GE Healthcare
Life Sciences

Instructions 28-9955-34 AB Hydrophobic interaction media

Capto™ Butyl

Capto Butyl is a hydrophobic interaction chromatography (HIC) medium used in the capture and intermediate stages of protein purification.

Capto Butyl combines high capacity, high flow rate and low backpressure reduces process cycle times and increases productivity. The Capto product range comprises modern media that meet the demands of large-scale biopharmaceutical manufacturing today.

Capto Butyl offers the following benefits:

- Improved productivity and process economy in downstream operations
- Very high flow rates and large sample volume processing
- Excellent chemical stability

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1 BioProcess™ Media

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 Properties of Capto Butyl

Capto Butyl is a hydrophobic interaction chromatography (HIC) medium used in the capture and intermediate stages of protein purification.

Capto Butyl is based on a highly cross-linked agarose matrix with high physical and chemical stabilities which gives excellent flow properties. Such high flow rates permit rapid processing of large sample volumes with only moderate reductions in binding capacity.

Capto Butyl displays a different selectivity compared to Butyl Sepharose™ Fast Flow which is due to greater cross-linking of the agarose base matrix of Capto media. Characteristics of Capto Butyl medium are listed in Table 1.

Capto Butyl is intended for initial and intermediate step purifications requiring a chromatography medium with low to medium hydrophobicity. Capto Butyl often works efficiently with rather low salt concentrations. The mechanism of binding and elution onto the butyl ligand is different from that onto the phenyl ligand, giving a different selectivity. Performance during sample run and selectivity are similar to that of Butyl Sepharose 4 Fast Flow. Higher flow rates can be used with Capto Butyl during equilibration, wash and regeneration.

Table 1. Characteristics of Capto Butyl.

Ligand	Butyl
Matrix	Highly cross-linked agarose
Particle size, d_{50v}[1]	75 µm
Ligand density	≈53 µmol/ml medium
Dynamic binding capacity at QB10%	27 mg BSA/ml medium
Recommended liquid velocity[2]	150 to 350 cm/h
Maximum liquid velocity	At least 600 cm/h in a 1 m column with 20 cm bed height at 20ºC using process buffers with the same viscosity as water; corresponds to a residence time of 2 min
pH working range	3 to 13
pH stability[3] **- short term** **- long term**	 2 to 14 3 to 13
Storage	4ºC to 30ºC in 20% ethanol

[1] d_{50v} is the average particle size of the cumulative volume distribution.

[2] Water at room temperature.
Note: For viscous buffers and samples - the liquid velocity must be optimized and starting with a low flow rate is recommended in order not to exceed pressure limits.

[3] Short term pH: pH interval where the medium can be subjected to cleaning- or sanitization-in-place without significant change in function.
Long term pH: pH interval where the medium can be operated without significant change in function.

3 Method optimization

Separation of biomolecules on HIC media is based on interplay between the hydrophobicity of the medium, the nature and composition of the sample, the prevalence and distribution of surface-exposed hydrophobic amino acid residues, and the type and concentration of salt in the binding buffer. Unlike reversed phase chromatography (RPC), which is a separation method closely related to HIC, the binding of biological solutes to HIC media is promoted, or otherwise modulated, by the presence of relatively high concentrations of anti-chaotropic salts such as ammonium sulfate and sodium sulfate (Figure 1). Elution of bound solutes is achieved simply by stepwise or gradient elution with buffers of low salt content.

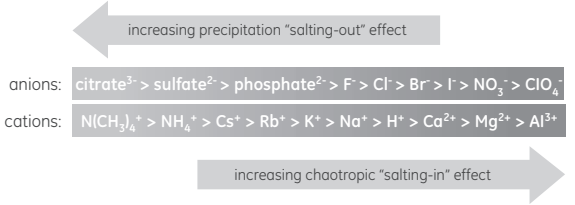

Fig 1. The Hofmeister series of some anions and cations arranged according to their effects on the solubility of protein in aqueous solutions. Increasing the salting-out effect promotes hydrophobic interactions and increases the binding capacity of the HIC medium for proteins. The opposite situation dominates when the chaotropic effect of the salts is increased.

HIC media available from GE Healthcare are produced as a graded series of hydrophobic media based on alkyl or aryl ligands attached to a hydrophilic base matrix, for example Capto and Sepharose. In each instance, the type and concentration of ligand has been optimized to cover the range of hydrophobicities of the proteins in a biological extract, varying from weak to moderate to strong hydrophobic proteins. This strategy results in HIC media for "all occasions" where the emphasis is on high recovery, purity, and reduced risk for denaturation of the target proteins in a biological extract.

Factors affecting HIC

The main parameters to consider when selecting a HIC medium and optimizing its chromatographic performance are:

- The nature of the base matrix (e.g., agarose, organic co-polymers, etc.)
- Structure of the ligand
- Concentration of the ligand
- Characteristics of the target protein and other sample components
- Type of salt
- Concentration of salt
- Temperature
- pH

Of these parameters, the *structure and concentration of ligand* as well as the *type and concentration of salt* added during the binding step are of highest importance in determining the outcome of an HIC event. In general, the type of immobilized ligand determines its binding selectivity toward the proteins in a sample while its concentration determines its binding capacity.

HIC media fall into two groups, depending on their interactions with sample components:

- Straight alkyl chains (butyl, octyl) show a "pure" hydrophobic character.
- Aryl ligands (phenyl) show a mixed mode behavior, where both aromatic and hydrophobic interactions as well as lack of charge play simultaneous roles.

The choice of ligand must be determined empirically through screening experiments for each individual separation problem.

Target protein

The target protein characteristics (in an HIC context) are usually not known since minimal data are available in this respect. There are some published data regarding the hydrophobicity indices for a number of purified proteins based on amino acid composition, the number and distribution of surface-exposed hydrophobic amino acids, and the order of their elution from RPC columns but

few, if any, have proved to be useful when purifying a protein in a real biological sample. For this and other reasons, the binding behavior of a protein exposed to an HIC medium has to be determined on a case-by-case basis.

Solvent

The solvent is one of the most important parameters, which influence the capacity and selectivity in HIC. In general, the binding process is more selective than the elution process. It is therefore important to optimize the start buffer with respect to pH, type of solvent, type of salt and concentration of salt.

Salts

The addition of various "salting-out" salts to the sample promotes ligand-protein interactions in HIC. As the concentration of salt is increased, the amount of bound protein increases up to the precipitation point for the protein. Each type of salt differs in its ability to promote hydrophobic interactions and it may be worthwhile testing several salts.

The most commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl and CH_3COONH_4. At a given concentration, ammonium sulfate often gives the best resolution of a mixture of standard proteins compared to other salts. If sodium chloride is used, a concentration of up to 3–4 M is usually needed. Due to instability, ammonium sulfate is not suitable when working at pH values above 8.0. Sodium sulphate is also a very good salting-out agent but protein solubility problems may exclude its use at high concentrations.

Protein binding to HIC adsorbents is promoted by moderate to high concentrations of "salting-out" salts, which also have a stabilizing influence on protein structure. Elution is achieved by a linear or step-wise decrease in concentration of the salt.

The HIC medium should bind the protein of interest at a reasonably low concentration of salt. Binding conditions are dependent on the salt chosen. The salt concentration should be below that which causes precipitation of proteins in the sample.

- If the substance does not bind, a more hydrophobic medium should be chosen.
- If the substance binds so strongly that non-polar additives are required for elution, a less hydrophobic medium should be tried.

The bound protein should be eluted from the column with high recovery.

pH

The effect of pH is not well established. In general, an increase in pH above 8.5 weakens hydrophobic interactions whereas a decrease in pH below 5.0 results in an apparent increase in the retention of proteins on HIC media. In the range of pH 5–8.5, the effect seems to be minimal or insignificant.

Temperature

It is generally accepted that the binding of proteins to HIC media is entropy driven, which implies that the solute-media interaction increases with increased temperature. In some instances, the reverse effect has been observed. In practical work, be aware that a purification process developed at room temperature might not be reproduced in the cold room, or vice versa. In other instances, temperature control is mandatory in order to obtain reproducible results from run to run.

Additives

Sometimes it is necessary to weaken the protein-ligand interactions by including different additives. Commonly used are water-miscible alcohols (propanol, ethylene glycol), detergents (SDS) and solutions of chaotrophic salts (lithium perchlorate, urea, guanidine hydrochloride).

Automated buffer preparation

Users of ÄKTA™ design chromatography systems with BufferPrep or BufferPro functionality can select from a range of buffer recipes to conveniently screen chromatography media over a range of pH values and elution conditions.

4 Scale-up

After optimizing the method at laboratory scale, the process can be scaled up. Scale-up is typically performed by keeping bed height and linear liquid velocity constant while increasing bed diameter and volumetric flow rate. However, since optimization is preferentially performed with small column volumes, in order to save sample and buffer, some parameters such as the dynamic binding capacity may be optimized using shorter bed heights than those being used in the final scale. As long as the residence time is kept constant, the binding capacity for the target molecule remains the same.

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

Select the bed volume according to required binding capacity. Keep sample concentration and gradient slope constant.

The larger equipment used when scaling up may cause some deviations from the method optimized at small scale. In such cases, check the buffer delivery system and monitoring system for time delays or volume changes. Different lengths and diameters of outlet tubing can cause zone spreading on larger systems.

5 Packing columns

Recommended columns

Table 2. Recommended columns for Capto Butyl

Column	Inner diameter (mm)	Bed volume[1]	Bed height (cm)
Lab scale			
HiScale™ 16/20	16	20–40 ml	max 20
HiScale 16/40	16	20–70 ml	max 35
HiScale 26/20	26	53–106 ml	max 20
HiScale 26/40	26	53–186 ml	max 35
HiScale 50/20	50	196–393 ml	max 20
HiScale 50/40	50	196–687 ml	max 35
Production scale			
AxiChrom™	50–200	0.2–12.5 l	max 40
AxiChrom	300–1000	7–314 l	max 40

[1] Bed volume range calculated from 10 cm bed height to maximum bed height.

All large-scale columns can be supplied as variable bed height columns. Do not choose large diameter columns if the bed height is low.

For practical instructions in good packing techniques, see the CD-ROM *Column Packing - The Movie* (18-1165-33).

For more details about packing HiScale columns, see instructions *HiScale™ columns (16, 26, 50) and accessories* (28-9674-70).

Packing HiScale columns

Packing preparations

Materials needed

Capto Butyl

HiScale column

HiScale packing tube (depending on bed height)

Plastic spoon or spatula

Glass filter G3

Vacuum suction equipment

Filter flask

Measuring cylinder

20% ethanol with 0.4 M NaCl

Equipment

ÄKTA systems, or a stand-alone pump such as Pump P-900, depending on the flow rate required, can be used for packing.

Equilibrate all materials to room temperature.

Definitions

The bed height of a gravity settled bed differs from the bed height of a bed settled at a given flow (consolidated). Therefore, the compression factor (CF) has to be separated from the packing factor (PF).

$L_{settled}$	Bed height measured after settling by gravity.
L_{cons}	Consolidated bed height Bed height measured after settling the medium at a given liquid velocity.
L_{packed}	Packed bed height
CF	Compression factor $CF = L_{settled}/L_{packed}$
PF	Packing factor $PF = L_{cons}/L_{packed}$
A_C	Cross sectional area of the column
V_C	Column volume $V_C = L_{packed} \times A_C$
C_{slurry}	Concentration of the slurry

Preparation of the slurry

To measure the slurry concentration, let the media settle in 20% ethanol at least overnight in a measuring cylinder or use the method for slurry concentration measurement described in application note 28-9259-32. This method can also be used with HiScale columns.

Washing the medium

Mount a glass filter funnel onto a filtering flask. Suspend the medium by shaking and pour into the funnel and wash according to the following instructions:

- 5 times with 5 ml 20% ethanol with 0.4 M NaCl/ml medium
- Gently stir with a spatula between additions.
- Move the washed medium from the funnel into a beaker and add 20% ethanol with 0.4 M NaCl to obtain a 50% slurry concentration.

Packing the column

Table 3. Main features of the packing method for HiScale 16/20 and HiScale 16/40

Column	HiScale 16/20	HiScale 16/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.10	1.10
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	25	25
Flow condition (cm/h)	750	750
Flow condition (ml/min)	25	25

Table 4. Main features of the packing method for HiScale 26/20 and HiScale 26/40

Column	HiScale 26/20	HiScale 26/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.15	1.10
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	66	66
Flow condition (cm/h)	750	750
Flow condition (ml/min)	66	66

Table 5. Main features of the packing method for HiScale 50/20 and HiScale 50/40

Column	HiScale 50/20	HiScale 50/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.15	1.15
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	250	250
Flow condition (cm/h)	750	750
Flow condition (ml/min)	250	250

Packing procedure

1. Assemble the column according to the column instructions (HiScale columns (16, 26, 50) and accessories, code no 28-9674-70).
2. Mount the column tube in a stand.
3. Connect the bottom adapter unit to the pump or a syringe and prime the bottom net with a slow flow of packing solution. This is easiest done if the nets are dry but if air is trapped under the net it can be removed by a light suction with a syringe.

4 Mount the bottom adapter unit in the bottom of the column tube and tighten the o-ring.

5 Fill the column with approximately 1 cm packing liquid using the pump/syringe. Disconnect the pump/syringe and put a stop plug on the outlet.

6 Mount the packing tube on top of the column tube.

7 Connect the top adapter to the pump and prime it with a slow downward flow. The net needs to be facing the roof as this is done. If air is trapped under the net it can be removed by a light suction with a syringe.

8 Fill the column with slurry suspended in packing solution. If needed, top up the slurry with extra packing solution so the top adapter dips into the slurry to avoid air under the net.

9 Mount the top adapter unit on top of the packing tube. Tighten the o-ring firmly and remove the bottom stop plug.

10 Start a downward flow with packing velocity according to Table 3, 4 and 5.

11 Let the flow run until the bed has consolidated.

12 Use the scale on the column to measure the bed height. There might be a build up of media at the column wall after the bed is consolidated and to easier see where the top of the bed is, a light source can be used.

13 Calculate the final bed height by dividing the consolidated bed height with the desired packing factor.
$L_{packed} = L_{cons}/PF$

14 Turn off the flow and put a stop plug in the bottom.

15 Dismount the top adapter from the packing tube.

16 Over a beaker or a sink, detach the packing tube from the column.

17 Remount the top adapter in the column tube. Make sure no air is trapped under the net and lower the adapter down to 1 to 2 cm above the bed, making sure the surface is not disturbed.

18 Tighten the O-ring on the adapter. Remove the bottom stop plug and carefully start turning the end cap down. While spilling out liquid through the bottom, proceed turning until the calculated final bed height is reached.

19 Make sure that the pressure peaks that occur during turning the end knob down do not exceed the pressure specifications of the media.

20 Start a downward flow to flow condition the bed. The flow rate is shown in Table 3, 4 and 5.

21 Let the flow run for about 10 column volumes. The column is ready to be tested.

Testing the packed column

See Chapter *Evaluation of column packing*.

6 Evaluation of column packing

Test the column efficiency to check the quality of packing. Testing should be done after packing, at regular intervals during the working life of the column or 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 (A_s). 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 0.8 M NaCl in water with 0.4 M NaCl in water as eluent.

For more information about column efficiency testing, consult the application note *Column efficiency testing* (28-9372-07).

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, liquid 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 liquid velocity 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 A_S

Calculate HETP and A_S from the UV curve (or conductivity curve) as follows:

$$HETP = \frac{L}{N}$$

$$N = 5.54 \times \left(\frac{V_R}{W_h}\right)^2$$

L = bed height (cm)

N = number of theoretical plates

V_R = volume eluted from the start of sample application to the peak maximum

W_h = peak width measured as the width of the recorded peak at half of the peak height

V_R and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance.

The reduced plate height, h, is calculated as follows:

$$h = \frac{HETP}{d_{50v}}$$

d_{50v} = mean diameter of the beads (cm)

As a guideline, a value of < 3 is very good.

The peak should be symmetrical, and the asymmetry factor as close to 1 as possible
(a typical acceptable range could be $0.8 < A_S < 1.8$).

A change in the shape of the peak is usually the first indication of bed deterioration due to excessive use.

Peak asymmetry factor calculation:

$$A_s = \frac{b}{a}$$

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

Figure 2 shows a UV trace for acetone in a typical test chromatogram from which the HETP and A_s values are calculated.

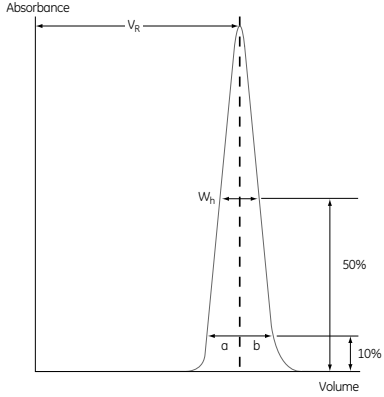

Fig 2. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

7 Cleaning-In-Place (CIP)

Correct preparation of samples and buffers should maintain columns in good condition. However, reduced performance, increased back pressure or blockage indicates that the chromatography medium needs cleaning.

CIP removes very tightly bound, precipitated or denatured substances from the medium. If such contaminants are allowed to accumulate, they may affect the chromatographic properties of the prepacked column, reduce the capacity of the medium and, potentially, come off in subsequent runs. If the fouling is severe, it may block the column, increase back pressure and reduce flow rate.

CIP should be performed regularly to prevent the build-up of contaminants and to maintain the capacity, flow properties and

general performance of prepacked columns. CIP is usually performed after the elution.

It is recommended to perform a CIP:

- When an increase in back pressure is seen.
- If reduced column performance is observed.
- Before first time use or after long term storage.
- Between runs when the same column is used for purification of different proteins to prevent possible cross-contamination.
- After every run with real feed.

CIP protocol

Capto HIC media can normally be regenerated by washing with distilled water, but are also alkali-tolerant media. The nature of the sample will ultimately determine the final CIP protocol so the CIP procedure below may require optimization. NaOH concentration, contact time and frequency are typically the main parameters to vary during the optimization of the CIP. The CIP procedure below removes common contaminants.

- Regular cleaning with 0.5–1.0 M NaOH. NaOH has the ability to dissolve proteins and saponify fats.
- Strongly bound substances can be removed by washing with 5–10 column volumes (CV) of up to 70% ethanol or 30% isopropanol.

Flow rate: It is recommended to use a lower flow rate than during the purification.

1 Wash with 3 CV of water or elution buffer.

2 Wash with 4 CV 0.5–1.0 M NaOH at a low flow rate (approximately 65–130 cm/h).

3 Wash with at least 3 CV of water.

8 Storage

Capto HIC media are supplied pre-swollen in a 20% ethanol solution. Recommended storage conditions are 20% ethanol at room temperature.

9 Ordering information

Product	Pack size	Code No
Capto Butyl[1]	25 ml	17-5459-01
	1 l	17-5459-03
	5 l	17-5459-04

[1] All bulk media products are supplied in suspension in 20% ethanol. For additional information, including data file, please contact your local GE Healthcare representative.

Related product	Quantity	Code No
HiScreen™ Capto Butyl	1x4.7 ml	28-9924-73
HiScale 16/20	1	28-9644-41
HiScale 16/40	1	28-9644-24
HiScale 26/20	1	28-9645-14
HiScale 26/40	1	28-9645-13
HiScale 50/20	1	28-9644-45
HiScale 50/40	1	28-9644-44

Related literature		Code No
Movie	Column Packing - The Movie	18-1165-53
Handbook	Hydrophobic Interaction Chromatography and Reversed Phase Chromatography, Principles and Methods	11-0012-69
Instructions	HiScale columns (16, 26, 50) and accessories	28-9674-70

10 Further information

Visit us at:www.gelifesciences.com/capto.

For technical support,
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