Eshmuno® S

Cat. No. 1.20078

Version 3.0

August 28th, 2013

CONTENT

1. Introduction

Eshmuno® S is a new and unique ion-exchange resin especially designed for highly productive downstream purification of monoclonal antibodies. This cation exchanger is the first product of the Eshmuno® family of smart resins and is highly productive in direct capture and post-protein A steps.

Eshmuno® S is a surface grafted rigid hydrophilic polyvinylether polymer bead for the purification of strong basic and neutral proteins.
Eshmuno® S process media provides high capacity and resolution along with all the advantages of polymerbased chromatographic beads such as high throughput, easy column packing, long life-time and high mechanical stability.
The most important advantage of the tentacle chemistry, where long, linear polymer chains ("tentacles") carry the functional groups, is the large number of sterically accessible ligands for the binding of biomolecules. All tentacles are covalently attached to the polyvinylether backbone and are chemically stable under conditions applied during chromatography, regeneration and sanitization.
Due to the titration behaviour the ion exchange capacity can be used from pH 2 up to pH 12. The separation of proteins is based on reversible electrostatic interactions between the positively charged regions of the proteins surface and the support. Proteins are retained efficiently on Eshmuno® S when the pH of the buffer is about 1 unit below their isoelectric points (pI).

2. General Properties of Eshmuno® S

Table 1: General properties of Eshmuno® S.

	Eshmuno® S
Mean particle size (D50)	85 µm
Type of chromatography	Strong cation exchange chromatography
Functional group	Sulfoisobutyl group
Ionic capacity	50 - 100 µeq/ml settled resin
Protein binding capacity (static)	135 - 195 mg lysozyme/ml settled resin
Polyclonal IgG dynamic binding capacity	≥ 60 mg/ml settled resin (2 min residence time, 10% breakthrough)
pK value	< 1
pH range (working)	pH 2 up to pH 12
pH range (CIP)	pH 0 up to pH 14
Elution conditions	high salt concentrations
Mechanical stability	8 bar
Pressure drop	≤ 1.0 bar (100x16mm, 5ml/min, 150 cm/h)
Operating temperature	4 °C to room temperature
Storage, preservative	20 % ethanol, 150 mmol/l NaCl
Regeneration	1 - 2 M NaCl
Sanitization	0.1 - 1.0 M NaOH
Linear flow rate	up to 1000 cm/h (< 2.5 bar net pressure) 20 x 10 cm i.d. column, 8% compression, 150 mM NaCl as mobile phase

3. Chromatographic Properties

3.1. Separation of Standard Proteins

A mixture of chymotrypsinogen A, cytochrome C, and lysozyme was separated under standard conditions for a Eshmuno® S. Absorption at 280 nm and conductivity were monitored.

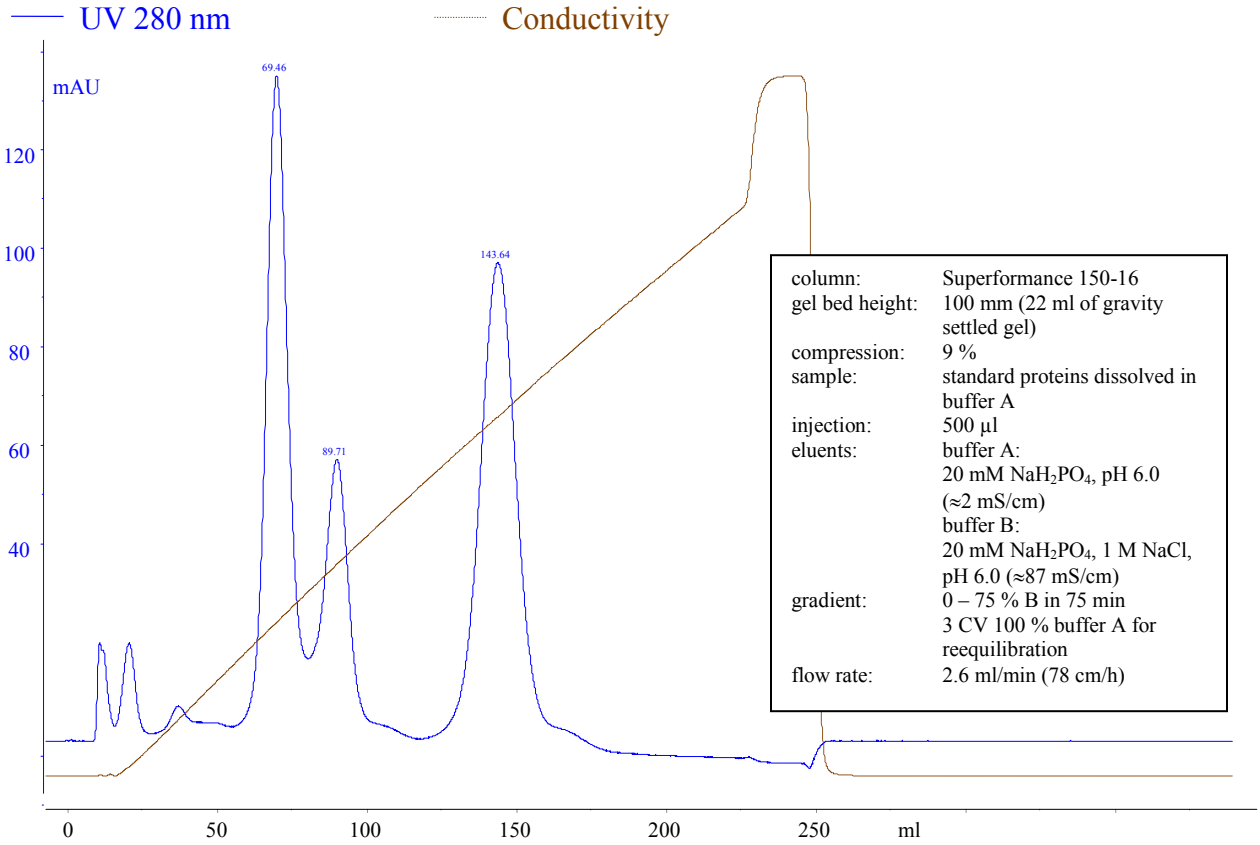

Figure 1: Separation of chymotrypsinogen A, cytochrome C, and lysozyme (5 mg/ml for each protein).

Conductivities at elution peak maxima:

Chymotrypsinogen A	22 mS/cm
cytochrome C	30 mS/cm
lysozyme	49 mS/cm

3.2. Pressure versus Flow Curves

Eshmuno® S was packed in 10 cm and 20 cm i.d. columns to 20 cm bed height and 8 % compression. Pressure versus flow curves were recorded according to the standard protocol.

Column:	Superformance®
Gel:	Eshmuno® S, lot no. 08RE052-08
Bed height:	20 cm
Compression:	8 % (sedimented gel volume is equivalent to 0 % compression)
Eluent:	150 mM NaCl

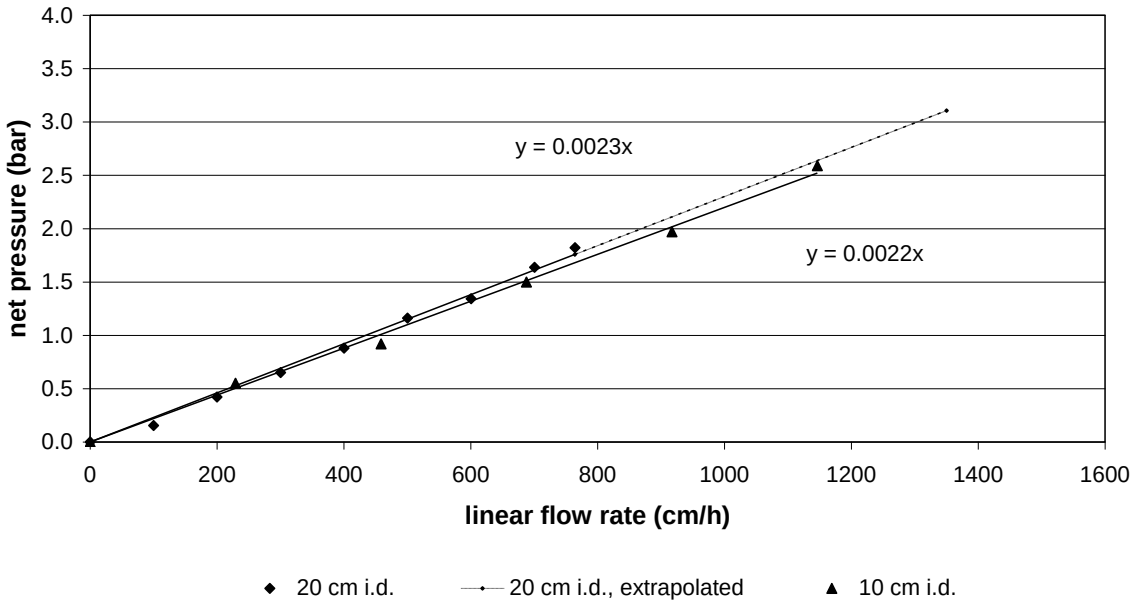

Figure 2: Pressure versus flow curve of Eshmuno® S (lot no. 08RE052-08).

Conclusion:
Excellent pressure flow behaviour for different column diameters.

3.3. Dynamic Capacity

To demonstrate the dependence of the binding capacity of Eshmuno® S on the residence time, the following dynamic experiments with polyclonal IgG were carried out. Binding capacities were calculated at 10 % breakthrough in the eluate as monitored at 280 nm.

Column:	1 ml Scout column (18.95 x 8.2 mm i.d.)
Gel:	Eshmuno® S, lot no. 08RE052-08
Binding buffer:	25 mM sodium acetate and 25 mM sodium phosphate, pH 5.0
Sample:	polyclonal IgG (Gammanorm, 5 mg/ml) in binding buffer
Eluent:	25 mM sodium acetate, 25 mM sodium phosphate and 1 M sodium chloride, pH 5.0

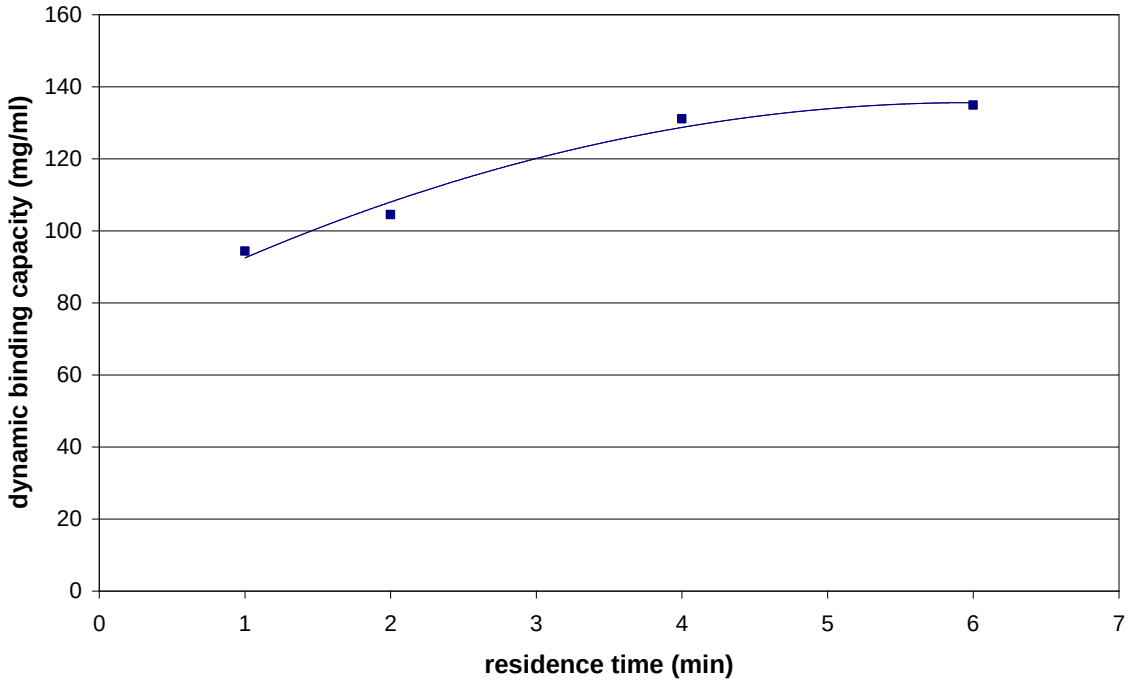

Figure 3: Influence of residence time on dynamic binding capacity of Eshmuno® S for polyclonal human IgG at 10 % breakthrough, 5 mg/ml pIgG in mixed buffer 25 mM acetate and 25 mM phosphate, pH 5.0.

Conclusion:
High dynamic IgG binding capacity even at low residence times.

3.4. Resolution versus Loading

To demonstrate the impact of loading on chromatographic resolution the following experiments with cytochrome C and lysozyme were carried out.

Column:	Superformance® 150-16
Gel:	Eshmuno® S, lot no. 09SAM001-08
Bed height:	10 cm
Compression:	10 %
Binding buffer:	20 mM sodium phosphate, pH 6.0
Sample:	Cytochrome C (2 mg/ml) and lysozyme (8 mg/ml) in binding buffer
Eluent:	20 mM sodium phosphate and 1 M sodium chloride, pH 6.0
Flow rate during elution:	150 cm/h

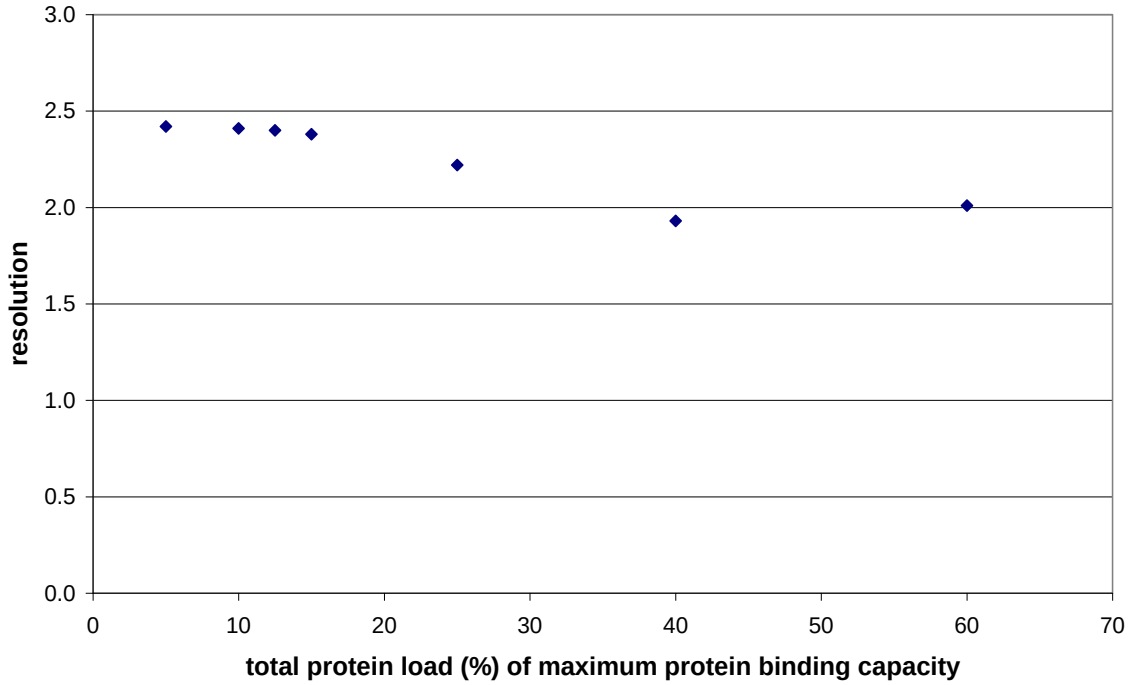

Figure 4: Cytochrome C and lysozyme were separated on Eshmuno® S at different loadings.

Conclusion:
High resolution on Eshmuno® S resin at high process load.

3.5. Resolution versus Flow Rate

To demonstrate the impact of flow rate on chromatographic resolution the following experiments were carried out with standard proteins at low and high loading.

Analytical load

Column:	Superformance® 150-16
Gel:	Eshmuno® S, lot no. 09SAM001-08
Bed height:	10 cm
Compression:	10 %
Binding buffer:	20 mM sodium phosphate, pH 6.0
Sample:	Chymotrypsinogen A (5 mg/ml), cytochrome C (5 mg/ml) and lysozyme (5 mg/ml) in binding buffer
Load:	500 µl
Eluent:	20 mM sodium phosphate and 1 M sodium chloride, pH 6.0

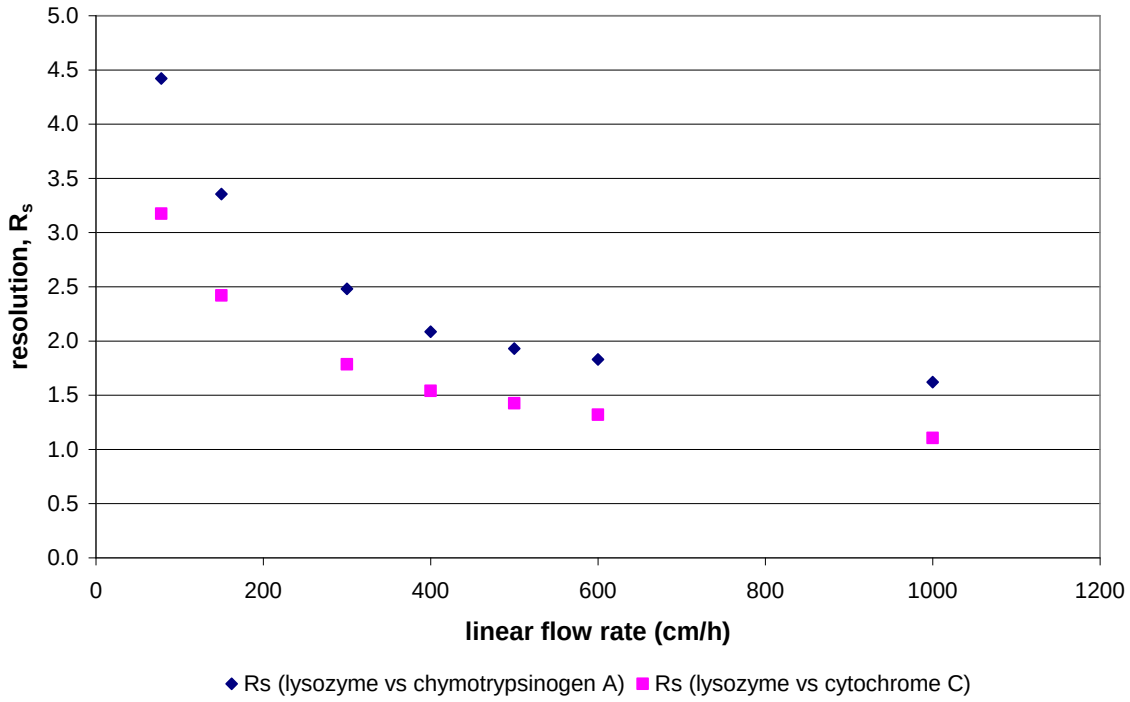

Figure 5: Chymotrypsinogen A, cytochrome C and lysozyme were separated on Eshmuno® S at different flow rates (load: 0.3 % of total column binding capacity based on lysozyme).

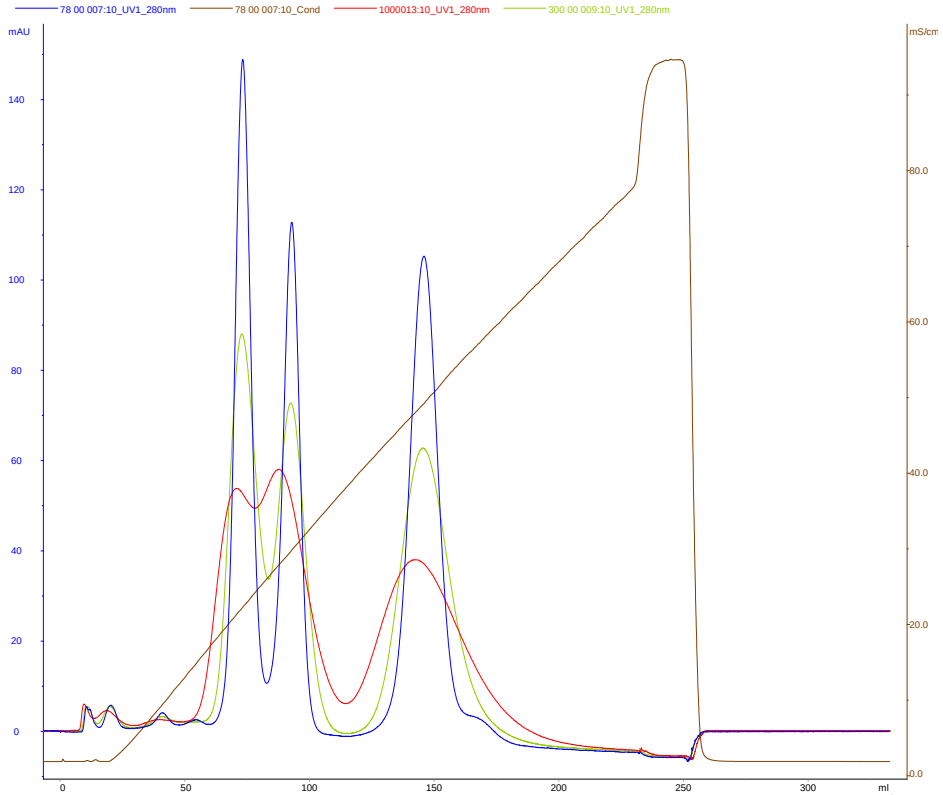

Figure 6: Separation of chymotrypsinogen A, cytochrome C and lysozyme on Eshmuno® S after loading the column to 0.3 % of total column binding capacity at 78 cm/h (blue), 300 cm/h (green) and 1000 cm/h (red curve).

Process load

Column:	Superformance® 150-16
Gel:	Eshmuno® S, lot no. 09SAM001-08
Bed height:	10 cm
Compression:	10 %
Binding buffer:	20 mM sodium phosphate, pH 6.0
Sample:	Cytochrome C (2 mg/ml) and lysozyme (8 mg/ml) in binding buffer
Load:	146 ml
Eluent:	20 mM sodium phosphate and 1 M sodium chloride, pH 6.0

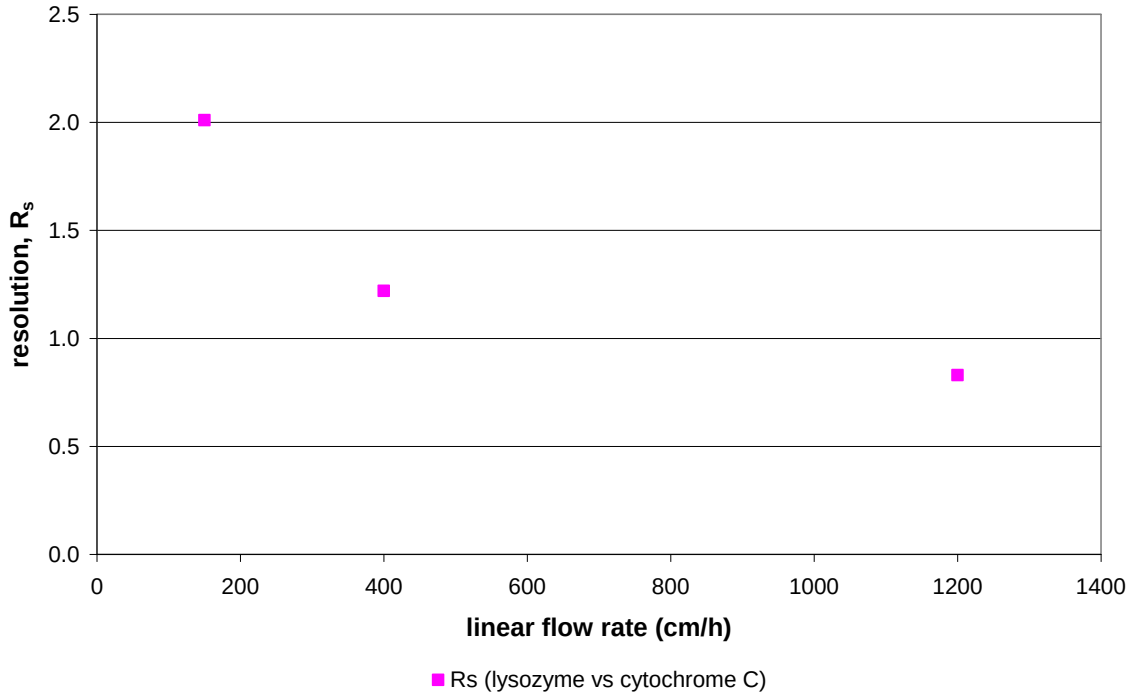

Figure 7: Cytochrome C and lysozyme were separated on Eshmuno® S at different flow rates (load: 60 % of total column binding capacity).

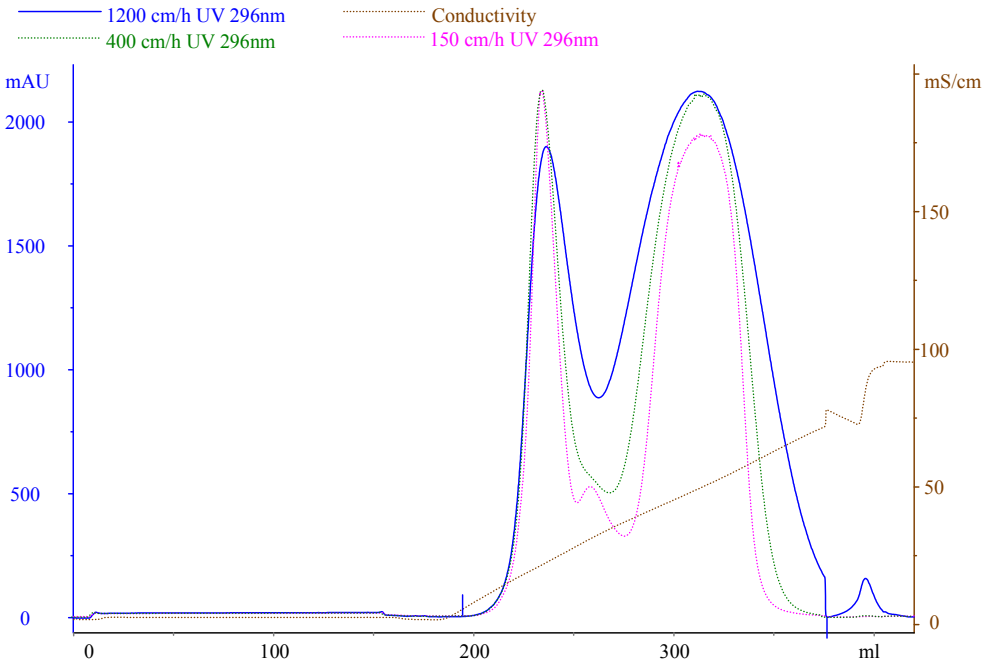

Figure 8: Separation of cytochrome C and lysozyme on Eshmuno® S after loading the column to 60 % total column binding capacity at 150 cm/h (magenta), at 400 cm/h (green) and 1200 cm/h (blue curve).

Conclusion:
High resolution even at high flow rates.

4. Chromatographic Stability

4.1. Reproducibility of 100 Cycles

To demonstrate the reproducibility of chromatography runs obtained with Eshmuno® S, separation of a protein mixture was repeated 100 times.

After each run with standard proteins, the column was cleaned with two column volumes (CV) of 1 M sodium hydroxide for 60 min at a flow rate of 20 cm/h, before re-equilibration with running buffer was performed.

Therefore, the total exposure time of the column to 1 M sodium hydroxide was at least 100 h.

Chromatographic conditions:

Column:	Superformance® 150-16
Gel:	Eshmuno® S, lot no. 09RE052-08
Bed height:	10 cm
Compression	10 %
Sample:	solution of chymotrypsinogen A (17.6 mg/ml), cytochrome C (8.8 mg/ml) and lysozyme (26.4 mg/ml) in buffer A (total protein concentration 52.8 mg/ml)
Sample volume:	5 ml (264 mg protein)
Buffer A:	20 mM NaH_2PO_4, pH 6.0
Buffer B:	20 mM NaH_2PO_4, 1 M NaCl, pH 6.0
Gradient:	linear, from 0 % B to 80 % B within 10.4 CV
Flow rate:	5 ml/min
Detection:	295 nm
Elution:	1 CV buffer B
CIP:	1 CV 1 M NaOH at 5 ml/min and 2 CV at 0.67 ml/min
Re-equilibration:	1 CV buffer B and 5 CV buffer A

The initial dynamic binding capacity at 600 cm/h and 10 % breakthrough for lysozyme was 109 mg/ml of packed resin, the final binding capacity after 100 cycles was determined to be 111 mg/ml.

Resolution

The figures show that the conductivities at peak maxima, retention times, and resolution for two different sample proteins are independent of the number of runs. Within a very narrow range the elution of the sample proteins remains the same for at least 100 chromatography cycles.

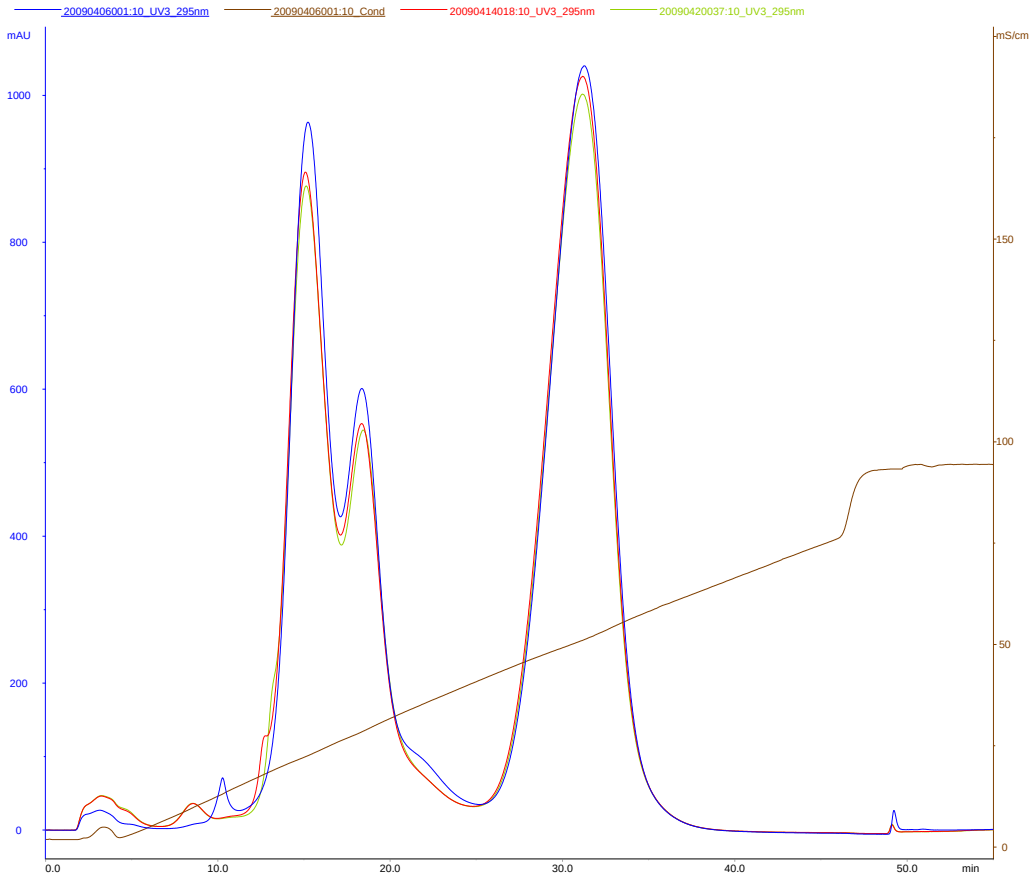

Figure 9: Separation of chymotrysinogen A, cytochrome C and lysozyme on Eshmuno® S, overlaid chromatograms of run 1 (blue) run 50 (red) and run 101 (green).

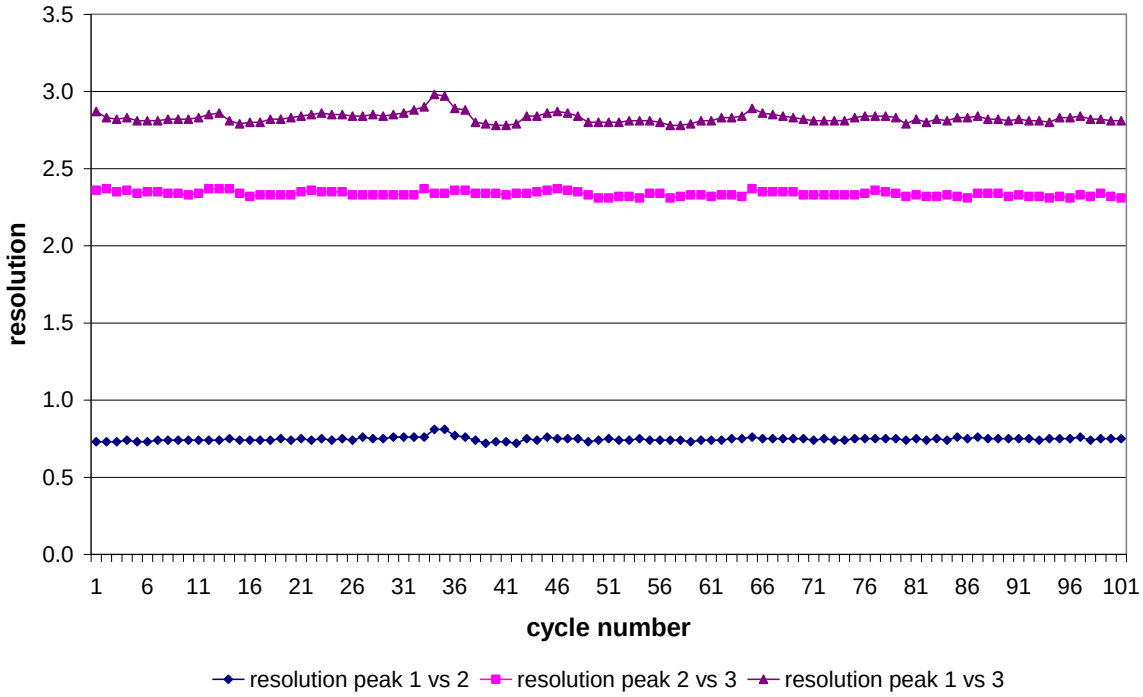

Figure 10: Resolution of chymotrypsinogen A (peak 1), cytochrome C (peak 2) and lysozyme (peak 3) after consecutive runs.

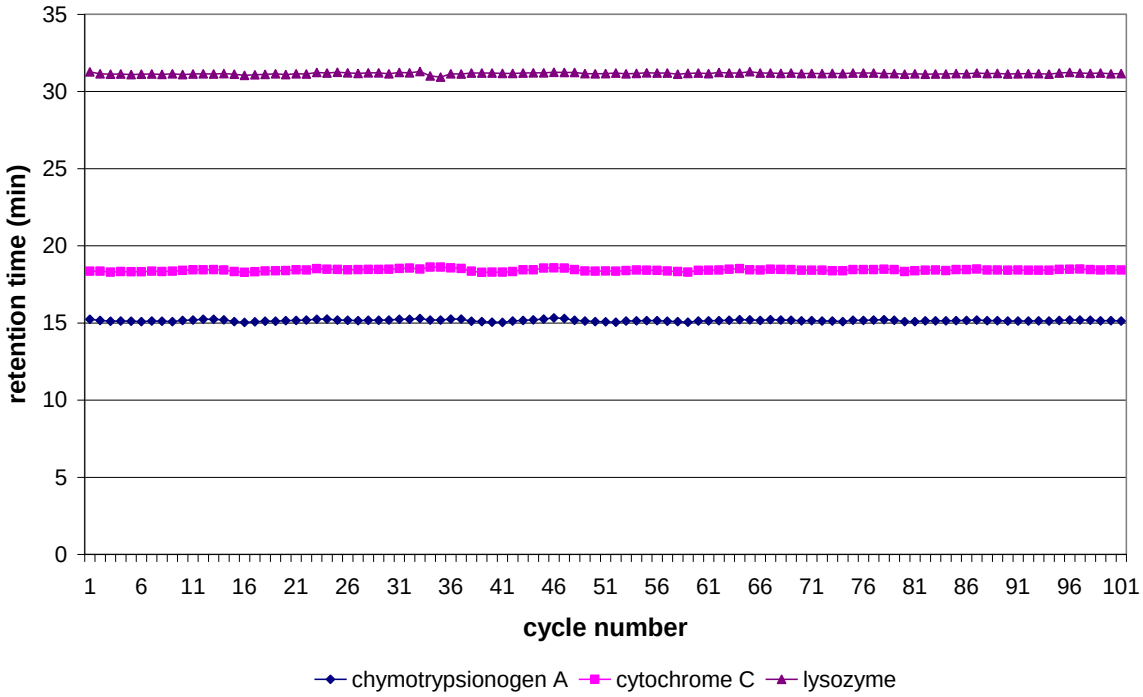

Figure 11: Retention times of peaks 1 - 3 after consecutive runs.

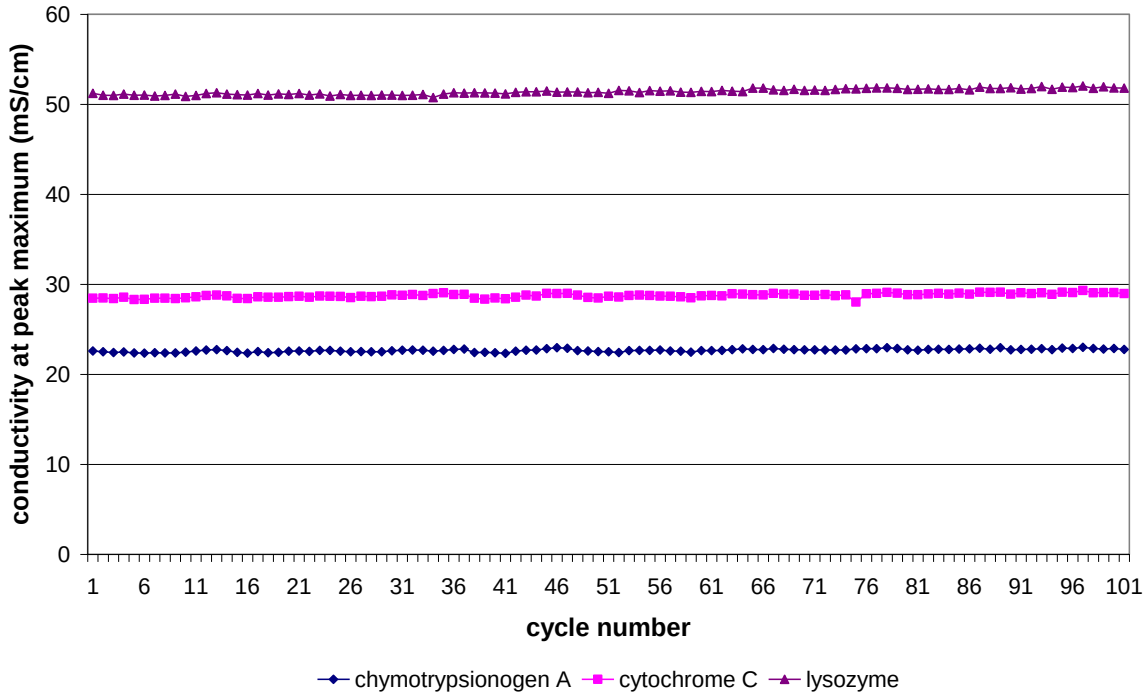

Figure 12: Conductivities at peak maxima of peaks 1 - 3 after consecutive runs.

Conclusion:

Within a narrow range the resolution of protein separation remains unchanged over a multitude of runs. The retention times and elution conductivities of peak maxima of the sample proteins remain the same for at least 100 chromatography cycles. The dynamic binding capacity for lysozyme remains unchanged after 100 chromatographic cycles.

5. Chemical Stability

5.1. Alkaline Stability

Due to the chemical structure of the Eshmuno® polymer matrix, which is a cross-linked rigid hydrophilic vinylether/urea copolymer and due to the surface modification with linear functionalized acrylamide polymers (graft polymer), the resulting Eshmuno® ion exchanger has a very high chemical stability even during prolonged exposure to usual clean-in-place conditions.

Both the polymeric matrix backbone, consisting of long alkyl chains with ether linkage to the hydroxyalkyl side groups, as well as the urea cross-linking units are very stable to caustic conditions. The same applies to the grafted functionalized polyacrylamide, which contain the ion exchanger ligands.

However, slow hydrolysis on the surface can occur by prolonged exposure of the support to strong alkaline conditions causing some release of hydrolysis products. These products could originate from the base matrix as well as in very small amounts from the graft polymer.

5.2. Stability against Sodium Hydroxide

Bulk resin (for measurement of protein binding capacity) and a packed column (for recording of chromatographic resolution and pressure versus flow curves) of Eshmuno® S, lot no. 08RE052-08, were stored in 1.0 M, 0.5 M and 0.1 M sodium hydroxide solution at room temperature.
For capacity testing, aliquots of the gel suspension were taken after specified time intervals, washed with water and neutralized with 100 mM sodium phosphate buffer pH 7.0. Protein binding capacities were determined according to the standard procedures described in the monograph.
For measurement of chromatographic resolution and pressure versus flow curves the resin was packed into a Superformance® 150-16 column to 10 cm bed height at 15 % compression. After testing the column according to the monograph the column was rinsed with sodium hydroxide and stored at room temperature. After specified time intervals, the column was rinsed with 2 column volumes (CV) of equilibration buffer, 2 CV of elution buffer at a flow rate of 150 cm/h (5 ml/min), followed by 3 CV of equilibration at a flow rate of 210 cm/h (7 ml/min), before the separation run. Chromatographic resolutions were determined according to the standard procedures described in the monograph (separation of chymotrypsinogen A, cytochrome C, and lysozyme). In addition, pressure versus flow curves were recorded after performing the chromatographic resolution. After testing the column was again rinsed with sodium hydroxide solution and stored at room temperature.

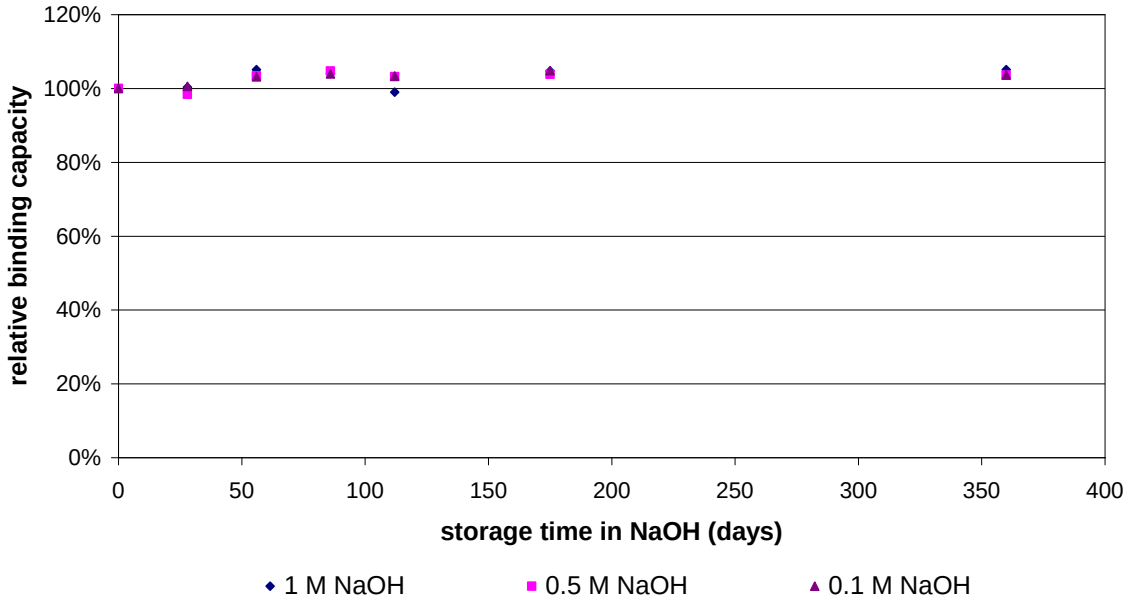

Figure 13: Relative static lysozyme binding capacity after prolonged treatment with 1.0 M, 0.5 M or 0.1 M sodium hydroxide.

Conclusion:
Eshmuno® S does not lose protein binding capacity even after exposure to 1 M sodium hydroxide solution for several months at room temperature. Concurrently, the resolution of standard proteins (see below) remains nearly unchanged in this period. Pressure versus flow properties (see below) are hardly altered during long term storage in 1 M sodium hydroxide solution.

5.2.1. Stability against 1 M Sodium Hydroxide

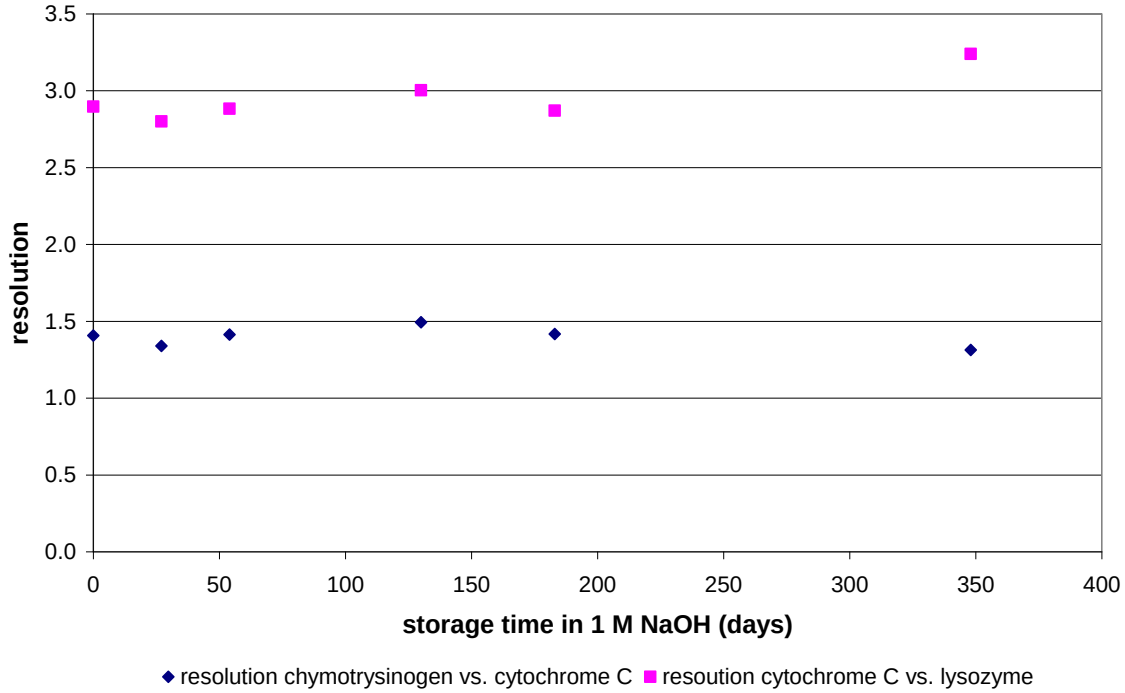

Figure 14: Resolution data for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 1 M sodium hydroxide for certain times after re-equilibration.

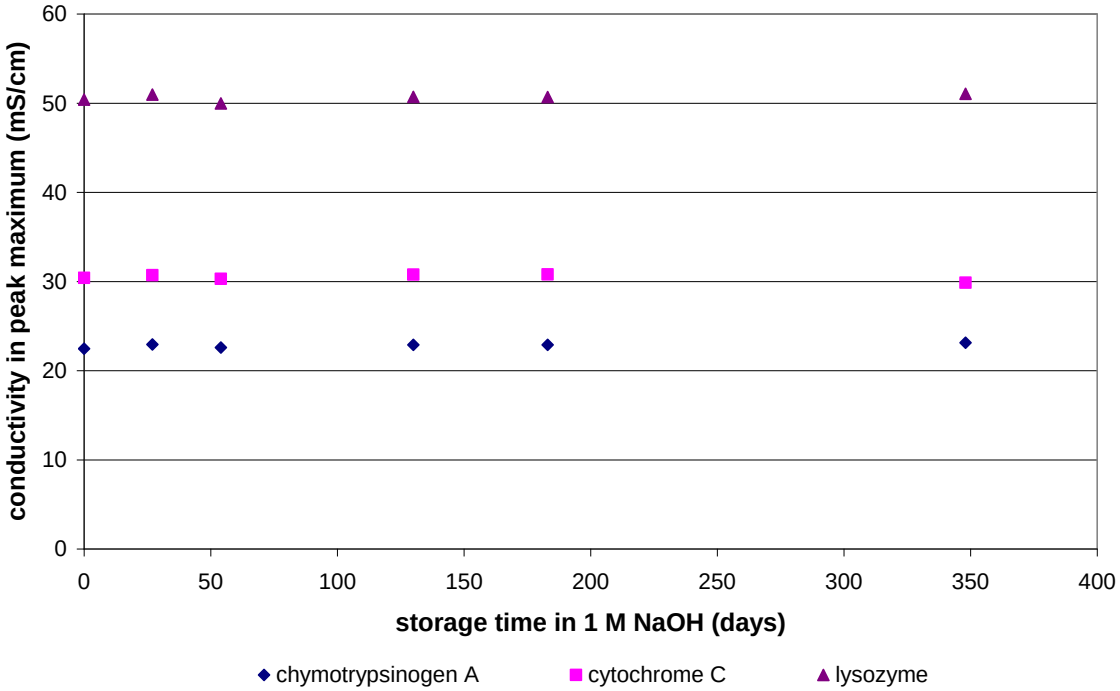

Figure 15: Elution conductivities at peak maxima for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 1 M sodium hydroxide for certain times after re-equilibration.

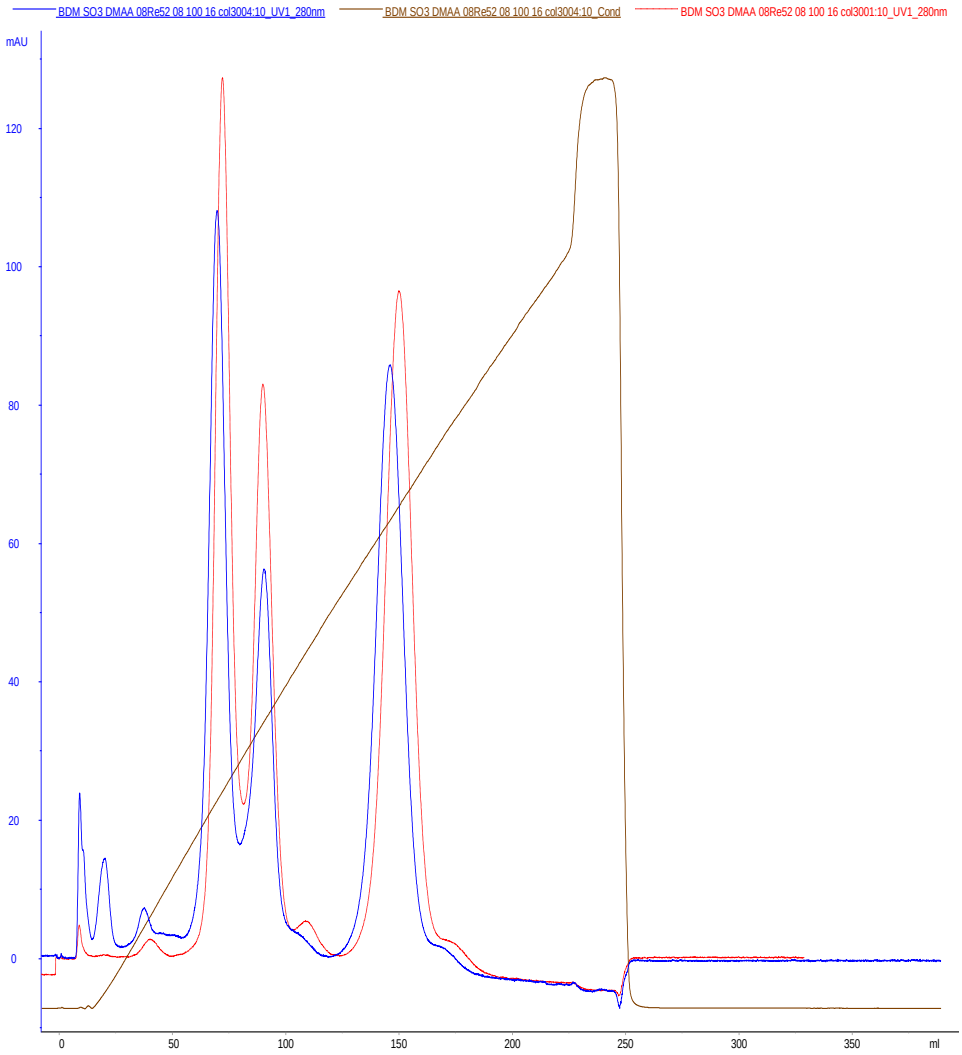

Figure 16: Chromatograms of the separation of of chymotrypsinogen A, cytochrome C, and lysozyme on a packed column (100 mm x 16 mm i.d., 10 % compression) with Eshmuno® S before (blue) and after storage in 1M NaOH (red, dashed line) for 348 days.

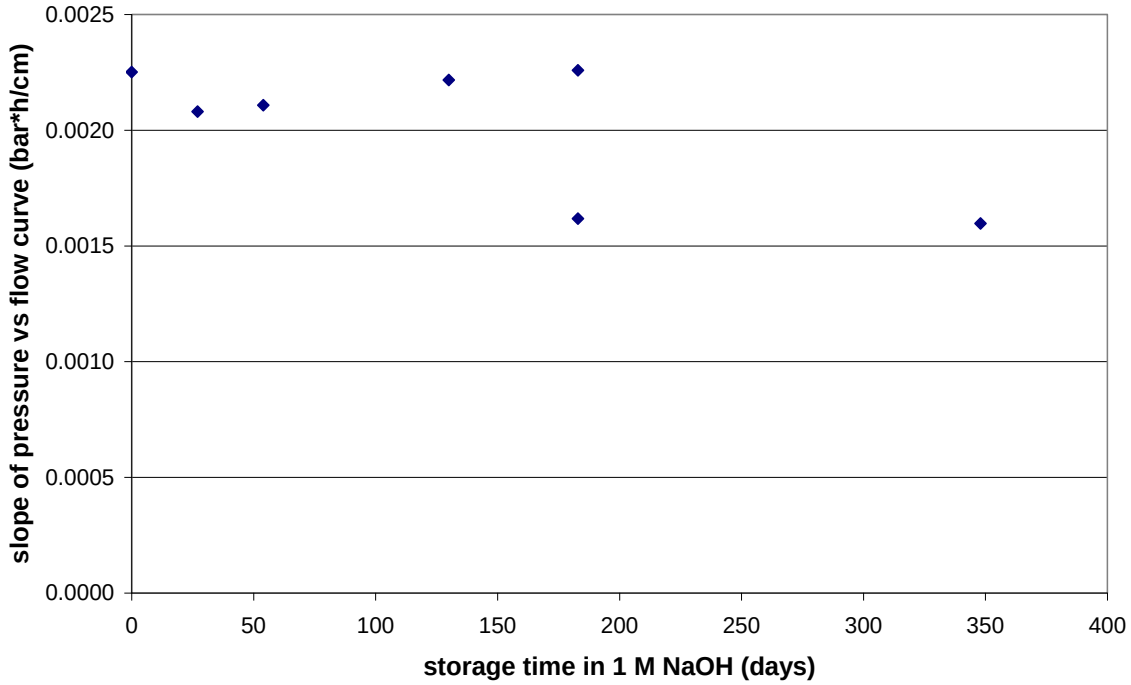

Figure 17: Slope of the brutto pressure drop versus flow curve after treatment with 1 M sodium hydroxide for certain times. Pressure versus flow curves were recorded in 100 x 16 mm i.d. columns packed to 10 % compression at 150 – 1200 cm/h. The slope of the brutto pressure drop versus flow curve after treatment with 1 M sodium hydroxide for certain times was determined by linear regression. The coefficient of determination (R^2) was always greater than 0.990. After 183 days fittings of the column were replaced. Slope of the pressure versus flow curve after 183 days is measured before and after exchange of the fittings.

5.2.2. Stability against 0.5 M Sodium Hydroxide

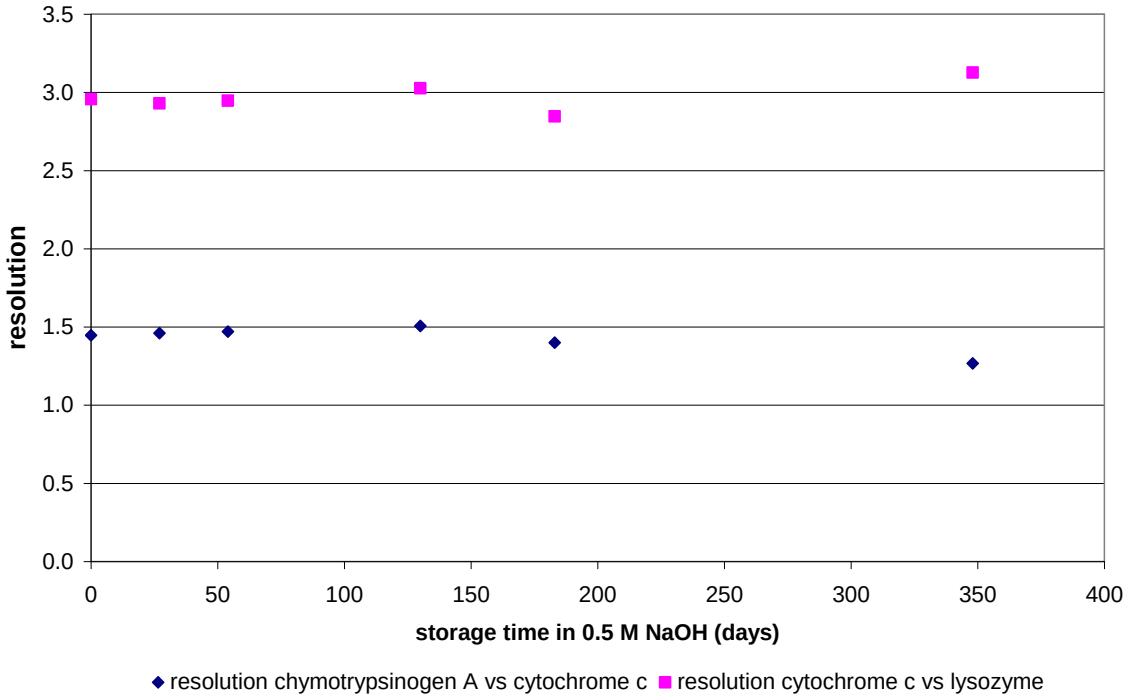

Figure 18: Resolution data for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 0.5 M sodium hydroxide for certain times after re-equilibration.

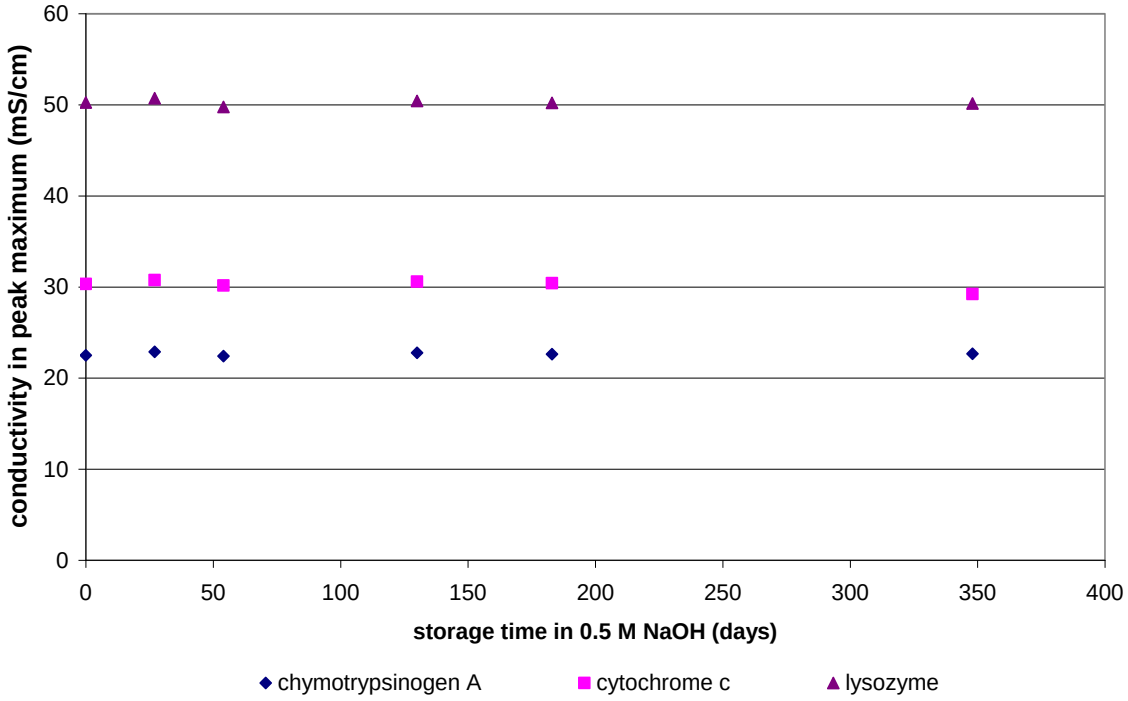

Figure 19: Elution conductivities at peak maxima for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 0.5 M sodium hydroxide for certain times after re-equilibration.

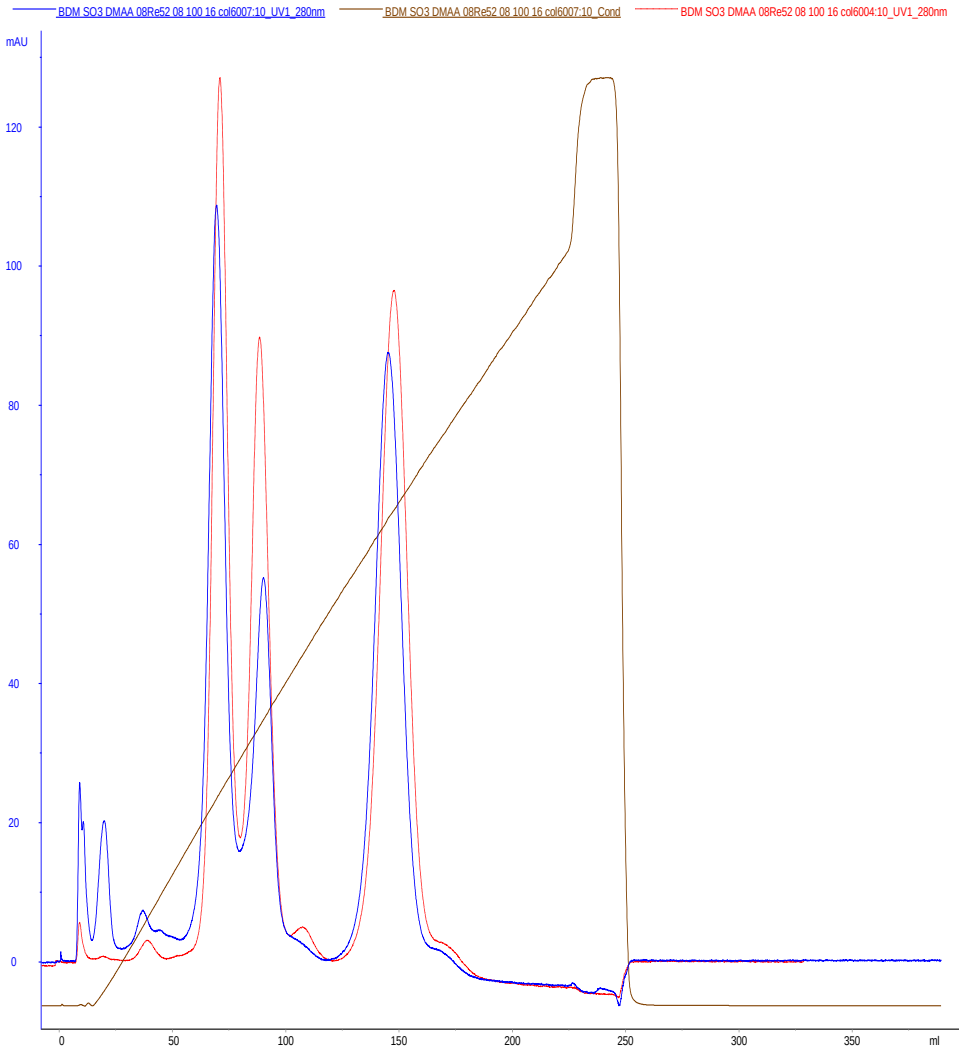

Figure 20: Chromatograms of the separation of of chymotrypsinogen A, cytochrome C, and lysozyme on a packed column (100mm x 16 mm i.d., 10 % compression) with Eshmuno® S before (blue) and after storage in 0.5 M NaOH (red, dashed line) for 348 days.

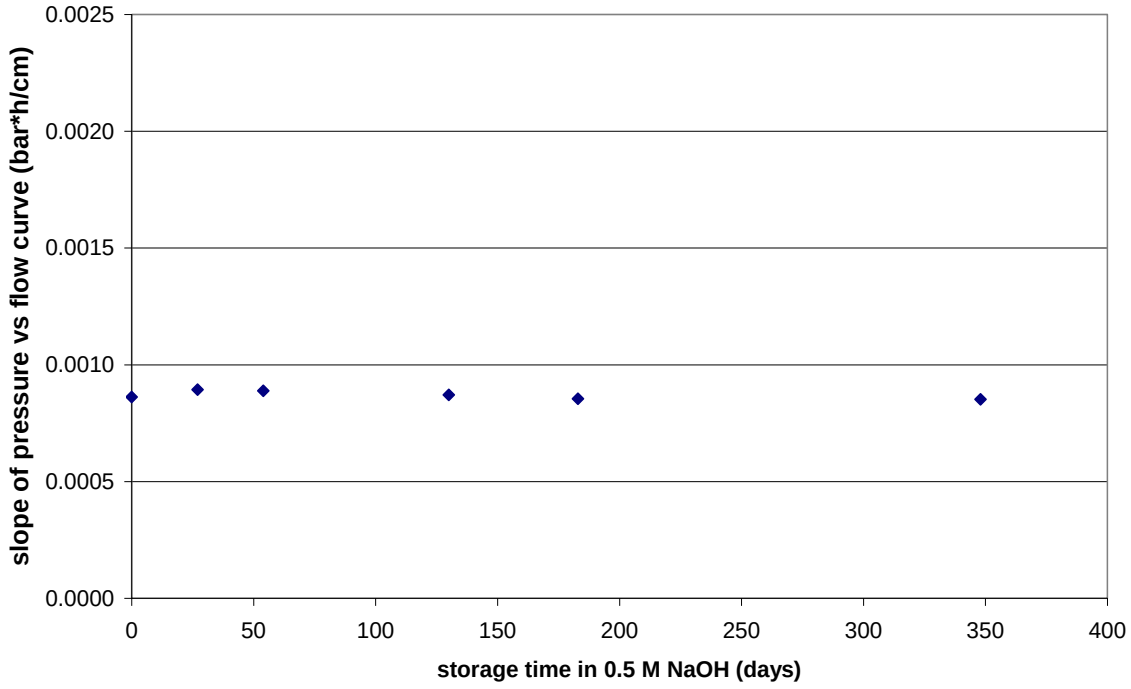

Figure 21: Pressure versus flow curves were recorded in 100 x 16 mm i.d. columns packed to 10 % compression (150 – 1500 cm/h). The slope of the net pressure drop versus flow curve after treatment with 0.5 M sodium hydroxide for certain times was determined by linear regression. The coefficient of determination (R^2) was always greater than 0.995.

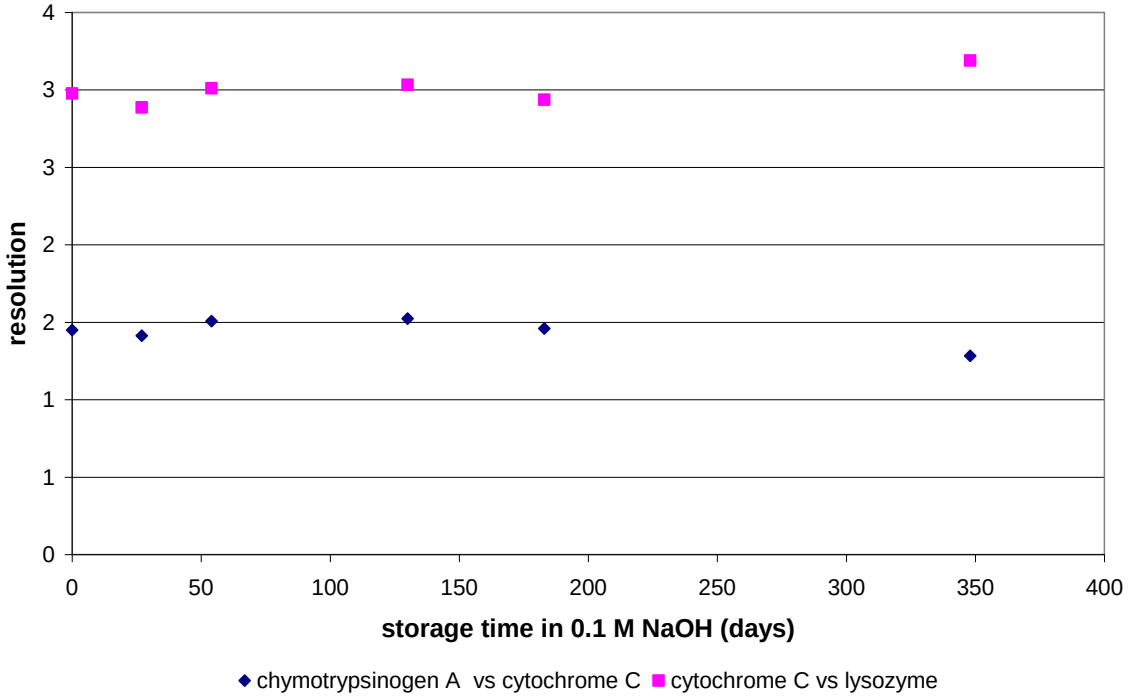

Figure 22: Resolution data for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 0.1 M sodium hydroxide for certain times after re-equilibration.

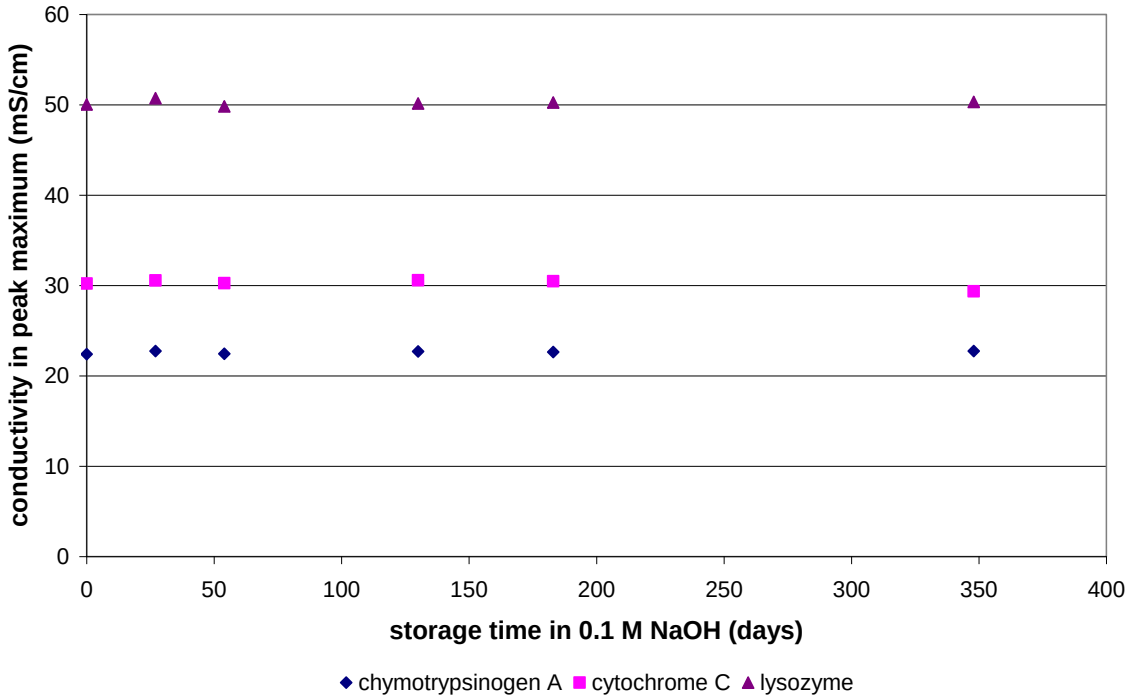

Figure 23: Elution conductivities at peak maxima for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 0.1 M sodium hydroxide for certain times after re-equilibration.

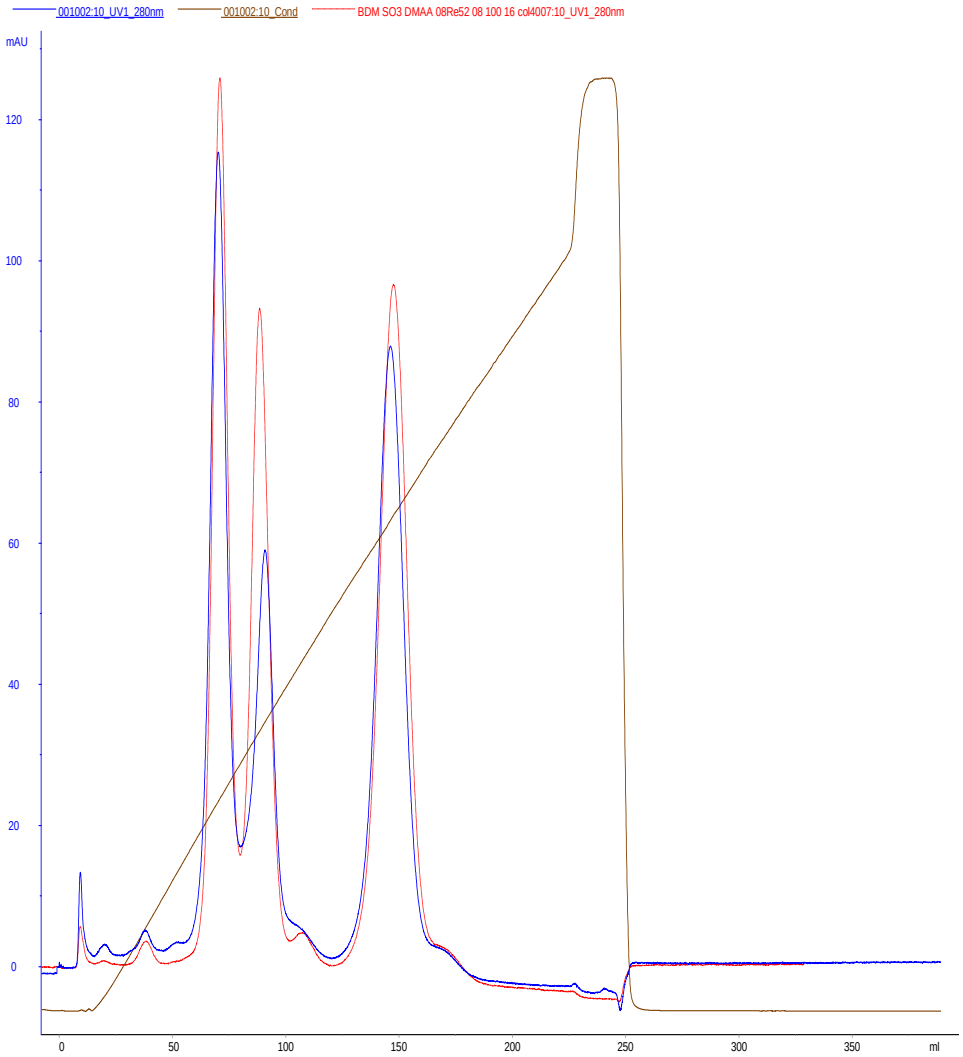

Figure 24: Chromatograms of the separation of of chymotrypsinogen A, cytochrome C, and lysozyme on a packed column (100mm x 16 mm i.d., 10 % compression) with Eshmuno® S before (blue) and after storage in 0.1 M NaOH (red, dashed line) for 348 days months.

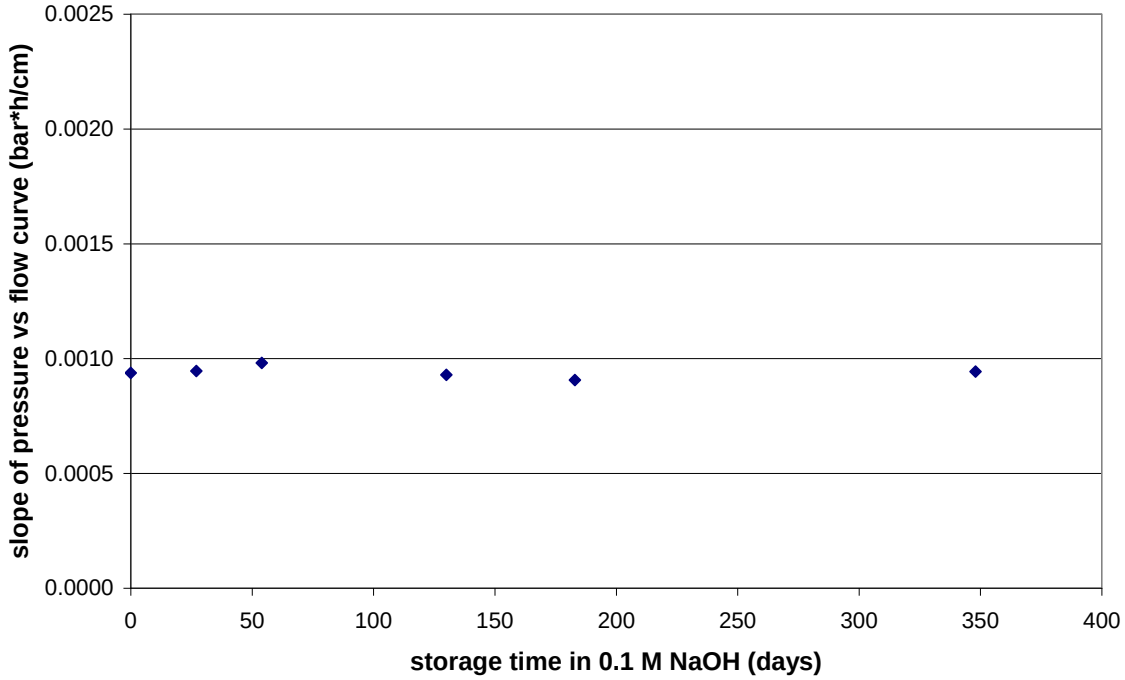

Figure 25: Pressure versus flow curves were recorded in 100 x 16 mm i.d. columns packed to 10 % compression (150 – 1500 cm/h). The slope of the net pressure drop versus flow curve after treatment with 0.1 M sodium hydroxide for certain times was determined by linear regression. The coefficient of determination (R^2) was always greater than 0.995.

5.3. Stability against Acids

According to the chemical structure of Eshmuno® S the amide groups present in the matrix can be expected to be prone to hydrolysis by strong mineral acids. As the functional ligands (sulfoisobutyl groups) are coupled to the polyacrylamide (tentacles), this would lead to release of soluble amine and, concomitantly, to the loss of ion exchanger functionality of the gel. However, acidic hydrolysis of the amide bond is an extremely slow reaction under normal chromatographic conditions. Of course, the functional ligands and the matrix can be hydrolyzed using harsh conditions, e.g. several hours treatment of the gel with 6 M hydrochloric acid at 120 °C under pressure.

5.3.1. Stability against 1 M Hydrochloric Acid

Bulk resin (for measurement of protein binding capacity) and a packed column (for recording of chromatographic resolution and pressure versus flow curves) of Eshmuno® S, lot no. 08RE052-08, were stored in 1.0 M hydrochloric acid solution at room temperature.
For capacity testing, aliquots of the gel suspension were taken after specified time intervals, washed with water and neutralized with 100 mM sodium phosphate buffer pH 7.0. Protein binding capacities were determined according to the standard procedures described in the monograph.
For measurement of chromatographic resolution and pressure versus flow curves the resin was packed into Superformance® 150-16 column to 10 cm bed height at 15 % compression. After testing the column according to the monograph the column was rinsed with hydrochloric acid and stored at room temperature. After specified time intervals, the column was rinsed with 2 column volumes (CV) of equilibration buffer, 2 CV of elution buffer at a flow rate of 150 cm/h (5 ml/min), followed by 5 CV of equilibration buffer at a flow rate of (2.6 ml/min), before the separation run. Chromatographic resolutions were determined according to the standard procedures described in the monograph (separation of chymotrypsinogen A, cytochrome C, and lysozyme). In addition, pressure flow curves recorded after performing the chromatographic resolution. After testing the column was again rinsed with hydrochloric acid and stored at room temperature.

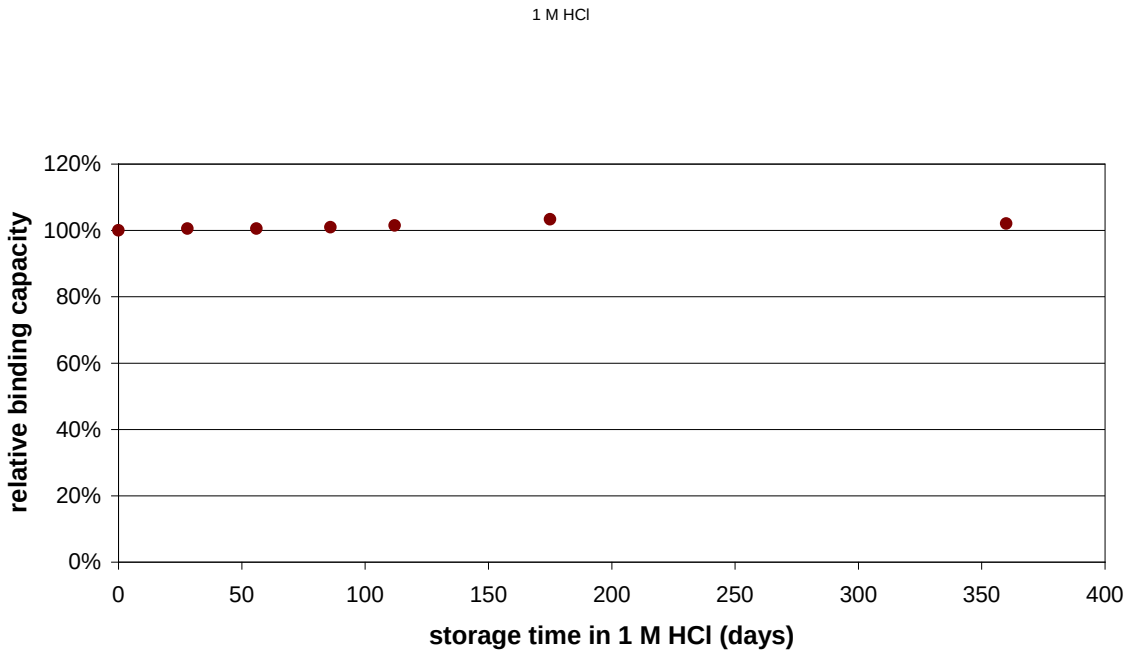

Figure 26: Relative static lysozyme binding capacity after prolonged treatment with 1.0 M hydrochloric acid.

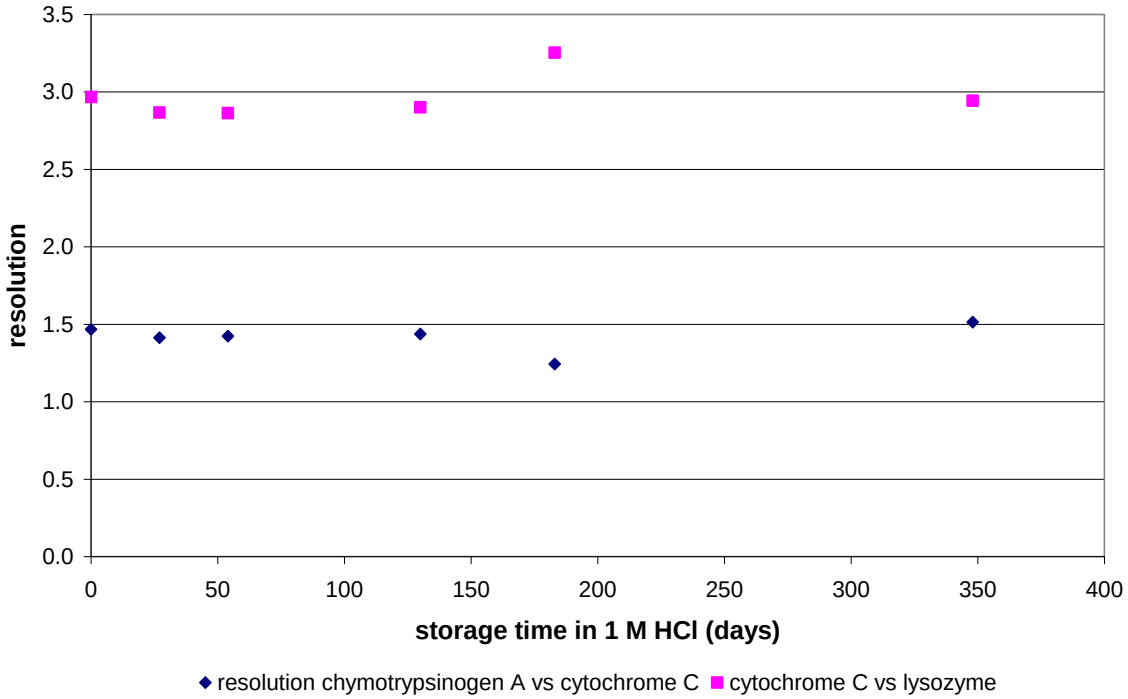

Figure 27: Resolution data for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 1.0 M hydrochloric acid for certain times after re-equilibration.

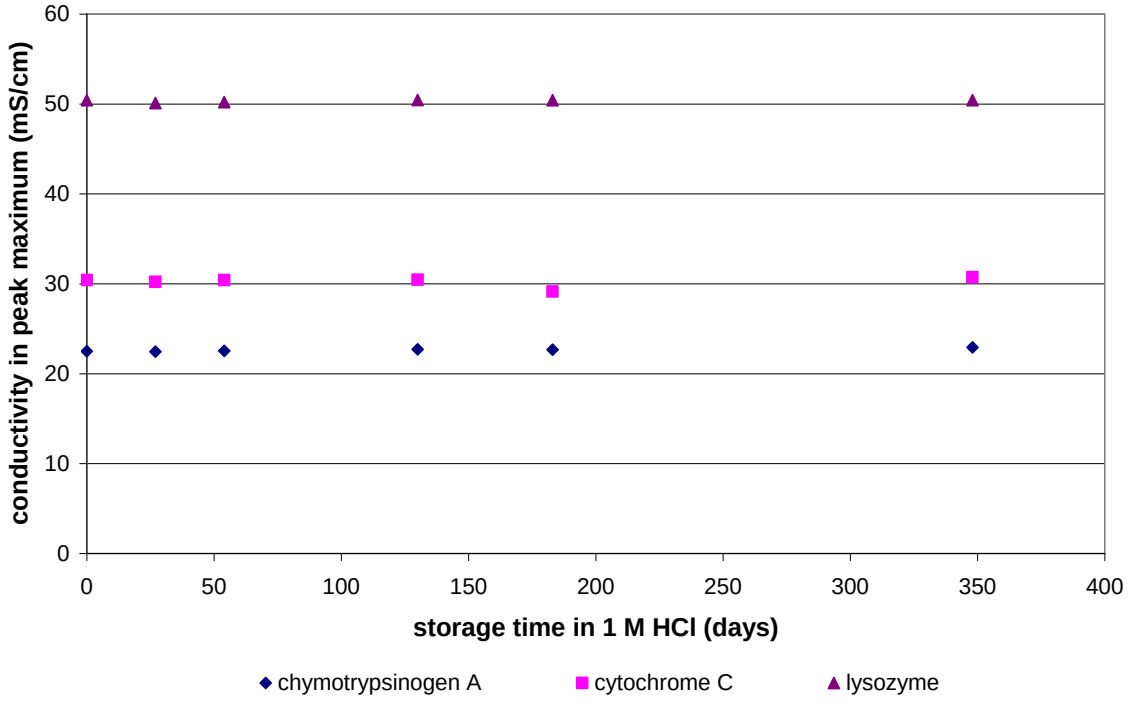

Figure 28: Elution conductivities at peak maxima for a standard separation on a packed column (100 mm x 16 mm i.d., 10 % compression) stored in 1.0 M hydrochloric acid for certain times after re-equilibration.

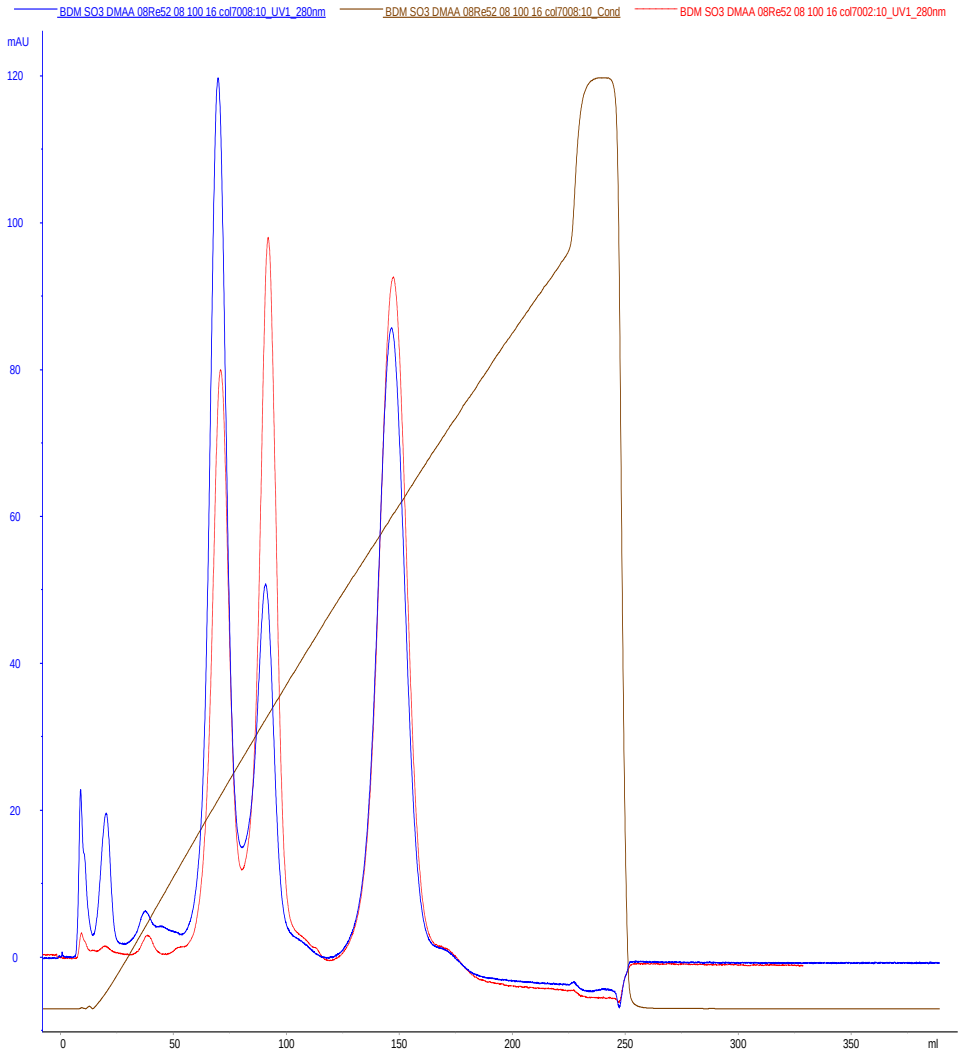

Figure 29: Chromatograms of the separation of of chymotrypsinogen A, cytochrome C, and lysozyme on a packed column (100mm x 16 mm i.d., 10 % compression) with Eshmuno® S before (blue) and after storage in 1.0 M hydrochloric acid (red, dashed line) for 348 days.

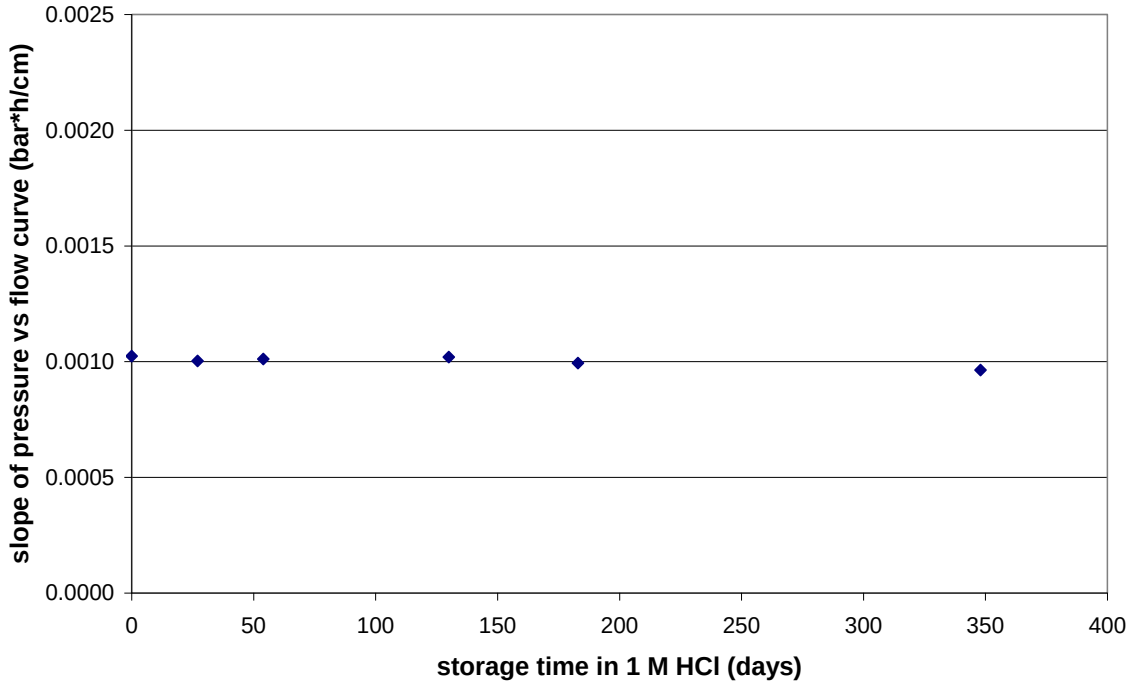

Figure 30: Pressure versus flow curves were recorded in 100 x 16 mm i.d. columns packed to 10 % compression (150 – 1500 cm/h). The slope of the net pressure drop versus flow curve after treatment with 1.0 M hydrochloric acid for certain times was determined by linear regression. The coefficient of determination (R^2) was always greater than 0.995.

Conclusion:

Eshmuno® S does not lose protein binding capacity after exposure to 1 M hydrochloric acid solution for several months at room temperature. Concurrently, the resolution of standard proteins remains nearly unchanged in this period. Pressure versus flow properties are hardly altered during long term storage in 1 M hydrochloric acid.

5.4. Stability against Various Chemicals

To demonstrate the compatibility of Eshmuno® S, columns packed with the resin were exposed to various chemicals commonly used in biochromatography. The chemicals are 5 M urea and 6 M guanidinium chloride (see tables below).

Experimental procedure:

Two 100 mm x 16 mm i.d. columns were packed with Eshmuno® S to 9 % compression according to the standard procedure. The columns were equilibrated to the respective chemical at 5 ml/min using ten column volumes. Afterwards the chemical was circulated through the column for about 16 hours at 6 ml/min.
After circulation with each chemical a pressure versus flow curve of the column was recorded with this chemical according to the packing protocol.
After this treatment the column was equilibrated with phosphate buffer and characterized by the separation of standard proteins as described in the monograph. Subsequently a pressure versus flow curve was performed using 150 mM sodium chloride eluent.

Results and Discussion:

After regeneration with buffer the chromatographic behaviour of Eshmuno® S was unchanged.

Table 2: Resolution data and elution conductivities at peak maxima for the separation of standard proteins before and after treamtment with particular chemicals.

untreated and regenerated column: 20 mM NaH_2PO_4, pH 6.0	elution conductivity (mS/cm)			resolution (EUP)	
	Chymotrypsinogen A	cyto-chrome C	lysozyme	chymotrypsinogen A vs.cytochrome C	cytochrome C vs. lysozyme
Untreated column 1	22.6	30.4	50.2	1.5	2.9
Column 1 after 5 M urea	22.4	30.3	50.1	1.5	3.0
Untreated column 2	22.7	30.0	50.6	1.5	2.8
Column 2 after 6 M guanidinium chloride	22.5	30.3	49.9	1.5	3.0
Untreated column 3	22.4	29.8	50.3	1.6	3.0
Column 3 30Vol% 2-propanol	22.4	30.4	49.9	1.5	3.3

The two chemicals listed in the table affect the slope of the pressure versus flow curves. After regeneration with aqueous buffer, the slope of the curves came back to the slope of the untreated column with some unavoidable deviations.

Table 3: Relative slope of the net pressure drop versus flow curve before, during and after treatment with particular chemicals. The slope was determined by linear regression and the coefficient of determination (R^2) was always greater than 0.97.

eluent A	Relative slope of pressure versus flow curve		
	before treatment (eluent 150 mM NaCl)	with eluent A	after regeneration (eluent 150 mM NaCl)
5 M urea	100 %	171 %	97 %
6 M guanidinium chloride	100 %	154 %	98 %
30 Vol% 2-propanol	100 %	317 %	97 %

Conclusion:

Treatment of a column packed with Eshmuno® S with various chemicals results in specific pressure versus flow properties. After regeneration of the column with buffer the pressure versus flow curves remain mostly unchanged when compared with the curve of the untreated column. There was no change in the chromatographic properties of the gel upon exposure to these chemicals after regeneration with buffer.

6. Removal of Ethanol

Complete removal of ethanol from bulk material is achieved by rinsing the gel with 7 column volumes of buffer or water.

The removal of ethanol is shown for a Superformance column 150-16 packed with Eshmuno® S equilibrated in 20 % ethanol. After washing with 3 column volumes of deionized water, the ethanol concentration had dropped to less than 0.05 % (figure 31). This method can also be scaled up to bigger columns.

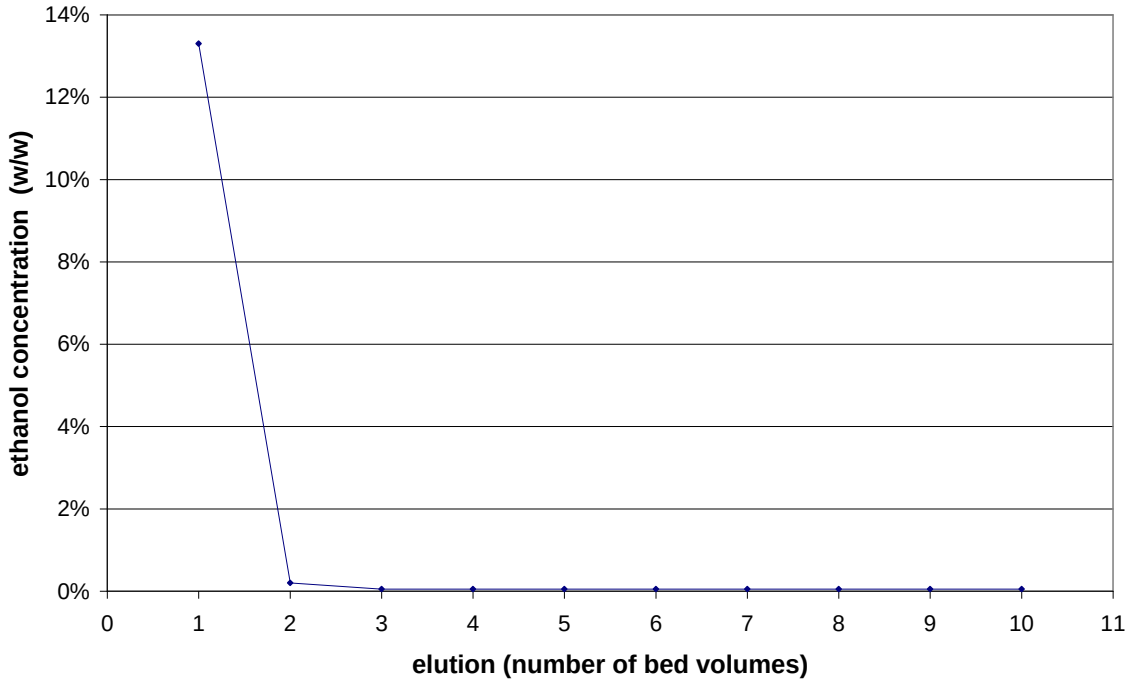

Figure 31: Ethanol concentration in the effluent.

Method:
A Superformance® 150-16 column was packed with Eshmuno® S to 15 % compression. After testing the packed column according to the standard packing procedure the gel bed was equilibrated with 4 bed volumes of storage solution (20 % ethanol/150 mM NaCl) at 100 cm/h. The gel was allowed to stand over night (20 h) in this solution. Subsequently, the column was again washed with deionized water, collecting the effluent in fractions of one bed volume. The flow rate was 100 cm/h.

The individual fractions were analyzed for ethanol content by gas chromatography (DB1

quartz capillary column, 30 m × 0.32 mm, equilibrated at 80°C, temperature programm: 5 min at 50°C, heating up to 240°C at a rate of 8°/min, hold at 240°C for 5 min; detector: FID; carrier gas: nitrogen. The detection limit was 0.01 mg ethanol per ml.).

To Place an Order or Receive Technical Assistance
In the U.S. and Canada, call toll-free: 1-800-645-5476

For other countries across Europe and the world, please visit:
www.emdmillipore.com/offices

For Technical Service, please visit:
www.emdmillipore.com/techservice