Separation of 5-Carboxyfluorescein (FAM) from Bovine Serum Albumin (BSA) using the OmniSep™ FPLC Desalting Column

by Trevor Hopkins, Global Product Manager, OmniSep™
Email: trevor.hopkins@omnisep.com

In the disciplines of proteomics and protein research – with particular emphasis on purification – laboratory sample clean-up and sample preparation involves a variety of techniques all designed to extract or purify protein samples, readying them for long-term storage or downstream processing applications.

Examples of the sample clean-up needs include:

1) Prior to a purification step to remove low-molecular-weight interferences, to change pH or exchange one buffer component for another;

2) Before Ion Exchange Chromatography (IEX) of the sample to reduce the ionic strength to permit binding of the sample to the media or after IEX to remove the salt used for elution;

3) After Affinity Chromatography to remove a low-molecular-weight component used for elution, which may be important if the substance interferes with stability or activity of the target protein or with subsequent purification;

4) After low-pH elution in a purification step, to restore pH;

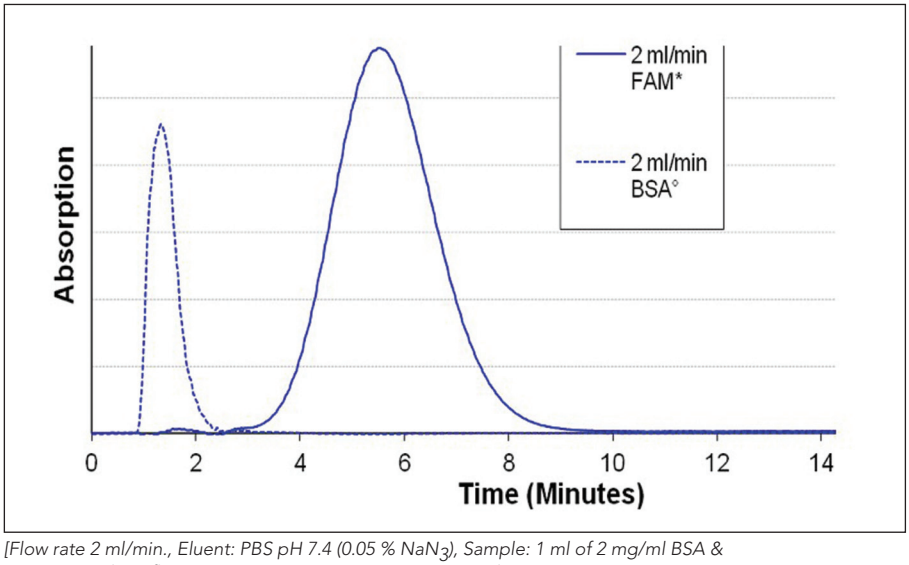
5) After purification to make a final adjustment of conditions of the purified protein; or

6) After labeling or other chemical modification of a protein, to stop the reaction or to remove excess reagents or inhibitors from enzymes

Dialysis, diafiltration and gel filtration (desalting, sample cleanup and buffer exchange) are the most broadly applicable methods for sample clean-up and preparation in laboratory and process applications. These techniques, with the incumbent pros and cons, have been used for decades and are based on the well-understood and documented principles of size exclusion which is non- adsorptive.

The removal of low-molecular-weight substances, salts, or buffer exchange by size exclusion chromatography (SEC) is often preferred because of the short time required, which reduces the risk of protein aggregation and degradation.

Desalting via SEC on a laboratory scale is well-proven, simple, and fast. In a single step SEC will rapidly remove low-molecular-weight contaminants and transfer the sample into the desired buffer. It can be used manually, together with a chromatography system, or in high-throughput applications.



*[Flow rate 2 ml/min., Eluent: PBS pH 7.4 (0.05 % NaN_3), Sample: 1 ml of 2 mg/ml BSA & 100µM 5-Carboxyfluorescein in PBS pH 7.4 (0.05 % NaN3) Abs. @ °280nm & *490nm]*

This study focuses on desalting via size exclusion chromatography and the validation of a commercially equivalent product to the market leader.

OmniSep™ 5ml Columns

Experimental Outline:

Variations of the bead size distribution were evaluated in order to observe its effect on the separation and commercial equivalence. A bead size distribution of OmniSep™D-25SF was selected.

Different media masses and packing methods were tested and the optimum media mass and packing process were selected based on the best column efficiency and reproducibility. Once the packing method had been

established and the gel mass and size distribution optimised, OmniSep™D-25SF in a commercially available column were prepared and evaluated.

Experimental Details:

Evaluation of column materials:

Commercially available plastic column parts were evaluated and compared to those of the 5mL Hi-Trap® Desalting columns available from GE Healthcare.

Evaluation of Media:

The Sephadex® G-25 media from several of the Hi-Trap® columns was dried and analysed with respect to its most important physical properties – particle size, particle size distribution, pore size, pore volume and matrix rigidity. The Hi-Trap® media properties were determined to be nearly identical to those of Sephadex® G25, Superfine grade (with a bead size distribution in the range of 20µm to 50µm) the only obvious difference was the lower particle size limit of the distribution which cut off at 15µm.

Comparative Testing:

Using a Shimadzu HPLC LC-10 System, with an SPD-M10Avp UV-Vis photodiode array detector, a flow rate of 10 mL/min (the maximum recommended for Hi-Trap® Desalting columns) and PBS as eluent, performance specifications of the Hi-Trap® were determined using a standard solution consisting of 2mg/mL bovine serum albumin (BSA) and 100µM 5-Carboxyfluorescein, single isomer (5-FAM) in phosphate buffered saline (PBS, pH 7.4 with 0.05% NaN_3). These Hi-Trap® performance specifications were then utilized as the standard for the commercial equivalence performance evaluation of combinations of the empty Hi-Trap® column, the empty commercially available column, the Hi-Trap® Matrix and the OmniSep™-25SF matrix.

Two matching batches of OmniSep™D-25SF were chosen for comparison study. In order to provide controls for evaluation, it was decided to evaluate the following configurations:

- Hi-Trap® Matrix in a commercially available column for evaluation and optimisation of column packing methods, independent of OmniSep™ performance, compared with an original Hi-Trap®.
- OmniSep™-25SF in a Hi-Trap® Column to evaluate OmniSep™-25SF performance directly with Hi-Trap® Matrix (identified as close to Sephadex® G25SF).
- OmniSep™-25SF in a commercially available FPLC Column for final column evaluation.

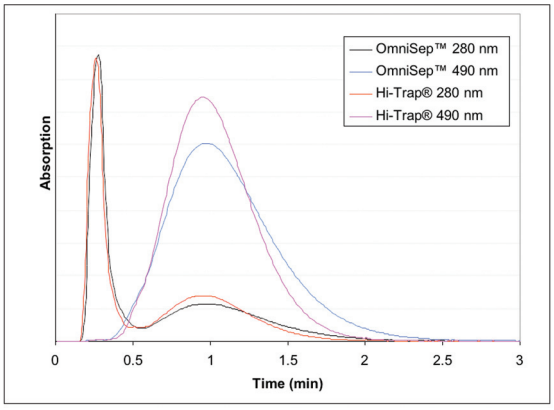
1) Separation of BSA and Fluorescein (Column Lot 1286-71):

a) Comparison of Hi-Trap® 5ML (Sephadex® G25 SF) and OmniSep™ 5ML (OmniSep™ 25 SF). Overlay of four runs. Spectra were measured at 280nm and 490nm each for Hi-Trap® and OmniSep™.

Figure 1a.

Hi-Trap®
vs.
OmniSep™

Column Lot
1286-71



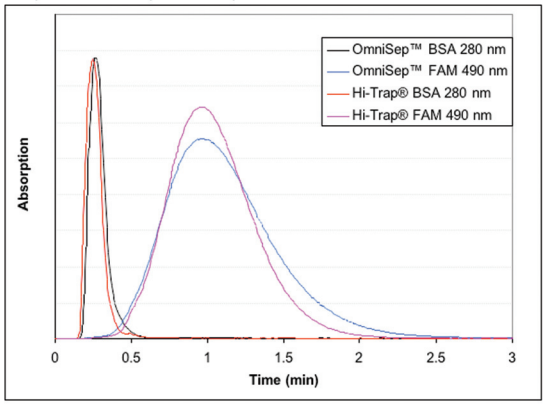
Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *10 ml/min*
Sample: *1 ml of 2 mg/ml BSA + 100 µM 5-Carboxyfluorescein (FAM) mixture in PBS pH 7.4 (0.05 % NaN_3)*

b) Comparison of Hi-Trap® 5ML (Sephadex® G25 SF) and OmniSep™ 5ML (OmniSep™ 25 SF). BSA and fluorescein run separately each on Hi-Trap® and OmniSep™. Overlay of four runs, measured at 280nm and 490nm.

Figure 1b.

Hi-Trap®
vs.
OmniSep™

Column Lot
1286-71



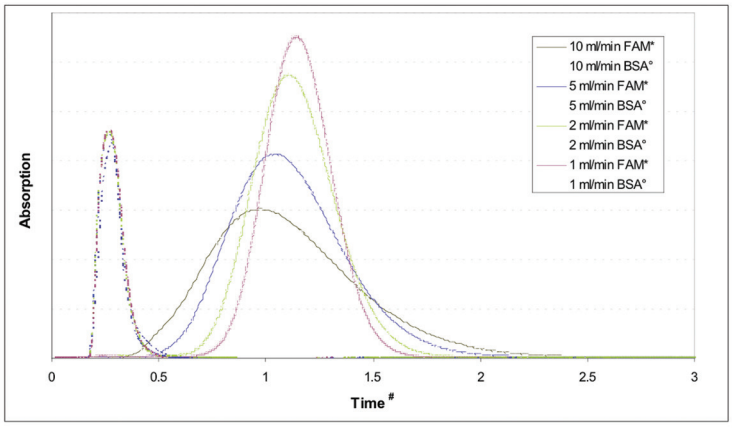
Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *10 ml/min*
Sample: *1 ml of 2 mg/ml BSA in PBS pH 7.4 (0.05 % NaN_3) @ 280 nm*
1 ml of 100 µM FAM in PBS pH 7.4 (0.05 % NaN_3) @ 490 nm

2) Dependence of Resolution on Flow Rates for OmniSep™ 5ML (Separation of BSA and Fluorescein)

Figure 2.

Flow Rate
and
Resolution

Column Lot
1286-71



Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *1 - 2 - 5 - 10 ml/min*
Sample: *1 ml of 2 mg/ml BSA + 100 µM 5-Carboxyfluorescein (FAM) mixture in PBS pH 7.4 (0.05 % NaN_3)*

** Absorption @ 490 nm*
° Absorption @ 280 nm (corrected for fluorescein absorption)
 # Time scale adjusted for comparison, original run times:
10 ml/min 3 minutes
5 ml/min 6 minutes
2 ml/min 5 minutes
1 ml/min 30 minutes

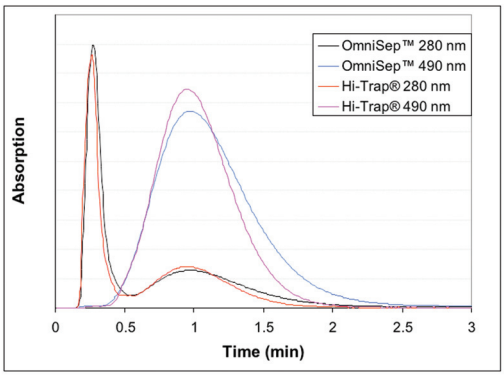
3. Separation of BSA and Fluorescein (Column Lot 1286-74):

a) Comparison of Hi-Trap® 5ML (Sephadex® G25 SF) and OmniSep™ 5ML (OmniSep™ D-25 SF). Overlay of four runs. Spectra were measured at 280nm and 490nm each for Hi-Trap® and OmniSep™.

Figure 3a.

Hi-Trap® vs. OmniSep™

Column Lot 1286-74



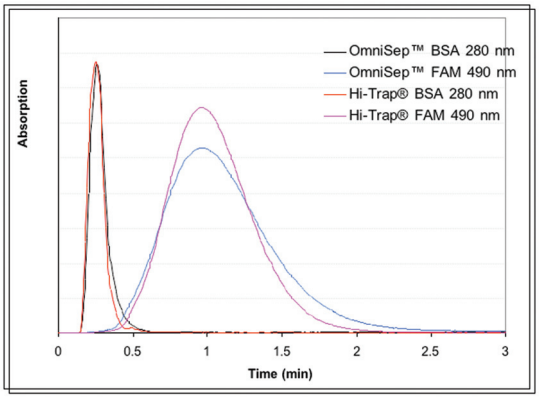
Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *10 ml/min*
Sample: *1 ml of 2 mg/ml BSA + 100 µM 5-Carboxyfluorescein (FAM) mixture in PBS pH 7.4 (0.05 % NaN_3)*

b) Comparison of Hi-Trap® 5ML (Sephadex® G25 SF) and OmniSep™ 5ML (OmniSep™ D-25 SF). BSA and fluorescein run separately each on Hi-Trap® and OmniSep™. Overlay of four runs, measured at 280nm and 490nm.

Figure 3b.

Hi-Trap® vs. OmniSep™

Column Lot 1286-74



Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *10 ml/min*
Sample: *1 ml of 2 mg/ml BSA in PBS pH 7.4 (0.05 % NaN_3) @ 280 nm*
1 ml of 100 µM FAM in PBS pH 7.4 (0.05 % NaN_3) @ 490 nm

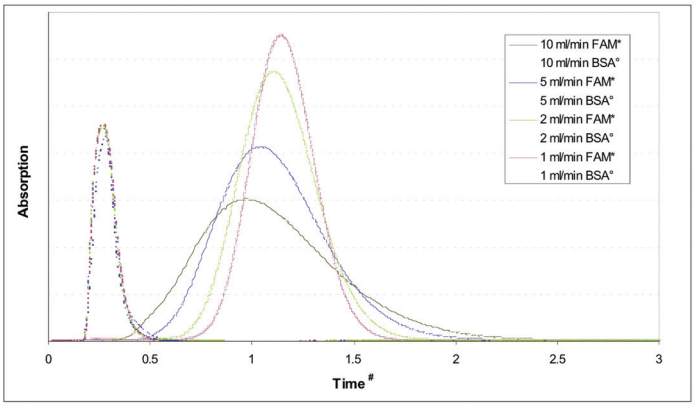
4. Dependence of Resolution on Flow Rates for OmniSep™ 5ML (Separation of BSA and Fluorescein)

Eluent: *PBS pH 7.4 (0.05 % NaN_3)*
Flow rate: *1 - 2 - 5 - 10 ml/min*
Sample: *1 ml of 2 mg/ml BSA + 100 µM 5-Carboxyfluorescein (FAM) mixture in PBS pH 7.4 (0.05 % NaN_3)*

Figure 4.

Flow Rate and Resolution

Column Lot 1286-74



* *Absorption @ 490 nm*
° *Absorption @ 280 nm (corrected for fluorescein absorption scale)*
\# *time scale adjusted for comparison, original run times:*
10 ml/min 3 minutes
5 ml/min 6 minutes
2 ml/min 15 minutes
1 ml/min 30 minutes

Results:

The results for two lots of OmniSep D media when manufactured into disposable 5ml devices are compared directly with 5ml Hi-Trap® Desalting columns using standard solutions of BSA and fluorescein and are exhibited in the following manner:

Figures 1a and 3a show the comparison of Hi-Trap® and OmniSep™ columns manufactured from OmniSep D-25 media lot 1286-71, clearly demonstrating an equivalent separation of BSA from fluorescein, as measured by dual detector absorbance at 280nm (protein) and 490nm (fluorescein) in separate runs and the spectra are overlaid.

Figures 1b and 3b show the comparison of Hi-Trap® and OmniSep™ columns manufactured from OmniSep D-25 media lot 1276-74, clearly demonstrating an equivalent separation of BSA from fluorescein, as measured by dual detector absorbance at 280nm (protein) and 490nm (fluorescein) in separate runs and the spectra are overlaid.

Figures 2 and 4 demonstrate the dependence of flow rate on separation resolution for the two lots of OmniSep D-25 media. As expected, significantly higher resolution is obtained for both lots of OmniSep D-25 media when using slower flow rates. Identical results are obtained with Hi-Trap® columns. The flow rate of 10mL/min is at the upper extreme limit of both the OmniSep™ and Hi-Trap® columns.

The results show the commercial equivalence for separation performance of OmniSep™ FPLC Desalting Columns manufactured from two lots of OmniSep D media when compared directly with Hi-Trap® desalting columns using standard solutions of BSA and fluorescein under normal use conditions and over a flow rate range.

Conclusions:

OmniSep™ D-25 FPLC desalting columns, manufactured using OmniSep™D-25 SF gel filtration matrix packed in a 5mL disposable FPLC column, demonstrate equivalent performance to that of a Hi-Trap® 5mL Desalting column from GE Healthcare with respect to separation of a 1 mL combined sample of 2mg/mL bovine serum albumin and 100µM fluorescein. Excellent resolution is obtained with a maximum flow rate of 10mL/min and superb separation at flow rates at 5mL/min and lower.

Trevor Hopkins is Global Product Manager for the OmniSep™ product line by Omnifit® Labware, manufactured by Diba Industries and available globally through Kinesis.