaptor.
11. Close the bottom outlet. Disconnect the column inlet and lower the adaptor approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

3.3 Packing process-scale columns

3.3.1 General packing procedures

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the 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).

Three types of packing methods are described:

- Pressure packing (for columns with adaptors)
- 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.

3.3.2 Determining optimal packing flow rates

The optimal packing flow rate depends 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 liter packed volume is approximately 1.15 liters sedimented medium.
2. Prepare the column 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 stabilized. 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 no greater than 70% of the packing flow rate/pressure.

3. 4 Packing methods

3.4.1 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 no air is 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 and start packing at the pre-determined packing flow rate (or pressure). Keep this 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 stabilized, 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 above 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 stabilized.
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).

3.4.2 Hydraulic packing

INdEX columns

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

In practice, 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.

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

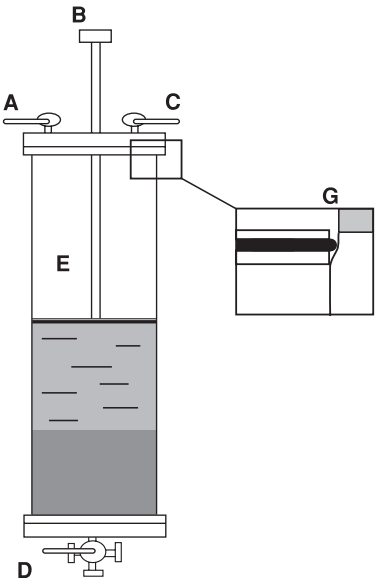


Fig. 2. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

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 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. Place the manometer 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 air trapped in the adaptor net.
7. Close (B) and open the elution inlet/outlet (D) to start packing. Apply a pre-defined constant hydraulic packing pressure.
8. When the bed has finally settled (no flow at the column outlet), stop the packing by closing (A) and (D). The adaptor stops moving when the hydraulic force acting downwards is equal to the mechanical force of the bed acting upwards.
9. Run the column with upward flow for a few minutes to remove residual air trapped in the adaptor. The column is now ready for use.
10. 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 to achieve the same flow properties as for columns packed with conventional techniques using a pump.

When packing Sepharose 6 Fast Flow media in INdEX columns to bed heights of 15 and 30 cm, the optimal hydraulic packing pressure is between 0.7 bar and 0.8 bar. The final mechanical compression at the end of the packing should be about 5 mm. The degree of mechanical compression is critical for the flow properties of the packed bed.

3.4.3 Packing Chromaflow columns

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 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). Restart the pump 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 a 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.

Note: You can also use a slightly different packing method where the amount of medium packed into the column compresses the bed. When all medium has entered the column, stop the pump, retract the top nozzle, close the bottom mobile phase valve and allow the medium to decompress within the column.

Packing position

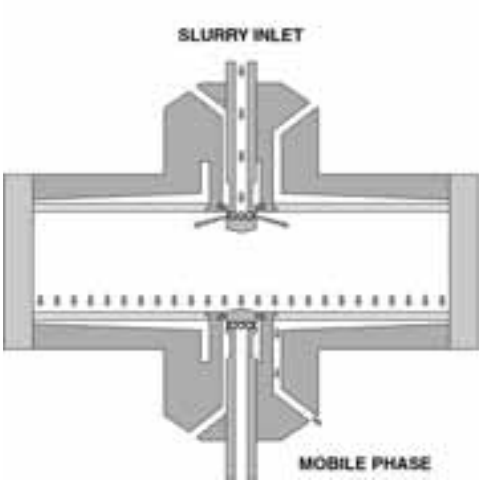
The top nozzle is extended part of the way (mid position) into the column. The bottom nozzle is fully retracted. Slurry enters the column via the top nozzle and excess liquid exits via the bottom mobile phase outlet. After packing, the slurry lines are isolated from the mobile phase and can be cleaned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

Running position

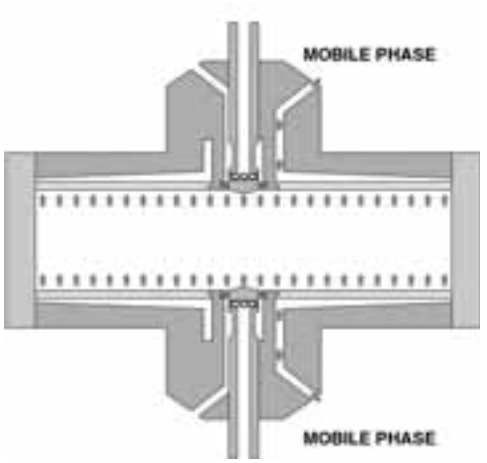
The bottom and top nozzles are retracted. Mobile phase enters the column directly into an annulus immediately behind the bed support. The annulus is cut at an angle to ensure that linear flow rate is kept constant during distribution of the mobile phase across the bed.

Unpacking position

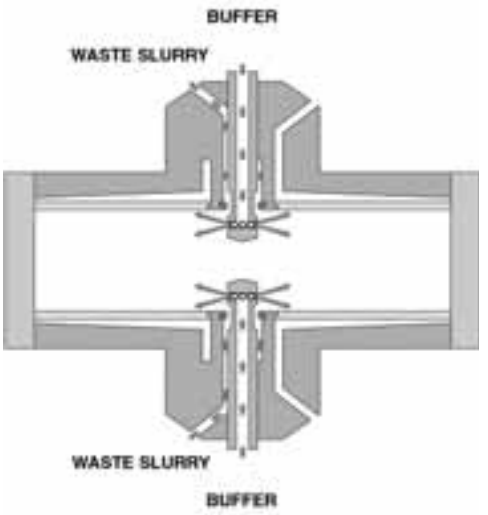
In this position, both bottom and top nozzles are fully extended into the column, thereby exposing a third passage through which medium leaves the column. Cleaning solution can be pumped through the nozzles and sprayed into the column. In this way, the column is easily and effectively cleaned without exposing the interior or the medium to the environment, or without dismantling the column.



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

4. Evaluation of column packing

To check the quality of the packing and to monitor it during the working life of the column, test column efficiency 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, A_s. These values are easily determined by applying a sample such as 1% v/v 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.

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:

$$HETP = L/N$$

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

where

L = bed height (cm)

N = number of theoretical plates

V_e = elution volume

W_h = peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparing column performance, reduced plate height is often used.

Reduced plate height is calculated as:

$$HETP/d$$

where d is the mean diameter of the beads. As a guide, a value below 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 = the distance between the peak front and the peak maximum measured at 10% of peak height.

b = the distance between the peak front and the peak end measured at 10% of peak height.

$$A_s = 0.8–1.8 \text{ (guide)}$$

Figure 4 shows a UV trace for acetone in a typical test chromatogram.

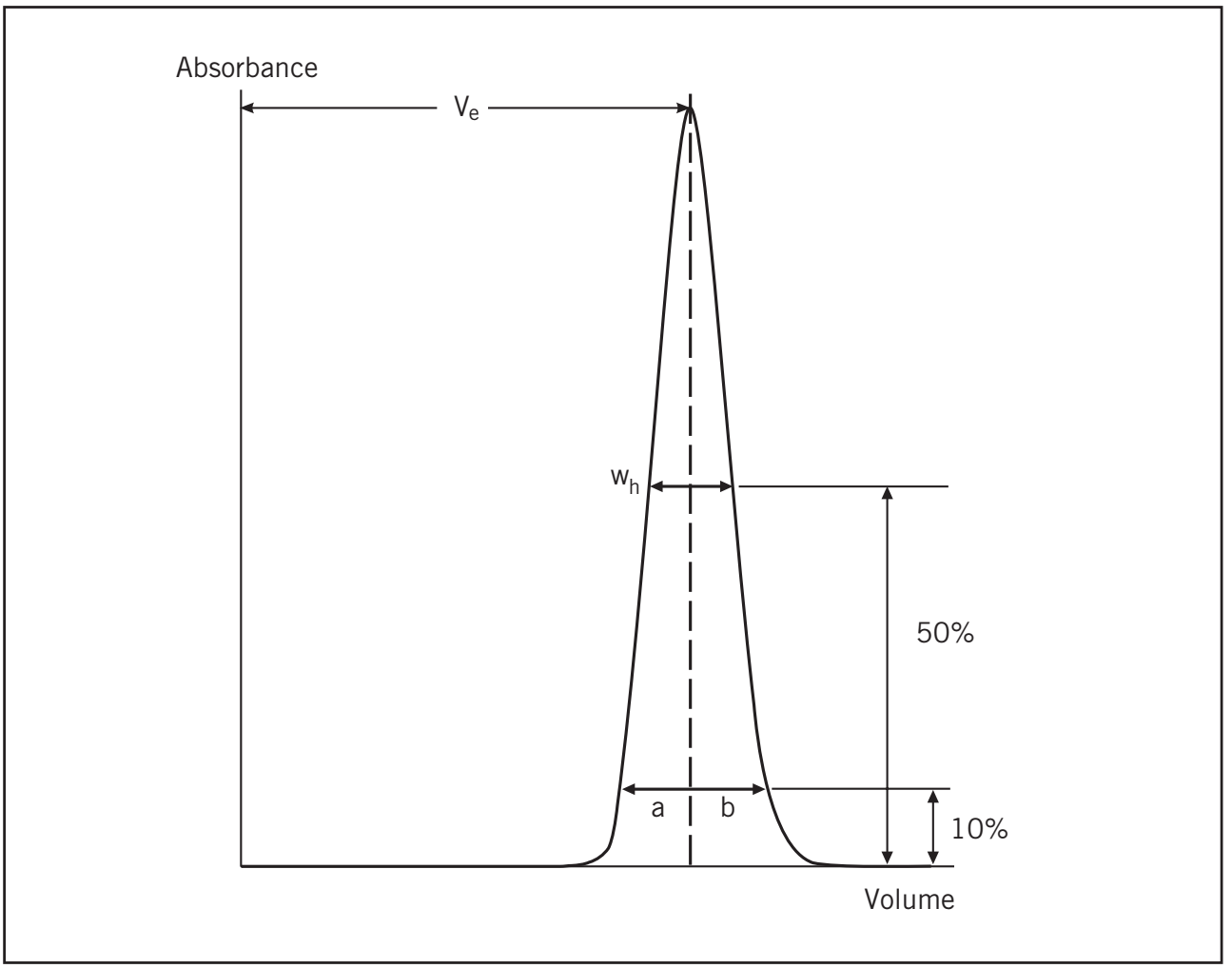


Fig. 4. UV trace for acetone.

5. Media and column maintenance

5.1 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.

5.2. 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.

5.3. Sanitization

Sanitization is the reduction 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.

5.4 Storage

Store Butyl-S Sepharose 6 Fast Flow in 20% ethanol at +4 to +30 °C to avoid microbiological growth.

6. Ordering information

Product	Pack size	Code No.
Butyl-S Sepharose 6 Fast Flow	25 ml	17-0978-10
	200 ml	17-0978-02
	1 liter	17-0978-03
	5 liters	17-0978-04
Empty laboratory columns	**Quantity**	
Tricorn 5/50	1	18-1163-09
Tricorn 5/100	1	18-1163-10
Tricorn 5/150	1	18-1163-11
Tricorn 10/50	1	18-1163-14
Tricorn 10/100	1	18-1163-15
Tricorn 10/150	1	18-1163-16
XK 16/20	1	18-8773-01
XK 26/20	1	18-1000-72
XK 50/20	1	18-1000-71

Handbook	Code No.
Hydrophobic Interaction Chromatography and Reversed Phase Chromatography: Principles and Methods	11-0012-69

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.

Process scale columns

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

Data File	Code No.
BPG 100, 140, 200, 300	18-1115-23
BPG 450	18-1060-59
INdEX	18-1115-61
Chromaflow	18-1138-92

Important information

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